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Exosomes Derived From T Regulatory Cells Suppress CD8+ Cytotoxic T Lymphocyte Proliferation and Prolong Liver Allograft Survival

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Background: CD8+ cytotoxic T lymphocytes (CTLs) have been proved to exert crucial roles in immunological rejection. Exosomes (EXOs) secreted by CD4+CD25+ regulatory T (Treg) cells is believed to be deeply involved in immune regulation. Nevertheless, whether immunomodulatory effect of CD4+CD25+ Treg cells on CD8+ CTL depends on EXOs remains unknown and needs to be explored.

Material/Methods: We purified CD4+CD25+ Treg cells followed by *in vitro* amplification. EXOs in culture supernatants of Treg cells was separated and identified. The effect of CD4+CD25+ Treg cells and CD4+CD25+ Treg cells-derived EXOs on CD8+ CTL viability, proliferation, cell cycle mRNA, intracellular cytokines, and protein expression were investigated.

Results: We successfully obtained EXOs from CD4+CD25+ Treg cells. The inhibition effect of EXOs on CD8+ CTL was concentration-dependent. In addition, the inhibition effect of CD4+CD25+ Treg cells could be reversed by GW4869, an EXOs inhibitor. The inhibition effect was associated with the regulation of IFN-γ and perforin. Our *in vivo* experiments proved that natural CD4+CD25+ Treg cells-released EXOs can prolong liver allograft survival.

Conclusions: CD4+CD25+ Treg cells-derived EXOs could become an alternative tool for manipulating the immune system to discover novel underlying immunomodulatory mechanisms.

MeSH Keywords: Allografts • CD8-Positive T-Lymphocytes • Exosomes

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Background

It has been generally recognized that organ transplantation is the most effective treatment for patients with end-stage organ failure [1]. However, due to the body's inherent immunological rejection of foreign organs, immunosuppressive drugs have to be used with transplantation. Although long-lasting immunosuppression has significantly increased graft survival in recent decades, it also increases the occurrence of opportunistic infections and cancer. It has become increasingly accepted that inducing allograft acceptance or tolerance is better than long-lasting administration of immunomodulatory molecules [2].

$CD8^+$ cytotoxic T lymphocytes (CTLs) have been proved to play a crucial role in immunological rejection. In contrast, $CD4^+CD25^+$ regulatory T (Treg) cells have previously been verified to alleviate this response by inhibiting immunological rejection and creating immune tolerance. By inhibiting the expansion of self-antigen-reactive T cells that are activated upon antigen simulation, natural polyclonal $CD4^+CD25^+$ Treg cells are deeply involved in the maintenance of self-tolerance [3]. Instead of secreting immune-suppressive cytokines, they exert non-specific suppression through an cell-to-cell contact mechanism [4]. Previous reports have revealed several cell contact-independent mechanisms responsible for Treg cells triggering suppression of T cell activation, including cytokines deprivation (such as IL-2) and inhibitor release [5,6]. In the last decade it has been also confirmed that Treg cells can release exosomes (EXOs) [7,8] to achieve intercellular communication without direct cell contact, by delivering suppressive messages to target cells.

EXOs are membrane-derived small vesicles that originate from endocytosis; they are rich in proteins and RNAs, with diameters 50–100 nm [9,10]. As a result, the EXOs can easily be delivered into plasma after endocytic-originated membrane fusing with the targeted cells [11,12]. The bioactive messengers within the EXOs dictate the subsequent behavior of target cells, and these effects are strictly dependent on the physiological status of the original cells that EXO vesicles are derived from [13]. There has been growing interest in the fact that immune cell-released EXOs show modulatory potential in immune responses [14]. Nevertheless, it remains unclear whether natural Treg cell-secreted EXOs can exert inhibitory effects on immune modulation.

