# Crosstalk Between Mesenchymal Stem Cells and T Regulatory Cells Is Crucially Important for the Attenuation of Acute Liver Injury

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One of the therapeutic options for the treatment of fulminant hepatitis is repopulation of intrahepatic regulatory cells because their pool is significantly reduced during acute liver failure. Although it is known that mesenchymal stem cells (MSCs), which have beneficent effects in the therapy of fulminant hepatitis, may promote expansion of regulatory T cells (Tregs) and regulatory B cells (Bregs), the role of these regulatory cells in MSC-mediated attenuation of acute liver injury is unknown. Herewith, we described the molecular mechanisms involved in the crosstalk between MSCs and liver regulatory cells and analyzed the potential of MSC-based therapy for the expansion of intrahepatic regulatory cells in mouse model of acute liver failure. MSC-dependent attenuation of α-galactosylceramide (α-GalCer)–induced acute liver injury in mice was accompanied with an increased presence of interleukin (IL) 10–producing $CD4^+CD25^+$ forkhead box $P3^+$ Tregs and IL10– and transforming growth factor β–producing marginal zone–like Bregs in the liver. Depletion of Bregs did not alter MSC-based alleviation of acute liver failure, whereas depletion of Tregs completely abrogated hepatoprotective effects of MSCs and inhibited their capacity to attenuate hepatotoxicity of liver natural killer T cells (NKTs), indicating that Tregs, and not Bregs, were critically involved in MSC-based modulation of acute liver inflammation. MSCs, in a paracrine, indoleamine 2,3-dioxygenase–dependent manner, significantly increased the capacity of Tregs to produce immunosuppressive IL10 and to suppress hepatotoxicity of liver NKTs. Accordingly, adoptive transfer of MSC-primed Tregs resulted in the complete attenuation of α-GalCer–induced acute liver failure. In conclusion, our findings highlighted the crucial importance of Tregs for MSC-based attenuation of acute liver failure and indicated the significance of MSC-mediated priming of Tregs as a new therapeutic approach in Treg-based therapy of acute liver injury.

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Liver transplantation is the most effective therapy for patients suffering from acute liver failure, a life-threating clinical condition characterized by progressive and extensive multilobular necrosis of hepatocytes and massive intrahepatic infiltration of inflammatory cells.[1] The increasing demand for liver transplantation and the decline in donor organs has highlighted the need for alternative therapies for the treatment of acute liver failure.

The liver contains different subsets of effector lymphocytes that are kept in check by a subpopulation of T cells known as regulatory T cells (Tregs). Because an increased Treg/T helper 17 ratio was associated with the better survival of acute-on-chronic liver failure patients,[2] an option for the therapy of acute liver injury is to enrich Tregs in the damaged livers and to alter the balance of effector and regulatory lymphocytes toward immunosuppressive Tregs.

*Abbreviations: 1-MT, 1-methyltryptophan; α-GalCer, α-galactosylceramide; ALT, alanine aminotransaminase; APC, allophycocyanin; AST, aspartate aminotransferase; Breg, regulatory B cell; CCR7, chemokine (C-C motif) receptor 7; CFSE, carboxyfluorescein diacetate succinimidyl ester; CY, cyclophosphamide; DC, dendritic cell; DMEM, Dulbecco's modified Eagle's medium; DP, dual-plate; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FOXP3, forkhead box P3; FSC-H, forward scatter height; H & E, hematoxylin-eosin; HGF,*

Even though passive transfer of Tregs showed beneficial effects in an animal model of acute liver failure,[3] Treg-based therapy has not yet been tested in patients suffering from this life-threating clinical condition. Most recently, a new concept of Treg-based therapy was brought to the table of operational tolerance in living donor liver transplantation.[3] However, it is still not verified whether administered Tregs actually reach the liver and maintain a stable phenotype over time.

In order to be present in an adequate number for therapeutic use, Tregs first have to be isolated from the peripheral blood of the patient, and then they have to be expanded in vitro.[4] This can represent a serious problem bearing in mind that in hyperacute cases of liver failure, encephalopathy and fatal outcome usually develop within a few days or a week after the onset of symptoms.[5] Thus, new approaches should be established with an aim to increase proliferation and activation of liver-resident Tregs in an optimal amount of time.

Indoleamine 2,3-dioxygenase (IDO) is a heme-containing enzyme critically involved in the induction of immune tolerance in the liver.[6] It converts tryptophan to kynurenine, which inhibits the proliferation of effector T cells and promotes generation of Tregs.[7] Most recently, by using α-galactosylceramide (α-GalCer)-induced hepatitis, well-established mice model of natural killer T cell (NKT)-dependent acute liver damage,[6] we highlighted the importance of mesenchymal stem cell (MSC)-derived IDO for the attenuation of acute liver failure.[8,9] MSCs protect from acute liver injury by reducing hepatotoxicity of liver NKT cells in IDO-dependent manner.[8,9]

Herewith, we describe an additional mechanism involved in MSC-mediated attenuation of acute liver failure that relies on the interaction between MSCs and Tregs in the injured livers. We showed that in the absence of Tregs, MSCs were not able to efficiently alleviate acute liver injury indicating the necessity and crucial importance of Tregs for the MSC-based attenuation of acute liver failure. Additionally, we described an additional, IDO-dependent mechanism by which MSCs promote expansion of Tregs and increased their capacity to produce interleukin (IL) 10, resulting in the suppression of NKT hepatotoxicity. Our data presented here indicate that MSC-based therapy could be used as a new therapeutic approach for the expansion of liver Tregs in the acute liver failure.

*hepatocyte growth factor; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon γ; IgM, immunoglobulin M; IL, interleukin; IP, intraperitoneally; MNC, mononuclear cell; MSC, mesenchymal stem cell; MSC-CM, mesenchymal stem cell–conditioned medium; MZ, marginal zone; NKT, natural killer T cell; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; RPMI-1640 medium, Roswell Park Memorial Institute 1640 medium; RTCA, real-time cell analyzer; SEM, standard error of the mean; SSC-H, side scatter height; TCR, T-cell receptor; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; Treg, regulatory T cell.*

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# Materials and Methods

## CELLS

Murine MSCs, isolated from bone marrow of C57BL/6 mice, were purchased from Gibco (Gibco-Life Technologies, Carlsbad, CA) (catalog number S1502-100). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL of penicillin G, and 100 μg/mL of streptomycin (Sigma-Aldrich, Munich, Germany), at 37° in a 5% $CO_2$ incubator. MSCs in passage 4 were used throughout the experiments.

## MOUSE HEPATOCYTE ISOLATION

Mouse hepatocytes were isolated as previously described.[10] Briefly, a cannula was inserted in the portal vein, and the liver was perfused for 15–20 minutes with T1 solution (0.9% NaCl, 0.05% KCl, 0.2% 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid, 0.08 mg/mL ethylene glycol tetraacetic acid, pH 7.4; all from Sigma-Aldrich, St. Louis, MO). As soon as the perfusion was started, the hepatic vein was immediately cut to allow the perfusate to run as a

waste. Then, the liver was perfused with T2 solution (0.6% NaCl, 0.05% KCl, 1.2% 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid, 0.07% CaCl2, 3 g/mL collagenase Type I, pH 7.4; all from Sigma-Aldrich, St. Louis, MO). The disaggregated liver tissue was collected with a curved spatula and transferred into a plate where the tissue was mechanically disrupted with a scalpel. Minced tissue was collected with 2 mL of DMEM (Sigma-Aldrich, St. Louis, MO), filtered through a 70-$\mu$m cell strainer, and centrifuged at 600 rpm for 4 minutes. The supernatant was removed; the pellet was resuspended in 3 mL of DMEM; and the cells were passed on 37.5% Percoll cushion (30 mL), centrifuged at 1050 rpm for 3 minutes, and resuspended in 2 mL of DMEM. Viable hepatocytes were counted.

