European Journal of Immunology

Gal-3 regulates the capacity of dendritic cells to promote NKT-cell-induced liver injury

Vladislav Volarevic[1], Bojana Simovic Markovic[1], Sanja Bojic[1], Maja Stojanovic[1], Ulf Nilsson[2], Hakon Leffler[3], Gurdyal S. Besra[4], Nebojsa Arsenijevic[1], Verica Paunovic[5], Vladimir Trajkovic[5] and Miodrag L. Lukic[1]

[1] Centre for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2] Centre for Analysis and Synthesis, Department of Chemistry, Lund University, Lund, Sweden
[3] Section MIG, Department of Laboratory Medicine, Lund University, Lund, Sweden
[4] School of Biosciences, University of Birmingham, Birmingham, UK
[5] Institute for Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Serbia

Galectin-3 (Gal-3), an endogenous lectin, exhibits pro- and anti-inflammatory effects in various disease conditions. In order to explore the role of Gal-3 in NKT-cell-dependent pathology, we induced hepatitis in C57BL/6 WT and Gal-3-deficient mice by using specific ligand for NKT cells: α-galactosylceramide, glycolipid Ag presented by CD1d. The injection of α-galactosylceramide significantly enhanced expression of Gal-3 in liver NKT and dendritic cells (DCs). Genetic deletion or selective inhibition of Gal-3 (induced by Gal-3-inhibitor TD139) abrogated the susceptibility to NKT-cell-dependent hepatitis. Blood levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-12) and their production by liver DCs and NKT cells were also downregulated. Genetic deletion or selective inhibition of Gal-3 alleviated influx of inflammatory $CD11c^+CD11b^+$ DCs in the liver and favored tolerogenic phenotype and IL-10 production of liver NKT and DCs. Deletion of Gal-3 attenuated the capacity of DCs to support liver damage in the passive transfer experiments and to produce pro-inflammatory cytokines in vitro. Gal-3-deficient DCs failed to optimally stimulate production of pro-inflammatory cytokines in NKT cells, in vitro and in vivo. In conclusion, Gal-3 regulates the capacity of DCs to support NKT-cell-mediated liver injury, playing an important pro-inflammatory role in acute liver injury.

Keywords: Dendritic cells · Gal-3 · Hepatitis · NKT cells · Regulatory T (Treg) cells

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Galectin-3 (Gal-3) is involved in several biological processes including regeneration, cell migration, as well as inflammatory and immune responses [1]. In the liver, Gal-3 was recently found to regulate hepatic progenitor cell expansion during liver injury [2]. It is also established that Gal-3 plays an important role in metabolic and inflammatory responses of this organ to various environmental challenges [3, 4]. Although it is well known that Gal-3, which is highly expressed in activated $CD4^+$ and $CD8^+$ T cells, macrophages, DCs, and natural killer (NK) cells, functions

Correspondence: Dr. Vladislav Volarevic
e-mail: drvolarevic@yahoo.com

as a key regulator of migration and function of these cells [5], the role of Gal-3 in NKT-cell activation and function is not fully understood.

Because NKT cells accumulate in the liver [6–8], in order to explore the role of Gal-3 in NKT-cell-dependent pathology, we induced hepatitis in wild-type (WT) and Gal-3-deficient (*Gal-3*$^{-/-}$) mice by using specific and strong ligands for NKT cells: α-galactosylceramide (α-GalCer), glycolipid antigen (Ag) presented by CD1d [9]. Recently, using Con A-induced liver injury, we showed that Gal-3 plays an important pro-inflammatory role in Con A-induced hepatitis [4], a well-established murine model of fulminant hepatitis [10], by promoting the activation of T lymphocytes, secretion of pro-inflammatory cytokines, and downregulation of M2 macrophage polarization in the liver. Although to some degree there is a mechanistic resemblance between Con A- and α-GalCer-induced hepatitis, there are several important differences between these models of immune-mediated liver injury [10, 11]. Con A binds mannose residues of many different glycoproteins and, thus, pan-activates lymphocyte populations irrespective of their Ag specificity, while α-GalCer is strictly dependent on the presentation of the MHC-homologous CD1d molecule and exerts its activating function via Ag-specific TCR recognition [11]. Thus, α-GalCer is considered as a surrogate for a physiologic Ag and, upon presentation by MHC-like CD1d to Ag-specific TCRs, induces onset of liver injury in an Ag-specific manner [11]. In addition, different immune cells had a crucial role in the pathogenesis of Con A- and α-GalCer-induced hepatitis. T lymphocytes, NKT cells, and macrophages are main immune cells involved in the pathogenesis of Con A-induced liver injury [4, 10]. On contrary, injection of α-GalCer induces liver injury in mice by activating liver NKT and DCs, while macrophages are dispensable for α-GalCer-mediated liver injury [11], suggesting α-GalCer-induced hepatitis as an ideal model for studying NKT and DC interplay in the liver, enabling the analysis of initial and fundamental events in the pathogenesis of NKT-comprising liver disorders.

Here, we provide evidence that Gal-3 is expressed on NKT cells and is involved in NKT and DC interaction in the liver, playing an important pro-inflammatory role in α-GalCer-induced acute liver injury. Our data indicate that maturation of DC and their capacity to promote NKT-cell-induced liver inflammation is Gal-3 dependent.

Results

α-GalCer induces expression of Gal-3 on liver DC and NKT cells

First, we investigated whether injection of α-GalCer would affect Gal-3 expression in the liver. As evaluated by immunohistochemistry and flow cytometry, 2 h after i.v. injection of α-GalCer, Gal-3 expression was strongly increased in the liver (Fig. 1A) and on the surface of CD3$^+$NK1.1$^+$ NKT and CD11c$^+$ DCs of α-GalCer-treated C57BL/6 mice, compared to untreated control mice (Fig. 1B).

Genetic deletion of Gal-3 abrogates susceptibility to α-GalCer-induced hepatitis

Next, to investigate the role of Gal-3 in NKT-cell-mediated hepatitis, we injected α-GalCer into WT and *Gal-3*$^{-/-}$ mice. Serum aspartate aminotransferase (AST) and ALT levels 16 h after α-GalCer injection were significantly lower in *Gal-3*$^{-/-}$ mice, compared to WT mice (Fig. 2A). Histopathological analysis of liver tissue sections confirmed that genetic deletion of Gal-3 abrogated α-GalCer-induced liver injury (Fig. 2C). Liver tissue sections in *Gal-3*$^{-/-}$ mice showed solitary areas of necrotic tissue characterized by standard morphologic criteria (loss of architecture, vacuolization, karyolysis, and increased eosinophilia). In contrast, liver tissue sections in WT mice showed widespread areas of necrosis with extensive infiltration of mononuclear cells (MNCs, Fig. 2B and C), indicating the ongoing inflammatory process. Extensive liver damage in WT mice was characterized by massive coagulative necrosis and cytoplasmic swelling of the majority of hepatocytes.

Deletion of Gal-3 modulates cytokine profile in α-GalCer-treated mice

A diminished inflammatory injury in α-GalCer-treated *Gal-3*$^{-/-}$ mice correlated with the cytokine production. Serum levels of TNF-α, IFN-γ, and IL-12 were significantly lower, while serum levels of anti-inflammatory IL-10 and TGF-β were significantly higher (Fig. 2D) in *Gal-3*$^{-/-}$ compared to WT mice 2 h after α-GalCer injection. There was no significant difference in the serum levels of IL-4 and IL-17 between α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice (Fig. 2D).

Gal-3 promotes production of pro-inflammatory cytokines in the liver NKT cells

The α-GalCer-triggered liver influx of NKT cells was significantly lower in *Gal-3*$^{-/-}$ compared to WT mice (Fig. 3A). The liver tissue of α-GalCer-treated *Gal-3*$^{-/-}$ mice contained significantly lower number of NKT cells expressing CXCR3 receptor (Fig. 3B), considered as a dominant chemokine receptor involved in NKT cell trafficking in inflammatory conditions [9]. Intracellular staining of liver NKT cells revealed significantly lower number of IFN-γ- and IL-17-producing NKT cells and significantly higher number of IL-10-producing NKT cells in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ mice (Fig. 3C and D), indicating that Gal-3 favors production of pro-inflammatory cytokines in the liver NKT cells. There was no significant difference in the number of TNF-α-, IL-4-, and IL-5-producing NKT cells between α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice (Fig. 3D). Intracellular staining of liver NK1.1$^+$ CD3$^-$ NK cells revealed significantly lower number of IFN-γ-producing cells in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ mice when compared to α-GalCer-exposed WT animals (Fig. 3E).

