Mesenchymal Stem Cells Attenuate Acute Liver Injury by Altering Ratio Between Interleukin 17 Producing and Regulatory Natural Killer T Cells

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Mesenchymal stem cells (MSCs) are, due to immunomodulatory characteristics, considered as novel agents in the treatment of immune-mediated acute liver failure. Although it is known that MSCs can regulate activation of T lymphocytes, their capacity to modulate function of neutrophils and natural killer T (NKT) cells, major interleukin (IL) 17–producing cells in acute liver injury, is still unknown. By using 2 well-established murine models of neutrophil and NKT cell–mediated acute liver failure (induced by carbon tetrachloride and α-galactoceramide), we investigated molecular and cellular mechanisms involved in MSC-mediated modulation of IL17 signaling during acute liver injury. Single intravenous injection of MSCs attenuate acute hepatitis and hepatotoxicity of NKT cells in a paracrine, indoleamine 2,3-dioxygenase (IDO)–dependent manner. Decreased levels of inflammatory IL17 and increased levels of immunosuppressive IL10 in serum, reduced number of interleukin 17–producing natural killer T (NKT17) cells, and increased presence of forkhead box P3 + IL10–producing natural killer T regulatory cells (NKTregs) were noticed in the injured livers of MSC-treated mice. MSCs did not significantly alter the total number of IL17-producing neutrophils, CD4+, and CD8 + T lymphocytes in the injured livers. Injection of mesenchymal stem cell–conditioned medium (MSC-CM) resulted with an increased NKTreg/NKT17 ratio in the liver and attenuated hepatitis in vivo and significantly reduced hepatotoxicity of NKT cells in vitro. This phenomenon was completely abrogated in the presence of IDO inhibitor, 1-methyltryptophan. In conclusion, the capacity of MSCs to alter NKT17/NKTreg ratio and suppress hepatotoxicity of NKT cells in an IDO-dependent manner may be used as a new therapeutic approach in IL17-driven liver inflammation.

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Interleukin (IL) 17 plays an important role in the pathogenesis of immune-mediated liver diseases.[1] Plasma levels of IL17 were significantly increased in patients suffering from alcoholic liver diseases, whereas IL17-producing T lymphocytes and neutrophils contributed to the inflammatory infiltrates in the livers of patients with alcoholic cirrhosis and hepatitis.[2] IL17 was shown as a key regulator in hepatic injury caused by neutrophil-induced inflammatory responses.[3] An increased hepatic expression of IL17 and elevated serum levels of IL17 correlated with the severity of hepatocyte damage and with the presence of IL17-expressing CD4 + T and CD3+NK1.1 + natural killer T (NKT) cells in the T cell model of acute liver failure.[4] Overexpression of IL17 resulted in massive hepatocyte necrosis, whereas blockage of IL17 signaling significantly ameliorated acute hepatitis, indicating that blockade of the IL17/IL17R signaling pathway

Abbreviations: 1-MT, 1-methyl-DL-tryptophan; α-GalCer, α-galactosylceramide; ALT, alanine aminotransaminase; APC, allophycocyanin; AST, aspartate aminotransferase; CCl_4, carbon tetrachloride; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FOXP3, forkhead box P3; H & E, hematoxylin-eosin; HepG2, liver hepatocellular carcinoma cells; HGF, hepatocyte growth factor; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon gamma; IL, interleukin; Ly6-G, lymphocyte antigen 6 complex locus G; MNC, mononuclear cell; MSC, mesenchymal stem cell; MSC-CM, mesenchymal stem cell–conditioned medium; NKp46, natural killer protein 46; NKT, natural killer T; NKT17, interleukin 17–producing natural killer T; NKTreg, natural killer T regulatory; PGE_2, prostaglandin E_2; RORγT, RAR-related orphan receptor gamma T; SEM, standard error of the mean; T_h, T helper; TCRγδ,

may represent a novel therapeutic approach in fulminant hepatitis.[4]

Mesenchymal stem cells (MSCs) are adult stem cells that can be found in almost all postnatal organs, including the liver.[5] MSCs can alter immune response through cell-to-cell contact or through the production of soluble factors.[6] Because of their immunomodulatory characteristics and because of their potential for differentiation into hepatocytes, MSCs may be considered novel therapeutic agents in the treatment of acute liver failure. Although it is well known that MSCs can regulate proliferation, activation, and effector function of T lymphocytes, professional antigen-presenting cells (dendritic cells, macrophages, B lymphocytes), and natural killer cells,[5,6] their capacity to modulate the function of neutrophils and NKT cells, major IL17-producing effector cells in acute liver injury, is still unknown.

By using 2 well-established murine models of neutrophil– and NKT cell–mediated acute liver failure (induced by carbon tetrachloride [CCl_4] and α-galactosylceramide [α-GalCer]), we investigated molecular and cellular mechanisms involved in MSC-mediated modulation of IL17 signaling during the pathogenesis of acute liver injury.

We provide the evidence that a single intravenous injection of MSCs attenuate acute hepatitis in a paracrine, indoleamine 2,3-dioxygenase (IDO)–dependent manner and that this effect was accompanied with decreased serum levels of IL17 and increased serum levels of IL10, reduced number of IL17-producing NKT cells, and an increased presence of forkhead box P3 (FOXP3) + IL10–producing NKT regulatory cells in the liver.

T-cell receptor gamma delta; TNFα, 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 (Catalog Number S10502-01). 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, St. Louis, MO), at 37 °C in a 5% CO_2 incubator. MSCs from passage 6 were used throughout these experiments. Human liver hepatocellular carcinoma cell line, liver hepatocellular carcinoma cells (HepG2) (ATCC HB-8065), were maintained in DMEM supplemented with 10% FBS, at 37 ° in a 5% CO_2 incubator.