## GENERATION OF MSC-CONDITIONED MEDIUM

MSCs seeded at a density of 10,000 cells/cm$^2$ in a T75 flask[11] were cultured until reaching 80% confluence, and the culture medium was changed every 48 hours. After the last change of the medium, the 80% confluent cells were further cultured for 48 hours, and the medium designated as mesenchymal stem cell–conditioned medium (MSC-CM) was collected.[12]

## PHARMACOLOGICAL INHIBITION OF IDO

MSCs were cultured for 48 hours in culture medium containing 1 mM of 1-methyltryptophan (1-MT; Sigma-Aldrich, St-Louis, MO), an inhibitor of IDO enzymatic activity.[13]

## ANIMALS

Male 6-8–week-old C57BL/6 mice were used. All animals received humane care according to the criteria outlined in Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, 1985 revision), and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences of the University of 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.

## MOUSE MODEL OF ACUTE LIVER FAILURE

C57BL/6 mice were given a single intravenous injection of α-GalCer (50 $\mu$g/kg) dissolved in 200 $\mu$L of saline.[14] MSCs ($5 \times 10^5$) were intravenously injected, via tail vein,[15] into C57BL/6 mice immediately after α-GalCer administration (α-GalCer+MSC–treated mice), while control animals received the appropriate amount of MSCs only or saline only. To demonstrate that soluble factors were responsible for the MSC-mediated inhibition of NKT function, mice were injected intravenously with 0.2 mL of MSC-CM or 0.2 mL MSC-CM+1-MT, immediately after α-GalCer administration. Control animals received 0.2 mL of saline only.

Serum levels of alanine aminotransaminase (ALT) and aspartate aminotransferase (AST) were measured before and 16 hours after α-GalCer administration, by a standard photometric method using the automated biochemistry analyzer Olympus AU 400 (Olympus Diagnostica GMBH, Hamburg, Germany) and Olympus AU reagents, according to the manufacturer's instructions.

## HISTOLOGICAL ANALYSES AND SEMIQUANTITATIVE ASSESSMENT OF LIVER INJURY

Histological analysis and semiquantitative determination of liver injury were performed as previously described.[16] Briefly, the isolated livers were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and consecutive 4-$\mu$m tissue sections were cut at various depths and mounted on slides. Sections were stained with hematoxylin-eosin (H & E) and examined under low-power (100×) light microscopy (Zeiss Axioskop 40, Jena, Germany) equipped with a digital camera. The area of necrosis was quantified in H & E–stained liver section using ImageJ Software and was shown as a percentage of the total section area.[17] Sections were examined and scored by 2 independent investigators in a blind manner.

## DEPLETION OF REGULATORY CELLS

For Treg depletion, mice were injected intraperitoneally (IP) with cyclophosphamide (CY; Galenika A.D., Belgrade, Serbia) at a dose of 10 mg/kg or anti-CD25 antibody (PC61 mitochondrial antibody, Sigma-Aldrich,

Munich, Germany) at a dose of 250 mg/mouse at 3 days before α-GalCer injection.[14,18] B cell depletion was accomplished by a single IP injection of anti-mouse CD20 antibody (Clone 5D2, Genentech, Inc., San Francisco, CA) at a dose of 10 mg/kg, and the same isotype immunoglobulin was used for control.[19]

## ISOLATION OF HEPATIC MONONUCLEAR CELLS AND ANALYSIS WITH FLOW CYTOMETRY

The isolation of liver-infiltrating mononuclear cells (MNCs) was conducted as previously described.[16] Hepatic MNCs were screened for various cell surface and intracellular markers with flow cytometry 2 hours after α-GalCer injection. Briefly, $1 \times 10^6$ MNCs were incubated with anti-mouse CD4, CD25, CD49b, CD23, CD21, immunoglobulin M (IgM), CD5, B220, CD19, CD11c, CD11b, and CD8 monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (APC; all from BD Biosciences, San Jose, CA) following the manufacturer's instructions. MNCs derived from the liver were concomitantly stained for the intracellular content of forkhead box P3 (FOXP3), IL10, transforming growth factor $\beta$ (TGF-$\beta$), IL6, and tumor necrosis factor $\alpha$ (TNF-$\alpha$) by using the BD fixation/permeabilization kit and anti-mouse monoclonal antibodies conjugated with FITC, PE, PerCP, and APC (BD Bioscience). For intracellular cytokine staining, cells were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin for 5 hours, and GolgiStop (BD Biosciences) was added. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analyzed by using the Flowing software analysis program (Turku Centre for Biotechnology, Turku, Finland).

## ISOLATION OF NKTS

NKTs were isolated from hepatic MNCs by magnetic cell sorting according to the manufacturer's instructions. Single-cell suspensions of liver MNCs were labeled with a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against NKp46, CD45R, CD8a, CD115, and TCR$\gamma\delta$ and MicroBeads conjugated to monoclonal anti-biotin antibody (Miltenyi Biotec). The labeled cells were subsequently depleted by separation over a MACS Column (Miltenyi Biotec), which was placed in the magnetic field of a MACS Separator (Miltenyi Biotec). In the second step, the NK1.1+ NKTs were labeled with monoclonal anti-mouse NK1.1 antibody conjugated to APC and microBeads conjugated to monoclonal anti-mouse anti-APC antibody and positively selected using MACS Column (Miltenyi Biotec) and MACS Separator (Miltenyi Biotec). Isolated NKTs were then used in the coculture experiments and cytotoxicity assay as purified NKTs.

## ISOLATION OF TREGS

$CD4^+CD25^+$ Tregs were isolated from mouse splenocytes by magnetic cell sorting according to the manufacturer's instructions. First, the spleens were minced in Roswell Park Memorial Institute 1640 medium (RPMI-1640 medium; Sigma-Aldrich, St. Louis, MO) and forced gently through a 40-mm cell-strainer nylon mesh using a sterile syringe plunger and centrifuged at 400*g* for 5 minutes. Pelleted spleen cells were incubated in 2 mL of NH4Cl/Tris–Cl (pH 7.2) for 5 minutes, supplemented with 1 mL FBS, centrifuged at 400*g* for 5 minutes, and then resuspended in RPMI-1640 medium with 10% FBS.[10] Non-$CD4^+$ were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against CD8, CD11b, CD45R, CD49b, Ter-119, and Anti-Biotin MicroBeads. The labeled cells were subsequently depleted over a MACS column. The enriched $CD4^+$ T cells were labeled with CD25 MicroBeads for subsequent positive selection of $CD4^+CD25^+$ Tregs. Cell viability was determined by trypan blue staining.

## CYTOTOXICITY ASSAY

The dual-plate (DP) version of the xCELLigence system (Roche, Basel, Switzerland) was used as previously described.[8] Briefly, 100 $\mu$L of complete medium was added to each well, and background impedance on the plates was measured on the xCELLigence real-time cell analyzer (RTCA) DP instrument at 37° and 5% $CO_2$. Effector (NKTs) to target (hepatocytes) ratio 10:1 was used.[20] Hepatocytes were resuspended in DMEM with 10% FBS at $4 \times 10^5$ cells/mL. A total of 100 $\mu$L hepatocytes were added to each well of the E16 plate, which was then placed in the xCELLigence RTCA DP. NKTs, isolated by magnetic separation, were counted and resuspended at a concentration of $4 \times 10^6$ cells/mL in DMEM+10% FBS media. Then 100 $\mu$L of NKTs or media alone was added to the respective wells. The E16 plates were placed in the

xCELLigence RTCA DP, and impedance measurements were recorded every 15 minutes for 24 hours at 37° and 5% $CO_2$. NKT-mediated death of tumor cells was monitored in real time and was indicated by a decrease in cell index. Data were analyzed with RTCA Software 1.2 (ACEA Biosciences, San Diego, CA).

