Galectin-3 Deficiency Prevents Concanavalin A–Induced Hepatitis in Mice

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We used concanavalin A (Con A)-induced liver injury to study the role of galectin-3 (Gal-3) in the induction of inflammatory pathology and hepatocellular damage. We tested susceptibility to Con A–induced hepatitis in galectin-3-deficient (Gal-3$^{-/-}$) mice and analyzed the effects of pretreatment with a selective inhibitor of Gal-3 (TD139) in wild-type (WT) C57BL/6 mice, as evaluated by a liver enzyme test, quantitative histology, mononuclear cell (MNC) infiltration, cytokine production, intracellular staining of immune cells, and percentage of apoptotic MNCs in the liver. Gal-3$^{-/-}$ mice were less sensitive to Con A–induced hepatitis and had a significantly lower number of activated lymphoid and dendritic cells (DCs) in the liver. The level of tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin (IL)-17 and -4 in the sera and the number of TNFα-, IFNγ-, and IL-17- and -4-producing cluster of differentiation (CD)4$^+$ cells as well as IL-12-producing CD11c$^+$ DCs were lower, whereas the number of IL-10-producing CD4$^+$ T cells and F4/80$^+$ macrophages were significantly higher in livers of Gal-3$^{-/-}$ mice. Significantly higher percentages of late apoptotic Annexin V$^+$ propidium-idodide$^+$ liver-infiltrating MNCs and splenocytes were observed in Gal-3$^{-/-}$ mice, compared to WT mice. Pretreatment of WT C57BL/6 mice with TD139 led to the attenuation of liver injury and milder infiltration of IFNγ- and IL-17- and -4-producing CD4$^+$ T cells, as well as an increase in the total number of IL-10-producing CD4$^+$ T cells and F4/80$^+$ CD206$^+$ alternatively activated macrophages and prevented the apoptosis of liver-infiltrating MNCs. *Conclusions*: Gal-3 plays an important proinflammatory role in Con A–induced hepatitis by promoting the activation of T lymphocytes and natural killer T cells, maturation of DCs, secretion of proinflammatory cytokines, down-regulation of M2 macrophage polarization, and apoptosis of MNCs in the liver. (HEPATOLOGY 2012;55:1954-1964)

Concanavalin A (Con A)-induced liver injury is a well-established murine model of T-cell-mediated hepatitis. Intravenous (IV) injection of Con A induces acute liver injury and systemic immune activation in mice that resembles the pathology of immune-mediated hepatitis in humans.[1] Activated T cells have a critical role in Con A–induced liver damage.[1] Cluster of differentiation (CD)4$^+$ T lymphocytes infiltrate the liver tissue and secrete large amounts of cytokines, such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin (IL)-2 and -6, and granulocyte macrophage colony-stimulating factor.[1,2] Apart from cluster of differentiation (CD)4$^+$ T cells, CD8$^+$ T cells, natural killer (NK), natural killer T (NKT) cells, and macrophages could induce hepatocyte cell death by either cell-to-cell contact, through the secretion of proinflammatory cytokines, or reactive oxygen species.[1-4]

Galectin-3 (Gal-3) is a member of the β-galactoside-binding lectin family that can modulate immune and inflammatory responses and plays an important role in the pathogenesis of inflammatory liver diseases and liver tumors.[5,6] Gal-3 is widely expressed in immune cells, including activated T and B cells, macrophages, dendritic cells (DCs), and NK cells.[7,8] Gal-3 functions as a key regulator of T-cell activation and function[9] and is highly expressed in activated CD4+ and CD8+ T cells, but not in resting T cells.[10] Gal-3 has been found to be widely distributed.[9] Different functions have been described for extracellular and intracellular Gal-3.[9] Extracellular Gal-3 induces apoptosis in T cells, attenuates T-cell-receptor signaling and promotes the migration of T cells. On the contrary, intracellular Gal-3 inhibits apoptosis in T cells and promotes T-cell growth.[9]

The role of Gal-3 in inflammatory disease is controversial. Gal-3 deficiency led to reduced inflammation in experimental models of pneumococcal pneumonia[11] and atherosclerosis.[12] On the contrary, Gal-3-deficient (Gal-3−/−) mice were reported to develop higher inflammatory response in the lung after infection with *Toxoplasma gondii*,[13] suggesting that the effect of Gal-3 on inflammation is organ and disease specific.

Gal-3 is involved in the pathogenesis of inflammatory and malignant liver diseases.[5,6] Gal-3 plays a major role in the removal of circulating advanced lipoxidation endproducts (ALEs) by the liver, and the deletion of Gal-3 accelerates nonalcoholic steatohepatitis (NASH) or prevents the development of ALE-induced liver injury.[5] Gal-3 is involved in the progression of hepatocellular carcinoma where a higher expression rate of nuclear Gal-3 shows a markedly worse prognosis in malignant and chronic inflammatory liver diseases, suggesting different roles of Gal-3 in tumors and autoimmunity.[5,6]

Here, we provide evidence that Gal-3 deficiency leads to a marked attenuation of Con A–induced hepatitis accompanied by reduced mononuclear cell (MNC) infiltration in the liver, decreased serum levels of TNFα, IFNγ, IL-17 and -4, accumulation of IL-10-producing CD4+ T cells, and alternatively activated M2 liver macrophages and enhanced apoptosis of liver-infiltrating MNCs. Also, we show that pretreatment of wild-type (WT) mice with synthesized selective inhibitor of Gal-3 (TD139) attenuated Con A–induced liver injury and reduces the number of CD4+ and CD8+ T cells, but favors the influx of IL-10-producing CD4+ T cells in the liver and alternative activation of macrophages, decreases serum levels of IFNγ and IL-17 and -4, and increases serum levels of IL-10 in Con A–treated animals.

Thus, our data demonstrate that Gal-3 plays an important role in Con A–induced hepatitis and therefore may be a potential target for therapeutic intervention in acute liver diseases.

Materials and Methods

Animals. We used 6-8-week-old male WT and Gal-3−/− C57Bl/6 mice (kindly provided by Dr. Daniel Hsu, University of California, Sacramento, Sacramento, CA) for the induction of Con A–induced hepatitis. Targeted disruption of mouse Gal-3 gene was performed in C57Bl/6 embryonic stem cells, and mice homozygous for disrupted gene were obtained.[14] WT Gal-3+/+ C57Bl/6 mice of the same substrain were maintained in our animal facilities. All animals received humane care, and all experiments were approved by, and conducted in accord with, the Guidelines of the Animal Ethics Committee of the Faculty of Medicine of the University of Kragujevac (Kragujevac, Serbia). Mice were housed in a temperature-controlled environment with a 12-hour light-dark cycle and were administered standard laboratory chow and water *ad libitum*.

Con A–Induced Hepatitis. WT and Gal-3−/− C57Bl/6 mice were given a single IV injection of Con A (Sigma-Aldrich, St. Louis, MO) at 12 mg/kg body weight dissolved in 250 μL of saline. Serum levels of alanine aminotransaminase (ALT) and asparate aminotransferase (AST) were measured as previously described.[3]

Gal-3 inhibitor (Gal-3-INH; 300 μg per dose) was intraperitoneally (IP) administered 2 hours before and immediately after Con A injection.

Histological Analyses and Semiquantitative Assessment of Liver Injury. Histological analysis and semiquantitative determination of liver injury were performed as previously described.[3,15]

Immunohistochemistry of Human Livers. For immunoperoxidase staining of Gal-3, formalin-fixed, paraffin-embedded human liver tissue sections obtained from the Department of Pathology University of

Kragujevac tissue collection and mouse antihuman Gal-3 antibody (catalog no.: ab58086; Abcam, Cambridge, UK) and the rabbit ABC Staining system (catalog no.: sc-2018; Santa Cruz Biotechnology, Santa Cruz, CA) were used according to manufacturer instructions.

