Dendritic cells originating exosomal miR-193b-3p induces regulatory T cells to alleviate liver transplant rejection

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Abstract

Background

Exosomes exert considerable influence in mediating regulatory T (Treg) cells differentiation, which attach great importance to attenuating acute cellular rejection after liver transplantation (LT). And, miRNAs are known to play essential roles in cell-cell communication delivered by exosomes. However, the function of exosomal miRNAs in regulating Treg cells after LT remains unknown. Here, we performed an expression profiling analysis of exosome-miRNAs from human plasma after LT and investigated their immunoregulatory effects on Treg cells.

Methods

Fifty-eight LT patients and nine donors were included in this report. miRNA profiles in plasma exosomes were analyzed using next-generation sequencing. Flow cytometry, HE and multiplex immunofluorescent staining were used to identify Treg cells in the liver and peripheral blood. A mouse LT model was employed to confirm the infiltration of Treg cells in the liver and circulation. A lentiviral vector system was used to overexpress miR-193b-3p in dendritic cells (DCs), and exosomes isolated from these transfected cells were co-cultured with spleen lymphocytes in vitro. A quantitative Real-time PCR and enzyme-linked immunosorbent assay were used to detect the expression of cytokines.

Results

Treg cell infiltration was increased in the liver along with Th17 and CD8+ T cell, and it was down-regulated in peripheral blood in the acute rejection group, an effect confirmed in the mouse LT model. High-throughput sequencing revealed that miR-193b-3p was markedly up-regulated in plasma exosomes of non-rejection LT patients. The NLRP3 inflammasome was screened as a target for miR-193b-3p based on target prediction and functional enrichment analyses. Exosomal miR-193b-3p derived from DCs increased Treg cells as demonstrated in vitro. miR-193b-3p overexpression down-regulated NLRP3 as well as the inflammatory cytokines IL-1β and IL-17A while increasing levels of the cytokines IL-10 and TGF-β.

Conclusion

DC deriving exosomal miR-193b-3p promoted Treg cells by inhibiting NLRP3 expression. These findings not only provide a new perspective on the mechanisms, but also hold great promise for the treatment or prevention of liver allograft rejection.

Introduction
Acute LT rejection, which occurs with an incidence of approximately 30%, is mainly associated with T lymphocytes\(^1\)\(^–\)\(^3\). It has been reported that the decreased frequency of Treg in peripheral blood is associated with acute rejection after liver transplantation\(^4\), while Treg transfer could reduce anti-donor T cell responses in a clinical study\(^5\). At the same time, intrahepatic Treg cells accumulation of great importance in attenuating acute rejection in rat LT model\(^6\). Upon activation, CD4\(^+\) T cells can be polarized into Treg, Th17, and Th1 cells, depending on the conditions of the microenvironment\(^7\). More specifically, it seems that skew toward Treg differentiation leading to a balance between inflammatory cells and Treg cells represents one of the pathological basis for LT tolerance\(^8\),\(^9\). Accordingly, by focusing on the induction of the Treg cells, it will be possible to achieve a better understanding of immune tolerance modulation after LT.

The donor major histocompatibility antigen complex (MHC) presented to host T cells via dendritic cells (DCs) represents a critical step with regard to LT rejection\(^1\),\(^10\). Many different types of DCs exist within the liver and, as these DCs serve as the most potent antigen-presenting cells, they can then play a crucial role in immunomodulation after transplantation. Previous work from our laboratory has revealed that the distribution of DCs in liver grafts differ when comparing that observed between rejection versus non-rejection groups. Immature DCs downregulate T-cell responses to induce/maintain immunologic tolerance, while mature DCs promote immunity\(^11\). Graft infiltrating host DCs are associated with high expressions of PD-L1 resulting in CD8\(^+\) T cell deletion and induction of immune tolerance as demonstrated in a LT model\(^12\). CD11c\(^+\)CD11b\(^+\) DCs produce elevated levels of IL-10 and TGF-β, effectively suppressing inflammatory responses and increasing the proportion of Treg cells, which has the effect of producing a tolerogenic condition\(^13\). Recently, a novel mode of intercellular communication mediated by exosomes, a class of extracellular vesicles, has been implicated in regulating Treg cells\(^14\). DC-derived exosomes can alleviate hepatic ischemia-reperfusion injury\(^14\) and prolong intestinal allograft survival by increasing the proportion of Treg cells\(^16\).

Exosomes are released by various cells and have a diameter between 30–200 nm. They are secreted into the extracellular space as well as into a variety of bodily fluids, such as plasma/serum, saliva, cerebrospinal fluid and urine\(^17\),\(^18\). Exosomes contain functional proteins, genomic DNA, mRNA, microRNAs (miRNAs), small non-coding RNAs (ncRNAs), lipids and metabolites derived from their parent cells, all of which enable them to act as signaling platforms for delivering messengers to other cells\(^19\),\(^20\). MHC-bearing exosomes have been shown to activate T cells after islet and kidney transplantation which is sufficient for sustaining rejection\(^10\). However, exosomes can also exhibit immunoinhibitory effects that can then influence the allograft immunogenicity of LT\(^21\). Exosome-mediated responses can be disease-promoting or restraining depending on their parentally-derived cell cargos\(^14\).

miRNAs are a group of small ncRNAs with 18–25 nucleotides. Following binding to the 3’ untranslated area of the target mRNA they can guide proteins involved in the function of RNA-induced silencing complexes. As a result, they can degrade complementary targeted mRNAs or block its translation at the
post-transcriptional level\cite{22}. Up to 50% of gene translation is regulated by miRNAs\cite{23}, which can then regulate many cellular functions, including apoptosis, proliferation, immunoregulation and inflammatory responses\cite{24,25}. It has been reported that miRNAs account for the majority of ncRNAs in plasma exosomes and may be selectively packed into exosomes\cite{26} and, exosomal miRNAs appear to play a critical role with regard to immunoregulation after pancreatic islet, heart and small intestine transplantation\cite{16,27}. However, the profile of plasma derived exosomal miRNAs after LT and its regulation of the Treg cell has yet to be fully illuminated.