## IN VITRO EXPERIMENTS

For contact-independent cocultures, cells were cultured and physically separated using a 0.4-$\mu$m porous transwell system (Corning Incorporated, Life Sciences, France).

Tregs, isolated from the untreated mice and in vitro stimulated with concanavalin A (2.5 lg/mL) and IL2 (50 ng/mL),[21] were placed in the lower chamber (24-well transwell plate) and cultured in the presence (TregsMSCs) or in the absence of MSCs (Tregs). MSCs were seeded in the transwell inserts, and the ratio between MSCs and Tregs was 1:10.[22] After 48 hours of culture, expression of CD62L and chemokine (C-C motif) receptor 7 (CCR7) on Tregs and $Tregs^{MSCs}$ were analyzed by flow cytometry. Capacity of Tregs and $Tregs^{MSCs}$ to produce IL10 and TGF-$\beta$ was determined by enzyme-linked immunosorbent assay (ELISA).

In order to evaluate the importance of IDO inhibition for MSC-mediated priming of Tregs, contact-independent cocultures of Tregs and MSCs were grown in the presence ($Tregs^{MSCs+1\text{-}MT}$) or in the absence of 1-MT ($Tregs^{MSCs}$).[23] After 48 hours of culture, regulatory cells were harvested for adoptive transfer experiments. Immunoregulatory effects of $Tregs^{MSCs}$ and $Tregs^{MSCs+1\text{-}MT}$ on NKTs were examined in a direct coculture, at ratio 1:1. Precisely, $1 \times 10^5$ $Tregs^{MSCs}$ or $Tregs^{MSCs+1\text{-}MT}$ were added to each well that contained $1 \times 10^5$ NKTs.[24] Cytotoxicity of NKTs ($4 \times 10^5$/well) against hepatocytes ($4 \times 10^4$/well) were evaluated after 48 hours of culture.[20]

## TRANSFER OF TREGS

Tregs or $Tregs^{MSCs}$ were intravenously injected ($1\times10^6$ cells resuspended in 0.2 mL of saline) into CY-treated animals 24 hours before α-GalCer administration.[25]

## INTRAHEPATIC DETECTION OF TREGS

Tregs or $Tregs^{MSCs}$ were fluorescence-labeled using preincubation with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) according to the manufacturer's instructions.[26] For homing assays, $1 \times 10^6$ CFSE-labeled Tregs or $Tregs^{MSCs}$ were injected into the tail veins of CY-treated mice 24 hours before α-GalCer administration. MNC suspensions were prepared from liver tissues 2 hours after α-GalCer injection and analyzed by flow cytometry.

## MEASUREMENT OF CYTOKINES

Levels of TNF-α, interferon γ (IFNγ), IL10, TGF-β, and hepatocyte growth factor (HGF) in the mouse serum and cell supernatants were measured using ELISA kits specific for the mouse cytokines (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.[8]

## DETECTION OF KYNURENINE

IDO activity was determined by spectrophotometric assay for kynurenine in the MSC-CM and MSC-CM+1-MT.[27]

## RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

Total RNA from mice livers were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The fold change of messenger RNA gene expression for IDO, IFNγ, and β-actin as a housekeeping gene (Invitrogen, Carlsbad, CA) was calculated as described.[28]

## STATISTICAL ANALYSIS

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

## MSC-DEPENDENT ATTENUATION OF ACUTE LIVER FAILURE IS ACCOMPANIED WITH AN INCREASED PRESENCE OF TREGS AND REGULATORY B CELLS IN THE LIVER

Single intravenous injection of MSCs efficiently attenuated acute liver failure in mice (Fig. 1). Serum

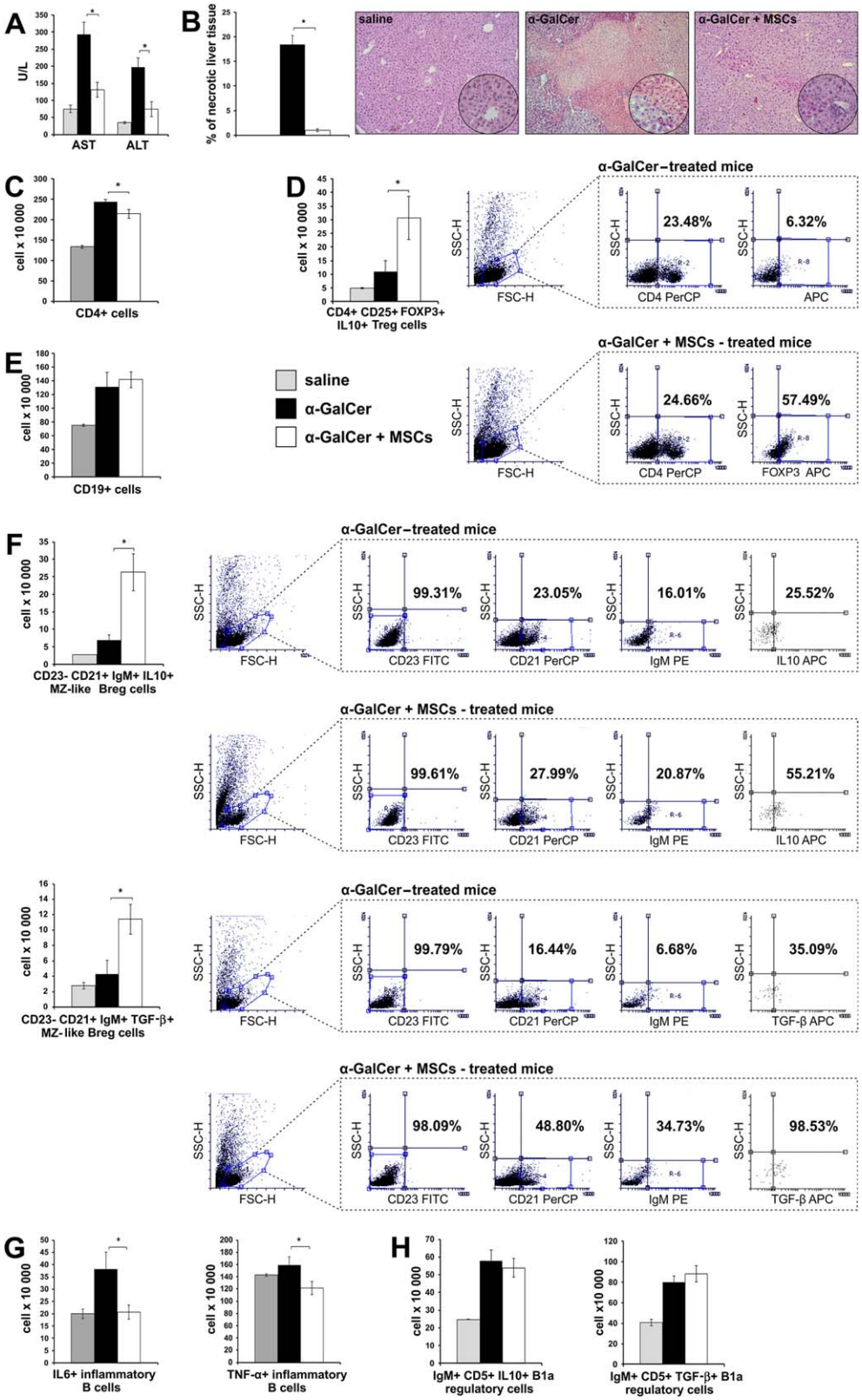


**FIG. 1.** MSC-dependent attenuation of α-GalCer–induced hepatitis is accompanied with an increased presence of Tregs and Bregs in the liver. (A) Serum AST and ALT levels; (B) histological examination was performed with H & E staining. Semiquantitative determination of liver injury estimated by ImageJ software. Total numbers of liver-infiltrating (C) CD4+ cells, (D) IL10-producing CD4+ CD25+FOXP3+ Tregs with representative flow cytometry dot plots, (E) CD19+ cells (F) IL10-producing and TGF-*β*–producing CD23−CD21+ $IgM^+$ MZ-like Bregs with representative flow cytometry dot plots and (G) IL6-producing and TNF-α–producing inflammatory B220+ cells in the livers of α-GalCer–treated and α-GalCer+MSC–treated mice are shown. (H) Total numbers of peritoneal B1a ($IgM^+CD5^+$) and B1b ($IgM^+CD5^-$) regulatory cells. Values are mean ± SEM (n = 10 per group). *$P < 0.05$.