GENERATION OF MESENCHYMAL STEM CELL–CONDITIONED MEDIUM

MSCs were seeded at a density of 10,000 cells/cm^2. In order to collect the mesenchymal stem cell-conditioned medium (MSC-CM), MSCs were first cultured in serum-containing complete medium and incubated at 37 °C in a humid atmosphere with 5% CO_2. At 80% confluence, the cells were washed twice with 1 × phosphate-buffered saline (Invitrogen, Carlsbad, CA), and the medium was then changed to serum-free medium. After 48 hours, the media were collected, centrifuged at 13,000*g* at 4 °C for 10 minutes, and stored at −80 °C until used.[7]

PHARMACOLOGICAL INHIBITION OF IDO

MSCs were cultured for 48 hours in culture medium containing 1 mM of 1-methyl-DL-tryptophan (1-MT; Sigma-Aldrich), an inhibitor of IDO enzymatic activity.[8]

ANIMALS

The 8-10–week-old male wild-type C57BL/6 mice were used for induction of acute liver injury. Mice were maintained in animal breeding facilities at the Faculty of Medical Sciences, University of Kragujevac,

Kragujevac, Serbia. All procedures were performed in accordance with the guidelines for the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals*, and all animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 86-23, 1985 revision). All experiments were approved by the Animal Ethical Review Board of the Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia. Mice were housed in a temperature-controlled environment with a 12-hour light-dark cycle and were administered with standard laboratory chow and water ad libitum. We used 10 mice per group.

INDUCTION OF ACUTE LIVER INJURY AND LIVER NECROSIS

Solution of CCl_4 (Sigma-Aldrich) and corn oil (Sigma-Aldrich) in ratio 1:1 and dose 2 μL/g body weight is applied intraperitoneally in a single dose.[9] All control mice were injected with corn oil only.

Alternatively, mice were given a single intravenous injection of α-GalCer (50 μg/kg) dissolved in 200 μL of saline.[10] Control animals received saline only.

Serum levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were measured 24 hours after CCl_4/corn oil treatment[9] or 16 hours after intravenous injection of α-GalCer.[10]

ADMINISTRATION OF MSCs

Also, 5×10^5 MSCs were intravenously injected into mice immediately after CCl_4 or α-GalCer administration.

HISTOLOGICAL ANALYSES AND SEMIQUANTITATIVE ASSESSMENT OF LIVER INJURY

Histological analysis and semiquantitative determination of liver injury were performed as previously described.[11] Briefly, the isolated livers were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and consecutive 4-μm 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 digital camera.

The area of necrosis was quantified using the Autodesk AutoCAD 2009 software application for design and drafting. Liver tissue sections were photographed, and each photograph of the tissue sample was imported into a newly created Autodesk AutoCAD 2009.dwg file. Using the "polyline" tool, "polyline" regions were drawn around the whole sample (marked with A) and around each of the necrotic areas in the photograph (marked with B). Then, the surface areas of the drawn regions were determined. First, the surface area of the A region was determined, and then the surface areas of each of the B regions (one by one) were determined. The surface area of each drawn region is presented as a unitless number in the Autodesk AutoCAD program. After all of the photographs from a whole liver tissue section were examined, the percentage of necrotic area was calculated by using the formula: $\mathbf{N = Bt \times 100/At}$, where N is the percentage (%) of necrotic area in the whole tissue section, At (A total) is the sum of the sample surface areas in the whole tissue section ($\mathbf{At = A1 + A2 + \ldots An}$, where is n is the number of photographs), Bt (B total) is the sum of the necrotic surface areas in the whole tissue section ($\mathbf{Bt = B1 + B2 + \ldots Bm}$, where m is the number of marked necrotic fields).[11]

MEASUREMENTS OF CYTOKINES IN SERUM

Commercial enzyme-linked immunosorbent assay (ELISA) sets (R&D Systems, Minneapolis, MN) were used to measure concentration of IL17, tumor necrosis factor α (TNFα), and IL10 cytokines according to the manufacturer's instructions. Blood samples were collected 24 hours after CCl_4 administration and 2 hours after α-GalCer injection from abdominal aorta during the euthanasia procedure. Serum was separated by centrifugation and stored at −80 °C.

MEASUREMENTS OF MSC-DERIVED IMMUNOSUPPRESSIVE FACTORS

IDO content of serum was determined using an ELISA kit (NeoBioLab) according to the manufacturer's instructions. Levels of hepatocyte growth factor (HGF) and prostaglandin E_2 (PGE_2) in the serum were measured using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

ISOLATION OF HEPATIC MONONUCLEAR CELLS AND FLOW-CYTOMETRIC ANALYSIS

The isolation of liver-infiltrating mononuclear cells (MNCs) was conducted as previously described.[12] Hepatic MNCs were screened for various cell surface and intracellular markers with flow cytometry 24 hours after CCl_4/corn, and 2 hours after α-GalCer injection. Briefly, isolated MNCs were incubated with murine anti-mouse CD3, CD4, CD49b, CD8, CD25, CD45, lymphocyte antigen 6 complex locus G (Ly6-G), and CD11b monoclonal antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, 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 IL17 and IL10 by using the fixation/permeabilization kit and conjugated anti-mouse monoclonal antibodies (BD Bioscience). For intracellular cytokine staining, cells were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate and 500 ng/mL of ionomycin for 5 hours, and GolgiStop (BD Biosciences) was added. Cells were fixed in Cytofix/Cytoperm, permeated with 0.1% saponin, and stained with fluorescent antibodies. Intracellular staining for FOXP3 was performed using the BD Bioscience fixation/permeabilization buffer kit following the manufacturer's instructions. After incubation, the cells were washed twice with phosphate-buffered saline containing 0.01% sodium azide followed by fixation in 200 μl of fixative solution (10 g/L of paraformaldehyde, 1% cacodylic acid, 6.65 g/L of sodium chloride, 0.01% sodium azide). Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analyzed by using the Flowing software analysis program.

