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RESEARCH ARTICLE

Mesenchymal stem cells protect from acute liver injury by attenuating hepatotoxicity of liver natural killer T cells in an inducible nitric oxide synthase- and indoleamine 2,3-dioxygenase-dependent manner

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Abstract

The effects of mesenchymal stem cells (MSCs) on the phenotype and function of natural killer T (NKT) cells is not understood. We used concanavalin A (Con A) and α-galactosylceramide (α-GalCer)-induced liver injury to evaluate the effects of MSCs on NKT-dependent hepatotoxicity. Mouse MSCs (mMSCs) significantly reduced Con A- and α-GalCer-mediated hepatitis in C57Bl/6 mice, as demonstrated by histopathological and biochemical analysis, attenuated the influx of inflammatory [T-bet$^+$, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ)-producing and GATA3$^+$, interleukin-4 (IL-4)-producing] liver NKT cells and downregulated TNF-α, IFN-γ and IL-4 levels in the sera. The liver NKT cells cultured *in vitro* with mMSCs produced lower amounts of inflammatory cytokines (TNF-α, IFN-γ, IL-4) and higher amounts of immunosuppressive IL-10 upon α-GalCer stimulation. mMSC treatment attenuated expression of apoptosis-inducing ligands on liver NKT cells and suppressed the expression of pro-apoptotic genes in the livers of α-GalCer-treated mice. mMSCs reduced the cytotoxicity of liver NKT cells against hepatocytes *in vitro*. The presence of 1-methyl-DL-tryptophan, a specific inhibitor of indoleamine 2,3-dioxygenase (IDO), or L-N^G-monomethyl arginine citrate, a specific inhibitor of inducible nitric oxide synthase (iNOS), in mMSC-conditioned medium injected into α-GalCer-treated mice, counteracted the hepatoprotective effect of mMSCs *in vivo* and restored pro-inflammatory cytokine production and cytotoxicity of NKT cells *in vitro*. Human MSCs attenuated the production of inflammatory cytokines in α-GalCer-stimulated human peripheral blood mononuclear cells in an iNOS- and IDO-dependent manner and reduced their cytotoxicity against HepG2 cells. In conclusion, MSCs protect from acute liver injury by attenuating the cytotoxicity and capacity of liver NKT cells to produce inflammatory cytokines in an iNOS- and IDO-dependent manner.

KEYWORDS

acute liver injury, IDO, immunosuppression, iNOS, mesenchymal stem cells, NKT cells

1 | INTRODUCTION

Mesenchymal stem cells (MSCs) are adult, multipotent cells that can be found in almost all postnatal tissues (Gazdic, Volarevic, Arsenijevic, & Stojkovic, 2015). Due to their immunomodulatory ability and capacity for self-renewal and differentiation into tissues of mesodermal origin, MSCs have been tested in many preclinical and clinical studies as possible new therapeutic agents for the treatment of immune disorders (Volarevic, Nurkovic, Arsenijevic, & Stojkovic, 2014). MSCs can alter the immune response and regulate the proliferation, activation and effector function of T lymphocytes, professional antigen-presenting cells [dendritic cells (DCs), macrophages, B lymphocytes] and natural killer

(NK) cells, through cell-to-cell contact or through the production of soluble factors (Gazdic et al., 2015). Prigione and colleagues recently described that human MSCs (hMSCs) could affect *in vitro* expansion of natural killer T (NKT) cells, in both a cell-to-cell contact and paracrine manner (Prigione et al., 2009), but MSC-mediated suppression of NKT cells *in vivo* has not been investigated.

NKT cells represent a unique T-cell subset expressing an invariant T-cell receptor (TCR) α chain (Vα14-Jα18 in mice; Vα24-Jα18 in humans) paired with a limited TCR Vβ chain repertoire (Vβ8.2, Vβ7 or Vβ2 in mice; Vβ11 in humans) together with NK cell-related markers (NK1.1 in mice; CD161 in humans) (Swain, 2008). NKT cells are the most abundantly present in the murine liver and are considered as the major effector cells in the pathogenesis of acute liver failure in mice (Biburger & Tiegs, 2005). To examine the MSC–NKT cell interaction *in vivo*, we used two well-established murine models of fulminant liver failure, concanavalin A (Con A)- and α-galactosylceramide (α-GalCer)-induced liver injury. Con A activates lymphocytes irrespective of their antigen specificity (Volarevic, Markovic, et al., 2014). NKT cells, T lymphocytes, and macrophages are the main immune cells involved in the pathogenesis of Con A-induced liver injury (Volarevic, Markovic et al., 2014). On the contrary, α-GalCer is dependent on the presentation of the Major histocompatibility complex (MHC)-homologous CD1d molecule and is known as a potent activator of NKT cells. Accordingly, 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 (Volarevic, Markovic et al., 2014), suggesting that α-GalCer-induced hepatitis as an ideal model for studying the pathogenesis of NKT-dependent liver disorders.

Herewith we provide the evidence that MSCs inhibit cytokine production and cytotoxic activity of NKT cells in an inducible nitric oxide synthase (iNOS)- and indoleamine 2,3-dioxygenase (IDO)-dependent manner and significantly attenuate Con A- and α-GalCer-induced NKT cell-dependent, acute liver injury. These findings could be helpful in highlighting the importance of NKT cells in immunomodulatory effects of MSCs in acute liver failure.

2 | MATERIALS AND METHODS

2.1 | Cells

Murine MSCs (mMSCs) isolated from bone marrow of C57BL/6 mice were purchased from Gibco Gibco/Invitrogen, Paisley, UK (no. S1502–100). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin G and 100 μg/ml streptomycin (Sigma-Aldrich, Munich, Germany), at 37°C in a 5% CO_2 incubator. mMSCs in passage 4 were used throughout the experiments. Consented (in accordance with the Guidelines of the Ethics Committee of the State University of Novi Pazar and Health Center of Novi Pazar) and characterized hMSCs isolated from human adipose tissue were obtained from Stem Cell Laboratory, Department of Biomedical Sciences, State University of Novi Pazar, Serbia (data not shown) (Nurkovic, Dolicanin, Tutic, & Kovacevic-Filipovic, 2013). hMSCs were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin G and 100 μg/ml streptomycin (Sigma-Aldrich) at 37°C in a 5% CO_2 incubator. hMSCs in passage 4 were used throughout these experiments. Human liver hepatocellular carcinoma cell line HepG2 cells (ATCC HB-8065) were maintained in DMEM supplemented with 10% FCS, at 37°C in a 5% CO_2 incubator.

2.2 | Hepatocyte isolation

Mouse hepatocytes were isolated as previously described (Volarevic et al., 2015). Briefly, a cannula was inserted in the portal vein and the liver was perfused for 15–20 min with T1 solution (0.9% NaCl, 0.05% KCl, HEPES 0.2%, 0.08 mg/ml EGTA, pH 7.4) (all from Sigma-Aldrich). As soon as the perfusion was started, the hepatic vein was immediately cut to allow perfusate to run as waste. The liver was then perfused with T2 solution (0.6% NaCl, 0.05% KCl, 1.2% HEPES, 0.07% $CaCl_2$, 3 g/ml collagenase type I, pH 7.4) (all from Sigma-Aldrich). 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 DMEM (Sigma-Aldrich), filtered through a 70 μm cell strainer and centrifuged at 600 rpm for 4 min. The supernatant was removed, the pellet was resuspended in 3 ml DMEM, cells were passed on 37.5% Percoll cushion (30 ml), centrifuged at 1050 rpm for 3 min and resuspended in 2 ml DMEM; viable hepatocytes were counted.

2.3 | Generation of MSC-conditioned medium (MSC-CM)

mMSCs or hMSCs were seeded at a density of 10 000 cells/cm^2. In order to collect the mMSC-CM or hMSC-CM, mMSCs or hMSCs 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, USA). The medium was then changed to serum-free medium. After 48 h, the media was collected, centrifuged at 13 000×*g* at 4°C for 10 min and stored at −80°C until used (Linero & Chaparro, 2014).

2.4 | Pharmacological inhibition of IDO and iNOS

mMSCs or hMSCs were cultured for 48 h in culture medium containing 1 mM 1-methyltryptophan (1-MT, Sigma-Aldrich), an inhibitor of IDO enzymatic activity (Yang et al., 2009).

To block iNOS activity, mMSCs or hMSCs were cultured for 48 h in the presence of 1 mM of an iNOS inhibitor, L-N^G-monomethyl arginine citrate (L-NMMA, Sigma-Aldrich) (Ren et al., 2008).