Isolation of Hepatic MNCs, Splenocytes, and Analysis With Flow Cytometry. The isolation of liver-infiltrating MNCs and splenocytes was conducted as previously described.[3] Hepatic MNCs of WT and Gal-3$^{-/-}$ mice were screened for various cell-surface and intracellular markers with flow cytometry before and at 8 hours after Con A injection. Briefly, 1 x 10^6 MNCs were incubated with antimouse CD3, antimouse CD4, antimouse CD25, antimouse CD8, antimouse CD19, antimouse NK1.1, antimouse CD11c, antimouse F4/80, and antimouse CD206 conjugated with fluorescein isothiocyanate (FITC; BD Biosciences, Franklin Lakes, NJ), phycoerythrin (PE; BD Biosciences), peridinin chlorophyll protein (BD Biosciences), or allophycocyanin (APC; BD Biosciences).

MNCs derived from livers were stained for different markers of cell subsets (i.e., CD4, CD8, NK1.1, CD11c, and F4/80) and concomitantly for the intracellular content of TNFα, IFNγ, and IL-17, -10, and -4. To this end, monoclonal antibodies were used as follows: antimouse TNFα and antimouse IL-10 conjugated with APC (BD Bioscience), antimouse IL-4, IFNγ, and IL-17 conjugated with PE.

Intracellular staining for forkhead box protein 3 (Foxp3) was performed using the BD Biosciences fixation/permeabilization buffer kit. Stained cells were counted using a BD Biosciences FACSCalibur, and the results were analyzed with WinMDI software.

Apoptosis Assays. For the detection of apoptosis, the Annexin V– binding capacity of liver MNCs and splenocytes was examined by flow cytometry using the Annexin V FITC Detection Kit (BD Pharmingen, San Jose, CA), as previously described.[16]

Measurement of Cytokines in the Serum. Individual mouse serum was collected, and serum levels of IFNγ, TNFα, and IL-17, -4, and -10 were measured by enzyme-linked immunosorbent assay (ELISA) using ELISA kits (R&D Systems, Minneapolis, MN).

Measurement of Cytokines in Supernatants of In Vitro Con A–Stimulated Splenocytes. Individual spleens of Con A–untreated WT and Gal-3$^{-/-}$ mice were collected. The single-cell suspension of splenocytes was cultured in 24-well plates at 4 x 10^6 cells per well and was stimulated with 5 μg/mL of Con A (Sigma-Aldrich). After 24 hours, supernatants were collected and cytokine concentrations were measured by ELISA kits (R&D Systems).

Statistical Analysis. All statistics were carried out using SPSS 13.0 for Windows software (SPSS, Inc., Chicago, IL). Results were analyzed using the Student t test. All data in this study were expressed as the mean ± standard error of the mean (SEM). Values of $P < 0.05$ were considered as statistically significant.

Results

Gal-3 Is Highly Expressed in the Livers of Patients Suffering From Acute Liver Disease. First, we investigated whether acute liver injury in humans would affect Gal-3 expression in the liver. Human liver tissue sections were obtained from patients suffering from acute liver disease induced by isoniazid or hepatitis B virus (HBV) and were compared to healthy controls. Compared to healthy controls (Supporting Fig. 1A), Gal-3 was strongly expressed in lining cells of hepatic sinuses both in patients with isoniazid-induced (Supporting Fig. 1B) and HBV-induced (Supporting Fig. 1C) fulminant hepatitis, suggesting a possible role of Gal-3 in liver inflammation.

Target Disruption of Gal-3 Gene Protects From Con A–Induced Liver Injury. Next, to investigate the role of Gal-3 in experimental fulminant hepatitis, we injected Con A into WT and C57Bl/6 mice with the targeted disruption of Gal-3 gene (Gal-3$^{-/-}$ mice). Serum AST and ALT levels after Con A injection were significantly lower in Gal-3$^{-/-}$, compared to WT, mice ($P < 0.05$) (Fig. 1A). Histological analyses of liver tissue sections also indicated that Gal-3$^{-/-}$ mice are less sensitive to Con A–induced hepatic injury (Fig. 1B). Liver tissue sections in Gal-3$^{-/-}$ mice showed several solitary areas of necrotic tissue characterized by standard morphologic criteria (i.e., loss of architecture, vacuolization, karyolysis, and increased eosinophilia). The majority of hepatocytes were not affected in livers of Gal-3$^{-/-}$ mice.

In contrast, liver tissue sections in WT mice showed widespread areas of necrosis with extensive infiltration of MNCs within liver lobules (Fig. 1Ba) and around the central veins and portal tracts (Fig. 1Ca), indicating the ongoing inflammatory process (Fig. 1B,C). Extensive liver damage in WT mice was characterized by massive coagulative necrosis and cytoplasmic swelling of the majority of hepatocytes. Nuclear chromatin condensation was found frequently, which may indicate hepatocyte apoptosis.

Consistent with those findings, the percentage of liver tissue with necrotic damage was markedly lower in Gal-3$^{-/-}$ mice (Supporting Fig. 2).
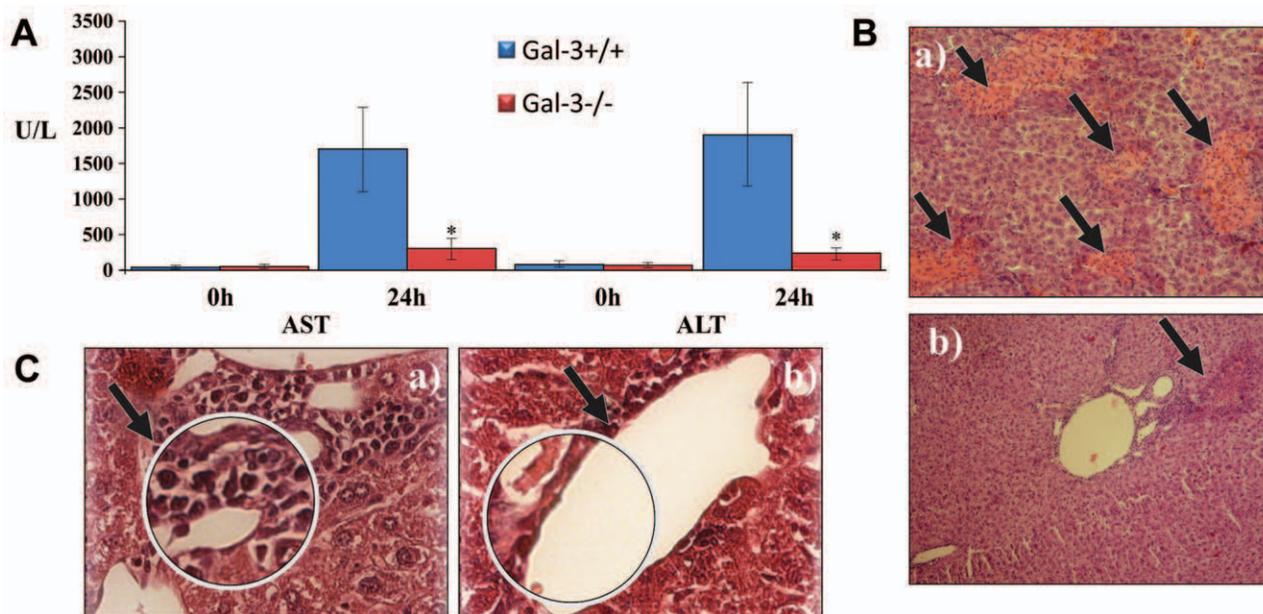
Fig. 1. Target disruption of Gal-3 gene protects from Con A–induced liver injury. (A) Serum AST and ALT levels at 24 hours after IV injection of Con A (12 mg/kg). Serum AST and ALT levels were significantly lower in Gal3$^{-/-}$ mice (red bars), compared to WT mice (blue bars). Combined results of two experiments with at least 10 animals per group (mean $\pm$ SEM; *P < 0.05). (B) Photomicrographs of representative H&E-stained mouse livers 24 hours after Con A injection. Massive hepatocyte necrosis (dark arrows) was observed in WT mice (a), in comparison, to a lesser extent, of liver damage observed in Gal3$^{-/-}$ mice (b). (C) Infiltration of MNCs around the central veins and portal tracts. Massive infiltration of MNCs was observed in WT mice (a), compared to Gal3$^{-/-}$ mice (b). H&E, hematoxylin and eosin.