ISOLATION OF NKT CELLS

NKT cells were isolated from hepatic MNCs by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions. Single-cell suspensions of MNCs derived from the liver were labeled with a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against natural killer protein 46 (NKp46), CD45R, CD8a, CD115, and T-cell receptor gamma delta (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$^+$ NKT cells 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). Purity of isolated NKT cells was checked by FACS analysis. Isolated NKT cells were then used in the coculture experiments and cytotoxicity assay as purified NKT cells.

CO-CULTURE OF MSCs AND NKT CELLS

NKT cells, in vitro stimulated with α-GalCer (100 ng/mL), were cultured alone and physically separated from MSCs using a 0.4-μm porous transwell system (Corning Incorporated, Life Sciences, France). Control cultures of NKT cells without α-GalCer stimulation were also included in all experiments. For contact-independent cocultures, NKT cells were placed in the lower chamber (24 wells) and MSCs were seeded in the transwell inserts, at ratio 10:1.[13] After 48 hours of culture, activated NKT cells were harvested for flow cytometry analysis or cytotoxicity assay.

CYTOTOXICITY ASSAY

HepG2 cells (4×10^4 HepG2 cell/well) were used as targets for NKT cells isolated from α-GalCer+MSC–treated mice and α-GalCer–treated mice, as well as for NKT cells isolated from livers of α-GalCer-, α-GalCer+MSC-CM-, and α-GalCer+MSC-CM+1-MT–treated mice that were restimulated with α-GalCer in complete medium, α-GalCer in MSC-CM, or α-GalCer in MSC-CM+1-MT, respectively. An effector to target ratio (E:T ratio) of 10:1 was used.[14] The E-plate 16 wells were placed in the xCELLigence RTCA DP (ACEA Biosciences, San Diego, CA), and impedance measurements were recorded for 6 hours at 37° and 5% CO_2. NKT cell–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, version 1.2 (Acea Biosciences, San Diego, CA).

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS, version 20.0 (IBM, Armonk, NY). Data are presented as means ± standard error of the mean (SEM). Statistical

significance was determined by Student t test and, where appropriate, using Mann-Whitney U test. Statistical significance was assumed at $P < 0.05$.

Results

SINGLE INJECTION OF MSCs EFFICIENTLY ATTENUATED CCL_4-INDUCED ACUTE LIVER INJURY, LIVER INFILTRATION OF INFLAMMATORY CELLS, AND SERUM LEVELS OF IL17

Intravenous injection of MSCs significantly attenuated CCl_4-induced hepatotoxicity, as determined by liver enzyme tests (Fig. 1A), macroscopic analysis, and histology (Fig. 1B). Serum AST ($P = 0.002$) and ALT levels ($P = 0.002$) were significantly lower in CCl_4+MSCs–treated mice compared with mice that received CCl_4 only (Fig. 1A). Macroscopically, livers of CCl_4-treated mice appeared larger and with a pale and irregular surface indicative of severe hepatocellular damage. CCl_4-induced macroscopic changes were significantly attenuated in MSC-treated animals. Livers obtained from CCl_4- and MSC-treated mice had a smooth surface with uniform and soft textures, similar to the livers of control animals (Fig. 1B, upper panel).

Histological analysis confirmed these findings (Fig. 1B, middle panel). Liver sections in CCl_4-treated mice showed widespread areas of necrosis characterized by standard morphologic criteria (ie, loss of architecture, vacuolization, karyolysis, and increased eosinophilia). Extensive liver damage in CCl_4-treated mice was characterized by massive coagulative necrosis and cytoplasmic swelling of the majority of hepatocytes. On the contrary, there were only several solitary areas of necrotic tissue in CCl_4-treated mice that received MSCs resulting in a significantly reduced total size of the necrotic areas (Fig. 1B, lower panel left; $P = 0.002$).

CCl_4-induced hepatocyte damage was accompanied with extensive infiltration of leukocytes within liver lobules and around the central veins and portal tracts, indicating the ongoing inflammatory process. As it is shown in Fig. 1B (lower panel right), infiltration of CD45 + leukocytes in the liver was significantly reduced in CCl_4-treated mice that received MSCs (Fig. 1B, lower panel right; $P = 0.04$).

A diminished inflammatory injury in CCl_4+MSCs–treated mice correlated with the production of IL17 (Fig. 1C). Serum levels of IL17 were significantly lower in CCl_4-treated mice that received MSCs compared with CCl_4-only treated animals ($P = 0.047$). This was associated with lower levels of hepatotoxic TNFα ($P = 0.03$) and significantly higher levels of immunosuppressive and hepatoprotective IL10 ($P = 0.04$) in sera of CCl_4+MSCs mice (Fig. 1C).