Material and Methods

Materials

The antibodies (details are provided in the corresponding sections) were from Santa Cruz Biotechnology (CA, USA) unless otherwise stated. All other chemicals and reagents, unless otherwise stated, were from Aladdin Chemical reagent Co., and were of analytical grade. All cells were cultured in RPMI 1640 medium (Sigma, USA) supplemented with 20 U/mL of IL-2 and T cell expander (Dynabeads™, Thermo-Fisher, USA) in a humidified atmosphere of 95% air/5% CO_2 in an incubator (Thermo Forma 311, Thermo Scientific, USA) at 37°C. Male BABL/c mice, Sprague-Dawley (SD) rats, and Wistar rats age 6–8 weeks were obtained from SLAC (Shanghai, China) and raised in the specific pathogen-free (SPF) II lab with free access to feed and water. All procedures were in accordance with the National Institutes of Health guidelines and ethics approval was received from the Ethics Committee of the First Affiliated Hospital of Kunming Medical University (Yunnan, China).

Isolation of cells

Lymphocytes (from spleens of BABL/c mice) were isolated by centrifugation using Lymphoprep. $CD8^+$ CTLs were prepared as previously described [15]. To obtain $CD4^+CD25^+$ Treg cells, cells were first incubated with antibodies (FITC anti-CD4 and PE anti-CD25) against surface markers, according to the manufacturer's instructions. Cells were separated using flow cytometry (Attune NxT, Thermo-Fisher, USA). Magnetic separation was performed according to a previous report [16] followed by cell sorting using a flow cytometer. The cell purity of the subsets after cell sorting was analyzed in the flow cytometer as part of the cell sorting procedure. Purified $CD4^+CD25^+$ Treg cells and $CD8^+$ CTLs were cultured for 1 week [17].

Exosomes (EXOs) isolation and purification

Culture medium was conditioned by $CD4^+CD25^+$ Treg cells for 48 h. Afterwards, the culture supernatant of was collected and subjected to serial centrifugation [16] to eliminate debris. Afterwards, the supernatants were again ultra-centrifuged at 110 000 g for 1 h at 4°C). The pellets were again washed in PBS and subjected to ultracentrifugation. The final pellets were re-suspended in appropriate amounts of PBS, filtered (0.22 μm), and stored (–80°C) until further use [17]. EXOs were observed by transmission electron microscopy (TEM, JEOL 1200EX, JEOL, Japan) as reported previously [18] and the size distribution of EXOs was determined using a Zeta/Particle Analyzer (Litesizer 500, Anton Paar, Austria) as reported previously [19].

Analysis of cell proliferation and cell cycle

The inhibition effect of $CD4^+CD25^+$ Treg cells on $CD8^+$ CTL proliferation was investigated. $CD8^+$ CTL cells were seeded into 24-well plates at a density of 1×10^6 cells/well. Afterwards, $CD4^+CD25^+$ Treg cells (1×10^6 cells/well, with/without 10 μM GM4869) and $CD4^+CD25^+$ Treg cells-derived EXOs (40 μg with/without GM4869 or 10 μg without GM4869, per well) were added to co-culture for 48 h. At predetermined time intervals,

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CD8+ CTL cells were collected and resuspended with an appropriate amount of culture medium. We used the Cell Counting Kit (CCK8, Beyotime, China) according to the manufacturer's instructions, and cell viability was determined at OD_{450} using a microplate reader (SpectraMax iD5, Molecular Devices, China). CD8+ CTLs without any treatment were selected as blank control.

Allogeneic mixed lymphocyte reaction (MLR) was conducted to further determine the proliferation of CD8+ CTLs as reported previously [20]. In brief, CD8+ CTLs were firstly labeled with 5 μmol/L Cell Proliferation eFlour 670 (Thermo Scientific, USA). In addition, CD4+CD25+ Treg cells were pre-processed with 25 μg/mL mitomycin-c for 30 min. Afterwards, CD8+ CTL were seeded into 24-well plates at the density of 1×10^6 cells/well and treated with the above-mentioned groups for 48 h of incubation. The proliferation of CD8+ CTLs was examined by flow cytometric measurement.