In this study, we report that Treg, Th17, and CD8\(^+\) T cell infiltrations were increased under conditions of a rejected liver, while Treg levels were significantly decreased in the peripheral blood, suggesting that a imbalance of intragraft Treg/inflammatory cells was involved in this acute rejection. Moreover, miR-193b-3p was markedly up-regulated in plasma-derived exosomes from non-rejection patients. When investigating the potential targets of miR-193b-3p and its effects on lymphocytes \textit{in vitro}, we found that exosomal miR-193b-3p could enhance Treg cells through NLRP3 inflamasomes inhibition. These results reveal a novel role for exosomal miRNAs in the regulation of immune responses after LT and highlight the foundation for the development of novel approaches in the treatment or prevention of LT rejection.

\section*{Methods}

\subsection*{Subjects and sample collection}

Consecutive LT patients were included in this prospective study which was conducted over the period from July 2020 to February 2021 at the Liver Transplantation Center of the Beijing Friendship Hospital, Capital Medical University. Patients with malignant tumors, concurrent autoimmune disease, any type of infection, or combined organ transplantation were excluded. The normal control group consisted of donors from living donor LT. To harvest plasma, blood samples were centrifuged at 2000 \(\times\) g for 10 min at 4\(^\circ\)C and 1-ml aliquots from the supernatant was transferred to a fresh 1.5-ml tube and stored at \(-80\)^\(\circ\)C prior to use. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density-gradient centrifugation using the Lymphocyte Separation Medium (Cat: 7111011, DAKEWE) and were analyzed with use of flow cytometry. Livers from donors as obtained during surgery and liver biopsy tissues of patients were used for histopathological studies. This protocol was approved by the Ethics Committee of the Beijing Friendship Hospital. Written informed consent was obtained from all participants or their guardians for the collection of plasma/liver samples and their clinical data to be used in this study.

\subsection*{Hematoxylin–eosin (HE) staining}

Liver tissues were fixed in 10\% neutral buffered formalin and embedded in paraffin. Sections (4-\(\mu\)m) were stained with hematoxylin–eosin (HE) for histological examination. The histological classification of hepatic HE staining was graded according to the Banff scheme. A rejection activity index (RAI) was
calculated from 3 individual scores (venous endothelial inflammation, bile duct damage and portal inflammation) as performed by a single-blinded pathologist. Acute rejection was defined as an RAI score of $\geq 3$.

**Immunohistochemistry (IHC)**

The 4-μm sections were deparaffinized and rehydrated, followed by microwave antigen retrieval in sodium citrate buffer (pH 6.0) for 15 min and cooling at room temperature. The slides were incubated in 3% H$_2$O$_2$ for 10 min and blocked with 5% bovine serum albumin for 10 min. Primary antibodies against CD4 (1:200, ab133616; Abcam), CD8α (1:200, ab237709; Abcam), forkhead box (Fox)p3 (1:200, ab22510; Abcam), IL-17A (1:200, ab79056; Abcam), and NLRP3 (1:200; ab214185; Abcam) were added and incubated at 4°C overnight. The sections were stained using a DAB kit (ZLI-9017, ZSGB-BIO, China).

**Multiplex immunofluorescent (mIF) and image analysis**

After testing all of the markers using chromogenic immunohistochemistry and uniplex IF staining, these markers were validated in liver samples as achieved using mIF staining. Tissue multiplex immunofluorescent (mIF) staining was performed with use of the Opal Polaris 7 Color IHC Detection Kit (Akoya Biosciences). Five-micrometer-thick formalin-fixed, paraffin-embedded tissue sections were baked for 2 h at 60°C before staining. Slides were rehydrated with graded ethanol in deionized water. Antigen retrieval was performed at pH 6 for 20 min at 95°C. The slides were serially stained with primary antibodies, consisting of CD4 (1:400, ab133616; Abcam), CD8 (1:200, ab178089; Abcam), forkhead box (Fox)p3 (1:200, ab215206; Abcam) and IL-17A (1:200, ab79056; Abcam) with incubation times being 1 h per primary antibody. Subsequently, anti-rabbit polymeric horseradish peroxidase (Opal IHC Detection Kit; Akoya Biosciences) was applied as a secondary label with an incubation time of 10 min. Following incubation of the slides for 10 min, antibody signaling was visualized with use of the corresponding Opal Fluorophore (Akoya Biosciences). Slides were mounted with anti-fade mounting medium (P36965; Life Technologies) and stored at 4°C before imaging. Image acquisition was performed using the Vectra Polaris multispectral imaging platform (Akoya Biosciences). Whole slide images were scanned and 5–7 representative regions of interest at $\times200$ resolution were chosen by the pathologist as multispectral images. Image analysis was performed using the InForm 2.4.8 Image Analysis Software (Akoya Biosciences).

**Exosome isolation and characterization**

The procedure for isolation of exosomes is shown in Figure 1A. Plasma samples or cell culture supernatants were centrifuged at 3,000 × g for 10 min at 4°C. Supernatants were transferred to a new tube and centrifuged at 90,000 × g for 30 min at 4°C. The supernatant was then filtered (0.22 μm) and the filtered culture solution was centrifuged at 100,000 × g for 150 min at 4°C. The pellets (exosomes) were resuspended in 200 μl phosphate-buffered saline. The concentration of exosomes was determined by analyzing protein concentrations as measured using the BCA Protein Assay Kit (PC0020, Solarbio). Nanoparticle tracking analysis (NTA) with ZetaView (Particle Metrix, GmbH, Meerbusch, Germany) was
used to track the number and size of exosomes. The morphology of exosomes was observed under transmission electron microscopy (TEM). Exosomes were deposited onto copper grids and negatively stained with 2% phosphotungstic acid for 2 min. After air-drying for 15 min at room temperature, the samples were observed under a transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan) at 80 kV.