2.5 | Animals

Male 6–8-week-old C57Bl/6 mice were used. All animals received human care 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 h light–dark cycle and were administered standard laboratory chow and water *ad libitum*. Per group we used 10 mice.

2.6 | Con A-induced hepatitis

Wild-type C57Bl/6 mice were given a single intravenous injection of Con A (Sigma-Aldrich) at 12 mg/kg bodyweight dissolved in 250 μl saline (Volarevic, Misirkic et al., 2015). mMSCs (5×10^5) were intravenously injected, via the tail vein, immediately after Con A administration (Con A + mMSC-treated mice). Control animals received the appropriate amount of mMSCs only or saline only. Serum levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were measured 24 h after Con A administration, by a standard photometric method using the automated biochemistry analyser Olympus AU 400 (Olympus Diagnostica GMBH, Hamburg, Germany) and Olympus AU reagents, according to the manufacturer's instructions (Volarevic, Milovanovic et al., 2012; Volarevic, Mitrovic et al., 2012).

2.7 | α-GalCer-induced hepatitis

Wild-type C57BL/6 mice were given a single intravenous injection of α-GalCer (50 μg/kg) dissolved in 200 μl saline (Volarevic, Markovic et al., 2014). mMSCs (5×10^5) were intravenously injected, via the tail vein, into C57Bl/6 mice immediately after α-GalCer administration (α-GalCer + mMSC-treated mice), whereas control animals received the appropriate amount of mMSCs only or saline only. To demonstrate that soluble factors were responsible for the mMSC-mediated inhibition of NKT cell function, mice were injected intravenously with 0.2 ml mMSC-CM, 0.2 ml mMSC-CM + 1-MT or 0.2 ml mMSC-CM + L-NMMA, immediately after α-GalCer administration. Control animals received 0.2 ml saline only. Serum levels of AST and ALT were measured 16 h after intravenous injection of α-GalCer (Volarevic, Markovic et al., 2014).

2.8 | Histological analyses and semi-quantitative assessment of liver injury

Histological analysis and semi-quantitative determination of liver injury were performed as previously described (Volarevic, Mitrovic et al., 2012). 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 haematoxylin and eosin and examined under low-power (100×) light microscopy (Zeiss Axioskop 40, Jena, Germany) equipped with a digital camera. Sections were examined by two independent investigators in a blind manner.

2.9 | Isolation of hepatic mononuclear cells (MNCs) and analysis with flow cytometry

The isolation of liver-infiltrating MNCs was conducted as previously described (Volarevic, Mitrovic et al., 2012). Hepatic MNCs were screened for various cell surface and intracellular markers with flow cytometry 8 h after Con A and 2 h after α-GalCer injection. Briefly, 1×10^6 MNC were incubated with R-PE-labelled murine CD1d tetramer preloaded with α-GalCer (ProImmune, Oxford, UK), anti-mouse CD4, CD3, CD8, CD25, F4/80, CD11c, CD80, CD86, CD19, FasL, CD107, TRAIL, perforin, granzyme B, NKG2D and KLRG1 monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC) (all from BD Biosciences, San Jose, CA, USA) following manufacturer's instructions. MNCs derived from the liver were concomitantly stained for the intracellular content of tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-10, IL-12, T-bet and GATA3 by using the 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 Phorbol myristate acetate (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 and stained with fluorescent antibodies. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analysed by using the Flowing software analysis program.

2.10 | Detection of mMSCs in the livers of con A- and α-GalCer-treated mice

mMSCs were fluorescence-labelled using pre-incubation with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, or, USA) according to the manufacturer's instructions, as described previously (Rüster et al., 2006). For homing assays, 5×10^5 CFSE-labelled mMSCs were injected into the tail veins of mice immediately after Con A or α-GalCer administration. MNC suspensions were prepared from liver tissues 8 h after Con A and 2 h after α-GalCer injection and analysed by flow cytometry.

2.11 | Isolation of NKT cells

NKT cells were isolated from hepatic MNCs by magnetic cell sorting according to the manufacturer's instructions. Single-cell suspensions of MNCs derived from the liver were labelled with a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against NKp46, CD45R, CD8a, CD115 and TCRγδ and MicroBeads conjugated to monoclonal anti-biotin antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The labelled 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 labelled with monoclonal anti-mouse NK1.1 antibody conjugated to APC and microBeads conjugated to monoclonal anti-mouse anti-APC antibody and positively selected using a MACS Column (Miltenyi Biotec) and a MACS Separator (Miltenyi Biotec). Isolated NKT cells were then used in the co-culture experiments and cytotoxicity assay as purified NKT cells. NKT cells isolated from livers of α-GalCer-, α-GalCer + mMSC-CM-, α-GalCer + mMSC-CM + 1--MT- and α-GalCer + mMSC-CM + L-NMMA-treated mice were restimulated with α-GalCer in complete medium, α-GalCer in mMSC-CM, α-GalCer in mMSC-CM + 1-MT or α-GalCer in mMSC-CM + L-NMMA, respectively. After 48 h, supernatants were collected for cytokine measurement and NKT were harvested for the cytotoxicity assay.

2.12 | Preparation of human peripheral blood mononuclear cells (PBMNCs)

The serum samples were obtained from fasting participants in the morning and PBMNCs were prepared using Histopaque

(SigmaAldrich) density gradient centrifugation, as previously described (Müller et al., 2006).

2.13 | Co-culture of mMSCs and NKT cells

NKT cells, stimulated *in vitro* with α-GalCer (100 ng/ml), were cultured alone and physically separated from mMSCs 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 co-cultures, NKT cells were placed in the lower chamber (24 well) and mMSCs were seeded in the transwell inserts, at a ratio of 10:1 (Krampera et al., 2013). After 48 h of culture, supernatants were collected and frozen at −20°C until cytokine concentrations were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA), while activated NKT were harvested for flow cytometry analysis or the cytotoxicity assay. hMSC and PBMNC interaction was examined in a similar set-up. PBMNCs, in complete medium supplemented with α-GalCer (100 ng/ml), were added to the lower chamber and hMSCs were added to the upper chamber, at a ratio of 10:1 (Prigione et al., 2009). The levels of cytokines in supernatants and the cytotoxicity of PBMNCs were evaluated after 48 h of culture. α-GalCer-stimulated PBMNCs (100 ng/ml) had been cultured for 48 h in the hMSC-CM in the presence or absence of 1 mM 1-MT or 1 mM L-NMMA (Sigma-Aldrich). Culture supernatants and cytokine-containing cells were collected for cytokine measurement and the cytotoxicity assay.

2.14 | Cytotoxicity assay

The DP version of the xCELLigence system (Roche CA, USA) was used in this study. The DP version comprises a measurement unit housed within a standard tissue culture incubator with three stations that each take E16 plates (each E16 plate has 16 wells). Complete medium (100 μl) was added to each well and background impedance on the plates was measured on the xCELLigence RTCA DP instrument at 37°C and 5% CO_2. HepG2 cells were used as targets for NKT cells. The seeding density of 4×10^4 HepG2 cells/well was considered optimal and used for all assays. An effector to target ratio (E:T ratio) of 10:1 was used (Wang et al., 2013). HepG2 cells were resuspended in DMEM with 10% FCS at 4×10^5 cells/ml. In total, 100 μl tumour cells were added to each well of the E16 plate, which was then placed in the xCELLigence RTCA DP. NKT cells, isolated from α-GalCer + MSC-treated mice and α-GalCer-treated mice, were counted and resuspended at a concentration of 4×10^6 cells/ml in DMEM +10% FCS media. Then, 100 μl NKT cells 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 min for 24 h at 37°C and 5% CO_2. NKT cell-mediated death of tumour cells was monitored in real time and was indicated by a decrease in cell index. Data were analysed with RTCA Software 1.2 (Acea Biosciences, San Diego, CA, USA).

2.15 | Measurement of cytokines

Levels of TNF-α, IFN-γ, IL-4 and IL-10 in the mouse serum and TNF-α, IFN-γ, IL-10, transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF) and prostaglandin E2 (PGE_2) in culture supernatants were measured using ELISA kits specific for the mouse cytokines (R&D Systems), according to the manufacturer's instructions. The IDO content of mouse serum and culture supernatants was determined using a mouse ELISA kit (no MI0064, NeoBioLab NeoBioLab, Massachusetts, USA).

Concentrations of TNF-α, IFN-γ and IL-4 in human cell culture supernatants were measured by ELISA kits specific for humans (R&D Systems), according to the manufacturer's instructions.