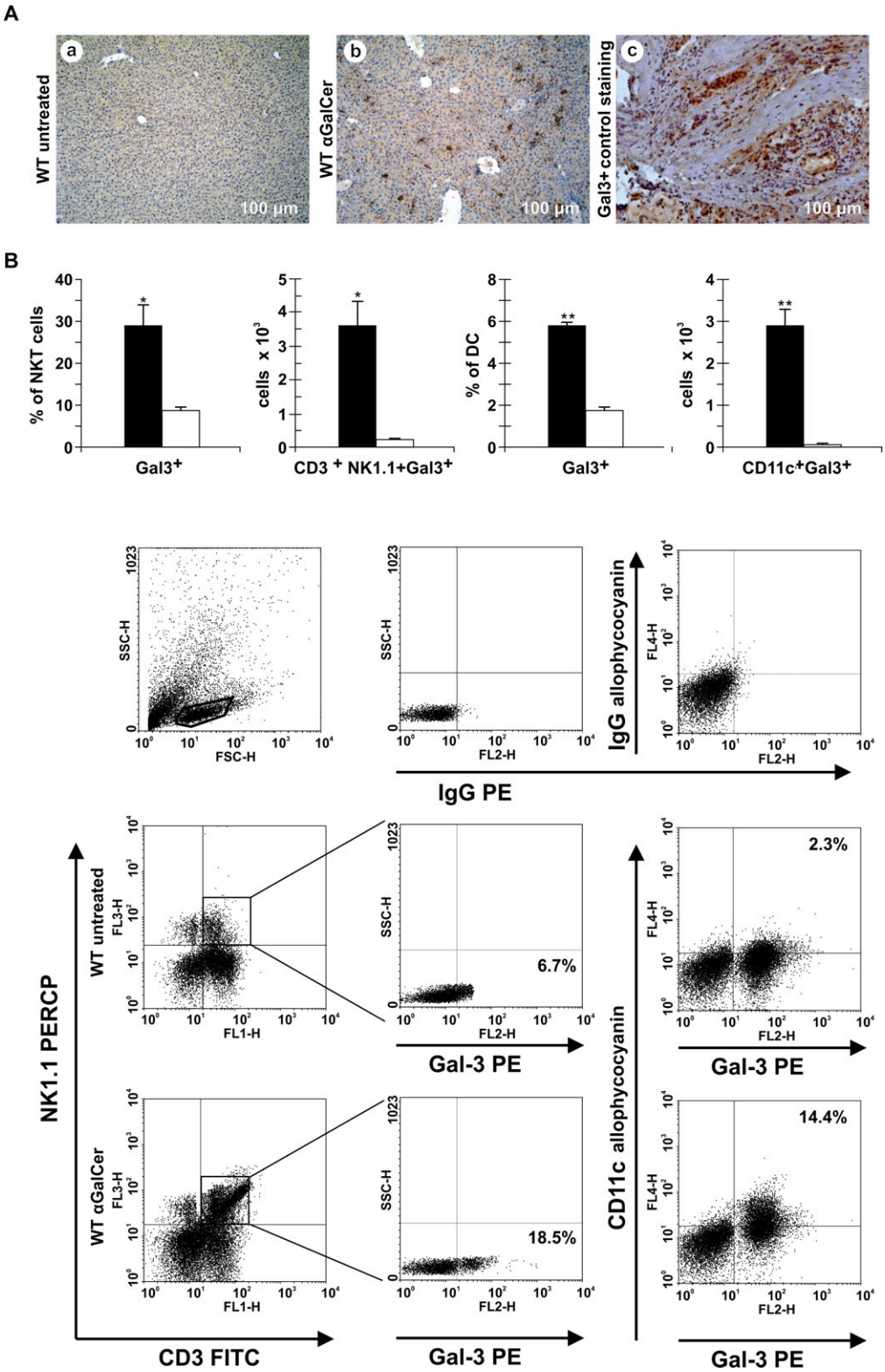

Figure 1. α-GalCer induces expression of Gal-3 on liver DC and NKT cells. (A) Immunohistochemical staining showing expression of Gal-3 in the liver samples obtained from (left panel) untreated and (middle panel) α-GalCer-treated WT C57BL/6 mice, collected 2 h after i.v. injection of α-GalCer (50 μg/kg) or vehicle (NaCl), are shown (right panel). Representative sample of human lung adenocarcinoma, used as positive control for Gal-3 immunohistochemical staining. Scale bar: 100 μm. Images are representative of two independent experiments performed, with $n = 12$. (B) The percentage, absolute number, and the gating strategy for enumerating live liver $CD3^+NK1.1^+$ NKT cells and $CD11c^+$ DC expressing Gal-3 are shown. Data are shown as the mean + SEM of 12 mice per group and pooled from two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t*-test.

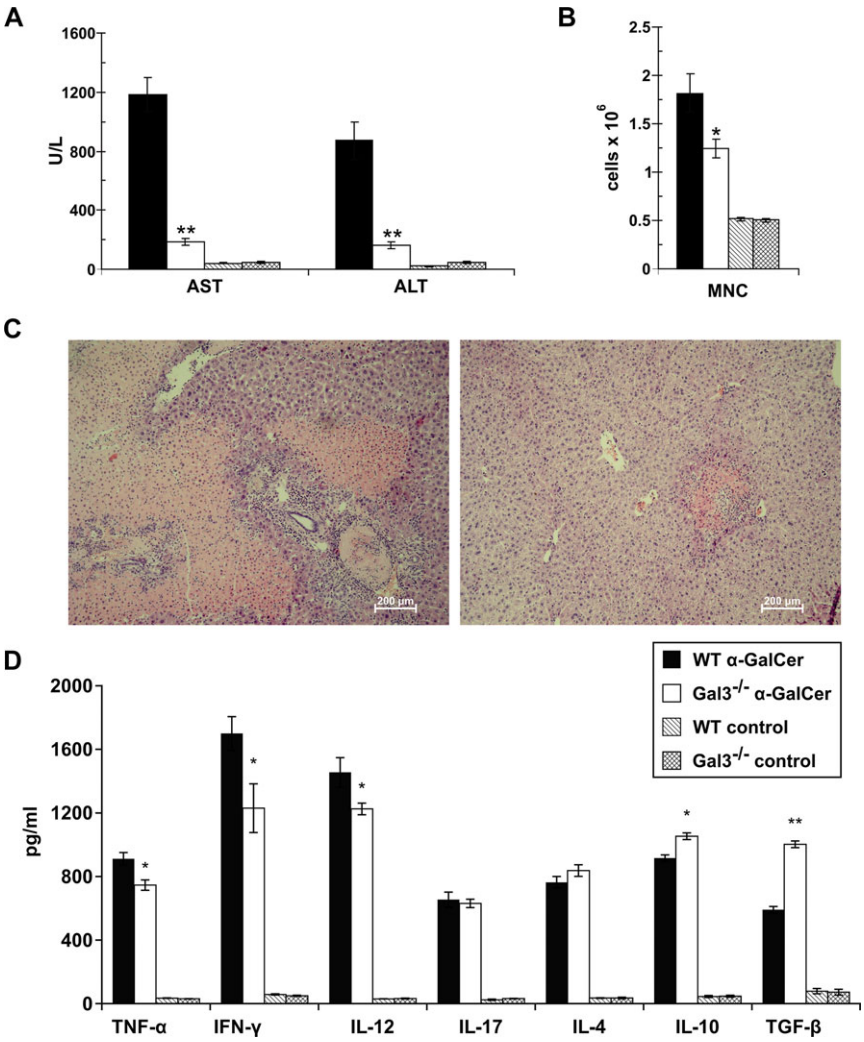

Figure 2. Targeted-disruption of *Gal-3* gene protects from α-GalCer-induced hepatitis. (A–D) WT C57BL/6 and *Gal3*$^{-/-}$ mice were injected i.v. with α-GalCer (50 μg/kg) or vehicle (NaCl). The samples for AST/ALT measurement and histological analysis were collected from WT C57BL/6 and *Gal3*$^{-/-}$ mice 16 h after injection, while absolute number of liver MNCs and serum cytokines were measured 2 h after administration of α-GalCer or vehicle. (A) Serum AST and ALT levels, (B) total number of MNCs in the liver, (C) hepatocyte necrosis, and (D) level of pro-inflammatory cytokines in the sera are shown. (C) Histological analysis shows liver tissue samples (H&E staining) and are representative of two independent experiments performed, with $n = 12$. Scale bar: 200 μM. (A, B, and D) Data are shown as the mean ± SEM of 12 mice per group and are pooled from two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; Student's *t*-test.

Deletion of Gal-3 favors a tolerogenic phenotype of liver DCs in α-GalCer-induced hepatitis

Gal-3 is important for the migration, adhesion, and maturation of mouse DCs [12]. Accordingly, we assessed the effects of Gal-3 deletion on influx, phenotype, and cytokine production of DCs in the injured liver. The percentage and absolute number of liver DCs were similar in untreated WT and *Gal-3*$^{-/-}$ mice, but the injection of α-GalCer induced a significantly higher influx of these cells in the liver of WT mice (Fig. 4A). Total number of CD11c^{+} and CD11c^{+}CXCR3^{+} DCs was significantly lower in the liver of α-GalCer-treated *Gal-3*$^{-/-}$ mice compared to WT animals (Fig. 4A and B), suggesting that Gal-3 is important for the expression of CXCR3 on DCs and their migration to injured liver. Importantly, we observed a significantly lower percentage and total number of CD11c^{+} DC expressing CD1d in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ mice compared to WT animals (Fig. 4C). Additionally, there was a significantly higher number of tolerogenic CD11c^{+}CD8^{+} and IL-10-producing DCs, and a significantly lower number of inflammatory CD11c^{+}CD11b^{+} DCs, CD11c^{+} DC expressing major histocompatibility complex (MHC) class II and costimulatory molecules CD80 and CD86, as well as IL-12-, TNF-α-, and IFN-γ-producing DCs in the liver of α-GalCer-treated *Gal-3*$^{-/-}$ compared to WT mice (Fig. 4D and E).