CD8+ CTLs subjected to the above-mentioned treatments for 48 h were collected, washed twice with precooled PBS, and resuspended in 250 μL PBS. Then, another 750 μL of ethanol was slowly added, mixed sufficiently, and frozen at –20°C overnight. We then added 500 μL propidium iodide (PI) and CD8-FITC to the mixed solution and incubated it at room temperature for 30 min. Finally, the cells were subjected to flow cytometry analysis.

mRNA validation assays

The mRNA was first reverse-transcribed to first-strand cDNA using a BeyoRT II cDNA first-strand synthesis kit (Thermo-Fisher Scientific). RT-qPCR was performed using the BeyoFast SYBR Green qPCR Mix (Thermo-Fisher Scientific) with the optimized procedure of denaturation at 95°C for 10 min at 1 cycle, 95°C for 30 s, and 58°C for 30 s (40 cycles). The reaction was run on an ABI 7500 and data analysis was performed using 7500 software v2.0 (ABI). Data were analyzed using the ΔΔCt method with snRNA U6 as the endogenous control. The following primers were used for the amplification of target transcripts:
GAPDH forward (5'-GGTTGTCTCCTGCGACTTCA-3') and reverse (5'-GGGTGGTCCAGGGTTTCTTA-3');
IFN-γ forward (5'-GAAAGCCTAGAAAGTCTGAATAACT-3') and reverse (5'-ATCAGCAGCGACTCCTTTTCCGCT-3');
perforin forward (5'-GATGTGAACCCTAGGCCAGA-3') and reverse (5'-GGTTTTTGTACCAGGCGAAA-3').

Western blot analysis

Total protein from the samples was extracted using RIPA lysis buffer (Thermo-Fisher Scientific) following the manufacturer's instructions, followed by concentration quantification using the Bradford protein concentration determination kit (Thermo-Fisher Scientific). Afterwards, Western blot analysis was performed strictly according to the method described in a previous report [21]. Briefly, samples were resolved by gel electrophoresis, transferred onto PVDF membranes, stained with corresponding primary antibodies, followed by incubation with IRDyeR680CW-labeled secondary antibody. Finally, the blots were observed using a densitometer (E-Gel Imager, Thermo-Fisher, USA). The results of protein expression were then quantified with Quantity One software (Bio-Rad, Hercules, CA, USA). The expression of beta actin was used as a control. Each sample was repeated in triplicate. The antibodies used were: LAMP-1 (catalog number: sc-71489), CD63 (catalog number: sc-365604), IFN-γ (catalog number: sc-12753), perforin (catalog number: sc-136994), and beta actin (catalog number: sc-376421).

Intracellular cytokine staining (ICS)

ICS was performed according to the method described in a previous report [22]. Briefly, CD8+ CTLs were subjected to the above-mentioned treatments for 48 h. Afterwards, Brefeldin A (10 μg/mL) was added for another 4 h of incubation. Then, the cells were stained with Live/Dead Fixable Red Stain dye (Thermo Scientific, USA) and fluorochrome-conjugated monoclonal antibodies for cell surface proteins, according to the manufacturer's instructions. The stained cells were fixed and permeabilized using a fixation/permeabilization buffer kit (BD Biosciences, USA) and stained for intracellular cytokines. The stained cells were subjected to flow cytometric analysis.

Orthotopic liver transplantation (OLT)

OLT was performed using a modified Kamada's technique, as previously reported [23]. Briefly, the liver was first perfused with lactated Ringer's solution (4°C) through the catheter and then stored in the same solution (4°C). After about 50 min of ischemic time, the allograft was transplanted orthotopically. The surgery was performed using an SD rat as donor and Wistar rat as recipient. The study was approved by the Ethics Committee of the Fist Affiliated Hospital of Kunming Medical University (approval number 201803138, dated 30 June 2018). A two-cuff technique was used in the reconstruction of portal vein and infrahepatic inferior vena cava. No antibiotics were used in the entire experiment. After OLT, 12 rats were chosen and randomly divided into 2 groups (A and B). Rats in group A received CD4+CD25+ Treg cells (6×10^7 cells of Wistar rat)-derived EXOs via intravenous injection (Day 1, 3, and 5 after transplantation) at a dose of 0.5 mL, while rats in group B received 0.5 mL of PBS (injected into the caudal vein) and were used as a blank control [24].