MSCs SIGNIFICANTLY REDUCE THE NUMBER OF IL17-PRODUCING NKT CELLS, BUT DID NOT ALTER THE PRESENCE OF IL17-PRODUCING NEUTROPHILS, $CD4^+$, AND $CD8^+$ T LYMPHOCYTES IN THE LIVER OF CCL_4-TREATED MICE

In order to dissect out cellular target of MSC-dependent modulation of IL17-driven inflammation in the CCl_4-induced liver injury, flow cytometry analysis of liver-infiltrated leukocytes was performed. Intracellular staining of hepatic-infiltrating neutrophils and MNCs obtained from CCl_4-treated mice revealed that the vast majority of IL17-producing inflammatory cells in the injured livers were NKT cells. Accordingly, MSC-mediated attenuation of IL17 production in CCl_4-induced liver damage was mainly a consequence of MSC-dependent down-regulated production of IL17 in the liver NKT cells. There was a significantly lower number of IL17-producing (CD3 + CD49b+) NKT cells ($P = 0.04$) in CCl_4+MSC–treated mice compared with animals treated with CCl_4 only (Fig. 2A). On the contrary, there was no significant difference in the total number of liver infiltrating IL17-producing (CD45+Ly6-G+) neutrophils (Fig. 2B), $CD4^+$, and $CD8^+$ (CD3+CD49b–) T lymphocytes between CCl_4 and CCl_4+MSC treated mice (Fig. 2C,D).

IN NKT-CELL DEPENDENT MODEL OF ACUTE LIVER FAILURE, MSCs REDUCED TOTAL NUMBER OF IL17 + NKT CELLS AND INCREASED PRESENCE OF FOXP3 + IL10+NKT CELLS

Because we have demonstrated that MSCs significantly attenuate IL17-driven acute liver inflammation mainly by affecting production of IL17 in NKT cells, we further investigated this phenomenon in α-GalCer–induced liver injury, a well-established model

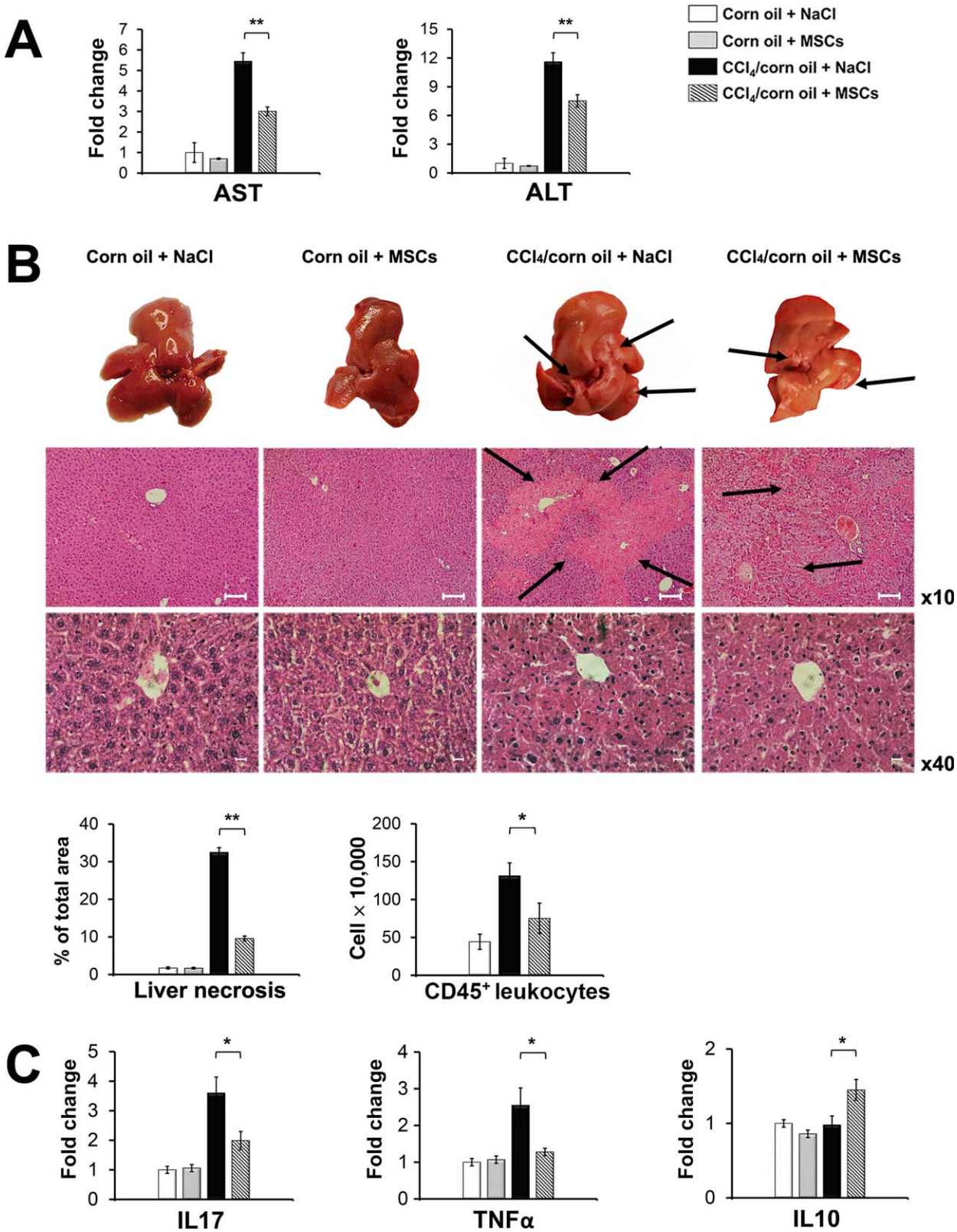

FIG. 1. MSCs ameliorate CCl_4-induced acute liver injury. (A) Serum AST and ALT levels, (B) Macroscopic appearance of the liver and representative H & E–stained mouse livers (upper panel; magnification × 10 and ×40). Semiquantitative determination of liver injury estimated by Autodesk AutoCAD 2009 software (lower panel left) and total number of CD45+leukocytes in the liver (lower panel right) as determined by flow cytometry. (C) The level of cytokines in serum. Data presented as mean ± SEM; n = 10 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