**miRNA library construction and sequencing**

Total RNAs were extracted from exosome pellets. Adaptors were added to the 3' and 5' ends, and then reverse transcribed into cDNA, followed by reverse transcription and polymerase chain reaction (PCR) amplification with a low number of cycles. The PCR products derived from the 18–30-base RNA molecules were purified by PAGE using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (Illumina, San Diego, USA). Purified library products were evaluated using the Agilent 2200 TapeStation, which were sequenced using the Illumina HiSeqTM 2500 platform. Adaptor sequences of the raw reads were trimmed and low-quality reads were excluded to obtain purified miRNAs for subsequent analysis. The clean reads were compared with the databases (miRBase version 22, Rfam12.1, piRNABank) to obtain non-coding (NC) RNAs. miRDeep2 was used to identify known mature miRNAs based on miRBase21 (www.miRBase.org) and predict novel miRNAs.

**Differential expression miRNAs target prediction and pathway enrichment analysis**

Differential expression (DE) miRNAs between two sets of samples were calculated using edgeR algorithm according to the criteria of |log2(Fold Change)| ≥ 1 and Q (corrected P) < 0.05. TargetScan 7.1, miRDB v21.0, miRTarBase and miRWalk were used to predict targets for the DE miRNAs. Prediction results from the four programs were further screened and sorted, and the prediction results of miRTarBase or miRDB, miRWalk and TargetScan were taken as candidate target genes of miRNA. When candidate target genes were not shared by two programs, the prediction results of a single database were selected. KOBAS3.0 (http://kobas.cbi.pku.edu.cn/) was used to performed KEGG and GO enrichment analysis of DE miRNA targets with a P < 0.05 required as the significance threshold.

**Exosomal miR-193b-3p overexpression in dendritic cells**

A lentiviral system was employed to overexpress miR-193b-3p in mouse DCs as a means to produce exosomes enriched in miR-193b-3p. DC2.4 was purchased from Procell Life Science & Technology (CL-0545). The plasmid pHBLCMVCZsGreen-T2A-Puro coupled with GFP (green) was used to construct the lentivirus vector containing miR-193b-3p according to the manufacturers’ instructions (Hanbio Biotechnology, China). In brief, DC2.4s were plated at 30–50% confluence and transfected with a previously prepared lentivirus consisting of a negative control vector or reconstructed vector containing a miR-193b-3p fragment. Following a 12 h incubation the culture medium containing the lentivirus was replaced with complete RPMI 1640 medium. Puromycin (15μg/ml) was used as a means to screen the DCs that were successfully transfected. At 48–60 h later, the DC2.4s transfected with lentivirus and exosomes secreted into the supernatant were collected for further analysis.
**LT mouse model**

SPF-grade male C3H and C57BL/6j mice (7-8 weeks old) served as LT recipients and donors, respectively. The mice were purchased from SiPeiFu (Beijing) Biotechnology Co., LTD [license SYXK (Beijing) 2017-0010]. The orthotopic LT model employed was generated using the established “two-cuff” technique as described previously[28]. Samples from these mice were obtained at seven and ten days after treatment. Mice were housed in SPF animal rooms in the Experimental Animal Center at the Beijing Friendship Hospital. All experiments in this study were performed according to the Beijing Friendship Hospital Guide for Laboratory Animals.

**Preparation of cells**

Mice were euthanized using carbon dioxide and the blood, liver and spleen were removed. Isolation of PBMC was the same as that described above. Lymphocytes from the liver and spleen were obtained following procedures contained in a previous report[28]. Erythrocytes were lysed using erythrocyte lysis buffer (R1010, Solarbio, China). The cells were filtered through a cell strainer (70 μm). Lymphocytes from the spleens of normal mice were plated at a density of 2×10⁵ cells/ml in a 96-well plate, which was previously coated with anti-CD3 (1μg/ml, BioLegend, USA) and anti-CD28 (1μg/ml, BioLegend) antibodies. IL-2 (100U/ml, #0717108, PEPROTECH) and TGF-β (20ng/ml, abs04222, absin, China) were added into the culture to induce Treg. These cells were then divided into three groups: 1) Exo-miR-193b-3p - cells co-cultured with 20 μg/ml Exo-miR-193b-3p or exo-NC, 2) miR-193b-3p mimics - cells transfected with 50 nM miR-193b-3p mimics or mimic-NC using RiboFECT™ CP Reagent according to the manufacturer’s protocol and 3) NLRP3-siRNA - cells treated with NLRP3 siRNA or NC.

**Flow cytometry**

The flow cytometry data were determined using Attune (Thermo, USA). Fluorescein isothiocyanate (FITC)-conjugated anti CD3 (300306, Biolegend), V500-conjugated anti CD45 (560777, Bioscience), Alexa fluor 700A (AF700)-conjugated anti CD4 (344622, Biolegend), V450-conjugated anti CD25 (560355, Bioscience), BV421-conjugated anti CD127 (351316, Biolegend), Allophycocyanin (APC)-conjugated anti CD196 (CCR3) and Phycoerythrin/Cyanine7 (PE-Cy7)-conjugated anti CD183 (CXCR6) were used to distinguish human Treg and Th17 cells. Phycoerythrin (PE)-conjugated anti CD11b (24965S, CST), allophycocyanin (APC) conjugated anti CD11c (33293S, CST), fluorescein isothiocyanate (FITC)-conjugated anti CD45 (62307S, CST), BV421-conjugated anti CD80 (104725, BioLegend) and BV605-A conjugated anti CD86 (105037, BioLegend) were used to characterize DCs. PerCP-Cy5.5-conjugated anti CD3 (100218, BioLegend), PE-conjugated anti CD4 (26589S, CST), AF700A-conjugated anti CD8α (100730, BioLegend), APC-conjugated anti CD25 (36055S, CST), PE-Cy7-conjugated anti Foxp3 (65210S, CST) were used to distinguish mouse Treg cells. FITC conjugated anti Ki-67 (11-5698-80, eBioscience) was used to evaluate cell proliferation, while the Live/Dead™ Fixable Aqua Dead Cell Stain Kit (L34965, Invitrogen) distinguished dead cells. For Foxp3 staining, the eBioscience™ Foxp3
fixation/permeabilization buffer kit was used following instructions of the manufacturer (00-5523-00; ebioscience). FlowJo version 10.8 (Tree Star Inc., Ashland, OR, USA) was used to analyze the results.