Results

To design an optimal protocol to satisfy the successful *ex vivo* expansion of purified CD4+CD25+ Treg cells from spleen lymphocytes, various culture conditions were tested using CD4+CD25+

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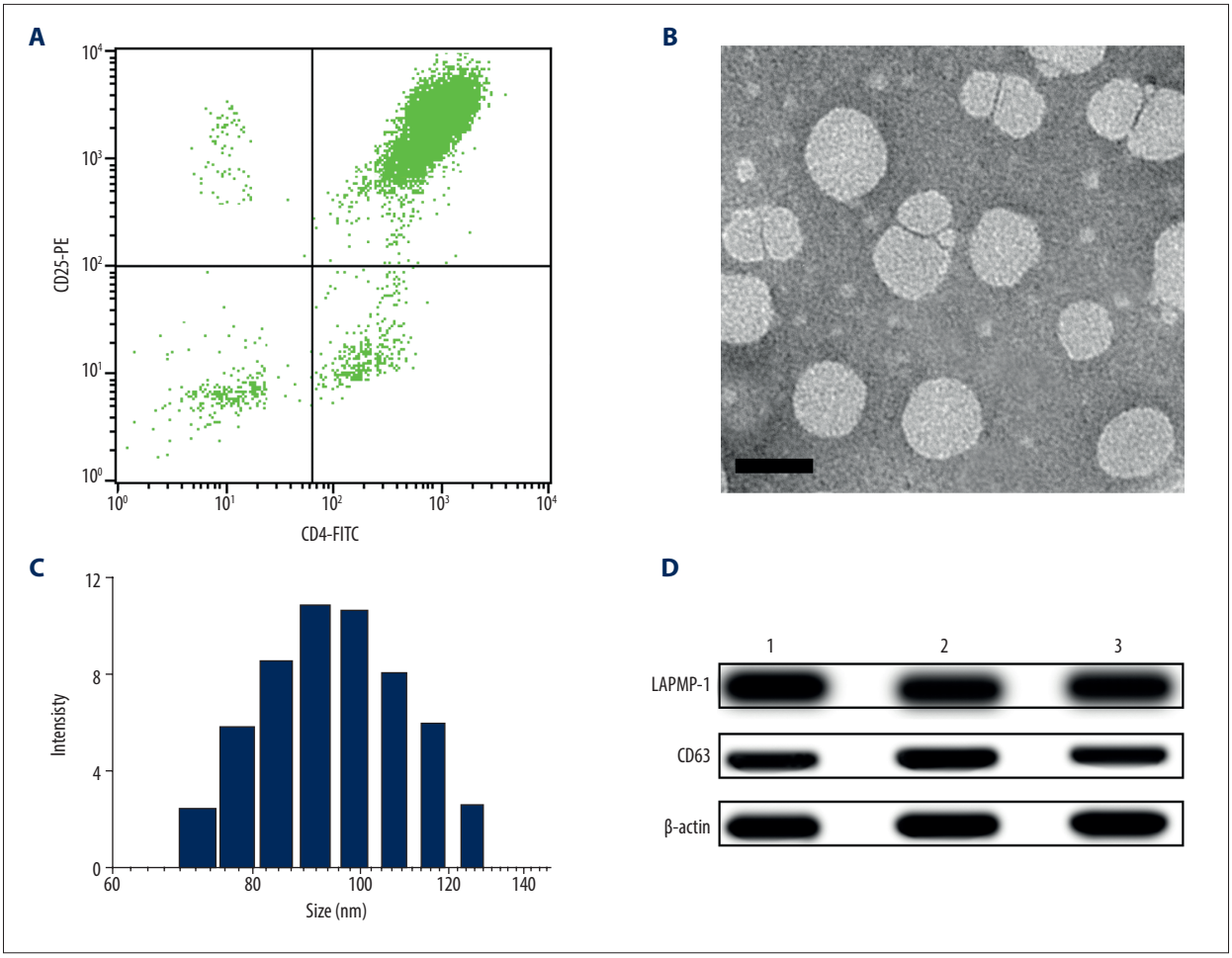

Figure 1. (**A**) Flow cytometric analysis of purity CD4+CD25+ Treg cells. (**B**) Electron micrograph of EXOs. Scale bar: 100 nm. (**C**) Size distribution of the EXOs. (**D**) Western blot analysis of EXOs. All 3 representative experiments were shown.