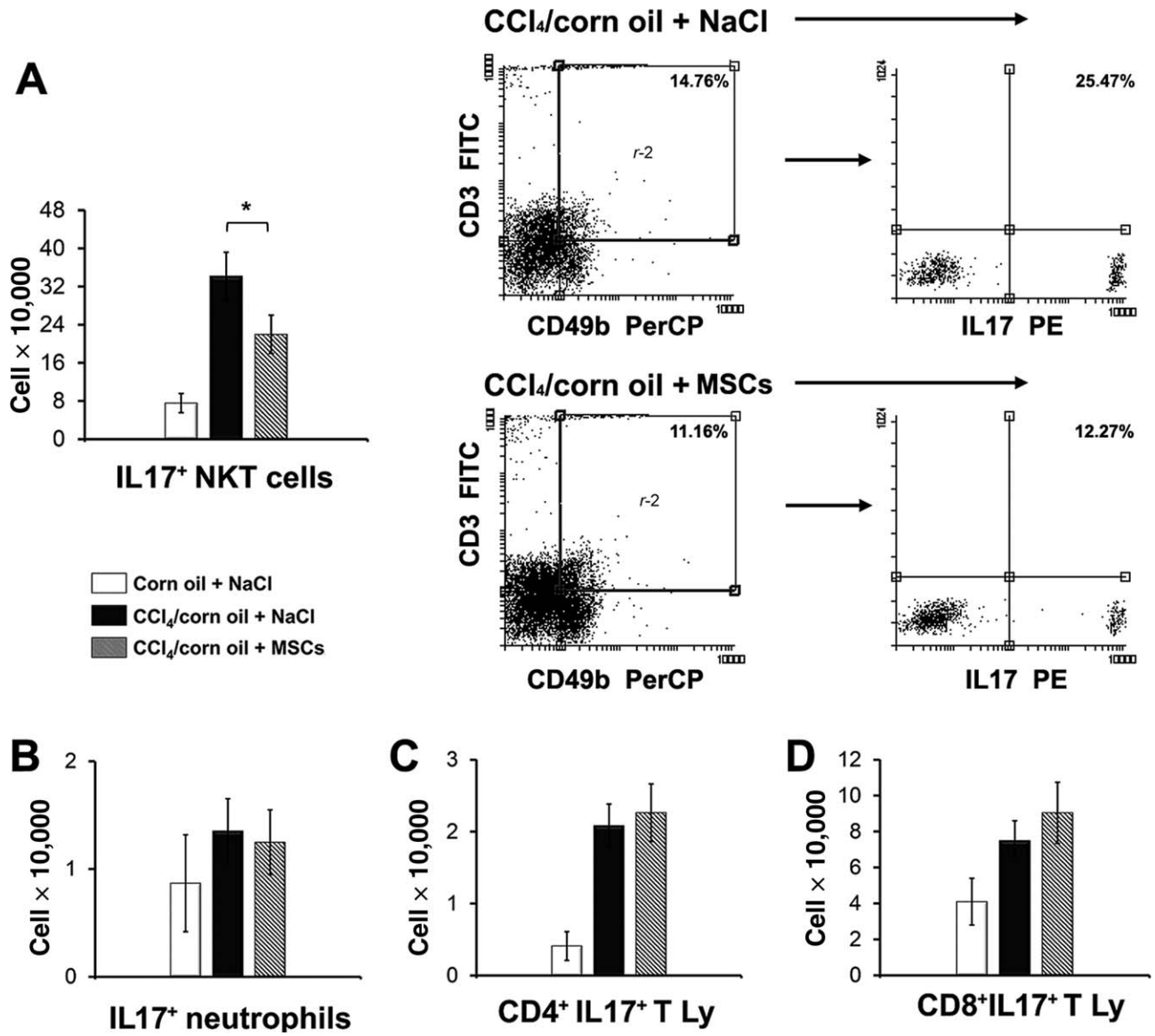

FIG. 2. MSCs reduce influx of IL17-producing NKT cells in the liver of CCl_4-treated mice. (A) Total numbers of liver infiltrating IL17-producing $CD3^+CD49b^+$ NKT cells with representative dot plots; total numbers of liver infiltrating IL17-producing (B) CD45+Ly6-G + neutrophils, (C) $CD3^+CD49b^-CD4^+$ and (D) $CD3^+CD49b^-CD8^+$ T cells in CCl_4 and CCl_4+MSC–treated mice. Data presented as mean ± SEM; n = 10 mice per group. $^*P < 0.05$.

of acute liver failure that is mediated by α-GalCer–activated liver NKT cells.[10]

A single intravenous administration of MSCs significantly reduced NKT-cell dependent liver damage, as confirmed by liver enzyme tests and histological analysis. As it is shown in Fig. 3A, MSCs significantly decreased serum levels of ALT ($P = 0.04$) and AST ($P = 0.01$) in α-GalCer–treated mice that correlated with markedly reduced necrosis of hepatocytes (Fig. 3B).