**Western blot**

Cell proteins were extracted on ice for 30 min using RIPA buffer with a protease inhibitor (P0013B; Beyotime, Shanghai, China). Protein samples were separated with 8–12% SDS-PAGE (Bio-Rad, Redmond, WA, USA), electroblotted onto polyvinylidene difluoride membranes (Billerica, MA, USA) and then incubated with primary anti-CD81 rabbit antibody (1:2000, ab109201; Abcam), anti-Alix rabbit antibody (1:2000, ab186429; Abcam), anti-TSG101 rabbit antibody (1:2000, ab125011; Abcam), anti-NLRP3 rabbit antibody (1:2000, ab263899; Abcam), anti-STAT3 rabbit antibody (1:2000, #30835; CST), anti-phospho-STAT3 rabbit antibody (1:2000, #34911; CST), anti-Caspase1 rabbit antibody (1:2000, ab138483; Abcam) or anti-β-actin rabbit antibody (1:2000, #4970; CST) at 4°C overnight. Horseradish-peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody (1:5000; Cell Signaling Technology). Antibody binding was detected using a chemiluminescence system (Tanon-5200 Multi; Shanghai, China).

**Quantitative reverse transcription (qRT)-PCR**

For qRT-PCR, the ABI7500 system (Applied Biosystems) was used. Extracted miRNA (100 ng) was reverse transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA Kit (KR211-02; Tiangen, Beijing, China) and qRT-PCR was performed with use of the miRcute Plus qPCR Kit (FP411; Tiangen). Total RNA (1500 ng) was reversed using the TRUEscript 1st Strand cDNA Synthesis Kit (PC1802; Aidlab, China) and qRT-PCR was performed with use of 2x Sybr Green qPCR Mix (low ROX) (PC60; Aidlab). mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with ΔCt calculated as Ct (mRNA of target) – Ct (reference gene). Primers are listed in Supplementary Data Table S9.

**Enzyme-Linked Immunosorbent Assay**

Cytokine levels of IL-17A, IL-2, IL-10, IL-6 and IL-1β and transforming growth factor (TGF)-β were assayed using ELISA according to instructions provided by the manufacturer (all ELISA kits from Anoric Biotechnology, Tianjin, China). All samples were measured in duplicate.

**Statistical analysis**

Data are shown as means ± SEMs. Comparisons among the three groups were performed using the Kruskal–Wallis test with the Mann-Whitney U-test or ANOVA with the Tukey test used for post-hoc pairwise comparisons of subgroups. Student’s $t$ tests were used for comparisons of data involving two groups. Data were tested for normality and log normality before use of parametric assessments. All analyses were conducted using GraphPad Prism version 8.0 (GraphPad Software). A $P < 0.05$ was required for results to be considered as being statistically significant.

**Results**
Characteristics of participants

A total of 67 participants were included in the study, with 9 cases in the normal control (NC) group, 25 in the rejection (R) group and 33 in the non-rejection (NR) group. The RAI score in the R group was 4.2±1.3. Aminotransferase levels in the R group were significantly greater than that in the other groups. There were no statistically significant differences in age, sex and follow-up times among the three groups. Detailed information on the characteristics of participants is contained in Table S1.

Characteristics of plasma-derived exosomes in human patients

Exosomes were extracted from the plasma of rejection (R) and non-rejection (NR) patients proved by biopsy and normal control (NC) from living-related LT donors (n=3 for each condition). The isolation procedure for exosomes is shown in Figure 1A. Results of TEM revealed cup- or oval-shaped exosome vesicles with diameters of approximately 100 nm (Figure 1B). Flow cytometry analysis indicated that exosomes were positive for CD81 and CD63 in the R group (Figure 1C), while western blots revealed that isolated exosomes were positive for Alix, TSG101 and CD81 (Figure 1D). NTA was used to determine the number and size distribution of particles in the purified exosomes with average particle sizes being within the expected range of 30–200 nm (Figure 1E).

Plasma-derived exosomal miRNA expression profiles in LT patients and donors

miRNA sequencing was used to profile exosomes within NC, R and NR groups with the reads mapped to miRNA being 19.69%, 27.46% and 42.13%, respectively (Figure 2A). We detected 825 miRNAs between the R and NR groups and 517 between the R and NC groups (Figure 2B). Compared to the NC group, there were five up-regulated and seven down-regulated differential expression (DE) miRNAs in the R group, and five up-regulated and eight down-regulated DE miRNAs as compared to the NR group (Figure 2C). Detailed information on DE miRNAs and RNA-seq are contained in Supplementary data-Table S2-S7. The GO and KEGG enrichment analysis of DE miRNAs between the R and NR group are shown in Figure S1A. To validate these miRNA sequencing data, 7 DE miRNAs were selected and their expressions quantified using qRT-PCR among three groups. With this analysis we found that the expressions of miR-3180-3p and miR-6739-5p were significantly up-regulated in the R group, while miR-193b-3p and miR-1295b-3p were markedly down-regulated, indicating that these miRNAs may be closely associated with T cell regulation after LT (Figure 2D). As miR-193b-3p has been reported to be involved with liver disease, we selected the highly abundant, miR-193b-3p, for use in subsequent investigations.