AST and ALT levels 16 hours after α-GalCer injection were significantly decreased in α-GalCer+MSCs–treated mice, compared with mice that received α-GalCer only ($P < 0.05$; Fig. 1A). Histopathological analysis of the liver confirmed these findings (Fig. 1B). Liver tissue sections in α-GalCer–treated mice showed widespread areas of massive coagulative necrosis with extensive infiltration of MNCs, within liver lobules and around the central veins and portal tracts, indicating the ongoing inflammatory process (Fig. 1B). In contrast, there were only several solitary areas of necrotic tissue in α-GalCer–treated mice that received MSCs resulting in significantly reduced total size of the hepatic necrotic areas (Fig. 1B).

The decrease in liver damage by MSCs was associated with the extensive infiltration of hepatoprotective regulatory cells within the liver (Fig. 1D,F). The flow cytometric analysis revealed a significant decrease in total number of CD4+ cells ($P < 0.05$; Fig. 1C) and significant increase in the total number of IL10-producing Tregs ($CD4^+CD25^+FOXP3^+$) in the livers of α-GalCer+MSC–treated mice compared with animals treated with α-GalCer only ($P < 0.05$; Fig. 1D). Although there was no significant difference in the total number of liver-infiltrated CD19+ B cells between experimental groups ($P > 0.05$; Fig. 1E), MSC treatment expanded regulatory and attenuated inflammatory B cells in the livers of α-GalCer–treated mice (Fig. 1F,G). The total number of IL10-producing and TGF-β–producing marginal zone (MZ)–like regulatory B cells (Bregs) ($CD23^-CD21^+IgM^+$) was significantly higher ($P < 0.05$; Fig. 1F), whereas the total number of IL6 and TNF-α–producing inflammatory B cells was notably lower in the livers of α-GalCer–treated mice that received MSCs ($P < 0.05$; Fig. 1G). There was no significant difference in the total number of liver-infiltrating IL10-producing and TGF-β–producing peritoneal B1a ($IgM^+CD5^+$) and B1b regulatory cells ($IgM^+CD5^-$) between the experimental groups ($P > 0.05$; Fig. 1H), suggesting that MSC-mediated attenuation of α-GalCer–induced liver damage may be a consequence of an increased presence of MZ-like Bregs and Tregs in the injured liver. Because both regulatory cell populations were notably lower in the spleens of α-GalCer+MSC–treated mice (Supporting Fig. 1), we assumed that MSCs promoted migration of these regulatory cells from the spleen into the injured livers where they suppress inflammation.[19]

## B CELL DEPLETION DID NOT AFFECT MSC-MEDIATED SUPPRESSION OF α-GALCER–INDUCED HEPATITIS

In the presence of MSCs, plasmablast formation is reduced and development of IL10-producing Bregs is induced.[19,29] In order to delineate the importance of Bregs in MSC-mediated attenuation of acute liver injury, we analyzed the effects of B cell depletion in α-GalCer–treated animals. Anti-CD20 antibody treatment efficiently depleted B cells in the livers of α-GalCer–treated animals (Supporting Fig. 2A, left panel). Depletion of Bregs (Supporting Fig. 2A, right panel) significantly aggravated liver failure in anti-CD20+α-GalCer–treated mice (Supporting Fig. 2B,C), indicating the importance of Bregs in attenuation of NKT-mediated acute liver injury. However, depletion of Bregs did not affect the capacity of MSCs to ameliorate acute liver failure, as determined by liver enzyme tests (Fig. 2A) and histological analysis (Fig. 2B). As shown in Fig. 2A, MSCs decreased serum levels of AST ($P < 0.05$) and ALT ($P < 0.05$) in anti-CD20+α-GalCer–treated mice in a similar manner as in α-GalCer–treated animals. Histopathological analysis confirmed these findings (Fig. 2B). Liver damage was reduced in a similar manner in the livers of anti-CD20+α-GalCer+MSC– and α-GalCer+MSC–treated mice (Fig. 2B).

In line with these findings, there was no significant difference in the serum levels of inflammatory cytokines (TNF-α, IFNγ) and anti-inflammatory IL10 between anti-CD20+α-GalCer+MSC–treated and α-GalCer+MSC–treated mice ($P > 0.05$; Fig. 2C), indicating that Bregs were not directly involved in MSC-mediated attenuation of acute liver inflammation.

Additionally, NKTs isolated from the livers of anti-CD20+α-GalCer+MSC–treated mice were as hepatotoxic as liver NKTs derived from α-GalCer+MSC–treated animals (Fig. 2D), confirming that depletion of Bregs did not affect MSC-mediated suppression of NKT hepatotoxicity.

## TREGS ARE CRITICALLY INVOLVED IN MSC-MEDIATED SUPPRESSION OF α-GALCER–INDUCED LIVER INJURY

In order to determine whether Tregs had a crucial role in MSC-dependent attenuation of acute liver failure,