Gal-3$^{-/-}$ Mice Have Lower Serum Levels of Proinflammatory Cytokines After Con A Injection. There was conflicting evidence of whether Gal-3 deficiency attenuates both T-helper cells (Th)1 and 2 or only Th1 activity.[9,17] We found that, after Con A injection, mice lacking endogenous Gal-3$^{-/-}$ had significantly lower serum levels of both Th1 and 2 cytokines, in comparison to WT mice.

Serum levels of TNFα, IFNγ, and IL-17 and -4, 8 hours after Con A injection, were significantly lower in Gal-3$^{-/-}$, compared to WT, mice (TNFα, P < 0.01; IFNγ, P < 0.05; IL-17, P < 0.05; IL-4, P < 0.01), whereas there was no significant difference in the level of serum IL-10 between WT and GAL-3$^{-/-}$ mice (Supporting Fig. 3A).

Despite the fact that we found the difference in cytokine levels between WT and Gal-3$^{-/-}$ mice after *in vivo* treatment with Con A, there was no significant difference in levels of TNFα, IFNγ, and IL-17, -4, and -10 in supernatants of *in vitro* Con A–stimulated
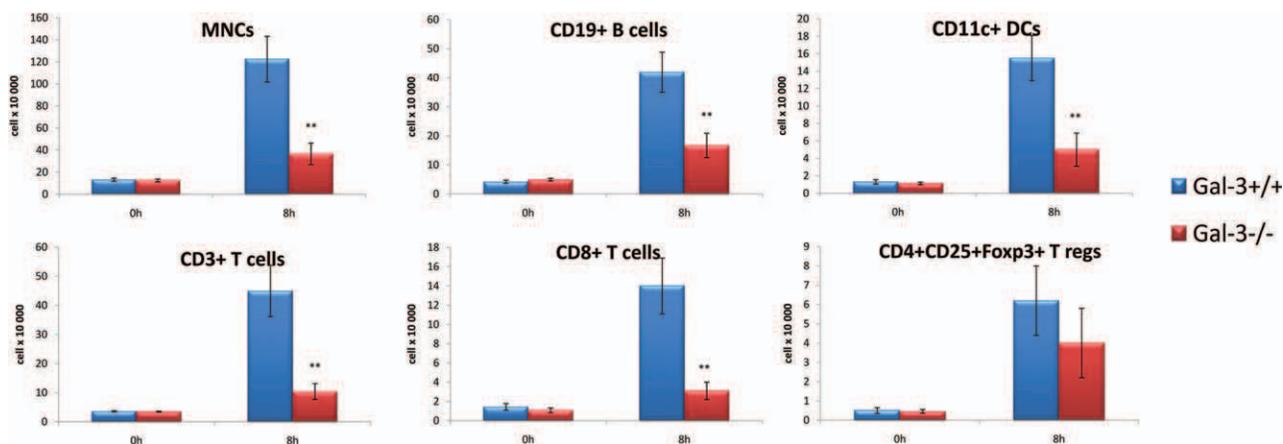
Fig. 2. Gal-3 deletion reduced the influx of activated lymphoid and DCs in the liver after Con A injection. A markedly decreased total number of MNCs in livers of Gal-3$^{-/-}$ (red bars) versus WT mice (blue bars) 8 hours after Con A injection, including CD3$^+$ and CD8$^+$ T cells, CD19$^+$ B lymphocytes and CD11c$^+$ DCs. There was no significant difference in the total number of CD4$^+$CD25$^+$Foxp3$^+$ Tregs between WT and Gal-3$^{-/-}$ mice. Combined results of two experiments with at least 8 animals per group (mean $\pm$ SEM; *P < 0.05; **P < 0.01).

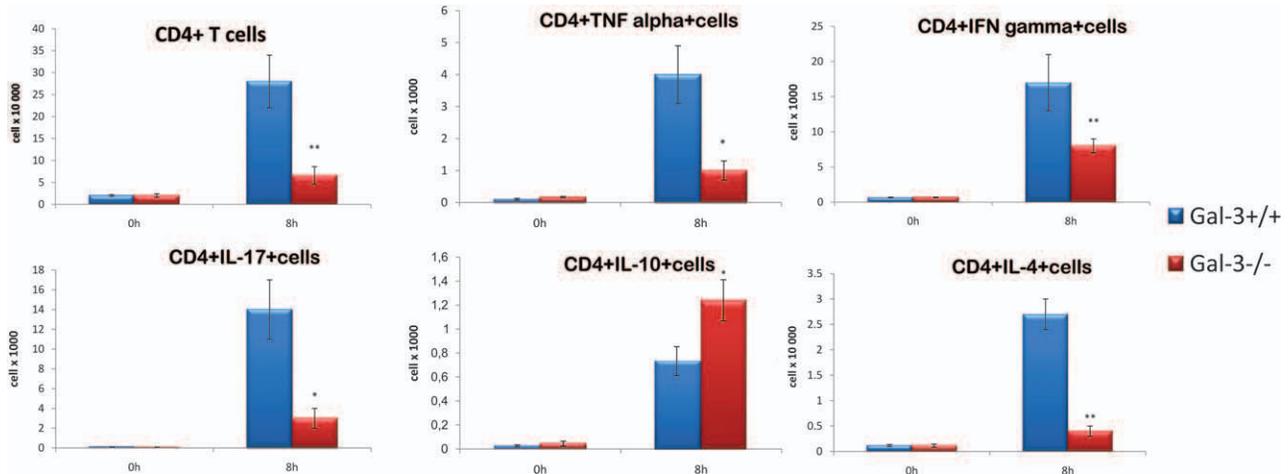Fig. 3. Gal-3 deletion significantly reduces influx and affects cytokine production in CD4$^+$ T cells. Significant decrease in the total number of TNFα-, IFNγ-, and IL-17- and -4-producing CD4$^+$ cells in Gal-3$^{-/-}$ mice (red bars), when compared to WT mice (blue bars), 8 hours after Con A injection. On the contrary, the total number of IL-10-producing CD4$^+$ T cells was significantly higher in livers of Gal3$^{-/-}$ Con A–treated mice. Combined results of two experiments with at least 8 animals per group (mean ± SEM; *$P < 0.05$; **$P < 0.01$).

splenocytes of WT and Gal-3$^{-/-}$ mice (Supporting Fig. 3B).

Influx of Activated Lymphoid and DCs in the Liver Is Reduced in Gal3$^{-/-}$ Mice. After Con A injection, there was an influx of MNCs in the liver. However, the number of MNCs in livers of Gal-3$^{-/-}$ mice was significantly lower then in WT mice (Fig. 2).

Eight hours after Con A injection, there was a decrease in the total number of liver-infiltrating lymphoid cells (e.g., CD3$^+$, CD4$^+$, and CD8$^+$ T cells, CD19$^+$ B cells, and NK1.1$^+$ NK and NK1.1$^+$CD3$^+$ NKT cells) (Figs. 2-4). All differences reached the level of statistical significance ($P < 0.01$).

Also, the number of CD11c$^+$ DCs ($P < 0.01$) and CD11c$^+$CD80$^+$CD86$^+$-activated DCs ($P < 0.05$) was lower in livers of Gal-3$^{-/-}$, compared to WT, mice (Figs. 2 and 4). However, there was no significant difference in the total number of T regulatory cells (Tregs; CD4$^+$CD25$^+$Foxp3$^+$) between WT and Gal-3$^{-/-}$ mice (Fig. 2). Also, the total number of F4/80$^+$ macrophages was similar in livers of Gal-3 mice and WT controls (Fig. 5A).