Treg cells are decreased in the peripheral blood of acute rejection patients

Peripheral blood mononuclear cells (PBMC) of patients and donors were analyzed using flow cytometry to assess the proportions of Treg and Th17 cells after LT. Compared with the NC and NR group, the percent of CD25^{hi}CD127^{low} Treg cells in the R group were significantly decreased (Figure 3A, B). Although levels of CD196^{hi}CD183^{low} Th17 cells in the R and NR groups were decreased as compared with the NC group, these differences failed to achieve statistical significance (Figure 3A, B). Lowest ratios of Treg to
Th17 cells were observed in the R group followed by the NC and then the NR group, however only the differences between R and NR groups was statistically significant (Figure 3B). Expressions of the plasma cytokines, IL-10 and TGF-β, in the R group were markedly decreased as compared with that of the NR group, while secretion of IL-2 was increased in the R group (Figure 3C). These results suggest that decreased Treg cells within the peripheral blood leading to a Treg/Th17 ratio are associated with acute rejection after LT. These findings are inconsistent with those of a previous report indicating that Treg was down-regulated and levels of Th17 cells were significantly increased in the peripheral blood of acute rejection LT patients[30]. Due to the differences of lymphocytes present in the circulation and allografts, we speculate that the decreased proportion of Treg cells in the peripheral blood might result from a decrease in Treg production and increased infiltration into the liver.

**T cell infiltration increase in LT patients**

When comparing HE staining results from liver samples in the NC and NR groups, those of the R group showed substantial morphological changes in hepatic tissue. Hepatocytes in the R group showed severe balloon-like degeneration, were swollen and necrotic, portal areas were infiltrated with many lymphocytes and neutrophils and sinusoidal endothelial cells were clearly injured (Figure 4A). In the rejection group, increased expressions of IL-17A, CD8, CD4 and Foxp3 within lymphocytes of same slide were present as compared with that observed in the normal and non-rejection groups (Figure S1B). To further understand the spatial distribution and changes of T cells in the process of acute rejection, mIF was used to assess the infiltration of T lymphocytes in the liver samples of three rejection and non-rejection patients and two normal control donors. The exact phenotype of T lymphocytes infiltrated within the graft after LT was identified from the liver samples. Four markers, CD4, CD8α, Foxp3, IL-17A and DAPI were used to identify Treg and Th17 cells. Hepatic sinusoidal endothelial cells, Kupffer cells and T cells expressed CD4, while T lymphocytes expressed CD8. We found that infiltration of CD4+ and CD8+ T cells were increased in the R group as compared with that in the other groups. IL-17A was mainly expressed in the cytoplasm of liver cells, endothelial cells and immune cells, while Foxp3 was mainly expressed in the nucleus of T cells. As expected, we detected simultaneous increases in CD4+Foxp3+ Treg, CD4+IL17A+ Th17, and CD8+ T cell infiltration in the acute rejection and non-rejection groups as compared with the normal control group, with maximal infiltration observed within the rejection group (Figure 4B, C). Moreover, we found a decrease in the proportion of Treg to Th17 and CD8+ T cells in the rejection group compared with the non-rejection group (Figure 4D). Taken together, we suppose that acute rejection results from an intrahepatic imbalance of Treg and inflammatory cells rather than a reduction in the number of Treg cells.

**Dynamic changes in Treg cells within the LT mouse model**

Results from HE staining indicated that acute rejection was present at seven days after LT in our mouse model and was accompanied with an infiltration of a large number of inflammatory cells, hepatocyte edema and microstructure damage within the liver. A further, and significantly worse, degree of rejection was observed at ten days post-LT (Figure 5A). Following LT, the proportion of Treg cells in the spleen increased when compared with that in normal mice, while the proportion of these cells decreased as a...
function of the degree of rejection present. Meanwhile, greater numbers of Treg cells in the blood and liver were present as a function of increases in the severity of acute rejection (Figure 5B). In both LT patients and our animal model of LT, increased numbers of Treg cells were recruited in the liver as a compensatory mechanism during acute rejection. Combining these results, we suppose that under conditions of acute rejection, increased numbers of Treg cells are activated and “homing” into the liver to negatively regulate pro-inflammatory cells.

**NLRP3 is a target gene of miR-193b-3p**

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, TargetScan, miRDB, miRWalk, and miRTarBase were used to predict potential miR-193b-3p target genes (Supplementary data-Table S8). Among the possible candidates, IL17RD, NLRP3, TGFBR3, LEF1 and TCF7L2 were all reported to be associated with CD4+ T cell function. As it had been reported that NLRP3 was related to immunological responses after transplantation and IHC staining revealed that NLRP3 expression was significantly increased in acute rejection patients, it seems likely that NLRP3 plays an important role in the immune rejection associated with LT (Figure S2A). Therefore, we examined whether NLRP3 may serve as a target for miR-193b-3p (Figure 6A). In addition, to determine the regulatory effects of miR-193b-3p on NLRP3, miR-193b-3p mimics were transfected into T cells (Figure 6B). Results of these experiments revealed that miR-193b-3p mimics increased CD25+Foxp3+ Treg cells (Figure S2B), while western blot results indicated a significant downregulation in NLRP3 protein levels (Figure 6C). In order to study the regulation of exosomal miR-193b-3p on NLRP3, we constructed a dendritic cell line with a high expression of miR-193b-3p. To achieve this goal, DC2.4, an immortalized dendritic cell line was used for transfection, and then exosomes in the culture supernate were isolated following the ultracentrifugation procedure (Figure S3). Exosomes (20 μg/ml) derived from DCs were co-cultured with T cells for 72 h to verify the transport of miR-193b-3p into target cells and their ability to inhibit NLRP3 expression. In response to such procedures we found that miR-193b-3p expression was upregulated in T cells (Figure 6D), while both mRNA and protein levels of NLRP3 were downregulated (Figure 6E, F). Based on these findings, we focused on NLRP3 as a target of miR-193b-3p and potential regulator of Treg cells.