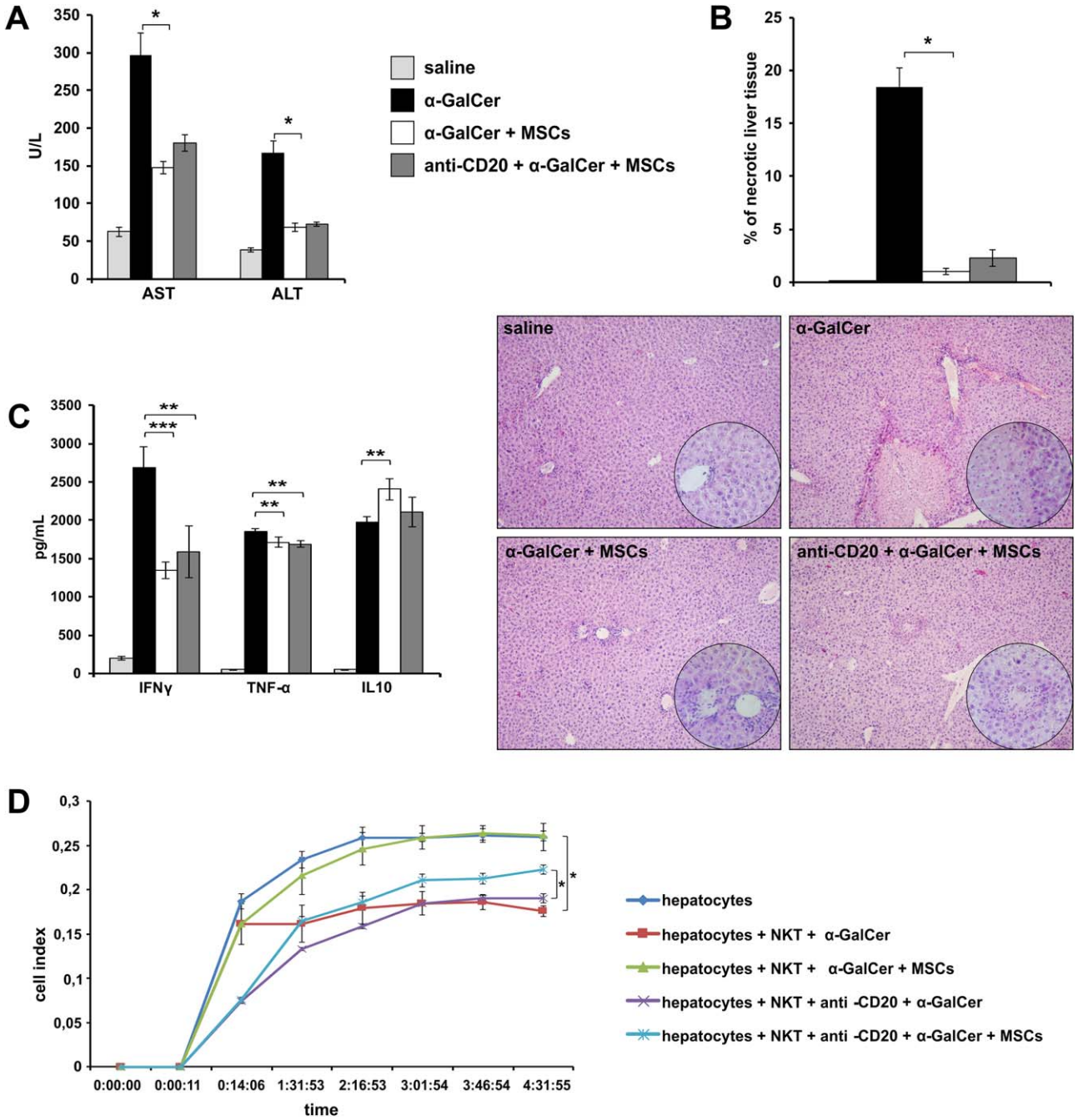


**FIG. 2.** B cell depletion did not affect MSC-mediated attenuation of acute liver inflammation. (A) Serum AST and ALT levels; (B) representative H & E–stained mouse livers. Semiquantitative determination of liver injury estimated by ImageJ software. (C) The level of cytokines in serum; (D) cytotoxic potential of NKTs against the hepatocytes in vitro as determined by the xCELLigence system. Values are mean ± SEM (n = 10 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the effects of CY and anti-CD25 antibody-induced depletion of Tregs were analyzed in α-GalCer+MSCs–treated mice (Fig. 3A). CY selectively depleted Tregs (Supporting Fig. 3A) without affecting immunosuppressive (CD11c+CD11b–CD8+IL10+) and inflammatory (CD11c+CD11b+CD8–) subpopulations of liver-infiltrated CD11c+ dendritic cell (DCs) (Supporting Fig. 3B). CY as well as anti-CD25

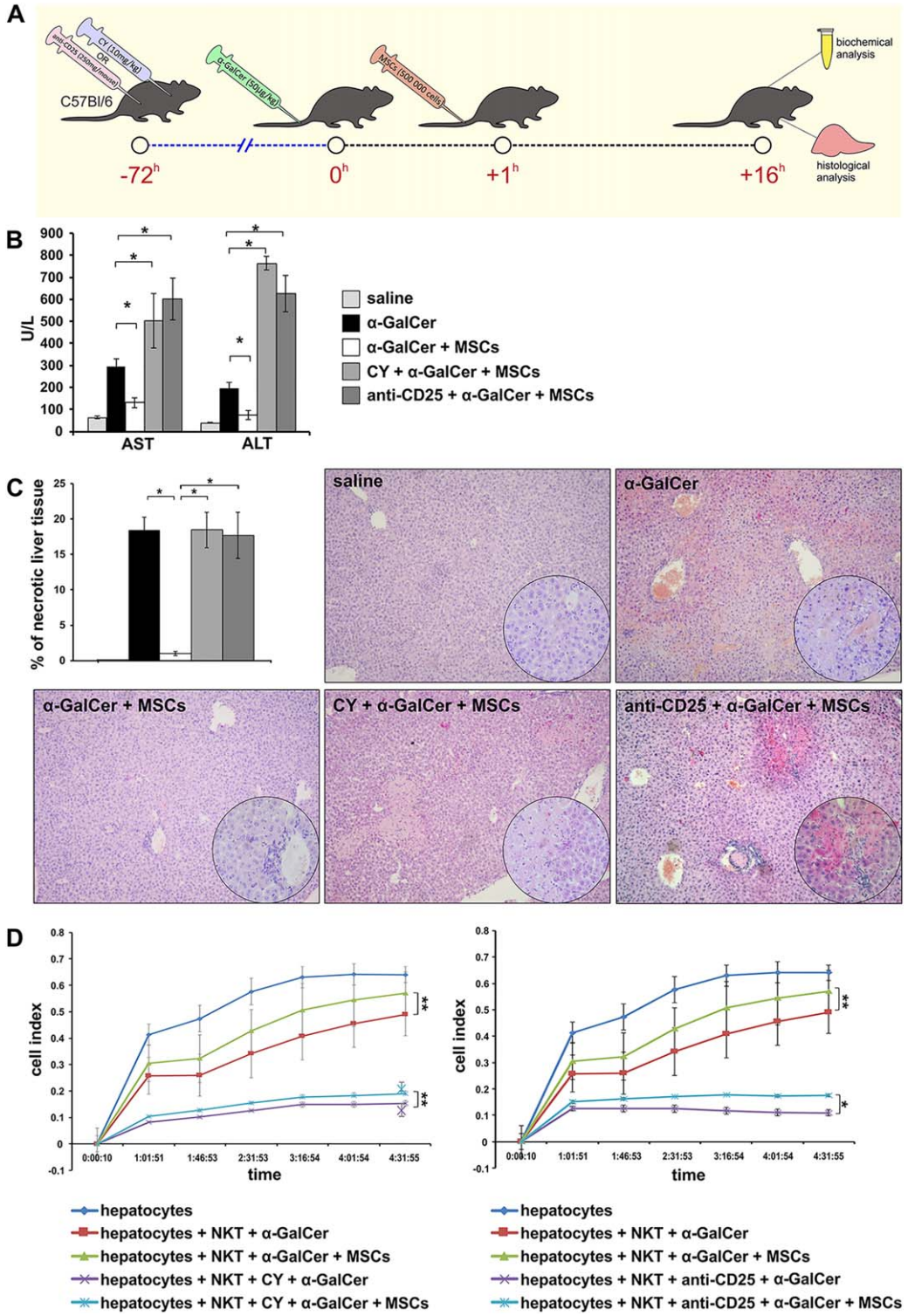


**FIG. 3.** Tregs are critically involved in MSC-mediated suppression of α-GalCer–induced liver injury. (A) C57BL/6 mice were given an IP injection of CY (10 mg/kg) or anti-CD25 monoclonal antibody (250 mg/mouse), 72 hours before α-GalCer administration (50 μg/kg). MSCs ($5 \times 10^5$) were intravenously injected into the tail vein of C57BL/6 mice 1 hour after α-GalCer application. Biochemical and histological analyses were performed 16 hours after treatment. (B) Serum AST and ALT levels, (C) representative H & E–stained mouse livers. Percentage of necrotic liver tissue, estimated by ImageJ software. (D) The results obtained by the xCELLigence system showed less hepatotoxicity of liver NKTs isolated from α-GalCer+MSC–treated mice than NKTs isolated from the livers of CY+α-GalCer+MSC–treated (left panel) and anti-CD25+α-GalCer+MSC–treated animals (right panel).