Intracellular staining of liver MNCs revealed significantly lower numbers of TNFα- ($P < 0.05$), IFNγ- ($P < 0.01$), and IL-17- ($P < 0.05$) and -4-producing CD4$^+$ cells ($P < 0.01$) in Gal-3$^{-/-}$, when compared
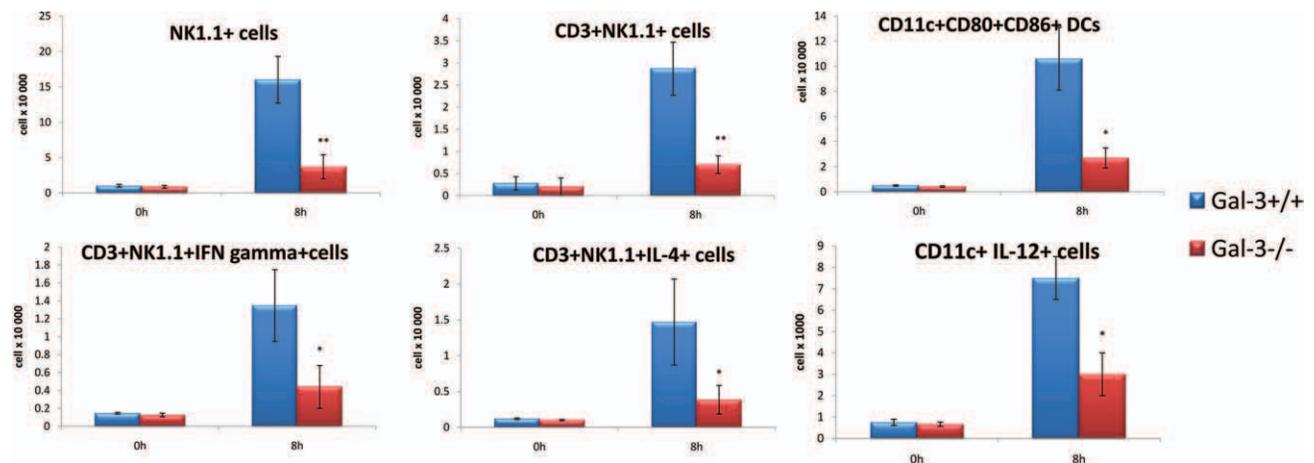
Fig. 4. Gal-3 deletion markedly decreases liver infiltration and cytokine production in NK, NKT, and activated DCs. The total number of liver-infiltrating NK1.1$^+$ NK, NK1.1$^+$CD3$^+$ NKT, and CD11c$^+$CD80$^+$CD86$^+$ DCs were significantly lower in Gal 3$^{-/-}$ mice (red columns), compared to WT mice (blue columns). In addition, a significant decrease in the total number of IFNγ- and IL-4-producing NK1.1$^+$CD3$^+$ NKT cells and IL-12-producing DCs was observed in Gal 3$^{-/-}$ mice 8 hours after Con A injection. Combined results of two experiments with at least 8 animals per group (mean ± SEM; **$P < 0.01$).

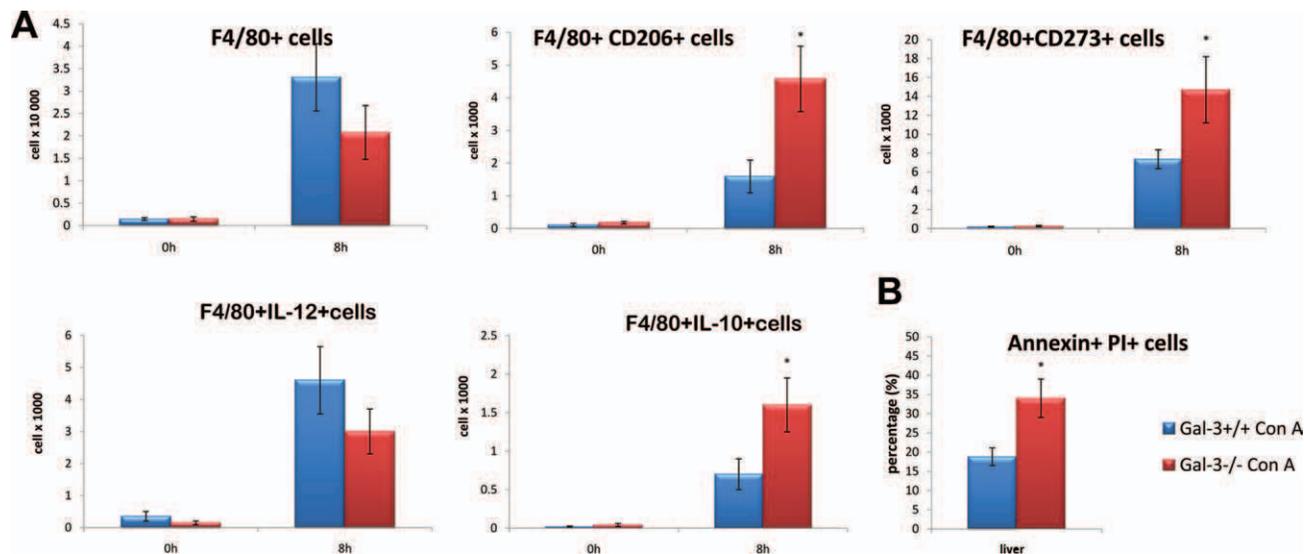Fig. 5. Gal-3$^{-/-}$ deletion favors the alternative activation of macrophages and enhances the apoptosis of liver-infiltrating MNCs in Con A–induced hepatitis. (A) Significantly higher percentages and total numbers of F4/80$^+$ CD206$^+$ alternatively activated (i.e., M2-polarized) and IL-10 producing F4/80$^+$ macrophages were found in livers of Gal-3$^{-/-}$ mice (red bars), compared to WT mice (blue bars), 8 hours after Con A injection. There was no significant difference in percentage and total number of F4/80$^+$ macrophages between WT and Gal-3$^{-/-}$ mice. Combined results of two experiments with at least 8 animals per group (mean $\pm$ SEM; *$P < 0.05$; **$P < 0.01$). (B) Significantly higher percentage of late apoptotic Annexin V$^+$ PI$^+$ liver-infiltrating MNCs was observed in Gal-3$^{-/-}$ mice (red bars), compared with WT mice (blue bars), 8 hours after Con A injection. Combined results of two experiments with at least 8 animals per group (mean $\pm$ SEM; *$P < 0.05$).

to WT, mice 8 hours after Con A injection (Fig. 3). Furthermore, the total number of IL-12-producing CD11c$^+$ DCs as well as IFNγ- and IL-4-producing NKT cells were significantly lower ($P < 0.05$) in livers of Gal-3$^{-/-}$, when compared to WT, mice (Fig. 4). There was no significant difference in the total number of IFNγ-producing CD8$^+$ T and NK cells and IL-10-producing CD11c$^+$ DCs (data not shown) between WT and Gal-3$^{-/-}$ mice.

Interestingly, the total number of IL-10-producing CD4$^+$ T cells and F4/80$^+$ macrophages was significantly higher ($P < 0.05$) in livers of Gal-3$^{-/-}$, compared to WT, mice (Figs. 3 and 5). Additionally, the ratio between the total number of IL-10- and IFNγ-producing CD4$^+$ T cells was significantly higher ($P < 0.05$) in livers of Con A–treated Gal-3$^{-/-}$, compared to WT, mice (4.53 $\pm$ 0.74 Gal3$^{-/-}$ versus 2.35 $\pm$ 0.56 WT).