2.16 | Detection of kynurenine

As IDO catalyses the metabolism of tryptophan in the kynurenine pathway, IDO activity was determined by spectrophotometric assay for kynurenine in the serum of Con A- and α-GalCer-treated animals and in culture supernatants (Ling et al., 2014).

2.17 | Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of apoptosis- and autophagy-related genes

Total RNA was extracted using TRIZOL and reverse transcribed with M-MuLV reverse transcriptase and random hexamers (all from Life Technologies, Carlsbad, CA, USA). The real-time RT-PCR analysis was performed in a Realplex Mastercycler (Eppendorf, Hamburg, Germany) using TaqMan Master Mix and the following TaqMan primers and probes from Life Technologies: Atg4b (no. Mm00558047_m1), Atg5 (Mm00504340_m1), Atg7 (Mm00512209_m1), Atg12 (Mm00503201_m1), beclin-1 (Mm01265461_m1), p53-upregulated modulator of apoptosis (Puma; Mm00519268_m1), Noxa (Mm00451763_m1), B-cell lymphoma 2 (Bcl-2; Mm00477631_m1), Bcl-2-associated X protein (Bax; Mm00432051_m1), Bcl-2-associated death promoter (Bad; Mm00432042_m1), p53 (Mm01731287_m1), phosphatase and tensin homologue (Pten; Mm00477208_m1), apoptotic protease activating factor 1 (Apaf1; Mm01223702_m1), Bcl-xL (Mm00437783_m1) and X-linked inhibitor of apoptosis protein (XIAP; Mm00776505_m1). The reaction conditions were as recommended by the manufacturer. The threshold cycle (Ct) values of the housekeeping gene (18 s RNA) were subtracted from the Ct values of target genes to obtain ΔCt. The relative gene expression is presented as $2^{-\Delta Ct}$ value normalized to the treatment without mMSCs.

2.18 | Statistical analysis

Results were analysed using Student's *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.

3 | RESULTS

3.1 | mMSCs ameliorate Con A-induced hepatitis

mMSCs migrated in the livers of Con A-treated mice (Figure 1A) and efficiently ameliorated Con A-induced acute liver injury as determined by liver enzyme tests (Figure 1B) and histology (Figure 1C). Serum AST and ALT levels were significantly lower ($p < 0.05$) in Con A + mMSC-

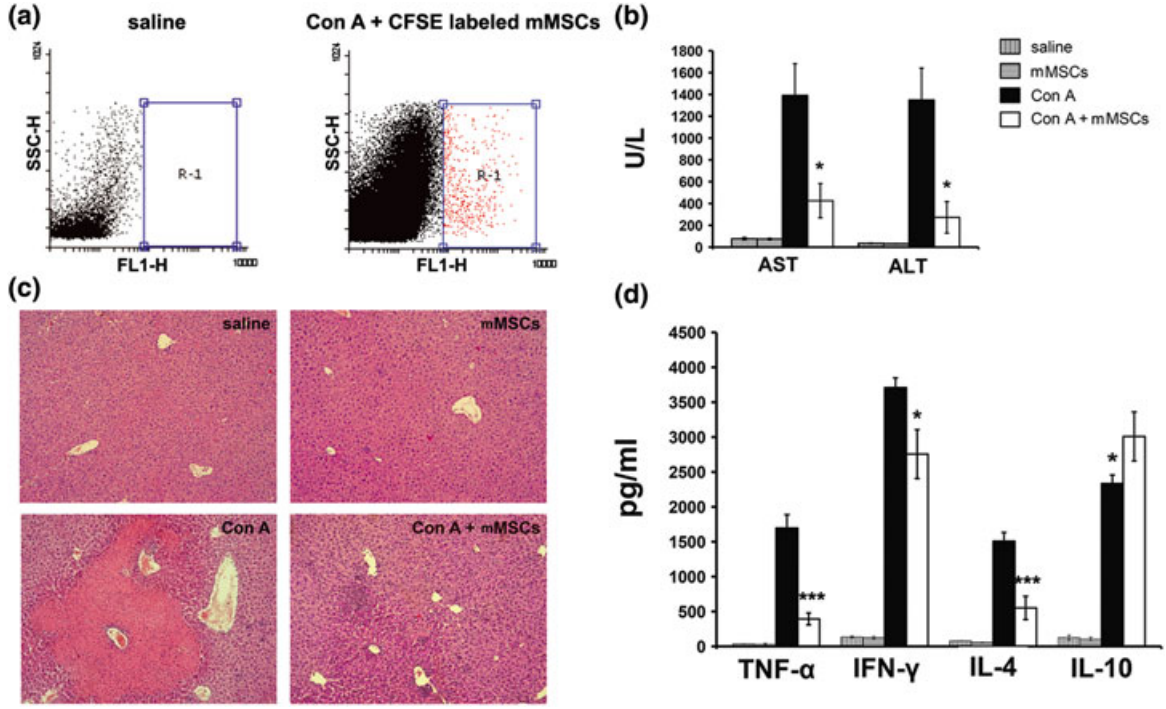

FIGURE 1 Mouse mesenchymal stem cells (mMSCs) ameliorate concanavalin a (con a)-induced hepatitis. Mice were injected intravenously with 0.2 ml saline, 5×10^5 mMSCs suspended in 0.2 ml saline, con a (12 mg/kg) or 5×10^5 mMSCs suspended in 0.2 ml saline immediately after con a administration. The samples for aspartate aminotransferase/alanine aminotransaminase (AST/ALT) measurement and histological analysis were collected after 24 h, whereas sera for cytokine measurement and livers for fluorescence-mMSCs detection were collected 8 h after systemic treatment. (A) detection of carboxyfluorescein diacetate succinimidyl ester-labelled mMSCs in the liver, (B) serum AST and ALT levels, (C) a histological examination was performed with haematoxylin and eosin (H&E) staining. H&E staining images of representative liver tissue samples are shown at the same magnifications (×100). (D) concentration of serum cytokines. Data presented as mean ± standard error of the mean; n = 10 mice per experimental group. $^*p < 0.05$; $^{***}p < 0.001$

treated mice compared with mice that received Con A only (Figure 1B). Histological analysis indicated that mMSC-treated mice were less sensitive to Con A-induced hepatic injury (Figure 1C). Liver tissue sections of Con A + mMSC-treated mice showed several solitary areas of necrotic tissue characterized by standard morphological criteria (i.e. loss of architecture, vacuolization, karyolysis and increased eosinophilia). By contrast, liver tissue sections in Con A-treated mice showed widespread areas of necrosis with extensive infiltration of MNCs within liver lobules and around the central veins and portal tracts, indicating the ongoing inflammatory process (Figure 1C). In accordance with the histological analysis, mMSCs significantly attenuated production of inflammatory cytokines. The concentrations of TNF-α, IFN-γ and IL-4 cytokines were significantly lower (TNF-α, $p < 0.001$; IFN-γ, $p < 0.05$; IL-4, $p < 0.001$), whereas the concentration of anti-inflammatory IL-10 was significantly higher ($p < 0.05$) in sera of Con A-treated mice that received mMSCs (Figure 1D).

3.2 | mMSC treatment attenuates the presence of hepatotoxic TNF-α-, IFN-γ- and IL-4-producing NKT cells in the liver of Con A-treated mice

The cellular make-up of the liver revealed a significantly lower number of TNF-α- ($p < 0.01$) and IFN-γ-producing ($CD4^+$ α-GalCer-loaded CD1d Tetramer$^+$) NKT cells that expressed T-bet ($p < 0.05$) (Figure S1A–C) in the livers of Con A + mMSC-treated mice compared with animals treated with Con A only. Also, the total number of hepatotoxic IL-4-producing NKT cells that expressed Th2 transcription factor GATA3 (Figure S1E, F) was significantly lower ($p < 0.05$) in the livers of Con A + mMSC-treated mice. The percentage of protective IL-10-producing NKT cells was significantly higher ($p < 0.05$) in the livers of Con A + mMSC-treated mice, suggesting a possible role of mMSCs in polarization of liver NKT cells (Figure S1D). However, the difference in the number of IL-10-producing NKT cells did not reach statistical significance ($p > 0.05$) (data not shown). Although there was no statistical difference ($p > 0.05$) in the number of liver infiltrated $CD8^+$ T and $CD19^+$ B cells between experimental groups, mMSCs treatment significantly ($p < 0.05$) attenuated the total number of inflammatory IL-12-producing macrophages and DCs (Figure S2), indicating a possible impact of mMSCs on the function of antigen presenting cells in the liver.