Intracellular staining revealed that MSC treatment managed to significantly reduce production of all inflammatory cytokines (IL17, interferon gamma [IFNγ], IL4) in liver NKT cells and that most of the NKT cells produce IL17 (Fig. 3C). Accordingly, MSCs attenuated the total number of liver-infiltrating IL17-producing NKT cells ($P = 0.02$; Fig. 3D) and down-regulated serum levels of IL17 ($P = 0.04$; Fig. 3E).

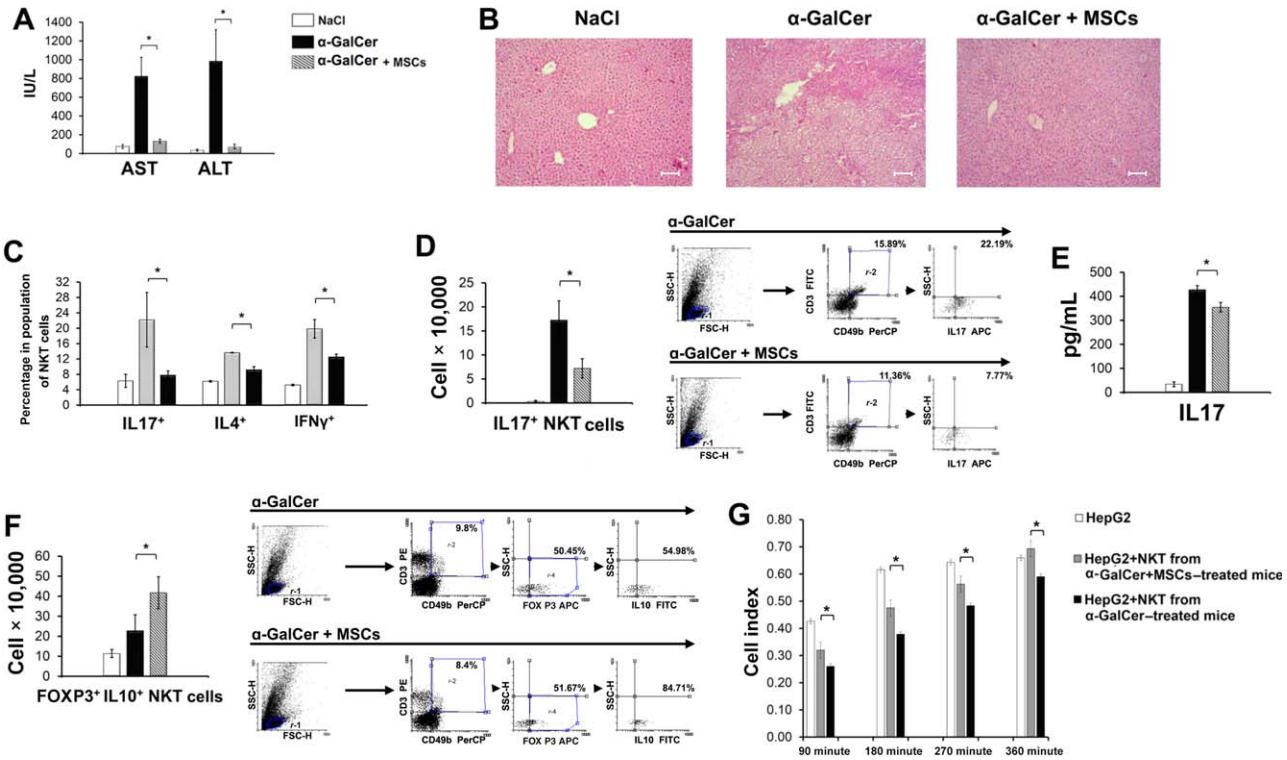

FIG. 3. MSC treatment attenuates the presence of hepatotoxic IL17 + NKT cells and increases the presence of protective FOXP3 + IL10+NKT cells in NKT cell–dependent model of acute liver failure. (A) Serum AST and ALT levels and (B) representative H & E–stained mouse livers. (C) Percentage of IL17, IL4, and IFNγ producing cells in the population of NKT cells; (D) Total number of IL17 producing NKT cells in liver with representative dot plots; (E) Serum levels of IL17; (F) Total number of liver-infiltrating IL10-producing FOXP3$^+$ regulatory NKT cells with representative dot plots; (G) The results obtained by xCELLigence system showed less cytotoxic activity of liver NKT cells isolated from mice treated with α-GalCer and MSCs. Data presented as mean ± SEM; n = 10 mice group. *$P < 0.05$.

Interestingly, a reduced number of IL17-producing NKT cells in the livers of α-GalCer+MSC–treated mice was accompanied with a significantly higher number of FOXP3$^+$ IL10$^+$ regulatory NKT cells ($P = 0.04$; Fig. 3E), indicating that MSC-mediated beneficial effects were related to the MSC-dependent modulation of the balance between interleukin 17–producing natural killer T (NKT17) and regulatory NKT cells within the liver.

MSCs SIGNIFICANTLY REDUCED HEPATOTOXICITY OF LIVER NKT CELLS

In order to further demonstrate that MSC-mediated suppression of IL17 production in NKT cells correlate with their capacity to directly induce damage of hepatocytes, we analyzed the cytotoxicity of NKT cells in vitro by using the xCELLigence system for monitoring real-time cytotoxicity. As it is shown in Fig. 3G, NKT cells isolated from α-GalCer+MSC–treated mice were significantly ($P = 0.03$) less cytotoxic against HepG2 cells then NKT cells isolated from animals treated with α-GalCer only, indicating that MSC treatment managed to significantly reduce hepatotoxic potential of liver NKT cells as well.