Gal-3$^{-/-}$ DCs have lower pro-inflammatory cytokine production and reduced capacity for liver damage

We next looked at the capacity of DCs isolated from livers of WT and *Gal-3*$^{-/-}$ mice to produce pro- and anti-inflammatory cytokines involved in liver pathology (TNF-α, IFN-γ, IL-12, IL-4, and IL-10) after α-GalCer stimulation. Significantly lower amounts of TNF-α, IL-12, and IFN-γ, and higher amounts of IL-10 were noticed in supernatants of in vitro α-GalCer-stimulated *Gal-3*$^{-/-}$ DCs co-cultured with liver WT MNCs compared to WT DCs co-cultured under the same condition (Fig. 5A).

Intracellular staining revealed that liver DC isolated from healthy *Gal-3*$^{-/-}$ mice, compared to liver DC obtained from healthy WT animals, expressed significantly higher amount of IL-10 and significantly lower amounts of IL-12 and IFN-γ (Fig. 5B,

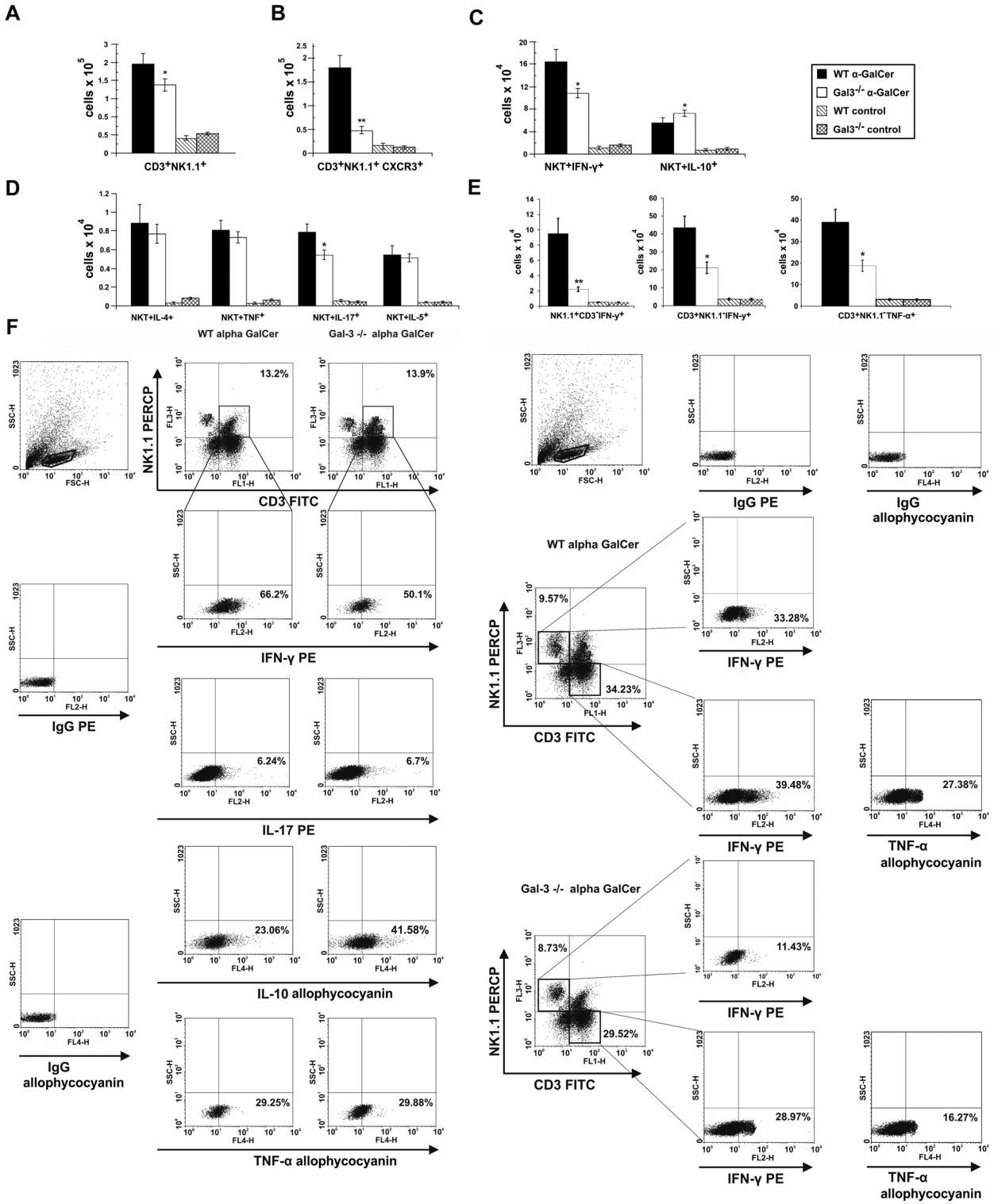

Figure 3. Gal-3 promotes production of pro-inflammatory cytokines in liver NKT cells of α-GalCer-induced hepatitis. (A–F) Livers of healthy and α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice were analyzed by intracellular cytokine staining and FACS analysis 2 h after administration of α-GalCer (50 μg/kg) or vehicle (NaCl). For intracellular cytokine staining, isolated cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 5 h, and GolgiStop was added. The absolute numbers of (A) $CD3^+NK1.1^+$ NKT cells, (B) $CD3^+NK1.1^+CXCR3^+$ NKT cells, (C) IFN-γ- IL-10-producing NKT cells, (D) IL-4-, TNF-α-, IL-17-, IL-5-producing NKT cells, (E) IFN-γ-producing $CD3^-NK1.1^+$ NK cells and $CD3^+NK1.1^-$ T lymphocytes are shown. (A–E) Data are shown as the mean ± SEM of 12 mice per group and are pooled from two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t*-test. (F) Representative flow cytometry dot plots show percentages of IFN-γ-, IL-10-, TNF-α-, IL-17-positive cells in the population of liver $CD3^+NK1.1^+$ NKT cells (left panel) and percentage of IFN-γ positive cells in the population of liver $CD3^-NK1.1^+$ NK cells and percentage of IFN-γ and TNF-α positive cells in the population of liver $CD3^+NK1.1^-$ T lymphocytes (right panel) 2 h after i.v. injection of α-GalCer. Plots are representative of two independent experiments.