Gal-3$^{-/-}$ Deletion Favors Alternative Activation of Macrophages and Enhances Apoptosis of Liver-Infiltrating MNCs and Splenocytes in Con A–Induced Hepatitis. We did not find any difference in the total number of liver F4/80$^+$ macrophages between WT and Gal-3$^{-/-}$ mice, but we noticed a significant difference in the total number of IL-10-producing F4/80$^+$ cells (Fig. 5A).

We found a significantly higher percentage and total number of F4/80$^+$ CD206$^+$ alternatively activated

(i.e., M2-polarized) macrophages in livers of Gal-3$^{-/-}$, compared to WT, mice (Fig. 5A). Thus, it appears that Gal-3 deletion favors the differentiation of IL-10-producing macrophages.

We assumed that apoptosis of infiltrating cells may contribute to the lower number of MNCs in livers of Gal-3$^{-/-}$ mice. Indeed, we found enhanced apoptosis of liver-infiltrating MNCs and splenocytes in Gal-3$^{-/-}$, compared to WT, mice (Fig. 5B; Supporting Fig. 5) 8 hours after Con A injection.

Both in livers and spleens, the majority of MNCs were in the stage of late apoptosis (Annexin V$^+$ propidium iodide [PI]$^+$ cells; Fig. 5B; Supporting Fig. 5). Significantly higher percentages of Annexin V$^+$ PI$^+$ liver-infiltrating MNCs ($P < 0.05$) and splenocytes ($P < 0.05$) were observed in Gal-3$^{-/-}$, mice compared to WT, mice (percentage of apoptotic cells in liver: 34% Gal3$^{-/-}$ versus 18.8% WT; in spleen: 38.7% Gal3$^{-/-}$ versus 17.3% WT).

Gal-3-INH Prevents Con A–Induced Liver Injury in C57BL/6 Mice. To further elucidate the role of Gal-3 in Con A–induced liver injury, we pretreated WT C57BL/6 mice with TD139, competing for the saccharide-binding site, 2 hours before and immediately after Con A injection.

We found that the administration of TD139 prevented the increase of serum liver transaminases (Fig. 6A). This
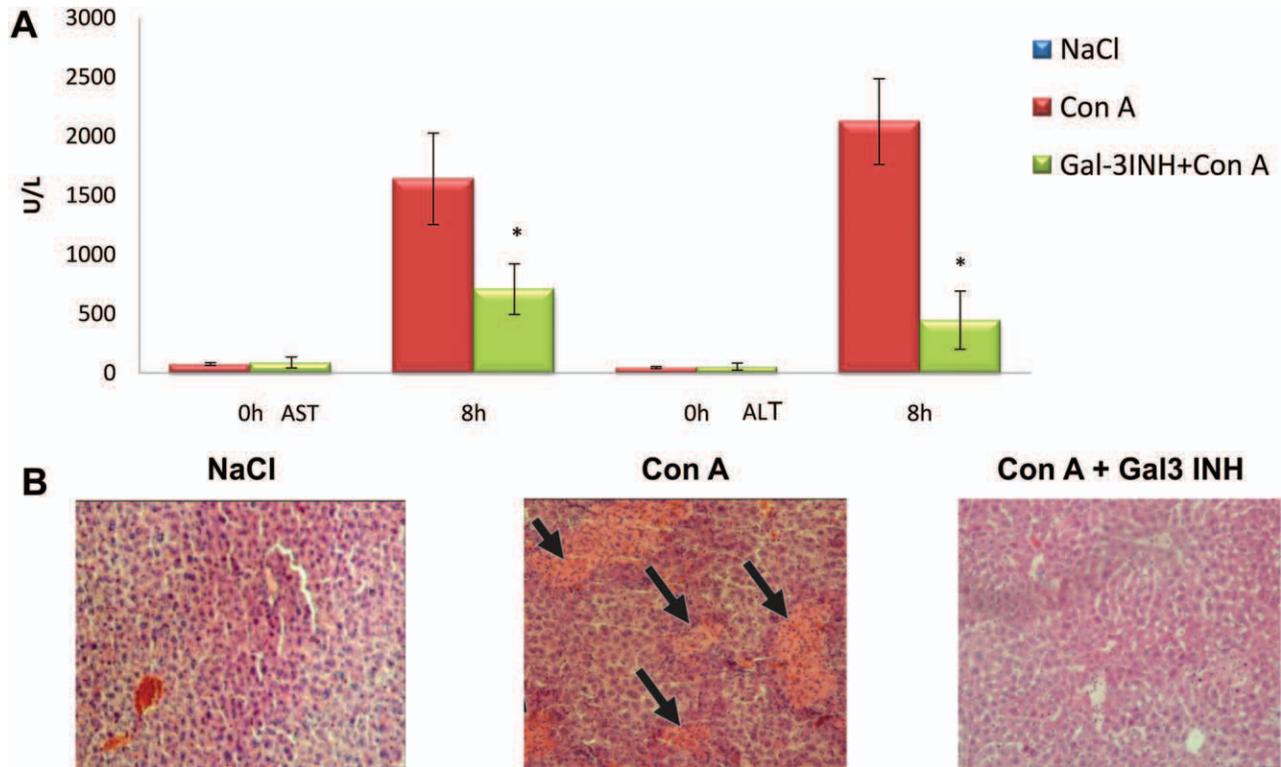
Fig. 6. TD139 prevents Con A–induced liver injury in C57BL/6 mice. (A) Absolute value of serum AST and ALT levels 24 hours after Con A injection in untreated mice (blue bars), Con A–only treated mice (red bars), and TD139 pretreated mice (green bars). Combined results of two experiments with at least 8 animals per group (mean ± SEM; *P < 0.05). (B) Histopathological examination of Con A–induced liver injury. Light micrographs of H&E liver sections were visible from untreated mice (a), treated with Con A only (c), and pretreated 2 hours and immediately after Con A injection with TD139 (c). Large necrotic areas (dark arrows) were visible in Con A–treated animals (b) and were markedly reduced in mice pretreated with TD139 (c). Original magnification, 400×. H&E, hematoxylin and eosin.

finding was consistent with scarce necrotic areas observed in the livers of pretreated animals, in contrast to significantly larger necrotic areas in liver parenchyma of mice treated with Con A and vehicle (Fig. 6B). IP injection of TD139 in Con A–untreated animals did not alter the serum level of liver enzymes (data not shown).

Levels of IFNγ and IL-17 and -4 were significantly lower in the sera of TD139-treated mice (P < 0.05) (Supporting Fig. 6). In contrast, IL-10 was significantly lower in mice that received Con A only (P < 0.05), whereas there was no significant difference in the serum level of TNFα between experimental groups.

We also examined whether TD139 would affect the infiltration of MNCs into the livers of mice after Con A injection (Fig. 7). Although there was no significant difference in the total number of liver-infiltrating MNCs, CD3$^+$ T cells, NK1.1$^+$ NK and CD3$^+$NK1.1$^+$ NKT cells, CD11c$^+$ DCs and CD11c$^+$CD80$^+$CD86$^+$ activated DCs, F4/80$^+$ macrophages (data not shown), and Gal-3 INH profoundly reduced CD4$^+$- and CD8$^+$ T-cell infiltration

into the liver parenchyma (Fig. 7). The total number of IFNγ- and IL-17- and -4-producing CD4$^+$ T cells and IFNγ-producing CD8$^+$ T lymphocytes was significantly lower in TD139-treated mice (Fig. 7). However, the total number of CD4$^+$ IL-10-producing cells was significantly higher in TD139-treated mice (P < 0.05). There was no difference in the total number of TNFα- producing CD4$^+$ T cells, IFNγ- and IL-4-producing CD3$^+$NK1.1 NKT cells, IFNγ-producing NK1.1$^+$ NK cells, IL-12- and -10-producing CD11c$^+$ DCs, and F4/80$^+$ macrophages between TD139-treated mice and mice that received Con A only (data not shown).