Treg cells from healthy individuals. Finally, $CD4^+CD25^+$ Treg cells were isolated from spleen lymphocytes by flow cytometric cell sorting. Results showed that the purity of $CD4^+CD25^+$ Treg cells was 93.2% (Figure 1A).

Figure 1B depicts an acquired TEM image of EXOs, demonstrating that EXOs secreted from $CD4^+CD25^+$ Treg cells had a typical round shape or exosomal "saucer" with a diameter of around 100 nm. The size distribution pattern of EXOs is displayed in Figure 1C, showing that the EXOs were narrowly distributed around 100 nm, which was consistent with results obtained by TEM. We chose LAMP-1 and CD63 as 2 different indicating proteins to verify the successful preparation of EXOs. Western blot analysis showed the simultaneous presence of LAMP-1 and CD63 (Figure 1D).

We tested the proliferation inhibition effect of $CD4^+CD25^+$ Treg cells, as well as $CD4^+CD25^+$ Treg cells-derived EXOs, on CD8+ CTL. As shown in Figure 2A, after 48 h of co-incubation, the cell viability of $CD4^+CD25^+$Treg cells-treated $CD8^+$ CTLs was only 52.23%, which was shorter than in untreated cells. Interestingly, we found that $CD4^+CD25^+$ Treg cells-derived EXOs also inhibited $CD8^+$ CTLs in a concentration-dependent manner. Low-concentration EXOs showed a much higher cell viability (81.34%) while high-concentration EXOs showed a stronger inhibition effect on $CD8^+$ CTLs, with 60.37% cell viability at 48 h after incubation, which was comparable to the inhibition effect of $CD4^+CD25^+$ Treg cells. In addition, it was noted that the inhibition effect of $CD4^+CD25^+$ Treg cells was reversed by GW4869, an EXOs inhibitor [25]. It was interesting to observe that when incubated with EXOs, the inhibition effect of EXOs was not significantly affected. As displayed in Figure 2B, and in line with the results in Figure 1A, the MLR of $CD8^+$ CTLs was significantly inhibited by $CD4^+CD25^+$ Treg cells, and this effect was reversed by GW4869. More importantly, the $CD4^+CD25^+$ Treg cells-derived EXOs showed comparable effects to $CD4^+CD25^+$ Treg cells, and this effect was not affected by GW4869.

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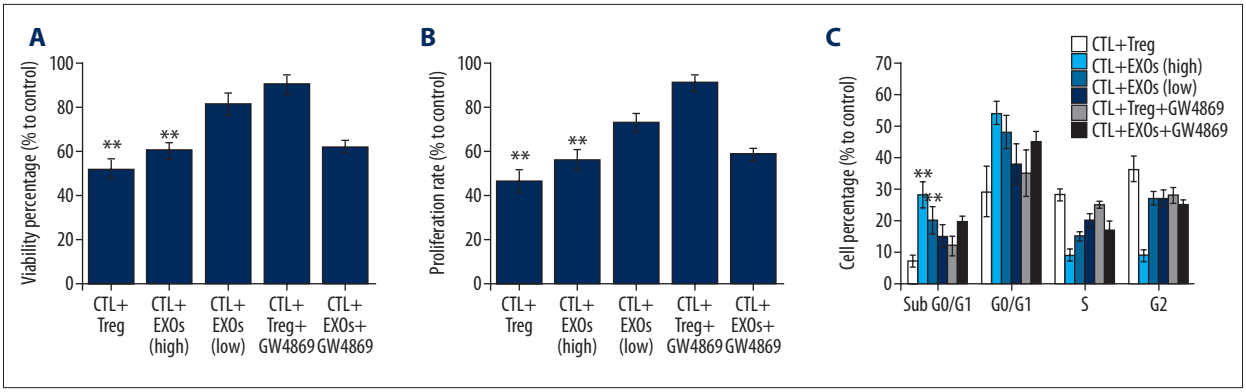