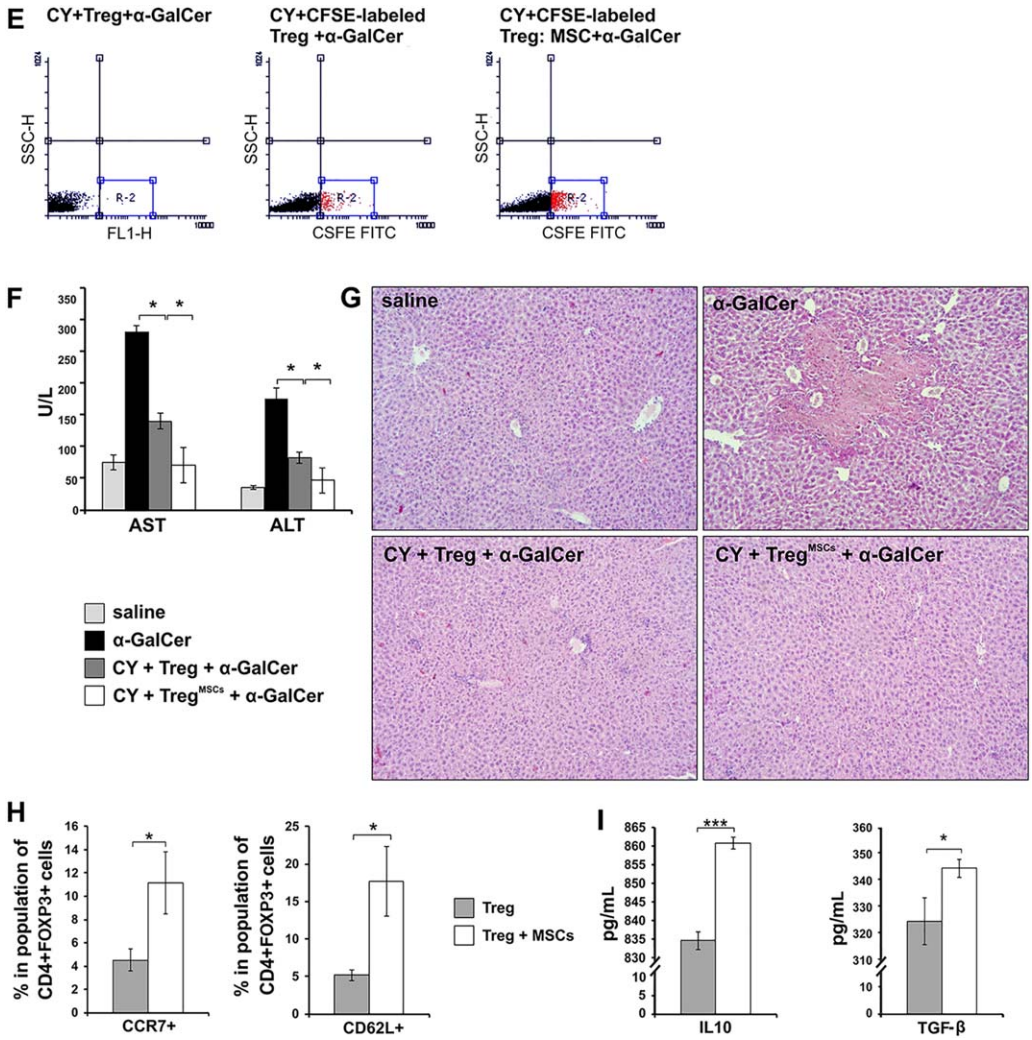


**FIG. 3.** (E) Detection of CFSE-labeled Tregs and Tregs$^{MSCs}$ in the liver; (F) passive transfer of MSC-primed Tregs significantly decreased serum level of transaminases in CY+α-GalCer–treated mice; and (G) H & E staining showing reduced liver damage in CY+Tregs$^{MSCs}$+α-GalCer–treated animals. (H) The percentage of CCR7 and CD62L expressing cells in a population of CD4$^+$FOXP3$^+$ Tregs, (I) The level of IL10 and TGF-$\beta$ cytokines in supernatants. Values are mean ± SEM (n = 10 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

antibody-induced depletion of Tregs significantly aggravated acute liver failure in α-GalCer–treated animals indicating the importance of Tregs for the suppression of acute liver injury (Fig. 3B,C). Importantly, depletion of Tregs completely abrogated the hepatoprotective activity of MSCs in α-GalCer–induced acute liver failure (Fig. 3B,C). A significantly higher serum levels of AST and ALT ($P < 0.05$; Fig. 3B) and a significantly higher percentage of liver parenchyma with necrotic damage ($P < 0.05$; Fig. 3C) accompanied with an extensive infiltration of MNCs were noticed in the livers of CY+α-GalCer+MSC–treated and anti-CD25+α-GalCer+MSC–treated mice compared with α-GalCer+MSC–treated animals.

Depletion of Tregs significantly reduced MSC-mediated attenuation of NKT hepatotoxicity (Fig. 3D). NKTs isolated from the livers of α-GalCer+MSC–treated mice were significantly less cytotoxic against hepatocytes than NKTs isolated from the livers of CY+α-GalCer+MSC–treated ($P < 0.05$;

Fig. 3D, left panel) and anti-CD25+α-GalCer+MSC–treated animals ($P < 0.05$; Fig. 3D, right panel).

In order to confirm that MSC-Treg interplay is directly responsible for MSC-mediated modulation of α-GalCer–induced liver injury, CY+α-GalCer–treated animals received either Tregs or Tregs cocultured in a transwell system with MSCs ($Tregs^{MSCs}$).

Intravenously injected Tregs and $Tregs^{MSCs}$ migrated in the livers of CY+α-GalCer–treated mice (Fig. 3E) and managed to attenuate acute liver injury (Fig. 3F,G). Importantly, this phenomenon was remarkably intensified in CY+α-GalCer–treated animals that received $Tregs^{MSCs}$ (Fig. 3F,G), indicating that MSCs increased the capacity of Tregs to migrate in the injured liver and to attenuate acute liver failure. In line with these findings, a significantly higher expression of CD62L and CCR7, involved in MSC-regulated migration of Tregs,[30] was noticed on $Tregs^{MSCs}$ when compared with Tregs ($P < 0.05$; Fig. 3H). Similarly, MSCs notably increased the capacity of Tregs to produce immunosuppressive IL10 ($P < 0.001$) and TGF-$\beta$ ($P < 0.05$; Fig. 3I).

## IN ACUTE LIVER FAILURE, MSCS PROMOTE EXPANSION OF TREGS IN A PARACRINE IDO-DEPENDENT MANNER

In order to explore whether MSCs are able to induce expansion of Tregs in vivo, in the same paracrine manner as in vitro, the therapeutic effects of MSC-CM were analyzed in α-GalCer–treated mice (Fig. 4). MSC-CM treatment managed to significantly attenuate α-GalCer–induced hepatocyte damage as evaluated by biochemical parameters (Fig. 4A) and histological analysis (Fig. 4B). A diminished inflammatory injury in α-GalCer+MSC-CM–treated mice correlated with the significantly higher presence of IL10-producing $CD4^+CD25^+FOXP3^+$ Tregs in the livers of α-GalCer+MSC-CM–treated mice when compared with α-GalCer–treated animals ($P < 0.05$; Fig. 4C), confirming that MSC-derived soluble factors are responsible for an increased number of Tregs in injured livers.