In line with results obtained from Gal3$^{-/-}$ mice (Fig. 5A), we found a significantly higher percentage and total number of F4/80$^+$ CD206$^+$ alternatively activated (i.e., M2-polarized) macrophages in livers of TD139-treated mice, compared to mice treated with Con A only (Fig. 8A). However, there was no difference in the total number of IL-12- and -10-producing macrophages (data not shown).
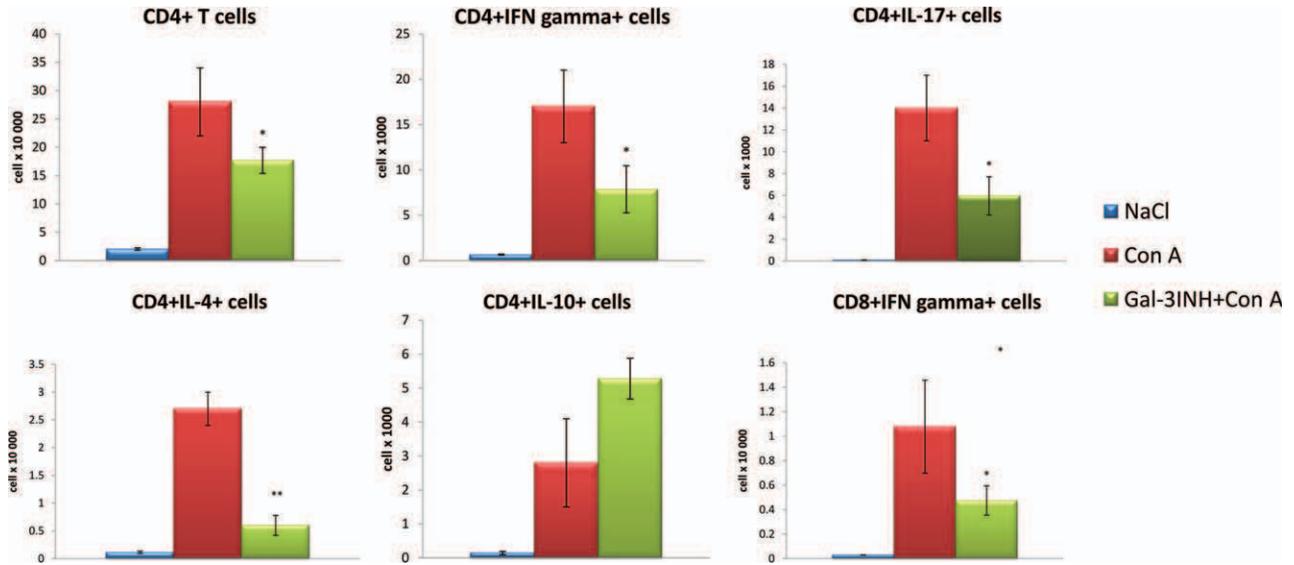
Fig. 7. TD139 significantly reduces influx of cytokine-producing T cells. TD139 markedly decreased the liver infiltration of CD4$^+$ T lympho-cytes, including IFNγ- and IL-17- and -4-producing CD4$^+$ T cells. Total number of IL-10-producing CD4$^+$ cells was significantly higher in livers of TD139-treated mice. The total number of liver-infiltrating IFNγ-producing CD8$^+$ T cells was significantly lower in mice that received TD139 before Con A injection. Combined results of two experiments with total of 8 animals per group (mean ± SEM; untreated mice, [blue bars], Con A–only treated mice [red bars], and TD139 treated mice [green bars]; *$P < 0.05$; **$P < 0.01$).

We also found that TD139 prevented apoptosis of liver-infiltrating MNCs (Fig. 8B) 8 hours after Con A injection. Significantly lower percentages of Annexin V$^+$ PI$^+$ liver-infiltrating MNCs ($P < 0.01$) were observed in TD139-treated mice, compared to mice treated with Con A only.
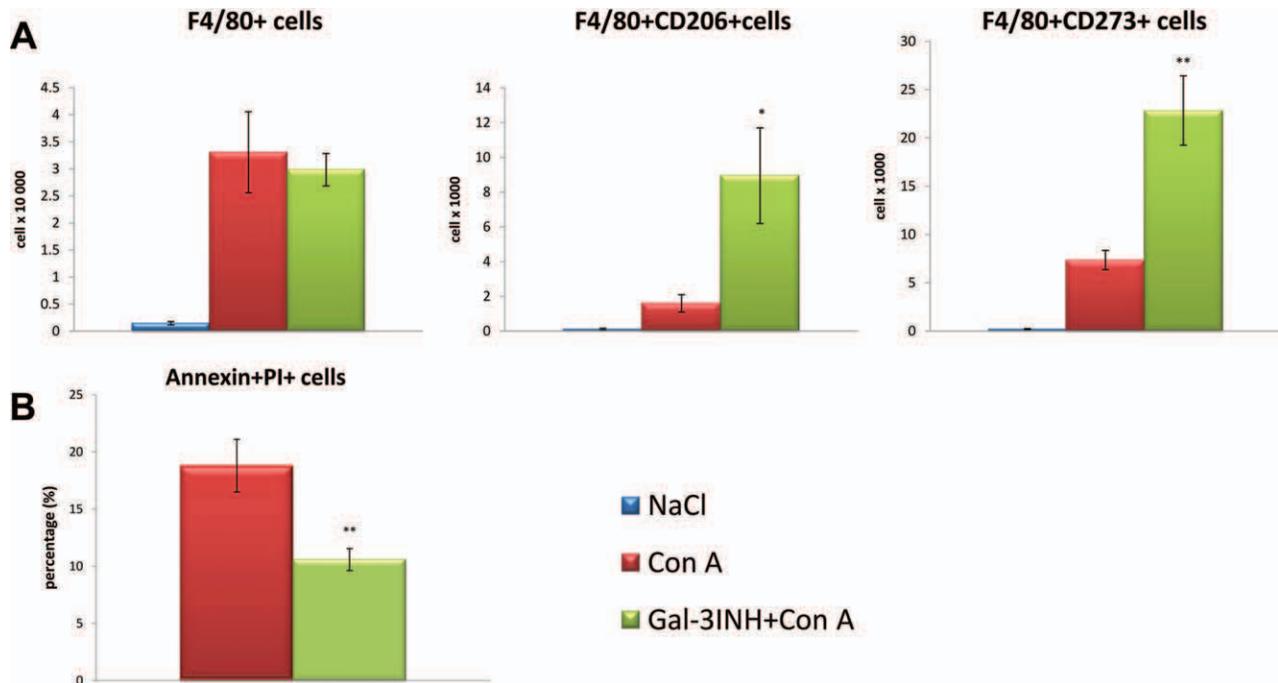

Fig. 8. TD139 significantly increases the number of alternatively activated macrophages and prevents apoptosis of liver-infiltrating MNCs. (A) TD139 favors alternative activation of macrophages. Significantly higher percentage and total number of F4/80$^+$ CD206$^+$ alternatively activated (i.e., M2-polar-ized) macrophages were found in livers of TD139-treated mice, compared to mice treated with Con A only. Combined results of two experiments with total of 8 animals per group (mean ± SEM; untreated mice [blue bars], Con A–only treated mice [red bars], TD139-treated mice [green bars]; *$P < 0.05$; **$P < 0.01$). (B) TD139 prevents apoptosis of liver-infiltrating MNCs in Con A–treated mice. Significantly lower percentage of Annexin V$^+$ PI$^+$ liver-infiltrating MNCs were observed in TD139-treated mice, compared to mice treated with Con A only. Combined results of two experiments with total of 8 animals per group (mean ± SEM; untreated mice [blue bars], Con A–only treated mice [red bars], and TD139 treated mice [green bars]; **$P < 0.01$).