3.3 | Single injection of mMSCs significantly reduced α-GalCer-induced liver injury

In order to provide evidence that mMSCs suppress NKT cell activity *in vivo*, directly and independently from DCs and/or macrophages, we used α-GalCer-induced hepatitis. mMSCs migrated in the livers of α-GalCer-treated mice (Figure 2A) and prevented the increase in serum liver transaminases (Figure 2B). This finding was consistent with scarce necrotic areas observed in the livers of α-GalCer + mMSC-treated animals, in contrast to significantly larger necrotic areas in liver parenchyma and massive infiltration of MNCs within liver lobules and

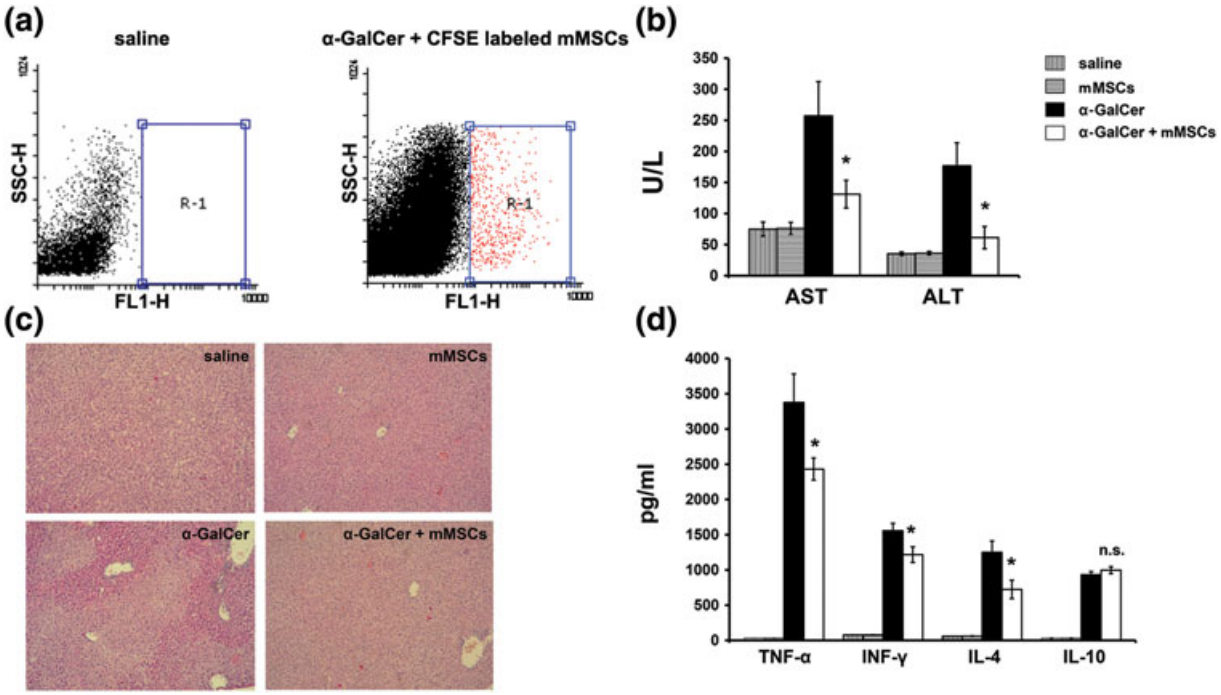

FIGURE 2 A single injection of mouse mesenchymal stem cells (mMSCs) significantly reduces α-galactosylceramide (α-GalCer)-induced liver injury. Mice were injected intravenously with 0.2 ml saline, 5×10^5 mMSCs suspended in 0.2 ml saline, α-GalCer (50 μg/kg) or 5×10^5 mMSCs suspended in 0.2 ml saline immediately after α-GalCer administration. The samples for aspartate aminotransferase/alanine aminotransaminase (AST/ALT) measurement and histological analysis were collected after 16 h, whereas sera for cytokine measurement and livers for fluorescence-labelled mMSCs detection were collected 2 h after the induction of hepatitis. (A) detection of carboxyfluorescein diacetate succinimidyl ester-labelled mMSCs in the liver, (B) serum AST and ALT levels, (C) a histological examination was performed with haematoxylin and eosin (H&E) staining. H&E staining images of representative liver tissue samples are shown at the same magnifications (×100). (D) concentration of serum cytokines. Data presented as mean ± standard error of the mean; n = 10 mice per experimental group. $^*p < 0.05$, $^{**}p < 0.01$

around the central veins and portal tracts of mice treated with α-GalCer only (Figure 2C). A diminished inflammatory injury in α-GalCer + mMSC-treated mice correlated with the cytokine production. The concentrations of inflammatory (TNF-α, IFN-γ and IL-4) cytokines were significantly lower ($p < 0.05$) in sera of α-GalCer-treated mice that received mMSCs, whereas there was no difference ($p > 0.05$) in serum concentration of anti-inflammatory IL-10 between experimental groups (Figure 2D).

3.4 | mMSCs reduce the number of TNF-α-, IFN-γ- and IL-4-producing NKT cells in the liver of α-GalCer-treated mice

Intracellular staining of liver NKT cells revealed a significantly lower total number of TNF-α- ($p < 0.05$), IFN-γ- ($p < 0.01$) and IL-4-producing NKT cells ($p < 0.05$) in α-GalCer + mMSC-treated mice, indicating that mMSCs attenuate production of pro-inflammatory cytokines in the liver NKT cells (Figure 3). There was no significant difference ($p > 0.05$) in the number of protective IL-10-producing NKT cells between experimental groups (Figure 3).and in the percentage (Figure S3) and total number (data not shown) of regulatory DCs ($CD11c^+CD8^+$) and IL-10-producing DCs, as well as inflammatory $CD11c^+$ DCs expressing CD11b, MHC class II, co-stimulatory molecules CD80 and CD86, and IL-12-producing DCs in the livers of α-GalCer + mMSC-treated mice compared with animals treated with α-GalCer only, suggesting that mMSCs modulate production of cytokines by acting directly on liver NKT cells.

3.5 | mMSCs attenuate expression of FasL, TRAIL and CD107 in liver NKT cells, reduce their hepatotoxicity and suppress the expression of pro-apoptotic genes in α-GalCer-induced hepatitis

mMSCs significantly attenuated the expression of the apoptosis-inducing ligands FasL ($p < 0.05$) and TRAIL ($p < 0.01$) and the degranulation marker CD107 ($p < 0.05$) on liver NKT cells of α-GalCer-treated mice (Figure 4A), whereas we did not find any difference in cytotoxic mediators (perforin and granzyme B) and receptors (NKG2D and KLRG1) expression between the experimental groups (Figure 4A). Accordingly, the results obtained by xCELLigence system for monitoring real-time cytotoxicity showed that NKT cells isolated from α-GalCer + mMSC-treated mice were significantly less cytotoxic against HepG2 cells and hepatocytes than NKT cells isolated from animals treated with α-GalCer only (Figure 4B, C), indicating that mMSC treatment significantly reduced the hepatotoxic potential of liver NKT cells. We next examined the influence of mMSCs on the expression of major apoptosis-regulating genes in α-GalCer hepatitis. mMSCs significantly reduced ($p < 0.05$) the expression of mRNA encoding pro-apoptotic mediators *Noxa*, *Bax*, *Bad* and *Apaf1* (Figure 4D) in the livers of α-GalCer-treated mice. The expression of other pro-apoptotic (*Pten*, *Puma*, *p53*) as well as anti-apoptotic genes (*Bcl-2*, *Bcl-xL*, *XIAP*) was