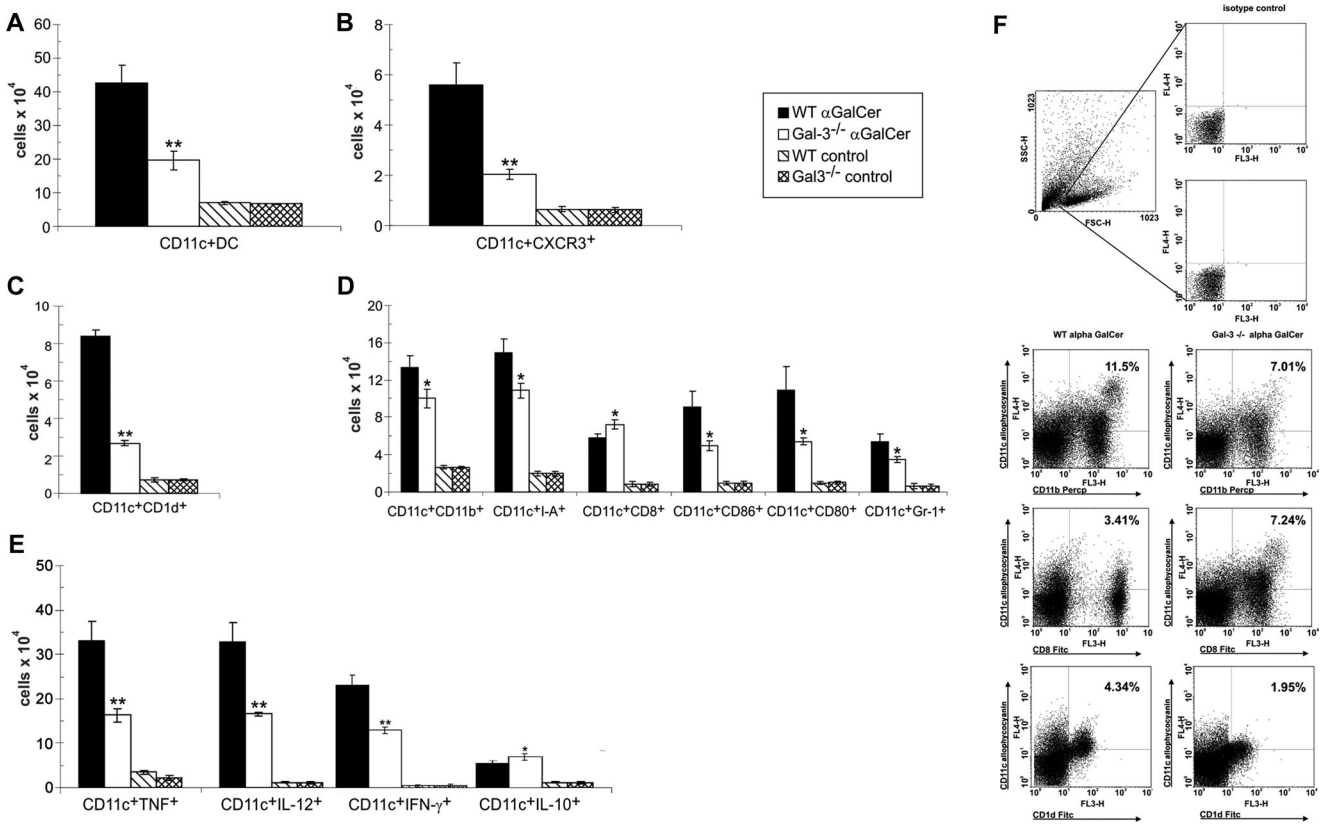

Figure 4. In α-GalCer-induced hepatitis, Gal-3 deletion favors a tolerogenic phenotype and the production of IL-10 in liver DCs. (A–F) Livers of healthy and α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice were administrated with α-GalCer (50 μg/kg) or vehicle (NaCl), and analyzed 2 h later by intracellular cytokine staining and FACS analysis. The absolute number of (A) CD11c$^+$ DCs; (B) CXCR3$^+$CD11c$^+$ DCs; (C) CD11c$^+$CD1d$^+$ DCs; (D) CD11c$^+$ DCs expressing CD11b, CD8, Gr-1, MHC class II and costimulatory molecules CD80, CD86; and (E) TNF-α-, IFN-γ-, IL-12-, IL-10-producing CD11c$^+$ DC are shown. For intracellular cytokine staining, isolated cells were stimulated with 50 ng/mL. (A–E) Data are shown as the mean ± SEM of 12 mice per group and are pooled from two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t*-test. (F) Representative flow cytometry dot plots show percentages of liver inflammatory CD11c$^+$CD11b$^+$ DC, regulatory CD11c$^+$CD8$^+$ DC and CD11c$^+$CD1d$^+$ DC that present α-GalCer to NKT cells, 2 h after i.v. injection of α-GalCer.

middle panel), indicating the important role of Gal-3 for the function of liver DCs. Accordingly, lower expression of IL-12 and IFN-γ and higher expression of IL-10 was seen in α-GalCer-stimulated *Gal-3*$^{-/-}$ DCs as well as in α-GalCer-stimulated *Gal-3*$^{-/-}$ DCs that were co-cultured with liver WT MNCs (Fig. 5B, middle panel).

To directly demonstrate that Gal-3 deficiency attenuates the capacity of DCs to support NKT-cell-induced liver damage, we adoptively transferred WT and *Gal-3*$^{-/-}$ liver DC to *Gal-3*$^{-/-}$ recipients. Serum AST and ALT levels upon α-GalCer treatment were significantly higher in *Gal-3*$^{-/-}$ mice that received DCs obtained from the livers of healthy WT mice, compared to those that received DC obtained from the livers of healthy *Gal-3*$^{-/-}$ mice (Fig. 5C, left panel).

Gal-3$^{-/-}$ DCs failed to optimally stimulate production of pro-inflammatory cytokines in NKT cells, in vitro and in vivo (Fig. 5B, upper panel, and C, right panel). Intracellular staining revealed that the total number of IFN-γ- and TNF-α-producing NKT cells was lower and IL-10-producing NKT cells were higher in the population of liver WT MNCs that were cultured with α-GalCer-pulsed DCs obtained from healthy *Gal-3*$^{-/-}$ mice compared to α-GalCer-pulsed DCs isolated from healthy WT animals (Fig. 5B, upper panel).

Transfer of liver DCs obtained from healthy WT mice aggravated α-GalCer-induced hepatitis in *Gal-3*$^{-/-}$ recipients by increasing the number of TNF-α- and IFN-γ-producing liver NKT cells, while transfer of liver DCs obtained from healthy *Gal-3*$^{-/-}$ mice enhanced the number of IL-10-producing NKT cells in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ animals (Fig. 5C, right panel).

CY enhances hepatitis without altering the differences between WT and *Gal-3*$^{-/-}$ mice

As it was recently published that Gal-3 negatively regulates the frequency and function of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells [1], we examined the influence of Gal-3 on liver Treg cells in α-GalCer hepatitis. As shown in Figure 6A, there was a significant increase in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ mice compared to WT animals. Interestingly, the percentage of TGF-β-producing Treg cells in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ mice was also higher, while