Discussion

Here, we provided the first evidence that Gal-3 plays an important role in the pathogenesis of Con A–induced hepatitis, a model of acute, fulminate hepatitis in humans.[1] Targeted disruption of Gal-3 gene attenuated liver injury by reducing the number of effector cells, including T lymphocytes (both CD4^{+} and CD8^{+}), B lymphocytes, DCs, and NK and NKT cells, and increasing the number of IL-10-producing CD4^{+} T cells and alternatively activated (i.e., M2-polarized) macrophages that was accompanied with lower serum levels of TNFα, IFNγ, and IL-17, but also IL-4. In addition, pretreatment of WT mice with TD139 attenuated Con A–induced liver injury (Fig. 6). IP injection of TD139 in WT mice 2 hours before and immediately after Con A injection suppressed the infiltration of IFNγ- and IL-17- and -4-producing CD4^{+} and IFNγ-producing CD8^{+} lymphocytes (Fig. 7), increased the total number of IL-10-producing CD4^{+} T cells (Fig. 7), attenuated serum levels of IFNγ and IL-17 and -4, elevated the serum level of IL-10 (Supporting Fig. 6), and increased the number of alternatively activated (i.e., M2-polarized) macrophages (Fig. 8A). It is interesting to note that the influx of NK cells producing IFNγ, induced by IL-18, leads to increased serum ALT activity.[18] Furthermore, treatment with IL-18-neutralizing antibody reduces the serum ALT level and inflammatory cell accumulation in the liver.[18]

Gal-3 activates DCs and macrophages, serves as a chemoattractant for these cells, and plays an important role in the proliferation of activated T lymphocytes.[10,19]

In line with these observations, we found that Gal-3^{−/−} mice exhibited a markedly reduced number of liver-infiltrating effector cells (Figs. 2-4), supporting a key role of Gal-3 in promoting liver inflammation. Significantly lower levels of TNFα, IFNγ, and IL-17 and -4 in the sera of Gal-3^{−/−}, compared to WT, mice (Supporting Fig. 3A) indicated that effector MNCs that infiltrated livers of WT and Gal-3^{−/−} mice were mostly TNFα-, IFNγ-, and IL-17- and -4-producing cells.

Indeed, there was a significantly lower number of TNFα-, IFNγ-, and IL-17- and -4-producing CD4^{+} T cells and a significantly higher number of IL-10-producing CD4^{+} T lymphocytes in livers of Gal-3^{−/−} and TD139-treated, compared to WT, mice (Figs. 3 and 7). It is known that CD4^{+} T lymphocytes are major effector cells involved in Con A hepatitis,[1] and that Con A–induced liver damage is driven by CD4^{+}

T-cell production of TNFα and IFNγ.[1,20] IL-17 has been reported to be both proinflammatory or without a direct inflammation-modulating role in Con A–induced hepatitis.[3] In our study, lower serum levels of IL-17 correlated with less-pronounced liver injury (Supporting Fig. 3A). Decreased levels of IL-17 that were found in the sera of Gal-3^{−/−} mice (Supporting Fig. 3A) correlated with reduced liver infiltration of IL-17-producing CD4^{+} T cells (Fig. 3).

It is well known that IL-10 has a hepatoprotective role in Con A–induced hepatitis through its suppressive property on proinflammatory cytokine production.[21,22] In Con A hepatitis, IL-10 deficiency is associated with a profound increase in the serum levels of IFNγ and TNFα.[21,22] In line with these observations, we found a significantly higher number of IL-10-producing CD4^{+} T lymphocytes in livers of Gal-3^{−/−} mice and Gal-3-INH-treated mice that correlated with reduced liver injury (Figs. 3 and 7). In addition, the ratio between the total number of IL-10- and IFNγ-producing CD4^{+} T cells was significantly higher in the liver of Con A–treated Gal-3^{−/−}, compared to WT, mice, suggesting that, in Con A hepatitis, Gal-3 affects IL-10 production in CD4^{+} T cells.

Although we found significantly lower levels of Th1 and 2 cytokines in the sera of Gal-3^{−/−} mice (Supporting Fig. 3A), there was no significant difference in the levels of TNFα, IFNγ, and IL-17, -4, and -10 in supernatants of *in vitro* Con A–stimulated splenocytes isolated from healthy WT and Gal-3^{−/−} mice (Supporting Fig. 3B). We therefore propose that the inflammatory milieu of the Con A–damaged liver is most likely responsible for the difference in cytokine production of liver-infiltrating MNCs of WT and Gal-3^{−/−} mice. Increased expression and secretion of Gal-3 have been observed in the inflammatory milieu of various tissues, and it is well known that Gal-3 promotes the influx of effector cells, particularly through affecting DCs and tissue-resident macrophages.[19]

Gal-3 is important for the migration, adhesion, and maturation of mouse DCs.[23] In line with these observations, our results showed that the total number of activated, mature CD11c^{+}CD80^{+}CD86^{+} DCs was significantly lower (P < 0.05; Fig. 4) in Con A–treated Gal 3[−/−], compared to WT, mice. In addition, Gal-3 expression in DCs greatly influences the strength of T-cell-mediated immune response triggered by DCs.[19] IL-12, mainly produced by DCs and macrophages, is essential for the onset of Con A–induced hepatitis, because IL-12 interacts directly with NKT cells, contributes to their recruitment to the liver, and enhances immune response through increased IL-4

production.[24] In line with these observations, our results show that attenuated liver injury noticed in Gal-3$^{-/-}$ mice correlates with a significantly reduced number of activated CD11c$^+$CD80$^+$CD86$^+$ DCs, IL-12-producing DCs, NK and NKT cells, and IL-4-producing CD4$^+$ T cells (Figs. 2-4), followed by a decreased serum level of IL-4 (Supporting Fig. 3A).

Gal-3 is abundantly expressed and secreted by macrophages.[25] Gal-3 is secreted into the extracellular compartment under cytokines, particularly IFNγ, overproducing pathological conditions, where it modulates inflammatory responses in tissue-resident macrophages.[24] M1 polarization and proinflammatory response of M1 macrophages is enhanced by IFNγ and/or IL-12,[26] whereas increased levels of IL-4 leads to M2 polarization of macrophages.[27] Macrophages are capable of diverse phenotypic heterogeneity, depending on their microenvironment, and their polarization is different in various tissues under various pathological conditions.[27] Some data suggest that increased expression of Gal-3 is a feature of the alternative (i.e., M2) macrophage phenotype, and that Gal-3 sustains and drives the M2 macrophage phenotype in the peritoneum and myocardium.[9,28] However, we present here, consistent with recently published results in animal models of diet-induced NASH,[6] that Gal-3 deletion attenuated both Th1 and 2 inflammatory responses in the liver and down-regulated the gene expression level of both Th1/M1 and Th2/M2 cells. Thus, it seems that reduced inflammation noticed in the livers of Gal-3$^{-/-}$ mice could be the result of both macrophage and T-cell attenuation. Accordingly, we found a decreased number of IL-12-producing CD11c$^+$ DCs in livers of Gal-3$^{-/-}$, mice compared to WT, mice (Fig. 4), suggesting that Gal-3 plays an important role in the antigen presentation and activation of T lymphocytes in Con A hepatitis.

IV-injected Con A is mainly phagocyted by liver macrophages and presented to CD4$^+$ Th lymphocytes, leading to their activation.[1] Knowing that Gal-3 has an important role in the phagocytic function of macrophages,[29] we assume that pretreatment with TD139 inhibited the expression of Gal-3 on macrophages, impaired phagocytosis of Con A, and reduced the activation of CD4$^+$ Th cells, which was manifested by the lower number of IFNγ- and IL-17- and -4-producing CD4$^+$ T cells and the higher number of CD4$^+$IL-10-producing T lymphocytes in livers of Con A–treated mice that received TD139 (Fig. 7).