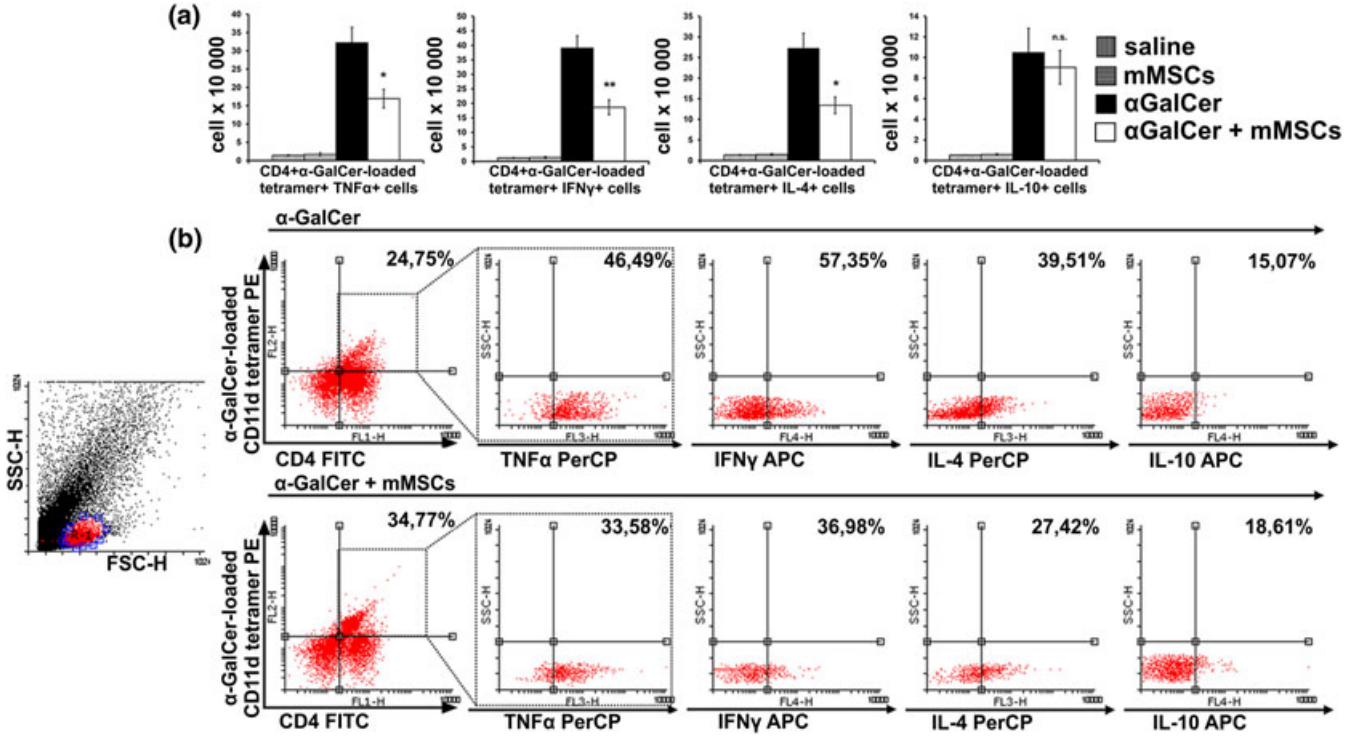

FIGURE 3 Mouse mesenchymal stem cells (mMSCs) reduce the influx of tumour necrosis factor-α (TNF-α)-, interferon-γ (IFN-γ)- and interleukin-4 (IL-4)-producing natural killer T (NKT) cells in the liver of α-galactosylceramide (α-GalCer)-treated mice. (A) Total numbers of TNF-α-, IFN-γ- and IL-4-producing ($CD4^+$ α-GalCer-loaded CD1d $tetramer^+$) NKT cells were significantly lower in the liver of mice treated with α-GalCer and mMSCs compared with mice treated only with α-GalCer. (B) representative flow cytometry dot plots show percentages of TNF-α-, IFN-γ-, IL-4-, IL-10-positive cells in the population of liver $CD4^+$ α-GalCer-loaded CD1d $tetramer^+$ NKT cells 2 h after systemic treatment. Values are mean ± standard error of the mean (n = 10 per group). $^*p < 0.05$, $^{**}p < 0.01$

not significantly affected by mMSCs ($p > 0.05$) (Figure 4D). The expression of genes required for the induction of autophagy (*atg4b*, *atg5*, *atg7*, *atg12*, and *Bcn1*) did not reach statistical difference ($p > 0.05$) in the liver of α-GalCer + mMSC-treated mice compared with α-GalCer-treated mice (Figure S4). These data indicate that mMSCs could alleviate α-GalCer hepatitis through the suppression of NKT cytotoxicity and subsequent decrease in apoptotic hepatocyte demise.

3.6 | mMSCs suppress activity of NKT cells in a paracrine manner

In order to investigate whether cell-to-cell contact was required for immunoinhibitory effects of mMSCs on NKT cells, co-cultures were performed in a transwell system where the two cell types are separated by a membrane permeable to soluble molecules. The significantly lower ($p < 0.05$) amounts of TNF-α and IFN-γ were noticed in supernatants of *in vitro* α-GalCer-stimulated liver NKT cells co-cultured with mMSCs in the transwell system when compared with α-GalCer-stimulated liver NKT cells that were cultured alone (Figure 5A). Intracellular staining revealed that *in vitro* α-GalCer-activated NKT cells co-cultured with mMSCs expressed significantly lower amounts of inflammatory cytokines (TNF-α, $p < 0.05$; IFN-γ, $p < 0.01$; IL-4, $p < 0.05$) and a significantly higher ($p < 0.05$) amount of immunosuppressive IL-10 (Figure 5B), indicating that mMSCs suppress activity of NKT cells in a paracrine manner. To directly demonstrate that soluble factors were responsible for the mMSC-mediated inhibition of NKT cell function, mice were intravenously injected with mMSC-CM, immediately after α-GalCer administration (α-GalCer + mMSC-CM-treated mice). mMSC-CM treatment significantly downregulated ($p < 0.05$) serum AST and ALT levels in α-GalCer-treated mice (Figure 5C). Additionally, liver NKT cells isolated from α-GalCer + mMSC-CM-treated mice were significantly less cytotoxic against HepG2 hepatocyte cells than NKT cells isolated from mice treated only with α-GalCer (Figure 5D).

3.7 | mMSCs suppress activity of NKT cells in an iNOS- and IDO-dependent manner

As IDO, PGE_2, HGF, IL-10 and TGF-β are well-known mediators of MSC-mediated inhibition (Meisel et al., 2004; Nasef et al., 2007; Sato et al., 2007), these immunosuppressive factors were measured in the sera of α-GalCer- and α-GalCer + mMSC-treated mice, as well as in the supernatants derived from liver NKT cells stimulated with α-GalCer in the presence or absence of mMSCs. There was no difference in the levels of PGE_2, HGF, IL-10 and TGF-β between groups (Figure 6 A, B). However, levels of IDO and kynurenine in the sera of α-GalCer + mMSC-treated mice (Figure 6A), as well as in the supernatants derived from NKT cells stimulated in the presence of mMSCs, were significantly higher ($p < 0.05$) (Figure 6B).

Additionally, kynurenine was also elevated in the serum of Con A + mMSC-treated mice (Figure S5A), suggesting that the production of IDO by mMSCs may be important for efficient and strong suppression of NKT cell effector functions in both models of acute liver injury.