Figure 2. (**A**) Cell viability and (**B**) MLR and (**C**) cell cycle of CD8+ CTL treated with CD4+CD25+ Treg cells (1×10^6 cells/well, with/without 10 μM GM4869) and CD4+CD25+ Treg cells-derived EXOs (40 μg with/without GM4869 or 10 μg without GM4869, per well) for 48 h. Untreated CD8+ CTL cultured for the same period of time was employed as control. ** p<0.01 *vs.* control. Values are expressed as the mean ± standard deviation (n=3).

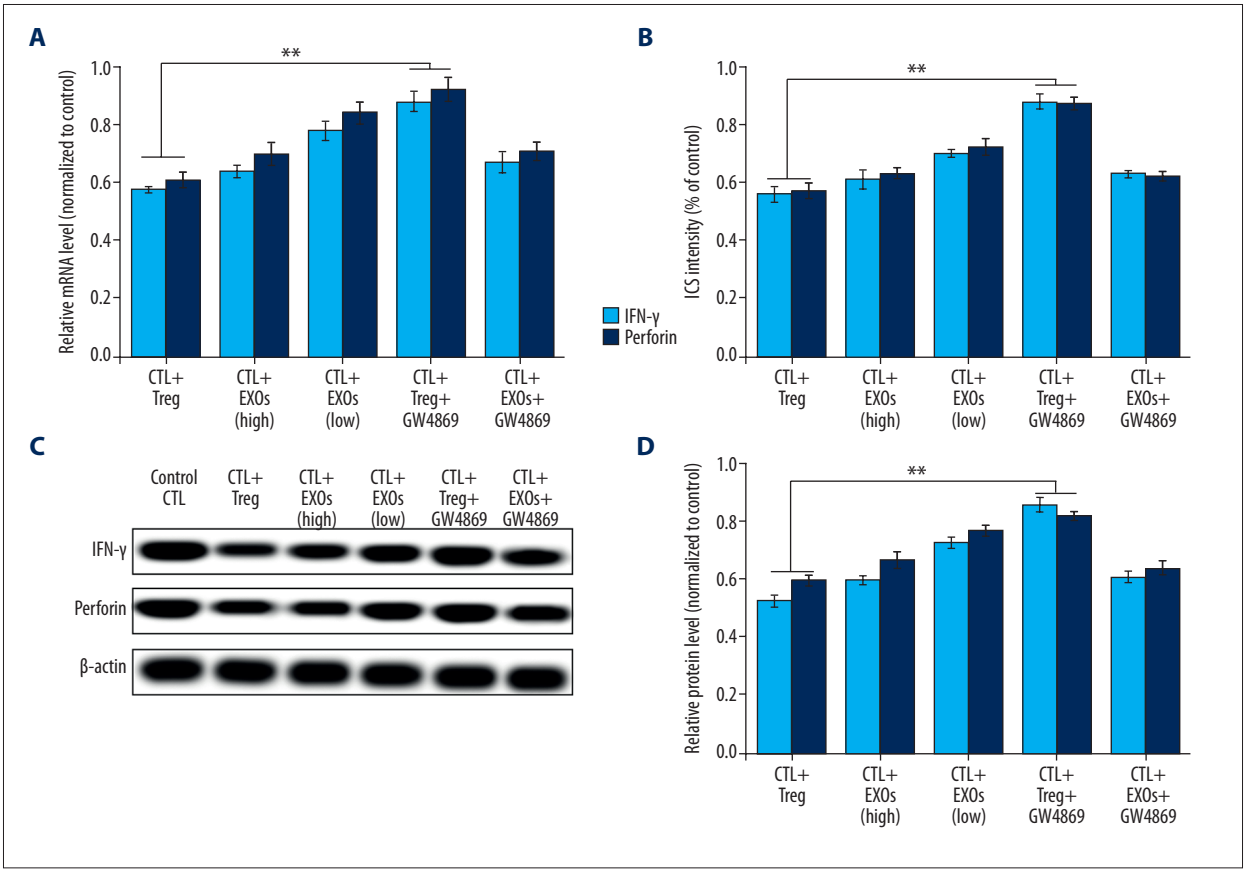

Figure 3. (**A–D**) (**A**) qtPCR, (**B**) ICS, and (**C**) Western blot analysis of perforin and IFN-γ expression in CD8+ CTL treated with CD4+CD25+ Treg cells (1×10^6 cells/well, with/without 10 μM GM4869) and CD4+CD25+ Treg cells-derived EXOs (40 μg with/without GM4869 or 10 μg without GM4869, per well) for 48 h. Untreated CD8+ CTL cultured for the same period of time was employed as control. ** p<0.01. Values are expressed as the mean ± standard deviation (n=3).

As shown in Figure 2C, compared with untreated CD8+ CTLs, CD4+CD25+ Treg cells-treated ones showed cell cycle arrest in G0/G1 phase, which indicated that cell proliferation in this group was suppressed. We also noted that the percentage of cells in sub-G0/G1 phase in this group was different from that in the control group. Subsequent experiments obtained results consistent to those of cell viability assays. CD4+CD25+ Treg cells-derived EXOs showed similar effect to CD4+CD25+ Treg cells,

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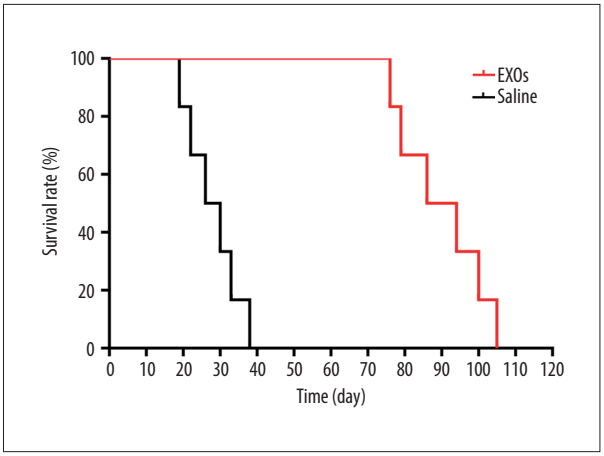

Figure 4. Kaplan-Meier curves for the survival time after OLT. The difference in overall survival time between the 2 groups was significant ($p<0.001$). (NS – not significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