Extensive apoptosis of liver MNCs in Gal-3$^{-/-}$ mice could also be one of the factors leading to the reduced number of effector cells in livers of Gal-3$^{-/-}$ mice after Con A injection.

It is well known that *in vivo* injection of Con A leads to increased apoptosis of thymocytes and splenocytes,[30] and that intra- and extracellular Gal-3 have opposite roles in the induction of T-cell apoptosis. Intracellular Gal-3 prevents the apoptosis of T lymphocytes, whereas extracellular Gal-3 induces the apoptosis of activated T cells.[9,10] Consistent with these findings, our results show that deletion of Gal-3 gene, because of the lack of intracellular (i.e., antiapoptotic) Gal-3, enhanced the apoptosis of MNCs, whereas injection of TD139 through the inhibition of extracellular (i.e., proapoptotic) Gal-3 prevented the apoptosis of MNCs in Con A–treated mice (Figs. 5B and 8B). However, the number of pathogenic IFNγ-producing CD4$^+$ T cells was affected by TD139 (Fig. 7).

In conclusion, we propose that Gal-3 plays an important proinflammatory role in Con A–induced hepatitis by promoting the activation of T lymphocytes, NKT cells, DCs, cytokine secretion, prevention of M2 macrophage polarization, and apoptosis of MNCs that leads to severe liver injury. Gal-3 may therefore be a potential target for therapeutic intervention in acute liver failure.

References

1. 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.
2. Mizuhara H, Uno M, Seki N, Yamashita M, Yamaoka M, Ogawa T, et al. Critical involvement of interferon gamma in the pathogenesis of T-cell activation-associated hepatitis and regulatory mechanisms of interleukin-6 for the manifestations of hepatitis. HEPATOLOGY 1996;23: 1608-1615.
3. Volarevic V, Mitrovic M, Milovanovic M, Zelen I, Nikolic I, Mitrovic S, et al. Protective role of IL-33/ST2 axis in Con A-induced hepatitis. J Hepatol 2011;56:26-33.
4. Takeda K, Hayakawa Y, Van Kaer L, Matsuda H, Yagita H, Okumura K. Critical contribution of liver natural killer T cells to a murine model of hepatitis. Proc Natl Acad Sci U S A 2000;97:5498-5503.
5. Matsuda Y, Yamagiwa Y, Fukushima K, Ueno Y, Shimosegawa T. Expression of galectin-3 involved in prognosis of patients with hepatocellular carcinoma. Hepatol Res 2008;38:1098-1111.
6. Iacobini C, Menini S, Ricci C, Fantauzzi CB, Scipioni A, Salvi L, et al. Galectin-3 ablation protects mice from diet-induced NASH: a major scavenging role for galectin-3 in liver. J Hepatol 2011;54: 975-983.
7. Dumic J, Dabelic S, Flogel M. Galectin-3: an open-ended story. Biochim Biophys Acta 2006;1760:616-635.
8. Radosavljevic G, Jovanovic I, Majstorovic I, Mitrovic M, Lisnic VJ, Arsenijevic N, et al. Deletion of galectin-3 in the host attenuates metastasis of murine melanoma by modulating tumor adhesion and NK cell activity. Clin Exp Metastasis 2011;28:451-462.
9. Hsu Dk, Chen HY, Liu FT. Galectin-3 regulates T-cell functions. Immunol Rev 2009;230:114-127.

10. Joo HG, Goedegebuure PS, Sadanaga N, Nagoshi M, von Bernstorff W, Eberlein TJ. Expression and function of galectin-3, a beta-galactoside-binding protein in activated T lymphocytes. J Leukoc Biol 2001; 69:555-564.

11. Nieminen J, St-Pierre C, Bhaumik P, Poirier F, Sato S. Role of galectin-3 in leukocyte recruitment in a murine model of lung infection by *Streptococcus pneumoniae*. J Immunol. 2008;180:2466-2473.

12. Nachtigal M, Ghaffar A, Mayer EP. Galectin-3 gene inactivation reduces atherosclerotic lesions and adventitial inflammation in ApoE-deficient mice. Am J Pathol 2008;172:247-255.

13. Bernardes ES, Silva NM, Ruas LP, Mineo JR, Loyola AM, Hsu DK, et al. *Toxoplasma gondii* infection reveals a novel regulatory role for galectin-3 in the interface of innate and adaptive immunity. Am J Pathol 2006;168:1910-1920.

14. Hsu DK, Yang RY, Pan Z, Yu L, Salomon DR, Fung-Leung WP, et al. Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. Am J Pathol 2000;156:1073-1083.

15. Volarevic V, Milovanovic M, Arsenijevic N, Lukic M. The new semiquantitative method for determination of liver damage after Concanavalin A administration. Ser J Exp Clin Res 2010;11:45-48.

16. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 1995;184:39-51.

17. Liu FT, Hsu DK. The role of galectin-3 in promotion of the inflammatory response. Drug News Perspect 2007;20:455-460.

18. Kimura K, Sekiguchi S, Hayashi S, Hayashi Y, Hishima T, Nagaki M, et al. Role of interleukin-18 in intrahepatic inflammatory cell recruitment in acute liver injury. J Leukoc Biol 2011;89:433-442.

19. Jeon SB, Yoon HJ, Chang CY, Koh HS, Jeon SH, Park EJ. Galectin-3 exerts cytokine-like regulatory actions through the JAK-STAT pathway. J Immunol 2010;185:7037-7046.

20. Robinson R, Wang J, Cripps J, Milks M, English K, Pearson T, et al. End-organ damage in a mouse model of fulminant liver inflammation requires CD4+ T cell production of IFN-γ but is independent of Fas. J Immunol 2009;182:3278-3284.

21. Louis H, Le Moine O, Peny MO, Quertinmont E, Fokan D, Goldman M, et al. Production and role of interleukin-10 in concanavalin A-induced hepatitis in mice. HEPATOLOGY 1997;25:1382-1389.

22. Erhardt A, Biburger M, Papadopoulos T, Tiegs G. IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice. HEPATOLOGY 2007;45:475-485.

23. Hsu DK, Chernyavsky AI, Chen HY, Yu L, Grando SA, Liu FT. Endogenous galectin-3 is localized in membrane lipid rafts and regulates migration of dendritic cells. J Invest Dermatol 2009;129:573-583.

24. Zhu R, Diem S, Araujo LM, Aumeunier A, Denizeau J, Philadelphe E, et al. The Pro-Th1 cytokine IL-12 enhances IL-4 production by invariant NKT cells: relevance for T cell-mediated hepatitis. J Immunol 2007;178:5435-5442.

25. Liu FT, Hsu DK, Zuberi RI, Kuwabara I, Chi EY, Henderson WR, Jr. Expression and function of galectin-3, a beta-galactoside binding lectin, in human monocytes and macrophages. Am J Pathol 1995;147: 1016-1028.

26. Hölscher C, Atkinson RA, Arendse B, Brown N, Myburgh E, Alber G, et al. A protective and agonist function of IL-12p40 in mycobacterial infection. J Immunol 2001;167:6957-6966.

27. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J Immunol 2005;175: 342-349.

28. MacKinnon A, Farnworth S, Hodkinson P, Henderson N, Atkinson K, Leffler H, et al. Regulation of alternative macrophage activation by galectin-3. J Immunol 2008;180:2650-2658.

29. Sano H, Hsu DK, Apgar JR, Yu L, Sharma BB, Kuwabara I, et al. Critical role of galectin-3 in phagocytosis by macrophages. J Clin Invest 2003;112:389-397.

30. Fayad R, Sennello JA, Kim SH, Pini M, Dinarello CA, Fantuzzi G. Induction of thymocyte apoptosis by systemic administration of concanavalin A in mice: role of TNF-alpha, IFN-gamma, and glucocorticoids. Eur J Immunol 2005;35:2304-2312.