MSCs AFFECT THE CAPACITY OF NKT CELLS TO PRODUCE IL17 IN PARACRINE MANNER

In order to investigate whether cell-to-cell contact was required for MSC-dependent suppression of IL17 production in NKT cells and their attenuated hepatotoxicity, cocultures were performed in a transwell system where MSCs and liver NKT cells were separated by a membrane permeable to soluble molecules.

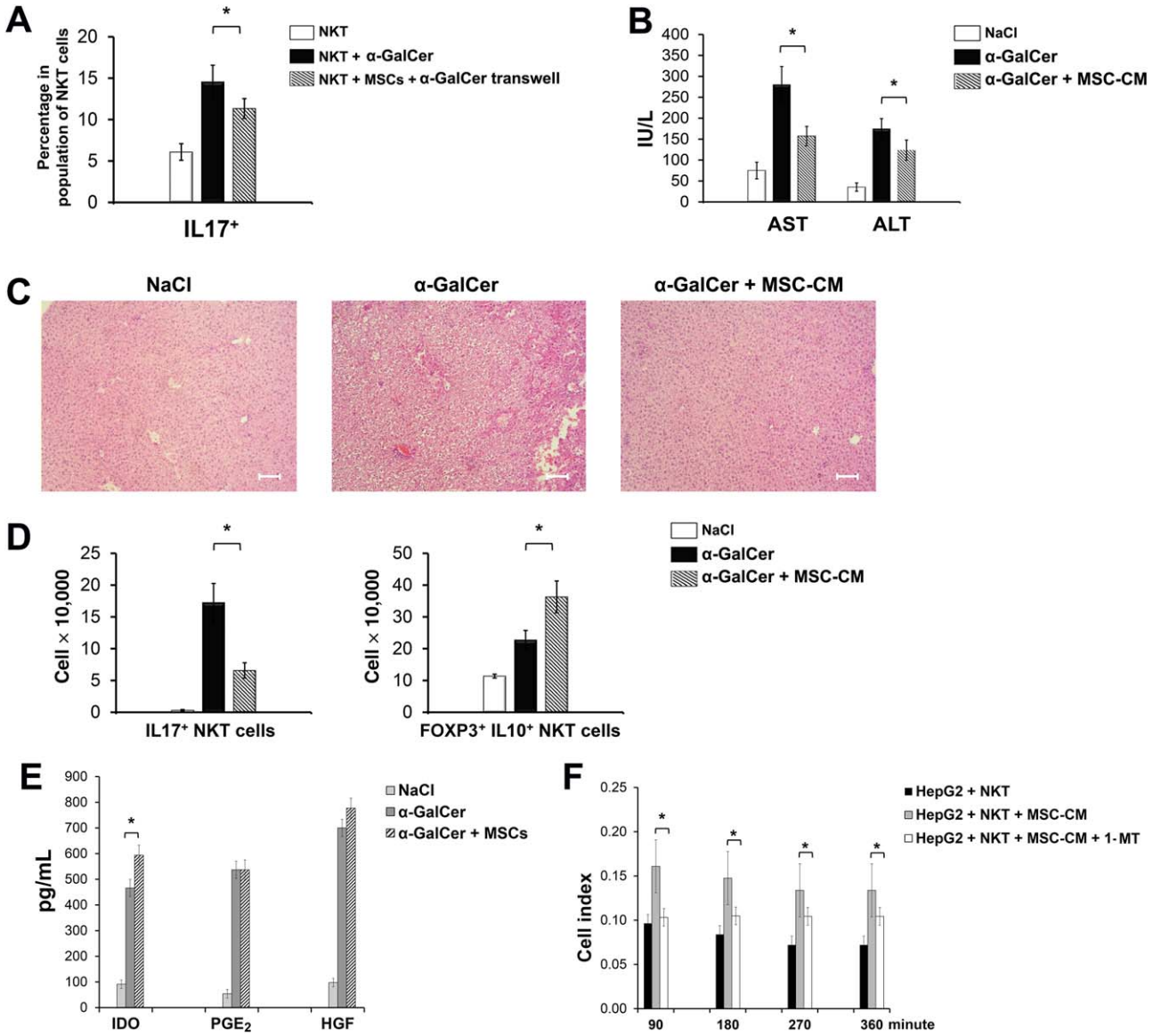

FIG. 4. MSCs affect the capacity of NKT cells to produce IL17 in an IDO-dependent manner. (A) Percentage of IL17-producing NKT cells cocultured with MSCs in transwell systems; (B) serum AST and ALT levels; (C) representative H & E–stained liver samples; (D) total number of liver infiltrating IL17-producing NKT cells; and (E) IL10-producing FOXP3 + regulatory NKT obtained from control, α-GalCer, and α-GalCer+MSC-CM treated mice; (F) Serum levels of IDO, PGE_2, and HGF in α-GalCer, and α-GalCer + MSC treated mice. (G) Cytotoxic potential of NKT cells against the HepG2 hepatocyte cells in vitro as determined by xCELLigence system. Data are shown as the mean ± SEM of 10 mice per group and are pooled from 2 independent experiments. $^*P < 0.05$.

Intracellular staining revealed that in vitro α-GalCer-activated NKT cells cultured with MSCs in a transwell system expressed significantly ($P = 0.02$) lower amounts of IL17 (Fig. 4A), indicating that MSCs reduce production of IL17 in NKT cells in paracrine manner.