**DC-derived exosomal miR-193b-3p increase Treg cells via the NLRP3/IL10/STAT3 signal pathway**

As a means to determine whether NLRP3 may contribute to the dynamic changes in Treg cells, we knocked down NLRP3 expression with use of small interfering RNA (siRNA). With this approach there was a significant increase in CD25+Foxp3+ Treg cells (Figure S2C). Whether DC-derived exosomes could be internalized by T cells and subsequently affect the frequency of CD25+Foxp3+ Treg was also investigated. Exosomes and target cells were labeled with PKH26 and CellTracker™ Green CMFDA, respectively, according to the manufacturer’s protocol and were then incubated for 72 h. After 8 h, the exosomes were mainly internalized by T cells (Figure 7A). Meanwhile, exosomal miR-193b-3p significantly increased the proportion of CD25+Foxp3+ Treg cells (Figure 7B, D). Compared to miR-193b-3p mimic/NC (Figure S2B), exosome-mediated miR-193b-3p delivery promotes the Treg cells more efficient. The Ki-67 index of Treg cells was increased, suggesting that exosomal miR-193b-3p activated
CD25+Foxp3+ Treg (Figure 7C). In addition, results from western blots revealed that exosomal miR-193b-3p decreased NLRP3 and Caspase-1 expressions while increasing phosph-STAT3 (p-STAT3) (Figure 7E). In the EXO-miR-193b-3p group mRNA levels of IL-1β, IL-17A and IL-6 were decreased, while those of TGF-β, IL-2 and IL-10 increased when compared with that of the negative control group (Figure 7F). Moreover, IL-17A and IL-1β secretions were decreased while those of TGF-β, IL-2 and IL-10 were increased in the EXO-miR-193b-3p versus EXO-NC group (Figure 7G). Related to these findings are reports indicating that miRNA reduces the expression of NLRP3 and increases the expression of IL-10 and STAT3, effects which can then activate Treg function and relieve inflammation[30]. Thus, we conclude that exosomal miR-193b-3p showing potential attenuates acute rejections of LT by increasing Treg cells via the NLRP3/IL10/STAT3 signal pathway.

Discussion

It has been well established that exosomes are critically related to the induction and maintenance of immune responses after transplantation. For example, findings from studies with transplant models have indicated that MHC molecules, carried by exosomes, represent key factors involved with initiating and maintaining homo-immune responses[33,34]. There is a 100- to 1000-fold increase in the number of T cells associated with allograft rejection as compared with that observed after microbial infection. This increase is attributable to the high frequency of T cells recognizing antigens, a process mainly mediated by exosomes and referred to as the “indirect” pathway. Donor serum-derived exosomes have the capacity to induce Treg cells and significantly prolong graft survival as demonstrated in a mouse model of heart transplantation[35]. And donor-derived exosomes found in blood samples of LT recipients can be captured by DCs through “cross-dressing” and subsequently induce tolerance[21]. DCs are the most potent antigen-presenting cells and play a pivotal role in T-cell function on adaptive immunity[36]. The regulation of CD86 and CD80 on T cells is bidirectional, and the percent of CD86 and CD80 expression in DCs is critical for maintaining the homeostatic balance between effector T and regulatory T cell populations[37]. CD80+CD86+ DCs inhibit inflammatory responses by releasing anti-inflammatory cytokines IL-10 and immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO)[38,39]. In addition to cytokines, the effect of immune regulation by DC is at least partly due to the role of exosomes. The capacity for suppressive activity of DC-derived exosomes as a means to reduce inflammation through interacting with CD4+CD25+ cells has been shown to depend on CD86 and CD80 in delayed-type hypersensitivity and rheumatoid arthritis models[37]. Exosomes derived from DCs have the ability to promote tolerance in disease models of cancer, transplantation and autoimmunity[40]. Immature DCs deriving exosomes can amplify Treg and prevent acute rejection in a rat LT model[41]. Our current results reveal the ability for DCs to promote Treg cells by delivering exosomal miR-193b-3p to T cells. The function of exosomes depends on the status of their parental cells and cargo.

Among the many components of exosomes, miRNAs represent essential messenger molecules[42] that could achieve post-transcriptional regulation. Approximately 70% of all miRNAs are expressed in the
liver\textsuperscript{[43]}. Accordingly, miRNAs serve as potential indicators of allograft function following LT. Free-circulating miRNAs (fc-miRNAs) are usually non-specific and unstable as they bind to lipoproteins or proteins released from cells either during apoptosis or necrosis and exhibit similar changes in a variety of disease or pathological states\textsuperscript{[44]}. In contrast to that of the low concentrations and poor stability of fc-miRNAs, exosomal miRNAs demonstrate a stable expression in bodily fluids\textsuperscript{[14]}, and some miRNAs have been shown to be preferentially released in exosomes\textsuperscript{[25, 45]}. Recent evidence has been presented showing that circulating exosomes can deliver miRNAs for intercellular communication as well as to regulate the expression of target genes and functions of recipient cells\textsuperscript{[46]}. Alexander et. al. reported that exosomes can modulate inflammatory responses via miRNAs between immune cells\textsuperscript{[46]}. Following LT, we identified 13 exosomal miRNAs between the non-rejection and acute rejection groups and 12 between normal controls and acute rejection group. Such results indicate that transplant-related miRNAs released into exosomes are specific and selective indirectly\textsuperscript{[48]}. In this report, the high expression of miR-193b-3p was selected for subsequent investigations as it has been reported to participate in liver diseases\textsuperscript{[49]}, and its capacity to regulate Treg cells was verified in \textit{in vitro}.

The most notable findings in this report include the revelation of the profile of exosomal miRNAs in response to LT and the influence of exosomal miR-193b-3p on the Treg cells, which may provide an index of tolerance induction after LT. When exosomes with increased miR-193b-3p were co-cultured with spleen lymphocytes \textit{in vitro}, we demonstrated that exosomal miR-193b-3p was effective in increasing Treg cells. Moreover, we found that not only could miR-193b-3p derived from DCs exosomes show similar biological function as that of miR-193b-3p mimics, but could also activate Treg cells as measured with Ki-67. At the same time, the promotion of Treg mediated by exosomal miR-193b-3p was more potent than that of miR-193b-3p mimics, suggesting the potentiation effect of exosomes. These results indicate that exosomal miRNA has the potential to prevent or attenuate allograft rejection by acting on Treg cells. The therapeutics targeting exosomal miRNAs hold great promise as a selective strategy for achieving immunosuppression.