and the effect was concentration-dependent. Once treated with GW4869, the cell cycle profile of $CD4^+CD25^+$ Treg cells became similar to that of untreated cells, while that in $CD4^+CD25^+$ Treg cells-derived EXOs showed no significant changes.

We used IFN-γ and perforin as 2 representative proteins to verify the activity of $CD8^+$ CTL. The corresponding mRNA level of these 2 proteins were first determined using qtPCR. As shown in Figure 3A, compared with untreated $CD8^+$ CTLs (control), the mRNA level in $CD4^+CD25^+$ Treg cells-treated $CD8^+$ CTL was much lower. It was calculated that the mRNA level was only 58% and 61% for IFN-γ and perforin, respectively, in this group. In addition, in line with results obtained from cell viability, proliferation, and cell cycle assays, qtPCR results showed that $CD4^+CD25^+$ Treg cells-derived EXOs inhibited $CD8^+$ CTLs in a concentration-dependent manner. High-concentration EXOs showed much more effective inhibition than low-concentration ones. When treated with GW4869, the inhibition effect of $CD4^+CD25^+$ Treg cells was reduced, as the mRNA levels of both proteins in CD8+ CTL returned to almost 90% that of normal expression. ICS was further performed to study the intracellular changes of both proteins. As displayed in Figure 3B, $CD4^+CD25^+$ Treg cells decreased the expression of both proteins via the secretion of EXOs. Finally, Western blot assay was performed to further confirm the above observations (Figure 3C, 3D). Western blot assay demonstrated trends similar to qtPRC assay results in the expression of both proteins.