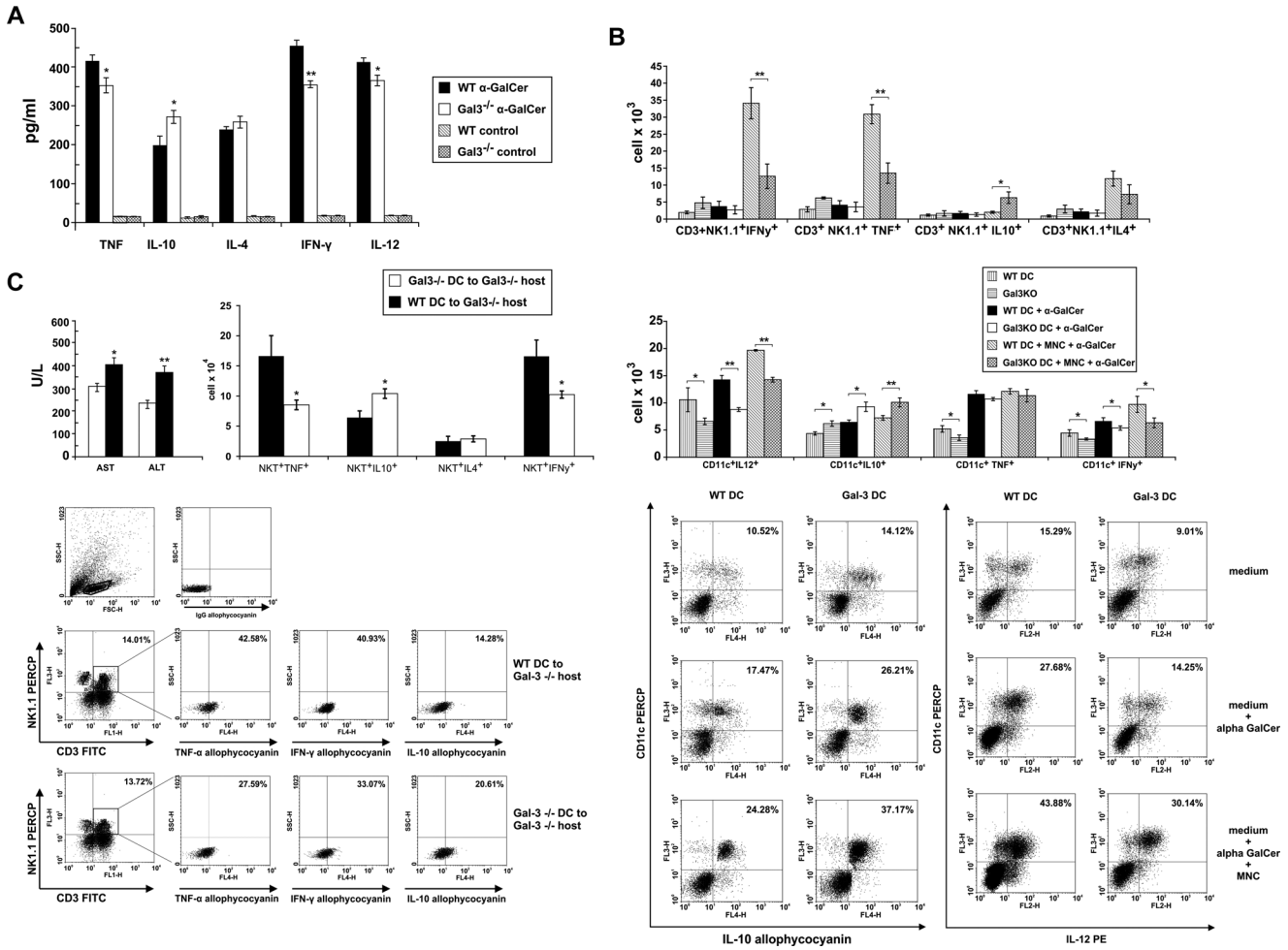

Figure 5. Gal-3 deletion attenuates production of pro-inflammatory cytokines by DCs and their capacity to support liver damage upon passive transfer. (A–C) Liver DCs (3×10^4 cells/well), derived from untreated WT and *Gal-3*$^{-/-}$ C57BL/6 mice were stimulated for 24 h with α-GalCer (100 ng/mL) or vehicle (NaCl) and for an additional 48 h, were co-cultured with liver MNCs (0.6×10^6 cells/well). The level of cytokines in (A) supernatants, (B) their intracellular expression in liver (top) NKT cells, (middle) DCs (bottom) and representative dot plots, are shown. (C) Transfer experiments of α-GalCer (50 μg/kg) treated *Gal-3*$^{-/-}$ mice that received liver DCs (i.v., 5×10^5 cells per mouse), isolated from untreated WT and *Gal-3*$^{-/-}$ mice are shown. (Left panel) Serum AST and ALT levels, (right panel) total number and representative flow cytometry dot plots of TNF-α-, IFN-γ-, IL-10-positive liver CD3$^+$NK1.1$^+$ NKT cells are shown. For intracellular cytokine staining, isolated NKT cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 5 h, and GolgiStop was added. The samples for AST and ALT measurements were collected 16 h after α-GalCer injection. The samples for the FACS analysis of liver NKT cells were obtained 2 h after α-GalCer injection. (A–C) Data are shown as the mean ± SEM of 12 mice per group and are pooled from two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t*-test. (B and C) Plots are representative of two independent experiments.

IL-10-producing Treg cells were present in similar number in livers of α-GalCer-treated *Gal-3*$^{-/-}$ and WT mice (Fig. 6B). An increase in the number of TGF-β-producing Treg cells correlated with a decrease in STAT-3 expression in liver-infiltrated CD4$^+$CD25$^+$Foxp3$^+$ Treg cells (Fig. 6C and D).

It has previously been shown that Treg cells protect against Con A-induced, T-cell-mediated hepatitis in a TGF-β-dependent manner [13]. Therefore, it was of interest to see if Treg cells play a similar TGF-β-dependent protective role also in α-GalCer-induced NKT-cell-mediated liver injury, as well as to further explore the influence of Gal-3 in their activity. The dose of 10 mg/kg cyclophosphamide (CY), which was reported to selectively deplete Treg cells but not NKT cells [14], did not induce liver damage as evaluated by liver function test and histology (data not shown). As shown in Supporting Information Figure 1A, serum AST and ALT levels were significantly higher in CY + α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice compared to treatment with α-GalCer only. We also found that CY + α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice, compared to treatment with α-GalCer only, had significantly elevated serum concentrations of TNF-α, IFN-γ, IL-12, IL-17, and IL-10, associated with massive MNC-infiltration in the liver (Supporting Information Fig. 1B and C). On the other hand, CY pretreatment resulted in a significant decrease in serum levels of TGF-β (Supporting Information Fig. 1B). CY also significantly reduced the number of TGF-β-producing Treg cells (Supporting Information Fig. 1D), which correlated with a higher influx of CD1d$^+$ DCs in the liver (Supporting Information Fig. 2A), indicating that the ability of TGF-β to inhibit CD1d expression in DCs [15] is preserved

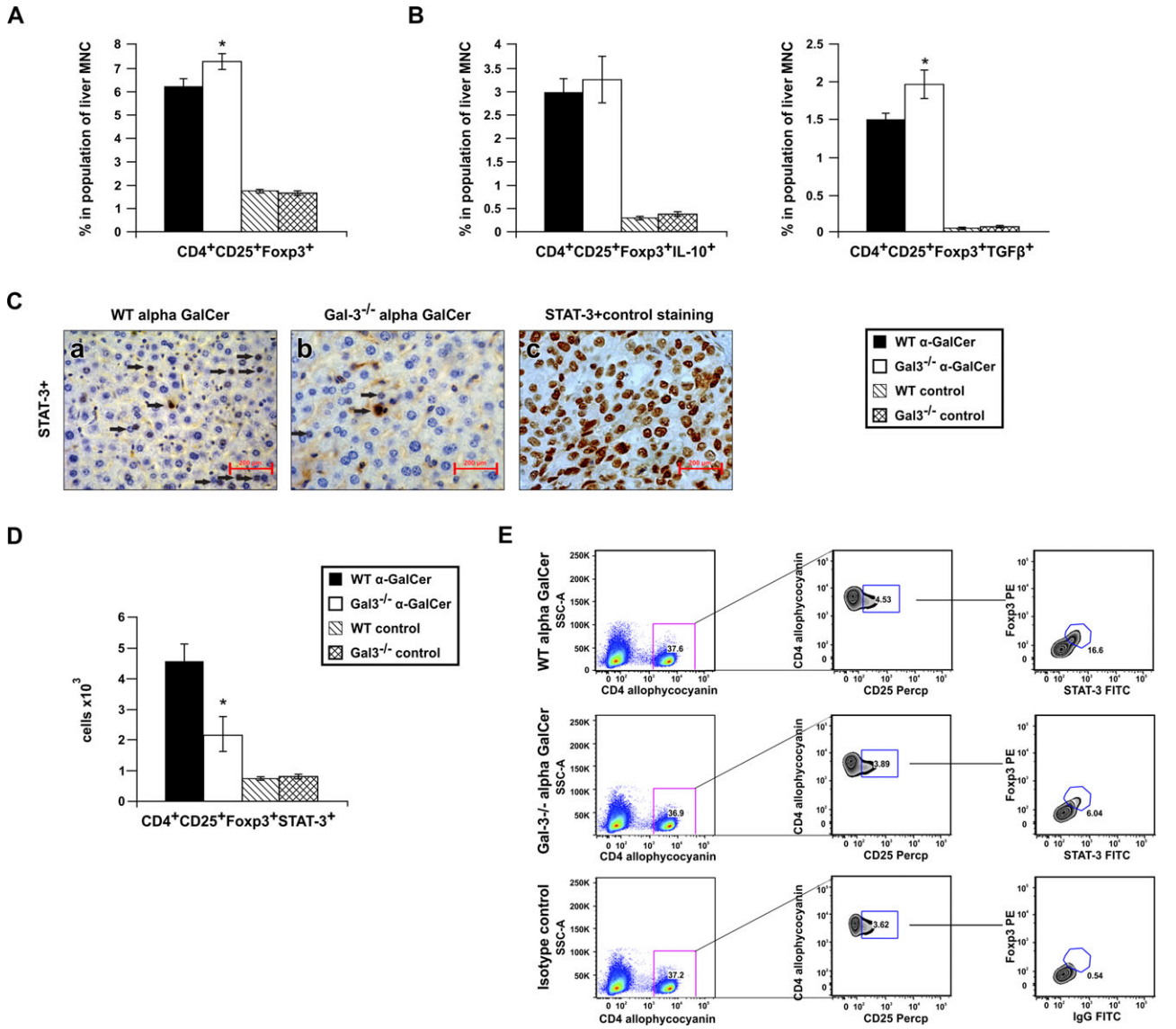

Figure 6. Deletion of Gal-3 promotes the induction of TGF-β-producing Treg cells in α-GalCer-induced hepatitis. (A–E) Livers of healthy and α-GalCer-treated WT and *Gal-3*$^{-/-}$ mice were treated with α-GalCer (50 μg/kg) or vehicle (NaCl) and samples were analyzed 2 h after administration by intracellular cytokine staining and FACS analysis. (A and B) The percentage of (A) $CD4^+CD25^+Foxp3^+$ Treg cells, and (B) IL-10- and TGF-β-producing $CD4^+CD25^+Foxp3^+$ Treg cells. (C) Immunohistochemical staining showing expression of STAT-3 in the liver samples obtained from α-GalCer-treated (left panel) WT C57BL/6 and (middle panel) *Gal-3*$^{-/-}$ mice, collected 2 h after i.v. injection of α-GalCer (50 μg/kg) or vehicle (NaCl), are presented. (Right panel) Representative sample of human poorly differentiated breast carcinoma, used as positive control for STAT-3 immunohistochemical staining, is shown. Scale bar: 200 μM. Images are representative of two independent experiments. (D) The absolute number of $CD4^+CD25^+Foxp3^+STAT3^+$ cells are shown (left panel). (E) Representative flow cytometry dot plots showed percentage of $Foxp3^+STAT\text{-}3^+$ cells gated on $CD4^+CD25^+$ cells, and are representative of two independent experiments. (A, B, D) Data are shown as the mean ± SEM of 12 mice per group and are pooled from two independent experiments. *$p < 0.05$; Student's *t*-test.

in α-GalCer-induced hepatitis. CY-induced depletion of Treg cells enhanced α-GalCer-hepatitis by promoting influx and activation of NKT and DCs, but did not alter the differences between WT and *Gal-3*$^{-/-}$ mice: the total number of IL-10-producing DCs and NKT cells were significantly higher (Supporting Information Fig. 2C and D), while those of TNF-α-, IL-12-, and IFN-γ-producing DCs (Supporting Information Fig. 2B) and IFN-γ- and IL-17-producing NKT cells (Supporting Information Fig. 2D) were significantly lower in livers of CY + α-GalCer-treated *Gal-3*$^{-/-}$ mice compared to WT animals.