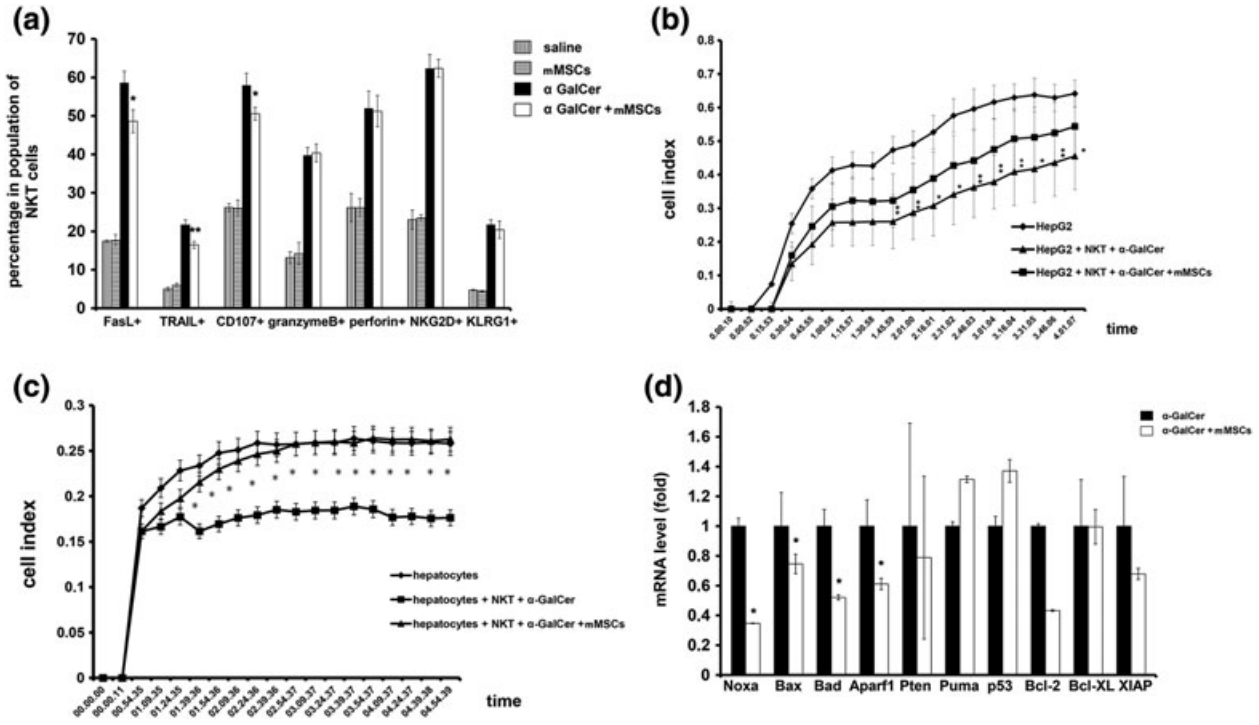

FIGURE 4 Mouse mesenchymal stem cells (mMSCs) attenuate the expression of FasL, TRAIL and CD107 on liver natural killer T (NKT) cells, reduce their hepatotoxicity and suppress the expression of pro-apoptotic genes in α-galactosylceramide (α-GalCer)-induced hepatitis. (A) the percentage of FasL, TRAIL and CD107 expressing NKT cells was significantly lower in the liver of mice treated with α-GalCer and mMSCs compared with mice treated with α-GalCer only. The results obtained by xCELLigence system showed less cytotoxic activity of liver NKT cells isolated from mice treated with α-GalCer and mMSCs against (B) HepG2 cells and (C) hepatocytes. (D) real-time reverse transcription-polymerase chain reaction analysis revealed that mMSCs decreased the expression of several pro-apoptotic genes (*Noxa*, *Bax*, *Bad* and *Apaf1*) but not anti-apoptotic genes (*Bcl-2*, *Bcl-xL* and *XIAP*) in the liver tissue of α-GalCer-treated mice. Values are mean ± standard error of the mean (n = 10 per group). $^{*}p < 0.05$, $^{**}p < 0.01$

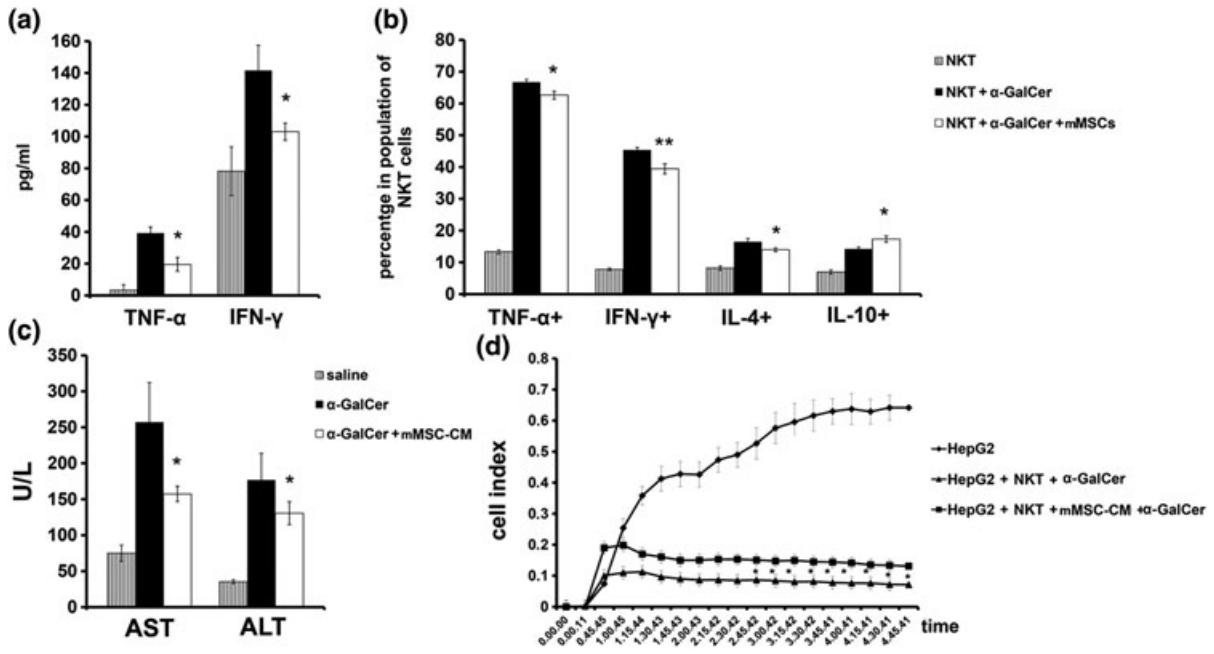

FIGURE 5 Mouse mesenchymal stem cells (mMSCs) suppress the activity of natural killer T (NKT) cells in a paracrine manner. (a, B) *In vitro* α-galactosylceramide (α-GalCer)-stimulated liver NKT cells isolated from healthy mice were co-cultured for 48 h with mMSCs in transwell systems at a ratio of 10:1. The level of cytokines in (a) supernatants and (B) their intracellular expression in liver NKT cells are shown. (C, D) mice were injected intravenously with saline (0.2 ml), α-GalCer (50 μg/kg) or mMSC-conditioned medium (mMSC-CM; 0.2 ml) immediately after α-GalCer administration. (C) serum aspartate aminotransferase and alanine aminotransaminase levels collected 16 h after the systemic treatment. (D) liver NKT cells isolated from α-GalCer + mMSC-CM-treated mice were significantly less cytotoxic against HepG2 hepatocyte cells than NKT cells isolated from mice treated only with α-GalCer. Data were shown as the mean ± standard error of the mean of 10 mice per group and are pooled from two independent experiments.$^{*}p < 0.05$, $^{**}p < 0.01$