Acute rejection seriously affects the quality of life and graft survival time after LT\textsuperscript{[1]}. Interestingly, an decrease of Treg cells is associated with allograft rejection as well as various inflammatory diseases, including autoimmune diseases and tumorigenesis\textsuperscript{[9]}. Results from clinical studies have revealed that, under conditions of acute rejection, the number of circulating Treg cells was significantly decreased, whereas Th17 cells were increased\textsuperscript{[7, 30, 50]}. However, the results of our study indicate that the percent of Treg cells were decreased in the peripheral blood when accompanied with rejection, while Th17 cells failed to show any substantial increase. As a result of these changes, the ratio of Treg to Th17 was lower in the rejection versus non-rejection group. The Treg related cytokines, TGF-β and IL-10, were found to be significantly increased in the non-rejection group. These data suggest that a decreased Treg/Th17 ratio in the peripheral blood was attributed to the reduction of Treg cells may serve as a potential indicator of acute rejection after LT\textsuperscript{[30]}. When gene expression profiles of liver and blood samples were determined following LT rejection, it was found that intrahepatic effector T cells and macrophages were significantly
increased, while CD8+ T cells significantly decreased in the blood[51]. The discrepancy that exists in lymphocytes between peripheral blood and grafts suggests that liver biopsy samples can provide increased levels of detailed information regarding the immune status after LT [52].

In the acute rejection group, a large number of lymphocytes and macrophages are accompanied with bile duct damage as observed with HE staining. However, it remains difficult to determine and differentiate the exact phenotypes and distribution of intrahepatic T cells from the same biopsy sample. With advances and widespread application of multiplexed imaging platforms, CD8+ T cells and subsets of CD4+ T cells, Th17 and Treg cells can now be revealed and differentiated within the same slide. With these techniques we found that the spatial distribution of CD8+ T, Th17 and Treg cells in hepatic lobules differed among the three groups[53]. In present study, the increased infiltration of CD4+ and CD8+ T cells within the liver emphasizes the functions exerted by T lymphocytes after acute rejection. Interestingly, an increased number of intrahepatic Treg cells was observed in the rejection group, however, the percent of Treg/inflammatory cells was decreased. In the mouse LT model, we found that acute rejection was associated with increases in Treg cells within the spleen. With the progression of deterioration resulting from rejection, there was a reduction of Treg cells in the spleen but a significant increase within the liver. These data provide evidence in support of our hypothesis that the body recruits more Treg cells into the liver, and therefore the reduction in circulating Treg cells represents a compensatory mechanism[4, 51, 54]. Such an effect may serve as the basis for the extensive infiltration of Treg cells within the liver following rejection. Taken together, we propose that the increased number of intrahepatic Treg cells was not comparable to that of Th17 and other inflammatory cells in the acute rejection group, and the Treg and inflammatory cells remained unbalanced.

Treg accumulation within the graft represents an important factor for tolerance in animal models[4, 51, 54], while our current results indicate that its infiltration increases with rejection. We have speculated that host T lymphocytes are primed after LT due to the molecular MHC of donors. When Treg and Th17/CD8+ T cells were in a dynamic balance, immune tolerance was induced without T lymphocyte infiltration in the liver[30]. However, when this balance was disrupted, Treg was no longer able to compensate for Th17 and other inflammatory cell functions. As a result, many pro-inflammatory cells from recipients such as Th17 and CD8+ T cells were activated and accompanied the infiltration of Treg cells within the liver. Immune tolerance requires a balance of the graft’s ongoing immunological activity, which can be accomplished with a transient and persistent trend toward the accumulation of Treg cells[52]. Sánchez-Fueyo has found that Treg transfer was able to reduce anti-donor T cell responses and facilitate the reduction of immunosuppression following liver transplantation, which showed the significant effect of Treg immunotherapy[52]. The dynamic increase of Treg cells can lead to an equilibrium of immunological activity after LT[7, 30]. We found that exosomal miR-193b-3p possesses the ability to restore the balance by increasing Treg cells. In this regard, the current study focused on the role of exosomes in the regulation of Treg cells as indicating a protocol with a great potential for clinical utility[15]. Further studies will be
required to generate a more comprehensive understanding of this phenomenon and its promise for clinical utilization.

miR-193b-3p can directly target cyclin D1 (CCND1) and ETS1, the regulators of Treg and CD8+ T cells\[56\] that participate in subarachnoid hemorrhage, ovarian cancer, breast cancer and hepatocellular carcinoma\(57^-60\). In this study, we report that an up-regulation of miR-193b-3p down-regulates the expression of NLRP3 along with increasing Treg cells, functions which are identical to that as obtained with NLRP3-siRNA. Simultaneously, the expressions of IL-1β/IL17A are decreased, and that of IL2/IL10/p-STAT3 increased. In addition to increasing the percent of Treg cells, exosomal miR-193b-3p also activates Treg function by increasing cytokine levels, which has significant implications for immune regulation after LT. Therefore, NLRP3 can function as a regulator of Treg cells. Upon activation, NLRP3 recruits ASC and procaspase-1, leading to caspase-1 activation and the processing of pro-IL-1β to produce mature IL-1β\[61\]. NLRP3 inflammasome-mediated release of IL-1β has been shown to skew CD4+ T cells toward Th17 differentiation leading to corneal graft rejection\[62\]. Inhibition of NLRP3 increases Treg\[63\] and reduces the incidence of bronchitis after lung transplantation\[64\], and silencing the activation of the NLRP3 inflammasome by miR-223-3p led to remarkable mitigation of fibrosis development and activation of hepatic stellate cells\[64\]. An up-regulation of the Treg cells in rheumatoid arthritis may result from the direct binding of miRNA-20a to the NLRP3 3′-UTR\[66\]. miR-520c-3p has the potential to inhibit NLRP3 inflammasome activation, thereby increasing IL-10 and STAT3 expression to activate Treg cell function\[31,32\]. Our current results suggest that exosomal miR-193b-3p can increase Treg cells by targeting the NLRP3/IL10/STAT3 signal pathway and, in this way, mitigate acute rejection after LT.