Pharmacological Gal-3 neutralization prevents development of α-GalCer-induced liver injury

To correlate the effects of Gal-3 genetic deletion with those of the selective Gal-3 inhibitor (TD139), we treated WT C57BL/6 mice with TD139 2 h before and immediately after α-GalCer injection. TD139 pretreatment prevented development of α-GalCer-induced hepatitis, as demonstrated by a significant decrease in the serum liver transaminases (Fig. 7A), which correlated with histological findings showing small necrotic areas in contrast to significantly

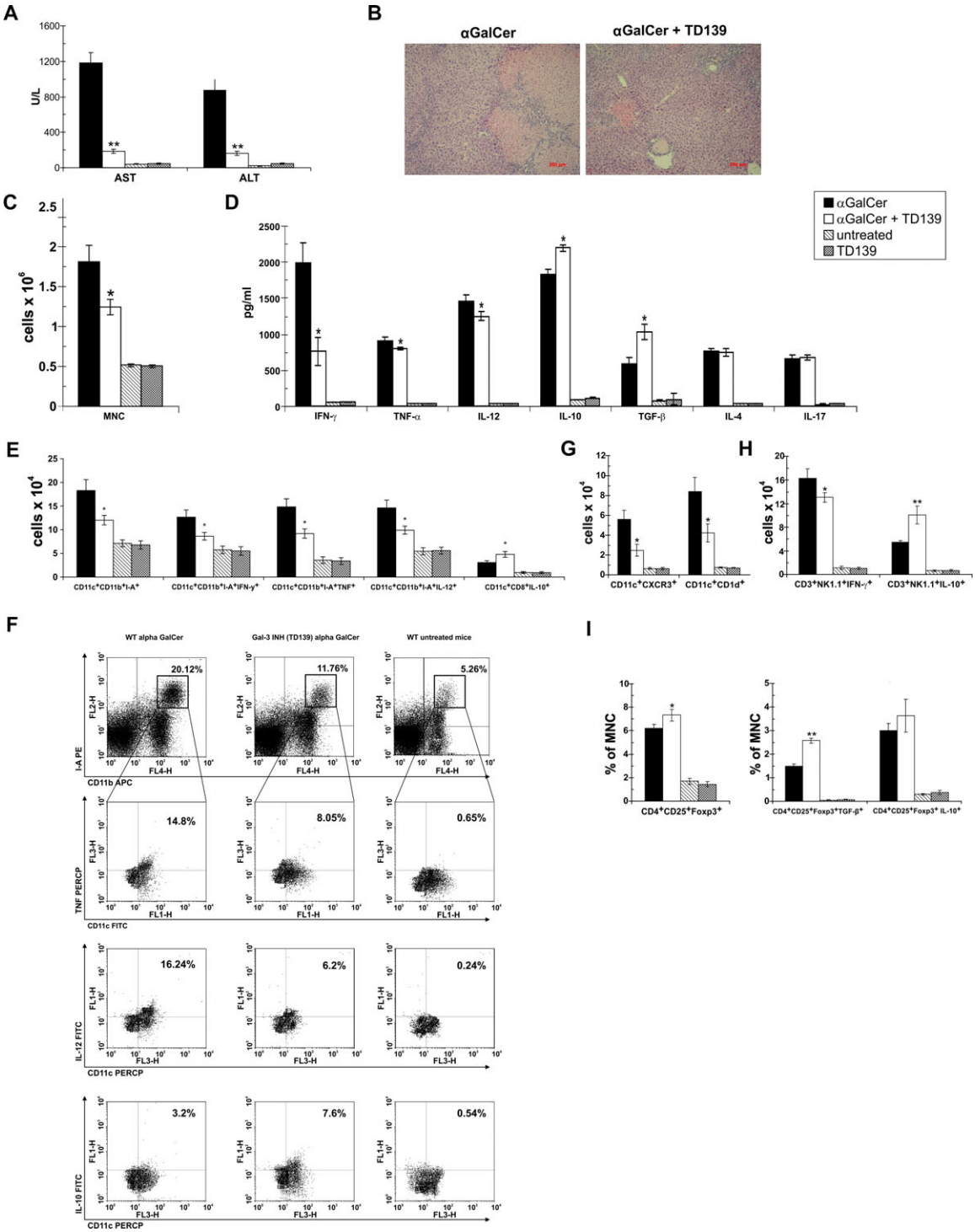

Figure 7. TD139 prevents α-GalCer-induced liver injury. (A–E) WT C57BL/6 mice were treated with α-GalCer (50 μg/kg), α-GalCer (50 μg/kg) + TD139 (15 mg/kg) or vehicle (NaCl). (A) Serum AST and ALT levels and (B) histological tissue samples were analyzed 16 h after treatment. (B) Histological analysis show liver tissue samples (H&E staining) from single experiment representative of two independent performed, with $n = 12$. Scale bar: 200 μM. (C and D) Absolute number of (C) liver MNCs and (D) serum cytokines is shown. (E) Total number of IL-12-, IFN-γ-, TNF-α-producing inflammatory $CD11c^+CD11b^+I\text{-}A^+$ DC, IL-10-producing regulatory $CD11c^+CD8^+$ DC. (F) Representative flow cytometry dot plots showed percentage of cytokine-producing DC in gated $CD11b^+I\text{-}A^+$ liver-infiltrating cells. (G) Absolute number of $CD11c^+$ DC expressing CXCR3 chemokine receptor and CD1d. (H) Total number of liver-infiltrating IFN-γ- and IL-10-producing $CD3^+NK1.1^+$ NKT cells. (I) Percentage of (left panel) $CD4^+CD25^+Foxp3^+$ Treg cells, and (right panel) IL-10- and TGF-β-producing Treg cells. (C–G) Data shown were obtained from the livers of WT C57BL/6 mice, 2 h after treatment. For intracellular cytokine staining, isolated MNCs were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 5 h, and then GolgiStop was added. (A, C–H) Data are shown as the mean ± SEM of 12 mice per group and are pooled from two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t*-test.

larger necrotic areas in liver parenchyma of mice treated with α-GalCer only (Fig. 7B). Additionally, TD139 markedly reduced MNC-infiltration into the liver parenchyma (Fig. 7C), inhibited the influx of $CD11c^+$ DC in a CXCR3-dependent manner (Fig. 7G), downregulated serum levels of pro-inflammatory cytokines TNF-α, IL-12, IFN-γ and increased those of the anti-inflammatory IL-10 and TGF-β (Fig. 7D). TD139 also decreased the liver infiltration of inflammatory $CD11c^+$ DCs expressing CD11b, MHC class II, costimulatory molecules CD80 and CD86 as well as TNF-α-, IL-12-, and IFN-γ-producing $CD11c^+CD11b^+I\text{-}A^+$ DCs (Fig. 7E). On the contrary, there was a significantly higher number of IL-10-producing $CD11c^+$ DC in the livers of TD139 + α-GalCer-treated mice compared to treatment with α-GalCer only (Fig. 7E). The lower number of TNF-α-, IL-12-, and IFN-γ- producing DCs, as well as $CD1d^+$ DCs (Fig. 7E–G), correlated with the increase in number of $IL\text{-}10^+$ NKT cells and decrease in number of $IFN\text{-}\gamma^+$ NKT cells in the livers of TD139 + α-GalCer-treated WT mice (Fig. 7H). In line with the results obtained in *Gal-3*$^{-/-}$ mice, TD139 pretreatment increased the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells and TGF-β-producing Treg cells (Fig. 7I), which correlated with a reduced liver influx of DCs expressing CD1d (Fig. 7G).

Discussion

Although NKT cells are the major T-cell subset within the liver, many aspects of their intrahepatic activation and their regulatory effect on other (innate or specific) components of the hepatic immune system are still unknown [16]. Here, we provide the first evidence that Gal-3 regulates the capacity of DCs to support NKT-cell-mediated liver injury.

A genetic deletion of Gal-3, as well as TD139-mediated selective inhibition of Gal-3, abrogates susceptibility to α-GalCer-induced hepatitis; downregulates serum levels of TNF-α, IFN-γ, and IL-12 (Figs. 2 and 7) and attenuates their production by liver DCs and NKT cells; reduces the liver influx of DCs; and favors tolerogenic phenotype and IL-10 production by liver DCs and NKT cells (Figs. 3, 4, and 7).

It is well known that both the expression of Gal-3 and its secretion into the extracellular compartment are significantly enhanced in inflammatory conditions [17]. In the present study, the injection of α-GalCer, a potent stimulator of NKT cells, enhanced the expression of Gal-3 in the liver NKT and DCs (Fig. 1), which is consistent with its proposed role in α-GalCer-induced liver pathology.