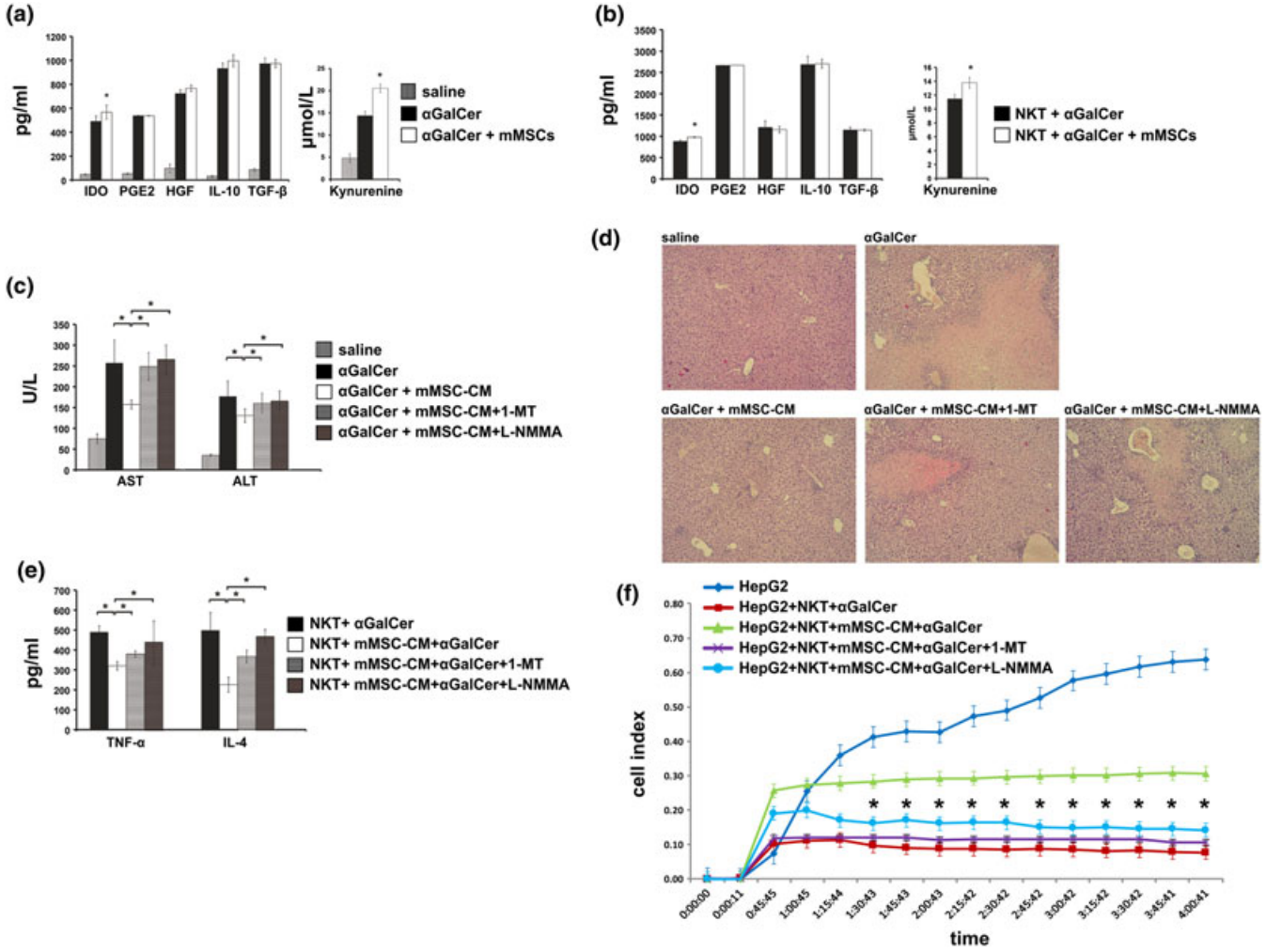

FIGURE 6 Mouse mesenchymal stem cells (mMSCs) suppress the activity of natural killer T (NKT) cells in an inducible nitric oxide synthase (iNOS)- and indoleamine 2,3-dioxygenase (IDO)-dependent manner. IDO, prostaglandin E2, hepatocyte growth factor, interleukin-10, transforming growth factor-β and kynurenine were determined in (a) mice sera and (B) supernatants by enzyme-linked immunosorbent assay. (C, D) C57BL/6 mice were treated with saline (0.2 ml), α-galactosylceramide (α-GalCer; 50 μg/kg), mMSC-conditioned medium (mMSC-CM; 0.2 ml), mMSC-CM (0.2 ml) + 1-methyltryptophan (1-MT; 1 mM) or mMSC-CM (0.2 ml) + L- N^G-monomethyl arginine citrate (L-NMMA; 1 mM) immediately after α-GalCer administration. (C) serum aspartate aminotransferase and alanine aminotransaminase levels and (D) histological tissue samples were analysed 16 h after treatment (haematoxylin and eosin staining). (E, F) *In vitro* α-GalCer-stimulated liver NKT cells were co-cultured for 48 h in mMSC-CM, mMSC-CM + 1-MT (1 mM) or mMSC-CM + L-NMMA (1 mM). (E) cytokine production and (F) cytotoxic potential of NKT cells against the HepG2 hepatocyte cells are depicted. Data are shown as the mean ± standard error of the mean of 10 mice per group and are pooled from two independent experiments.*$p < 0.05$, **$p < 0.01$

IDO plays a key role in hMSC-based immunosuppression, whereas mMSCs also use iNOS-dependent suppression of the immune response (Shi et al., 2012). Accordingly, blockade of iNOS by L-NMMA or IDO activity by 1-MT abrogated the hepatoprotective capacity of mMSCs-CM injected to α-GalCer-treated and Con A-treated mice. There were significantly elevated ($p < 0.05$) serum levels of AST and ALT, as well as widespread areas of necrosis with extensive infiltration of MNCs in the livers of α-GalCer + mMSC-CM + L-NMMA- and α-GalCer + mMSC-CM + 1-MT-treated mice compared with α-GalCer + mMSC-CM-treated animals (Figure 6C, D). Also, increased serum levels of AST and ALT (Figure S5B) and extensive hepatocytes necrosis (Figure S5C) were noticed in Con A + mMSC-CM + L-NMMA- and Con A + mMSC-CM + 1-MT-treated mice compared with mice that received Con A + mMSC-CM.

In line with results obtained *in vivo*, when liver NKT cells were cultured in mMSC-CM, a significant ($p < 0.05$) inhibition of TNF-α and IL-4 production, as well as the cytotoxicity against HepG2 cells, was observed (Figures S5D, E, 6E, F). The presence of L-NMMA or 1-MT in mMSCs-CM managed to counteract the inhibitory effect exerted by mMSC-CM (Figures S5D, E, 6E, F).

3.8 | hMSCs alter effector functions of α-GalCer-stimulated human PBMNCs in an iNOS- and IDO-dependent manner

In order to demonstrate the relevance of the data obtained in the animal model for hMSC suppression of NKT cells, we investigated whether hMSCs affect cytokine production and cytotoxic potential

of *in vitro* expanded and activated human NKT cells. As shown in Figure S6A, supernatants derived from α-GalCer-stimulated PBMNCs co-cultured with hMSCs in a transwell system showed significantly lower ($p < 0.05$) levels of IFN-γ and IL-4, compared with supernatants derived from stimulated PBMNCs cultured alone. The cytotoxicity of α-GalCer-stimulated PBMNCs cultured with hMSCs against target HepG2 cells was significantly lower ($p < 0.05$) when compared with the cytotoxicity of α-GalCer-stimulated PBMNCs cultured alone (Figure S6B). In line with the results obtained in mice, L-NMMA and 1-MT almost completely restored production of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-4) as well as the cytotoxic activity of α-GalCer-stimulated PBMNCs, suggesting iNOS and IDO as important factors for hMSC-mediated suppression of human NKT cells (Figure S6C, D).

4 | DISCUSSION

Although the efficacy of MSC-based therapy of acute liver diseases has been reported [reviewed in Volarevic, et al. (2014)], the effect of MSCs on the phenotype and function of liver NKT cells, major effector cells in acute liver injury, is not fully understood. Here we provide the evidence that MSCs protect from acute liver injury by attenuating cytotoxicity and the capacity of liver NKT cells to produce inflammatory cytokines in an iNOS- and IDO-dependent manner.

NKT cells are most abundantly present in the microvascular compartments of the liver. Therefore, these cells have been directly linked to the development of liver damage in a number of animal models of hepatitis, as well as in patients with autoimmune hepatitis (Swain, 2008). NKT cells appear to play crucial roles in the induction of hepatic injury by co-operating with conventional T cells, macrophages and DCs, and through the effector mechanisms involving the Fas/FasL interaction, perforin/granzyme system and the IFN-γ-, IL-4- and/or TNF-α-mediated system (Takeda et al., 2000).

Here we showed that mMSCs migrated in injured liver and attenuated Con A-induced liver injury by reducing the number of major effector cells, including TNF-α, IFN-γ- and IL-4-producing NKT cells, DCs and macrophages in the liver (Figures S1, 2), which was accompanied by lower serum levels of TNF-α, IFN-γ and IL-4, and a higher serum level of IL-10 (Figure 1D). These results hinted that it is important to distinguish the direct action of mMSCs on NKT cells from indirect effects mediated by other cell types contained in the liver.

Therefore, we used another animal model of NKT cell-mediated fulminant liver failure, induced by α-GalCer, to evaluate the effect of mMSCs on NKT cells in hepatitis. mMSC treatment led to a decrease in the total number of TNF-α-, IFN-γ- and IL-4-producing NKT cells followed by attenuation of α-GalCer hepatitis (Figure 3). We found that mMSCs reduced α-GalCer-induced liver injury by suppressing NKT cells without affecting cytokine production, polarization and antigen presentation of liver DCs (Figure S3).

It was reported that the administration of α-GalCer upregulated FasL and TRAIL in NKT cells, receptors responsible for NKT cell-mediated apoptosis and cytotoxicity, leading to liver injury (Huang et al., 2014). In acute liver injury, MSCs inhibit hepatocyte apoptosis by suppressing the expression of Bax and Bad (Cai et al., 2015). Bax interacts with Noxa, the expression of which is induced by IFN-γ in a p53-independent manner (Sun & Leaman, 2005). Accordingly, our findings show that mMSCs attenuate NKT cell hepatotoxicity through inhibition of the surface expression of FasL, TRAIL and CD107, reduce IFN-γ and suppress expression of pro-apoptotic *Noxa*, *Bax*, *Bad*, leading to the attenuation of hepatocyte damage (Figure 4).

Various mediators are proposed to be responsible for the immunosuppressive effects of MSCs, including nitric oxide (NO), IDO, TGF-β, HGF, PGE_2 and IL-10 (Meisel et al., 2004; Nasef et al., 2007; Sato et al., 2007). IDO plays a key role in hMSC-mediated immunomodulation, whereas mMSCs mainly use iNOS-dependent suppression of the immune response (Ren et al., 2009).

IDO produced by MSCs suppresses proliferation or induces apoptosis of T and B lymphocytes, suppresses IFN-γ production in NK cells, inhibits the cytotoxicity of $CD8^+$ T and NK cells and induces the differentiation of monocytes into immunosuppressive M2 macrophages (Corcione et al., 2006; François, Romieu-Mourez, Li, & Galipeau, 2012; Li et al., 2014; Rasmusson, Uhlin, Le Blanc, & Levitsky, 2007; Sotiropoulou, Perez, Gritzapis, Baxevanis & Papamichail 2006; Spaggiari et al., 2008).