This work is not without limitations. The fact is that many factors influence the exosomal miRNAs in the plasma. We thought some miRNAs could not be detected mainly due to the low concentration of plasma exosomes and the selectivity of miRNA secretion. However, the RNA-sequencing data was used to screen the differential expression miRNAs; meanwhile, the high abundance of miR-193b-3p in the non-rejection group as compared to the non-rejection group was confirmed using qRT-PCR. Notably, no longitudinal observations on respective Treg cell populations were performed in peripheral blood and liver samples. In addition, no effective models have been established to test the effects of exosomal miR-193b-3p \textit{in vivo}. Despite these limitations, our findings provide a foundation for further investigations directed at the effects of exosomal miRNAs on immune tolerance induction and graft survival after LT.

**Conclusion**

Our work demonstrates that acute LT rejection was attributed to increased T cells infiltration leading to an intrahepatic imbalance of Treg and inflammatory cells. We also found that exosomal miR-193b-3p derived from DCs could inhibit NLRP3 and promote Treg cells, as demonstrated \textit{in vitro}. Based upon these results, we conclude that exosomal miR-193b-3p has the potential to attenuate acute rejection and induce tolerance after LT.
Declarations

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FOOTNOTE:

Conflicts of Interest: The authors have no conflicts of interest to declare.

Data Availability Statement: For further details, the datasets analyzed during the current study are available from the corresponding author on reasonable request. Due to the perioperative data may be updated over time, only one years within the publication date is available. The RNA-sequence data reported in this paper have been deposited in the Genome Sequence Archive (GSA) for human of Beijing Institute of Genomics, Chinese Academy of Sciences, https://gsa.big.ac.cn/gsa-human/ (GSA-Human: HRA001934).

Ethics Statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee(s) and with the Helsinki Declaration. This study was approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University (2020-P2-059-01). We declare that no organs from executed prisoners were used at Beijing Friendship Hospital, Capital Medical University. All the operations were approved by the Ethical Committee of Beijing Friendship Hospital, Capital Medical University, and all living donors were voluntary and altruistic. Informed consent was obtained from all individual guardians of participants included in the study.

Author Contributions ZJZ, LYS and LW: study concept and design, supervised the study, and critically revised the manuscript. BC, SPL, XJC and JS: collected and analyzed data, conducted the experiments and statistical analysis; BC: drafted the manuscript. All authors read and approved the final manuscript.

References


**Figures**
Figure 1

Figure 2

**Differential expression of miRNAs in plasma exosomes.** A: Distribution of mappable non-coding miRNAs by next-generation sequencing in circulating exosomes. B: Point plot showing miRNA profiles in the R and NC or NR groups. C: Heatmap of differential expression (DE) miRNAs between plasma-derived exosomes from donors (NC), rejection (R) and non-rejection (NR) patients. Red indicates upregulation and green downregulation. N=3 in each group. D: DE miRNAs as measured using qRT-PCR. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3

**Decreased Treg cells in peripheral blood of acute rejection patients.** A: Representative flow cytometry plots of CD25^{Hi}CD127^{Lo} Treg cells and CD196^{Hi}CD183^{Lo} Th17 cells in plasma from liver transplantation patients and donors. B: Percent of CD25^{Hi}CD127^{Lo} Treg cells, CD196^{Hi}CD183^{Lo} Th17 cells, and their ratios in the three groups. C: IL-10, TGF-β and IL-2 cytokines in plasma were measured using ELISA. NC: Normal control; R: Rejection; NR: Non-rejection; *p < 0.05, **p < 0.01, ns: not significant.
Figure 4

**Increased T cells infiltration as related to acute rejection in liver transplantation patients.** A: HE staining in liver samples of the NC, R, and NR groups. B: Representative images of mIF staining, CD4 (red), CD8α (cyan), Foxp3 (green) and IL-17A (yellow) together with DAPI (blue) components in the three groups to distinguish between CD25⁺Foxp3⁺ Treg and CD4⁺IL17A⁺ Th17 cells. Scale bars, 100μm. C: Percent of CD25⁺Foxp3⁺ Treg, CD4⁺IL17A⁺ Th17 cells, and CD8⁺ T cells in liver tissue following mIF staining. D: The
ratio of Treg to Th17 and CD8\(^+\) T cells. NC: Normal control; R: Rejection; NR: Non-rejection; *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ns: not significant.

**Figure 5**

*Intrahepatic Treg cell infiltration increases in mice after liver transplantation.* A: HE staining in livers of mice in the normal control group (NC) and at seven and ten days after liver transplantation. B: Dynamic
changes in CD25+Foxp3+ Treg cells from the spleen, peripheral blood and liver of NC mice and at seven and ten days after liver transplantation.

Figure 6

Up-regulation of miR-193b-3p down-regulates NLRP3 expression. A: Bioinformatic analysis indicating that miR-193b-3p regulates the expression of 3'-UTR of NLRP3. B: High expressions of miR-193b-3p after
transfection of 50 nM miR-193b-3p mimics or NC into T cells. C: Transfection of miR-193b-3p mimics induced a significant downregulation of NLRP3. D: Expression of miR-193b-3p in T cells at 24 h after coculture with DCs derived exosomes. E: Exosomal miR-193b-3p downregulates the NLRP3 expression as quantified using qRT-PCR. F: Western blot showing that NLRP3 expression was decreased by exosomal miR-193b-3p. Data are presented as means ± SEMs. Data represent that as determined from three independent experiments. **P < 0.01, ***P < 0.001.
Exosomal miR-193b-3p induce Treg cells via regulating NLRP3 expression. A: Exosomes were stained with PKH26 red membrane dye and T cells counterstained with CellTracker™ Green CMFDA (blue). B: Representative flow cytometry of Treg cells. C: Representative flow cytometry of Ki-67 index in Treg cells. D: Frequency of Treg cells within spleen lymphocytes after cocultured with exosomal miR-193b-3p/NC. E: Western blot showing expressions of NLRP3, phosph-STAT3 (p-STAT3), STAT3 and Caspase-1 with β-actin as a control. F: Cytokines were measured using qRT-PCR. G: Results from Elisa confirmed the differential expression of cytokines. Data are presented as means ± SEMs. Data represent that as determined from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns: not significant.

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