It is well-known that, among all MSC-derived immunosuppressive factors, IDO, IL10, HGF, and TGF-$\beta$ are mainly responsible for MSC-based attenuation of acute liver injury.[31] Because IDO has been identified as a critical molecular switch that simultaneously blocks reprogramming of Tregs into effector T cells,[32] we examined the expression of IDO in the livers of α-GalCer–treated animals and evaluated the effects of IDO inhibitor (1-MT) in MSC-dependent modulation of acute liver failure.

Expression of IDO in the livers of α-GalCer–treated mice (Fig. 4D) negatively corresponded with the extent of liver injury (Fig. 4A,B), but positively correlated with the total number of liver-infiltrated Tregs (Fig. 4C). Application of 1-MT significantly down-regulated expression of IDO in the injured livers ($P < 0.05$; Fig. 4D), exacerbated α-GalCer–induced acute liver injury (Fig. 4A,B) and remarkably attenuated the total number of liver Tregs ($P < 0.05$; Fig. 4C), indicating the importance of IDO for Treg-mediated attenuation of acute liver failure.

Accordingly, we further analyzed effects of 1-MT on the immunosuppressive potential of MSC-CM and on MSC-CM–dependent expansion of Tregs and attenuation of α-GalCer–induced liver injury; 1-MT significantly attenuated concentration of kynurenine ($P < 0.05$; Supporting Fig. 4A), but it did not alter concentration of other immunosuppressive factors (IL10, HGF, and TGF-$\beta$) in MSC-CM ($P > 0.05$; Supporting Fig. 4B). Importantly, 1-MT completely abrogated the therapeutic effects of MSC-CM in vivo, as evaluated by remarkably higher serum levels of AST and ALT in α-GalCer+MSC-CM+1-MT–treated mice compared with α-GalCer+MSC-CM–treated animals ($P < 0.05$; Fig. 4A). Histological analysis confirmed these findings (Fig. 4B). H & E staining showed destruction of the hepatic architecture, massive infiltration of inflammatory cells, and extensive hepatocellular necrosis in the α-GalCer+MSC-CM+1-MT–treated group that was opposite to the attenuated liver injury and inflammation noticed in α-GalCer+MSC-CM–treated animals (Fig. 4B).

The total number of $CD4^+CD25^+FOXP3^+IL10^+$ Tregs was significantly attenuated in the livers of α-GalCer+MSC-CM+1-MT–treated mice compared with α-GalCer+MSC-CM–treated animals ($P < 0.05$; Fig. 4C), indicating the crucial importance of IDO for MSC-CM–dependent expansion of Tregs in injured livers of α-GalCer+MSC-CM–treated animals.

In order to confirm the importance of MSC-derived IDO for Treg-dependent suppression of NKT hepatotoxicity, in vitro experiments were performed (Fig. 4E). MSCs, in paracrine manner, significantly increased capacity of Tregs to produce immunosuppressive IL10 ($P < 0.001$; Fig. 4F), and this phenomenon was completely abrogated in the presence of 1-MT ($P < 0.001$; Fig. 4F). Accordingly, an addition of 1-MT in MSC-Tregs culture attenuated the capacity of Tregs to suppress hepatotoxicity of NKTs,

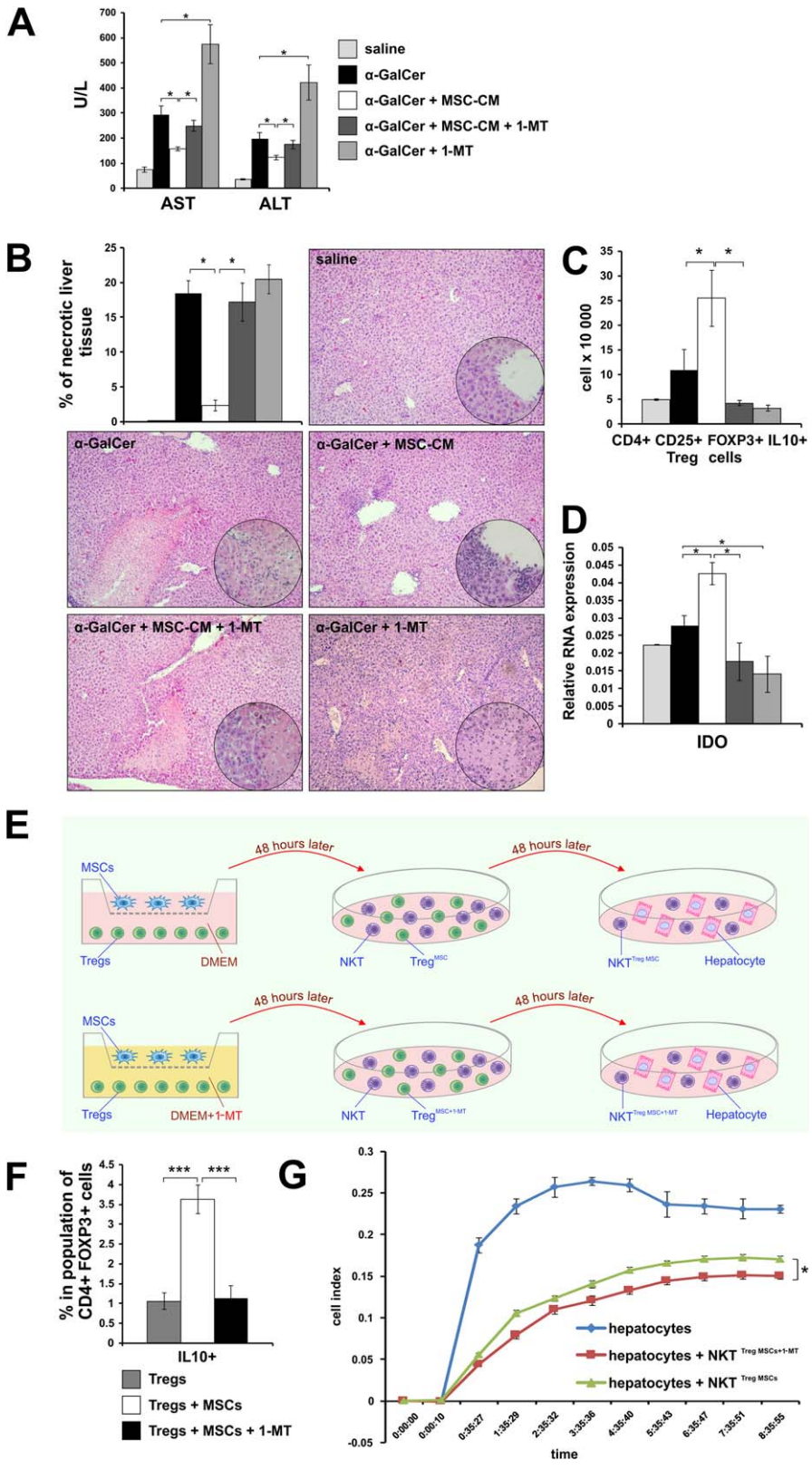


**FIG. 4.** In acute liver failure, MSCs promote expansion of Tregs in paracrine, IDO dependent manner. (A) Serum AST and ALT levels; (B) representative H & E–stained mouse livers. Percentage of necrotic liver tissue, estimated by ImageJ software, (C) total number of liver-infiltrating IL10-producing $CD4^+CD25^+FOXP3^+$ Tregs; (D) expression of IDO in the livers. (E) Scheme of experimental design. (F) The percentage of IL10-producing cells in a population of $CD4^+FOXP3^+$ Tregs cocultured with MSCs in transwell systems in the presence or in the absence of IDO inhibitor; (G) hepatotoxicity of NKTs against hepatocytes in vitro determined by the xCELLigence system.

indicating that MSC-mediated priming of Tregs is IDO dependent ($P < 0.05$; Fig. 4G).