MSC-derived IDO is an enzyme that has powerful immunomodulatory effects resulting from its enzymatic activity which leads to catabolism of the essential amino acid L-tryptophan to L-kynurenine (Ito et al., 2010). Metabolites of the L-kynurenine pathway have been shown to act as immunoregulatory molecules that have immunosuppressive effects in the tissue microenvironment (Ito et al., 2010). Accordingly herewith we showed that injection of mMSCs increased serum levels of kynurenine in Con A- and α-GalCer-treated mice accompanied by attenuated liver injury.

mMSCs mainly use an iNOS-dependent mechanism to produce NO and directly suppress proliferation and cytokine production in lymphocytes (Ren et al., 2009). As NO is highly unstable, it only acts locally and for NO-dependent suppression immune cells have to be recruited into close proximity to MSCs (Sato et al., 2007). When an injury is present, MSCs migrate in response to inflammatory cytokines and chemokines, produced by damaged hepatocytes and liver-infiltrated immune cells, and home to the sites of wounding (Rüster et al., 2006). Under inflammatory conditions, IFN-γ and TNF-α provoke mMSCs to express iNOS (Li et al., 2012). As, in the presence of IFN-γ, low molecular concentrations of NO increase IDO activity (López, Alegre, Díaz, Mugueta, González, 2006), we assumed that after α-GalCer or Con A stimulation, NKT cells increased production of IFN-γ, which provoked mMSCs to express iNOS and produce NO, which increased IDO activity and led to the attenuation of NKT cell cytotoxicity (Figure S7).

Accordingly, results obtained by pharmacological inhibition of iNOS and IDO activity in MSCs indicate that both iNOS and IDO signalling pathways are responsible for MSC-mediated suppression of NKT cell activity.

The presence of either L-NMMA, a specific iNOS inhibitor, or 1-MT, a specific inhibitor of IDO, in mMSC-CM injected into α-GalCer-treated mice, counteracted the hepatoprotective effect of mMSCs, elevated serum levels of AST and ALT and increased liver tissue loss and inflammatory cell infiltration (Figures S5B, C, 6C, D). In line

with results obtained *in vivo*, either L-NMMA or 1-MT almost completely restored pro-inflammatory cytokine production (TNF-α and IL-4) as well as cytotoxic activity of NKT cells against HepG2 hepatocyte cells *in vitro* (Figures S5D, E, 6E, F), suggesting the importance of both iNOS and IDO signalling for mMSC-mediated inhibition of NKT cell effector functions.B6

It was demonstrated that pharmacological inhibition of iNOS and IDO skewed the cytokine response of NKT cells towards Th1 profile (Ito, Ando, & Seishima, 2015; Molano, Illarionov, Besra, Putterman, & Porcelli, 2008). Reciprocally, NO and tryptophan-derived catabolites, L-kynurenine, 3-hydroxy-kynurenine or 3-hydroxy-anthranilic acid, shifted the cytokine balance towards a more Th2 pattern (Daniel et al., 2006; Molano et al., 2008). A decrease in the production of IL-4 in liver NKT cells of Con A + mMSC- and α-GalCer + mMSC-treated mice could be explained by the fact that, although IL-4 is considered a prototypic Th2-type (i.e. anti-inflammatory) cytokine, in the setting of NKT-dependent liver disease, IL-4 has pro-inflammatory properties (Swain, 2008).

5 | CONCLUSION

Our study provides the evidence that MSCs protect from acute liver injury by attenuating liver NKT cell cytotoxicity and their capacity to produce inflammatory cytokines in an iNOS- and IDO-dependent manner. Such novel findings could contribute to improve the potential of clinical therapeutic trials using MSC infusion for immunomodulation purposes.

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CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

MG, VT and VV wrote the manuscript. MG, BSM, LjV, TN and VV collected the data. MG, VT, MLL and VV were involved in the analysis and interpretation of the data. NA, VD,VT, MLL and VV contributed towards conception and design. All the authors have approved the final version of this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. mMSCs treatment attenuates the presence of hepatotoxic NKT cells in the liver of Con A-treated mice. Livers of saline-, mMSC-, Con A- and Con A + mMSC-treated mice were analysed by intracellular cytokine staining and FACS analysis 8 h after treatment. The total number of (A) TNF-α-producing ($CD4^+$ α-GalCer-loaded CD1d Tetramer$^+$) NKT cells, (B) IFN-γ-producing NKT cells, (C) $CD4^+Tbet^+$ cells, (E) IL-4-producing NKT cells, (F) $CD4^+Gata3^+$ cells was significantly lower in the livers of Con A + mMSC-treated mice. (D) The percentage of IL-10-producing NKT cells was significantly higher in the livers of Con A + mMSC-treated mice. Values are mean ± SEM (n = 10 per group). $^*p < 0.05$, $^{**}p < 0.01$

Figure S2. mMSCs treatment markedly decreases liver infiltration and cytokine production in macrophages and dendritic cells. (A) The total number of liver infiltrating $F4/80^+$, $F4/80^+$ IL-12- and $F4/80^+$ IL-10-producing macrophages was significantly lower in Con A + mMSC-treated mice compared witho Con A-treated mice. (B) In addition, a significant decrease in the total number of $CD11c^+$ and $CD11c^+$ IL-12-producing DCs was observed in Con A + mMSC-treated mice 8 h after treatment. (C) There was no statistical difference in the number of liver infiltrating $CD8^+$ T and $CD19^+$ B cells between experimental groups. Values are mean ± SEM (n = 10 per group). $^*p < 0.05$, $^{**}p < 0.01$

Figure S3. mMSCs did not significantly affect phenotype of liver-infiltrating dendritic cells in α-GalCer-induced hepatitis. The percentage of (A) inflammatory $CD11c^+$ DCs expressing CD11b, MHC class II, co-stimulatory molecules CD80 and CD86, and IL-12-producing DCs and (B) regulatory $CD11c^+CD8^+$ DCs and IL-10 producing DCs is shown. Values are mean ± SEM (n = 10 per group). $^*p < 0.05$, $^{**}p < 0.01$

Figure S4. mMSC treatment has no effect on autophagy in α-GalCer-treated animals. The expression of genes required for induction of autophagy (*atg4b*, *atg5*, *atg7*, *atg12* and *Bcn1*) is shown

Figure S5. mMSCs suppress activity of liver NKT cells in an iNOS- and IDO-dependent manner. (A) Kynurenine was determined in mice sera. (B, C) C57BL/6 mice were treated with saline (0.2 ml), Con A (12 mg/kg), mMSC-CM (0.2 ml), mMSC-CM (0.2 ml) + 1-MT (1 mM) or mMSC-CM (0.2 ml) + L-NMMA (1 mM) immediately after Con A administration. (B) Serum AST and ALT levels and (C) histological tissue samples were analysed 16 h after treatment (H&E staining). (D, E) *In vitro* α-GalCer-stimulated liver NKT cells were co-cultured for 48 h in mMSC-CM, mMSC-CM + 1-MT (1 mM) or mMSC-CM (0.2 ml) + L-NMMA (1 mM). (D) Cytokine production and (E) cytotoxic potential of NKT cells against the HepG2 hepatocyte cells are depicted. Data are shown as the mean ± SEM of 10

mice per group and are pooled from two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$

Figure S6. hMSCs alter effector functions of α-GalCer-stimulated human PBMNCs in an IDO-dependent manner. (A, B) *In vitro* α-GalCer-stimulated human PBMNC isolated from healthy donors were co-cultured for 48 h with hMSCs in transwell systems at ratio 10:1. (A) The level of cytokines in the supernatants and (B) cytotoxicity of α-GalCer-stimulated PBMNC are shown. (C, D) *In vitro* α-GalCer-stimulated PBMNC were co-cultured for 48 h in hMSC-CM, hMSC-CM + 1-MT (1 mM) or hMSC-CM + L-NMMA (1 mM). (C) Cytokine production and (D) cytotoxic activity of α-GalCer-stimulated PBMNCs. Values are mean ± SEM of triplicates of two independently performed experiments

Figure S7. iNOS and IDO signalling pathways are responsible for mMSC-mediated suppression of NKT cell activity in acute liver injury. After α-GalCer or Con A stimulation, NKT cells increased production of IFN-γ and TNF-α that provoke mMSCs to express iNOS. Under inflammatory conditions, low molecular concentrations of highly unstable NO act locally, increasing the IDO activity, which leads to the attenuation of NKT cell cytotoxicity

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