To directly demonstrate that soluble factors were responsible for the MSC-mediated inhibition of IL17 production in NKT cells in vivo, mice were intravenously given MSC-CM and effects were similar to those observed after injection of MSCs. Intravenous injection of MSC-CM significantly down-regulated serum levels of AST ($P = 0.02$) and ALT ($P = 0.01$; Fig. 4B) and reduced hepatocyte damage (Fig. 4C) in α-GalCer–treated mice. Additionally, MSC-CM managed to significantly attenuate the total number of

liver IL17-producing NKT cells (Fig. 4D) and significantly ($P = 0.04$) increased the presence of FOXP3+IL10 + regulatory NKT cells in the liver of α-GalCer–treated mice (Fig. 4E), confirming that MSCs affect production of IL17 and IL10 as well as expression of FOXP3 in NKT cells in paracrine manner.

MSC-MEDIATED ATTENUATION OF NKT CELL HEPATOTOXICITY IS IDO-DEPENDENT

Because there is growing evidence that among MSC-derived soluble factors, IDO, PGE_2, and HGF are main mediators of MSC-mediated inhibition of cytokine production in immune cells,[15-17] these immunosuppressive factors were measured in the sera of MSC-treated mice with acute liver injury (Fig. 4F). Serum levels of IDO were significantly ($P = 0.03$) higher in α-GalCer+MSC–treated mice, although there was no difference in the levels of PGE_2 and HGF between MSC-treated and non-treated animals with acute liver failure.

In line with results obtained in vivo, MSC-CM also significantly ($P = 0.03$) attenuated hepatotoxicity of NKT cells in vitro (Fig. 4G), confirming that MSCs attenuated hepatotoxicity of NKT cells in a paracrine manner. Importantly, blockade of IDO activity completely abrogated the hepatoprotective capacity of MSC-CM. MSC-CM that contained IDO inhibitor (1-MT) did not manage to attenuate cytotoxicity of liver NKT cells against HepG2 cells, suggesting that MSC-mediated attenuation of NKT cell hepatotoxicity is IDO-dependent.

Discussion

NKT cells, the most abundantly present in the murine liver,[18-20] are the major effector cells in the pathogenesis of acute liver failure. The population of IL17-producing NKT cells, characterized by expression of transcriptional factor RAR-related orphan receptor gamma T (RORγT), are known as NKT17 cells and are considered a major IL17-producing population of cells in the pathogenesis of acute hepatitis. Additionally, IL17 signaling is important for neutrophil, CD4+, and CD8 + T cell–mediated hepatotoxicity as well. In line with these findings, we showed that MSC-mediated attenuation of acute hepatitis, followed by down-regulated serum levels of IL17, were mainly a consequence of MSC-mediated suppression of IL17 production in liver NKT cells, whereas the capacity of neutrophils and T lymphocytes for IL17 secretion was not altered by MSC therapy.

NKT cells, activated with α-GalCer, rapidly produce IL17 that in turn affect infiltration of other immune cells in the liver.[21] Accordingly, transplantation of MSCs as well as injection of MSC-CM managed to attenuate the capacity of liver NKT cells to produce IL17, which was followed by reduced infiltration of inflammatory cells in the CCl_4-injured and α-GalCer–injured livers (Figs. 1B, 3C, and 4D). These findings indicate that MSCs may suppress NKT17 cells in a paracrine manner and that cell-to-cell contact was not required for their modulation of the IL17 pathway in the liver.

During α-GalCer–induced liver injury, IL10-producing FOXP3 + regulatory T cells (Tregs) have been recruited to the liver where they suppress hepatic inflammation and immune response.[22] It is well known that dynamic changes in the frequencies of T helper (T_h) 17 and Tregs significantly affect outcome of acute hepatitis[23] and that an imbalance of the Treg/T_h17 ratio is usually linked to the progression of hepatitis.[24] Herewith, we showed that MSC-dependent suppression of NKT17 cells in the liver was accompanied with increased presence of hepatoprotective FOXP3 + IL10 producing NKT regulatory cells (Fig. 3D) and elevated serum levels of immunosuppressive IL10 (Fig. 1C), indicating that beneficial effects of MSC therapy in acute hepatitis is closely related to the modulation of the balance between NKT17 and regulatory NKT cells within the liver.

Progressive inflammatory diseases are associated with the loss of T_h17 cells and a reciprocal increase in the fraction of the immunosuppressive T regulatory cells both in peripheral blood and in inflamed tissues.[25] IDO, a potent immunosuppressive enzyme, has been identified as a critical molecular switch that stimulates immunosuppressive properties of Tregs and simultaneously blocks reprogramming of Tregs into IL17-producing effector T cells.[26] IDO inhibition or genetic deletion led to the reduced generation of Tregs and an increase in T_h17 differentiation both in vitro and in vivo.[26]

Under T_h1 and T_h2 conditions, IDO plays a key role in human MSC-mediated immunomodulation, whereas murine MSCs have lower IDO activity and usually use inducible nitric oxide synthase–mediated immunomodulation.[16,27,28] However, under T_h17 conditions, murine MSCs do not produce nitric oxide, and their immunosuppressive effects are mediated through the production of other mediators, including

IDO. MSCs are able to suppress the generation of effector T_h17 cells in IDO-dependent manner, and IDO inhibitors could be used to restore MSC suppression of T_h17 differentiation.[29] In line with these findings, we found that a MSC-mediated increase of the natural killer T regulatory (NKTreg)/NKT17 ratio in acute hepatitis correlated with increased serum levels of IDO and that MSC-dependent attenuation of NKT cell hepatotoxicity is IDO dependent.

In conclusion, the capacity of MSCs to alter the NKT17/NKTreg ratio and suppress hepatotoxicity of NKT cells in an IDO-dependent manner may be used as a new therapeutic approach in IL17-driven liver inflammation.

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