# Discussion

Therapeutic potential of MSCs in the treatment of acute liver failure has already been well described by us and others.[1,8,9] It is well-known that MSCs promote induction and expansion of Tregs and Bregs,[33-39] but the impact of these regulatory cells in MSC-mediated attenuation of acute liver failure was unknown. Accordingly, herewith, we described the molecular mechanisms involved in the crosstalk between MSCs and liver regulatory cells during acute liver inflammation.

Since it was recently shown that Bregs play an important role in the attenuation of fulminant hepatitis[19] and that, under inflammatory conditions, MSCs reduce plasmablast formation and promote development of IL10-producing Bregs,[29,40] we first analyzed the interaction between MSCs and Bregs in the injured livers. Although a significantly higher number of MZ-like IL10-producing and TGF-$\beta$–producing Bregs was noticed in the livers of α-GalCer+MSCs–treated mice when compared with α-GalCer–treated animals (Fig. 1F), the increased presence of Bregs was not essential for MSC-mediated suppression of acute liver failure, because their depletion did not alter the capacity of MSCs to attenuate acute liver failure (Fig. 2). However, depletion of Tregs completely abrogated beneficial effects of MSCs (Fig. 3), indicating the necessity and importance of Tregs for MSC-mediated alleviation of acute liver failure.

Tregs are residing in the liver where they have a crucial role in the maintenance of immunologic tolerance.[41] As a result of the decreased number of intrahepatic Tregs, the liver rapidly switches from an immune privilege organ into the organ enriched with the inflammatory and hepatotoxic cells.[42] Because liver Tregs are mainly responsible for the suppression of immune cell–mediated damage of hepatocytes during acute liver injury,[43,44] their depletion resulted in the aggravation of acute liver failure (Supporting Fig. 3C,D). Accordingly, repopulation of intrahepatic Tregs is a crucially important step for the attenuation of acute liver inflammation.[41] MSCs are able to promote induction and expansion of Tregs in vitro.[45] In line with these findings, we demonstrated that a single intravenous injection of MSCs or MSC-CM managed to rapidly increase the total number of Tregs in the injured livers (Figs. 1D and 4C), resulting with the attenuation of acute liver failure. Importantly, depletion of Tregs completely abrogated hepatoprotective effects of MSCs (Fig. 3A-C) and inhibited their capacity to attenuate hepatotoxicity of liver NKTs (Fig. 3D), indicating that Tregs were critically involved in MSC-based modulation of acute liver inflammation.

It has already been shown by us and others that adoptive transfer of Tregs reduce hepatic damage in mice.[44,46] Herewith, we demonstrated that MSCs significantly enhanced immunosuppressive and hepatoprotective potential of Tregs (Fig. 3F-I). When Tregs were cocultured with MSCs, their capacity to migrate in the injured livers and suppress acute liver inflammation was significantly intensified (Fig. 3H,I). An increased expression of CD62L and CCR7 (Fig. 3H) and increased production of immunosuppressive IL10 and TGF-$\beta$ were noticed in MSC-primed Tregs (Fig. 3I). Because CD62L and CCR7 are involved in MSC-regulated migration of Tregs[30] and IL10 and TGF-$\beta$ are mainly responsible for Treg-dependent attenuation of acute liver injury,[25] adoptive transfer of MSC-primed Tregs resulted in the complete attenuation of acute liver failure (Fig. 3F,G).

Previously published studies showed that MSCs can promote generation and expansion of Tregs in a paracrine manner through the production of prostaglandin E2 and TGF-$\beta$.[37] Because we did not find any differences in the serum levels of prostaglandin E2 and TGF-$\beta$ between α-GalCer and α-GalCer+MSC–treated animals (Supporting Fig. 5A,B), but we noticed increased levels of IDO and kynurenine in α-GalCer+MSC–treated mice (Supporting Fig. 5C,D), we focused our attention on IDO, an important MSC-derived immunomodulatory factor. MSC-derived IDO inhibits the generation of cytotoxic T lymphocytes and attenuates cytotoxicity of natural killer cells.[47,48] Most recently, we described the importance of IDO for MSC-mediated suppression of NKTs, major effector cells in acute liver failure.[8,9] It is well-known that intravenously injected α-GalCer induces production of IFN$\gamma$ in liver NKTs.[8] Herewith, we showed that MSCs, under the influence of increased levels of IFN$\gamma$ (Supporting Fig. 5E), increased IDO activity (Supporting Fig. 5C,D) and in an IDO-dependent manner reduce hepatotoxicity of NKTs (Fig. 3) resulting with the attenuation of acute liver injury and inflammation (Fig. 1). MSC-derived IDO was crucially important for the expansion of Tregs in the livers of MSCs and MSC-CM–treated

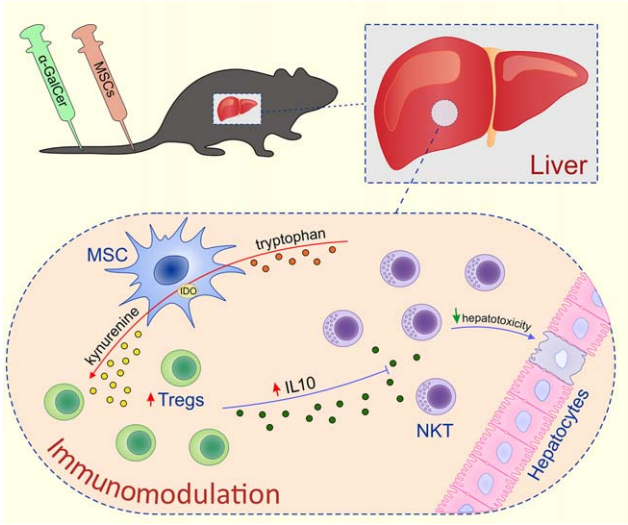


**FIG. 5.** Schematic diagram describing the mechanism responsible for MSC-mediated induction of Treg cells and attenuation of acute liver failure. MSCs, in paracrine manner, through the production of IDO, induced increased production of IL10 in Tregs, which in turn, in IL10-dependent manner, attenuated hepatotoxicity of liver NKTs, resulting with the attenuation of fulminant hepatitis.

mice with acute liver failure (Fig. 4). IDO-mediated regulation of tryptophan metabolism is a highly versatile modulator of cellular immunity.[6] IDO converts tryptophan to kynurenine, which inhibits the proliferation of effector T cells,[49] but promotes expression of Treg lineage-defining transcription factor FOXP3.[41] In line with these findings, injection of MSCs or MSC-CM significantly increased the total number of liver Tregs (Fig. 1D and 4C), a phenomenon that was completely abrogated when MSCs were cultured in the presence of IDO inhibitor (Fig. 4C). Moreover, an addition of 1-MT in MSC-Tregs coculture significantly attenuated the capacity of MSC-primed Tregs to suppress hepatotoxicity of NKTs (Fig. 4G).

Tregs suppress activation of liver NKTs in an IL10-dependent manner.[50] Intravenously injected IDO induces the production of immunosuppressive IL10 in activated lymphocytes, whereas 1-MT significantly reduces capacity of stimulated lymphocytes to secrete IL10.[51] Because MSCs, in a paracrine manner, significantly increased the capacity of Tregs to produce immunosuppressive IL10 and that this phenomenon was abrogated by 1-MT (Fig. 4F), we propose that MSCs, through the production of IDO, induced increased production of IL10 in Tregs, which in turn, in an IL10-dependent manner, attenuated hepatotoxicity of liver NKTs, resulting in the attenuation of acute liver failure (Fig. 5).

In conclusion, our data highlighted the crucial importance of Tregs for MSC-based attenuation of acute liver failure and indicated the significance of MSC-mediated priming of Tregs as a new therapeutic approach in Tregs-based therapy of acute liver failure.

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