The activation of DCs seems to be the critical initial trigger for the α-GalCer-induced hepatitis, in which liver DCs present α-GalCer in a CD1d-dependent manner to NKT cells [18]. It has been reported that α-GalCer treatment increases the number of liver DCs; enhances their maturation and expression of costimulatory molecules CD80, CD86, MHC class II; and stimulates them to produce large amounts of pro-inflammatory cytokines IL-12, TNF-α, and IFN-γ [18]. Gal-3 is required for these effects, serving as a chemoattractant for mouse DCs that is crucially important for their migration, adhesion and maturation [12]. In line with these observations, genetic deletion, as well as pharmacological inhibition of Gal-3 reduced the liver infiltration of DCs in α-GalCer hepatitis (Figs. 4 and 7), suggesting that Gal-3 is important for DC migration in this model of liver injury. In addition, our data demonstrate that Gal-3 is important for α-GalCer-induced cytokine production, polarization, and Ag presentation of liver DCs (Figs. 4, 5, and 7).

To directly demonstrate that Gal-3 deficiency attenuates the capacity of DCs to support NKT-cell-induced liver damage, we showed that WT liver DCs are more efficient than their *Gal-3*$^{-/-}$ counterparts in promoting α-GalCer-induced liver injury upon adoptive transfer to Gal-$3^{-/-}$ recipients (Fig. 5C). In line with these findings, we showed here that α-GalCer-pulsed Gal-$3^{-/-}$ DCs produce lower amounts of IL-12 and were not able to optimally stimulate production of TNF-α and IFN-γ in NKT cells in vitro and in vivo (Fig. 5).

Immature DCs constantly enter the liver from the blood and preferentially induce tolerance in the liver [19]. DCs need an additional early signal from the innate immune system, particularly from liver NKT cells, to mature and gain competence to prime immune response in the liver [16]. We assume that in the absence of Gal-3, DCs enter the liver in reduced number and fail to mature, thus preserving their immature/tolerogenic phenotype and produce more immunosuppressive IL-10 and less pro-inflammatory IL-12, TNF-α and IFN-γ. These events result in attenuated activation of NKT cells and reduced α-GalCer-induced liver injury. Since it is well known that both α-GalCer-activated IL-12-producing DCs and IFN-γ-producing NKT cells induce activation of liver NK cells and T lymphocytes [20], we noticed a decreased number of inflammatory NK and T cells in the livers of α-GalCer-treated *Gal-3*$^{-/-}$ mice (Fig. 3E). Thus, our results indicate that in α-GalCer hepatitis Gal-3 plays an important role in CD1d-dependant DC-mediated activation and polarization of NKT cells and consecutive activation of NK and T cells in the liver.

TGF-β, synthesized by liver Treg cells, inhibits CD1d expression on DCs [15]. Gal-3 negatively regulates the frequency and function of Treg cells in inflamed tissues [1] and enhances activation of STAT-3, which is known as a negative regulator of Treg function and TGF-β production [21]. Consistent with these findings, the injection of α-GalCer induced higher presence of TGF-β-producing but not IL-10-producing Treg cells in the injured livers of *Gal-3*$^{-/-}$ mice (Fig. 6B). Accordingly, there were less STAT-3-expressing Treg cells in the livers of *Gal-3*$^{-/-}$ mice (Fig. 6D). Despite the fact that TGF-β-producing Treg cells were more present in *Gal-3*$^{-/-}$ mice, after CY treatment α-GalCer-induced liver pathology was enhanced but the differences between WT and *Gal-3*$^{-/-}$ mice remained the same. This finding suggests that the difference in hepatitis is due to the attenuated interaction between liver DCs and NKT cells rather than due to the activity of TGF-β-producing Treg cells.

In conclusion, our data indicate that the capacity of liver DCs to promote NKT-cell-induced liver injury is Gal-3 dependent. Therefore, Gal-3 plays an important pro-inflammatory role in NKT-cell-dependent liver pathology.

Materials and methods

Animals

We used 6- to 8-week-old WT and *Gal-3*$^{-/-}$ C57BL/6 mice (breeding pairs kindly provided by Dr. Daniel Hsu, University of California, Sacramento, CA, USA). Targeted disruption of mouse *Gal*-3 gene was performed in C57BL/6 embryonic stem cells, and mice homozygous for the disrupted gene were obtained [22]. All experiments were approved by the Animal Ethics Committee of Faculty of Medicine (Kragujevac, Serbia) and performed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86–23 revised 1985).

Induction and evaluation of α-GalCer -induced hepatitis

WT and *Gal-3*$^{-/-}$ C57BL/6 mice were given a single intravenous injection of α-GalCer (50 μg/kg) dissolved in 200 μL of saline [11]. Since significant transaminase response could be detected 12–20 h after injection, peaking at 16 h after α-GalCer application [11], serum levels of alanine aminotransaminase (ALT) and AST were measured 16 h after i.v. injection of α-GalCer by standard photometric method using the automated biochemistry analyzer Olympus AU 400 (Olympus Diagnostica GMBH, Hamburg, Germany), and histological analysis of liver tissue was performed as previously described [4, 23].

Pharmacological inhibition of Gal-3

TD139 was i.p. administered (15 mg/kg) 2 h before and immediately after α-GalCer injection, as previously described [4]. Briefly, 4 mM stock solution of TD139 in 40% DMSO was made and stored in at −20°C. For each individual experiment, stock solution was diluted with buffer four times to obtain concentration of 1 mM TD139 in 10% DMSO (1 mM = 0.6489 μg/mL). Exactly 0.462 mL of this solution was i.p. injected in mice to give a dose of 15 mg/kg.

CY depletion of Treg cells

CY (Galenika A.D., Belgrade, Serbia) was dissolved in sterile distilled water and injected i.p. at a dose of 10 mg/kg, which affects Treg cells but not NKT cells [14], 3 days before α-GalCer injection.

Isolation of hepatic MNCs and flow cytometry analysis

The liver-infiltrating MNCs were isolated 2 h after α-GalCer application because already 3 h after α-GalCer injection, the majority of liver NKT underwent apoptosis [9]. The isolation of liver-infiltrating MNCs was conducted as previously described [4, 23]. Hepatic MNCs of WT and *Gal-3*$^{-/-}$ mice were screened for various cell surface and intracellular markers with flow cytometry 2 h after α-GalCer injection. Briefly, liver MNCs were stained with a combination of anti-mouse CD3, CD11c, CD1d, CD8, Gr-1, CD4, STAT-3, IL-12, and IL-10 Abs conjugated with fluorescein isothiocyanate (BD Bioscience); anti-mouse Gal-3, I-A, CD80, IFN-γ, TNF-α, IL-4, IL-5, IL-17, IL-12, and Foxp3 Abs conjugated with phycoerythrin (PE, BD Bioscience); anti-mouse NK1.1, CXCR3, CD11c, CD11b, CD25, and TNF-α Abs conjugated with peridinin chlorophyll protein (PerCP, BD Bioscience); and anti/mouse NK1.1, CD11c, CD11b, CD86, CD4, IL-10, TNF-α, IFN-γ, and TGF-β Abs conjugated with allophycocyanin (BD Bioscience). Intracellular staining for Foxp3 was performed using the BD Bioscience fixation/permeabilization buffer kit following manufacturer's instructions. For intracellular cytokine staining, cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 5 h, and GolgiStop (BD Biosciences) was added. Cells were fixed in Cytofix/Cytoperm, permeated with 0.1% saponin, stained with fluorescent Abs, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). CellQuest software (BD Biosciences) was used for data acquisition, and FlowJo and WinMDI software were used for analysis.

Serum cytokines and cytokine containing liver MNCs

Since cytokines revealed peak in sera of C57BL/6 mice about 2 h after α-GalCer application [11], serum levels of TNF-α, IFN-γ, IL-17, IL-12, IL-4, IL-10 and TGF-β were measured 2 h after α-GalCer injection by enzyme-linked immunosorbent assay (ELISA) using ELISA sets (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For the analysis of cytokine production in vitro, DCs were isolated from livers of untreated control WT and *Gal-3*$^{-/-}$ C57BL/6 mice, using Dynabeads Mouse DC Enrichment Kit (Invitrogen) and pulsed with 100 ng/mL of α-GalCer or vehicle alone for 24 h. After extensive washing, pulsed DCs (3×10^4 cells/well) were added to liver MNC (0.6×10^6 cells/well) cultured in 96-well plates [24]. Liver MNCs were co-cultured for an additional 48 h with vehicle- or α-GalCer-pulsed DCs, culture supernatants and cytokine containing cells were collected, and TNF-α, IL-12, IL-10, IFN-γ and IL-4 were measured by flow cytometry and ELISA sets (R&D Systems).

DC adoptive transfer

DC were isolated from livers of untreated WT and *Gal-3*$^{-/-}$ C57BL/6 mice, using Dynabeads Mouse DC Enrichment Kit (Invitrogen) and injected intravenously to *Gal-3*$^{-/-}$ mice (5×10^5 cells per mouse) immediately after α-GalCer injection. The samples for AST and ALT measurements were collected 16 h after α-GalCer injection and samples for the analysis of liver NKT cell activation were collected 2 h after α-GalCer injection. Liver damage was evaluated as described above.

Immunohistochemistry

In order to assess liver cells expressing Gal-3 and STAT-3, immunohistochemical (IHC) analysis was performed as previously described [4, 23]. Rabbit anti-mouse/human Gal-3 (ab53082, 1:100; Abcam Inc., Cambridge, MA, USA), rabbit anti/mouse/human STAT-3 (sc-135649, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) Abs, DAKO-labeled streptavidin biotin (LSAB) + system peroxidase (DAKO Corporation, Glostrup, Denmark) kit were used, according to the manufacturer's instructions.

Statistical analysis

Data were assessed for normality and equal variances and all data passed these criteria. Results were analyzed using the Student's *t*-test or one-way ANOVA. All data were expressed as the mean ± SEM. Values of $p < 0.05$ were considered as statistically significant.

Acknowledgments: This study was supported by the following grants: ON175069, ON175103, III41025 from the Ministry of Education, Science and Technology, Republic of Serbia, and MP01/12 from Faculty of Medical Sciences, University of Kragujevac, Serbia.

Conflict of interest: Ulf Nilsson and Hakon Leffler are co-founders and co-owners of the company Galecto Biotech AB involved in developing small-molecule galectin inhibitors as therapeutics.

References

1 **Fermino, M. L., Dias, F. C., Lopes, C. D., Souza, M. A., Cruz, A. K., Liu, F. T., Chammas, R. et al.,** Galectin-3 negatively regulates the frequency and function of CD4(+) CD25(+) Foxp3(+) regulatory T cells and influences the course of Leishmania major infection. *Eur. J. Immunol.* 2013. **43**:1806–1817.

2 **Hsieh, W. C., Mackinnon, A. C., Lu, W. Y., Jung, J., Boulter, L., Henderson, N. C., Simpson, K. J. et al.,** Galectin-3 regulates hepatic progenitor cell expansion during liver injury. *Gut* 2014. In press. DOI: **10.1136/gutjnl-2013-306290**.

3 **Iacobini, C., Menini, S., Ricci, C., Blasetti Fantauzzi, C., Scipioni, A., Salvi, L., Serino, M. 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.

4 **Volarevic, V., Milovanovic, M., Ljujic, B., Pejnovic, N., Arsenijevic, N., Nilsson, U., Leffler, H. et al.,** Galectin-3 deficiency prevents concanavalin A-induced hepatitis in mice. *Hepatology* 2012. **55**: 1954–1964.

5 **Radosavljevic, G., Volarevic, V., Jovanovic, I., Milovanovic, M., Pejnovic, N., Arsenijevic, N., Hsu, D. K. et al.,** The roles of galectin-3 in autoimmunity and tumor progression. *Immunol. Res.* 2012. **52**: 100–110.

6 **Johnston, B., Kim, C. H., Soler, D., Emoto, M. and Butcher, E. C.,** Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. *J. Immunol.* 2003. **171**: 2960–2969.

7 **Subramanian, M., Kini, R., Madasu, M., Ohta, A., Nowak, M., Exley, M., Sitkovsky, M. et al.,** Extracellular adenosine controls NKT-cell-dependent hepatitis induction. *Eur. J. Immunol.* 2014. **44**: 1119–1129.

8 **Nagy, L.,** Would eating carrots protect your liver? A new role involving NKT cells for retinoic acid in hepatitis. *Eur. J. Immunol.* 2012. **42**: 1677–1680.

9 **Osman, Y., Kawamura, T., Naito, T., Takeda, K., van Kaer, L., Okumura, K. and Abo, T.,** Activation of hepatic NKT cells and subsequent liver injury following administration of alpha-galactosylceramide. *Eur. J. Immunol.* 2000. **30**: 1919–1928.

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

11 **Biburger, M. and Tiegs, G.,** Alpha-galactosylceramide-induced liver injury in mice is mediated by TNF-alpha but independent of Kupffer cells. *J. Immunol.* 2005. **175**: 1540–1550.

12 **Hsu, D. K., Chernyavsky, A. I., Chen, H. Y., Yu, L, Grando, S. A. and Liu, F. T.,** Endogenous galectin-3 is localized in membrane lipid rafts and regulates migration of dendritic cells. *J. Invest. Dermatol.* 2009. **129**: 573–583.

13 **Wei, H. X., Chuang, Y. H., Li, B., Wei, H., Sun, R., Moritoki, Y., Gershwin, M. E. et al.,** $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells protect against T cell-mediated fulminant hepatitis in a TGF-beta-dependent manner in mice. *J. Immunol.* 2008. **181**:7221–7229.

14 **Rico, M. J., Rozados, V. R., Mainetti, L. E., Zacarias Fluck, M. F., Matar, P. and Scharovsky, O. G.,** Regulatory T cells but not NKT I cells are modulated by a single low-dose cyclophosphamide in a B cell lymphoma tumor-model. *Exp. Oncol.* 2012. **34**: 38–42.

15 **Ronger-Savle, S., Valladeau, J., Claudy, A., Schmitt, D., Peguet-Navarro, J., Dezutter-Dambuyant, C., Thomas, L. et al.,** TGFbeta inhibits CD1d expression on dendritic cells. *J. Invest. Dermatol.* 2005. **124**: 116–118.

16 **Trobonjaca, Z., Leithäuser, F., Möller, P., Schirmbeck, R. and Reimann, J.,** Activating immunity in the liver. I. Liver dendritic cells (but not hepatocytes) are potent activators of IFN-gamma release by liver NKT cells. *J. Immunol.* 2001. **167**: 1413–1422.

17 **Jeon, S. B., Yoon, H. J., Chang, C. Y., Koh, H. S., Jeon, S. H. and Park, E. J.,** Galectin-3 exerts cytokine-like regulatory actions through the JAK-STAT pathway. *J. Immunol.* 2010. **185**: 7037–7046.

18 **Sasakawa, A., Tatsumi, T., Takehara, T., Yamaguchi, S., Yamamoto, M., Ohkawa, K., Miyagi, T. et al.,** Activated liver dendritic cells generate strong acquired immunity in alpha-galactosylceramide treatment. *J. Hepatol.* 2009. **50**: 1155–1162.

19 **Yoneyama, H., Matsuno, K., Zhang, Y., Murai, M., Itakura, M., Ishikawa, S., Hasegawa, G. et al.,** Regulation by chemokines of circulating dendritic cell precursors, and the formation of portal tract-associatedlymphoid tissue, in a granulomatous liver disease. *J. Exp. Med.* 2001. **193**: 35–49.

20 **Nakano, M., Saeki, C., Takahashi, H., Homma, S., Tajiri, H. and Zeniya, M.,** Activated natural killer T cells producing interferon-gamma elicit promoting activity to murine dendritic cell-based autoimmune hepatic inflammation. *Clin. Exp. Immunol.* 2012. **170**: 274–282.

21 **Battaglia, A., Buzzonetti, A., Baranello, C., Fanelli, M., Fossati, M., Catzola, V., Scambia, G. et al.,** Interleukin-21 (IL-21) synergizes with IL-2 to enhance T-cell receptor-induced human T-cell proliferation and counteracts IL-2/transforming growth factor-β- induced regulatory T-cell development. *Immunology* 2013. **139**:109–120.

22 **Hsu, D. K., Yang, R. Y., Pan, Z., Yu, L., Salomon, D. R., Fung-Leung, W. P. and Liu, F. T.**, Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. *Am. J. Pathol.* 2000. **156**: 1073–1083.

23 **Volarevic, V., Mitrovic, M., Milovanovic, M., Zelen, I., Nikolic, I., Mitrovic, S., Pejnovic, N. et al.,** Protective role of IL-33/ST2 axis in Con A-induced hepatitis. *J. Hepatol.* 2012. **56**:26–33.

24 **Harada, M., Seino, K., Wakao, H., Sakata, S., Ishizuka, Y., Ito, T., Kojo, S, et al.,** Down-regulation of the invariant Valpha14 antigen receptor in NKT cells upon activation. *Int. Immunol.* 2004. **16**: 241–247.

Abbreviations: α**-GalCer**: α-galactosylceramide · **ALT**: alanine aminotransaminase · **AST**: aspartate aminotransferase · **CY**: cyclophosphamide · **Gal-3**: galectin-3 · **Gal-3**$^{-/-}$: Gal-3 deficient · **MNCs**: mononuclear cells · **TD139**: Gal-3 inhibitor

Full correspondence: Dr. Vladislav Volarevic, Centre for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, 69 Svetozara Markovica Street, 34 000 Kragujevac, Serbia
Fax: +38134306800-112
e-mail: drvolarevic@yahoo.com

Additional correspondence: Dr. Miodrag L. Lukic,
e-mail: miodrag.lukic@medf.kg.ac.rs

Received: 17/5/2014
Revised: 9/9/2014
Accepted: 28/10/2014
Accepted article online: 31/10/2014