To determine if $CD4^+CD25^+$ Treg cells-derived EXOs could prolong the survival of rats after liver transplantation, an OLT rat model was established and EXOs were subsequently administered into the recipients to observe the transplantation tolerance. As shown in Figure 4, the control group (group B) showed a medium survival of 28 days after transplantation, while the group treated with $CD4^+CD25^+$ Treg cells-derived EXOs (group A) had a clearly longer survival of 90 days.

Discussion

EXOs is a widely distributed nanovesicle that can be released from T cells [26]. To confirm that the EXOs used in our study were released from $CD4^+CD25^+$ Treg cells, a standardized method (ultracentrifugation after density gradient centrifugation) was used for EXOs purification from cell culture supernatant of $CD4^+CD25^+$ Treg cells. The globular vesicles obtained by this method had densities of 1.13–1.21 g/ml (floated on 30% sucrose/D_2O), which falls within the scope of EXOs according to a previous report [27]. The morphology of the obtained EXOs was observed using TEM [28], which demonstrated that EXOs secreted from $CD4^+CD25^+$ Treg cells had a typical round shape or exosomal "saucer" with a diameter of about 100 nm, which was in line with previous reports [27,29]. It has been well recognized that the particles about 100 nm in size around are best for *in vivo* distribution and long-term circulation, which are both conditions that allow EXOs to exert their functions [18,28].

It was reported that the membrane proteins most commonly associated with EXOs are tetraspanins, which includes CD82, CD63, and CD9, and are generally used as EXOs markers [30,31]. LAMP-1 is also considered another important indicator protein for EXOs identification [32]. The simultaneous presence of LAMP-1 and CD63 shown by Western blot analysis suggested that the obtained nanoparticles were indeed EXOs, and these were used in subsequent experiments.

The proliferation of $CD8^+$ CTL plays a crucial role in the immune system. Previous research has revealed that immunological rejection can be significantly alleviated by suppressing $CD8^+$ CTL proliferation [33]. We sought to further demonstrate the proliferation profile of $CD8^+$ CTLs after different treatments. MLR was performed to assess how $CD8^+$ CTLs react to different stimuli. The cell cycle represents the series of events within cells that leads to its division and DNA replication to produce 2 daughter cells [34]. To further study the inhibition effect of EXOs on $CD8^+$ CTL proliferation, cell cycle analysis was performed. It is generally recognized that cell cycle arrest usually indicates decreased cell viability or even cell apoptosis [35]. Results shown in Figure 2C suggested that $CD8^+$ CTL apoptosis also occurs after exposure to $CD4^+CD25^+$ Treg cells. Figure 2 shows that $CD4^+CD25^+$ Treg cells dramatically suppressed the proliferation of $CD8^+$ CTLs, which further confirmed that the inhibition effect of $CD4^+CD25^+$ Treg cells on $CD8^+$ CTL was dependent on the secretion of EXOs.

Perforin is a cytolytic pore-forming protein found in CTL [36]. It regulates the degranulation process of cells by binding to the

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target cell's plasma membrane, and then oligomerizes. This process is dependent on Ca^{2+}, and finally forms pores on the target cell. This allows for the passive diffusion of pro-apoptotic proteases (also known as granzymes) into the target cell [37] to create transmembrane tubules and to lyse a variety of target cells [38]. As a result, perforin is recognized as one of the main cytolytic proteins of cytolytic granules that serves as a key effector molecule for cytolysis mediated by T cells and natural killer cells [39]. IFN-γ is a dimerized soluble cytokine that is the only member of the type II class of interferons [40]. It is generally recognized as a cytokine that is critical for innate and adaptive immunity against viruses, as well as against some bacterial and protozoal infections [41].

qtPCR, ICS, and Western blot assays (Figure 3) demonstrated that EXOs excreted by $CD4^+CD25^+$ Treg cells were responsible for the decreased expression of perforin and IFN-γ in $CD8^+$ CTLs, and adds further evidence supporting the conclusion that the inhibition effect of $CD4^+CD25^+$ Treg cells on $CD8^+$ CTL depends on the secretion of EXOs. Finally, the liver transplantation experiment using an OLT rat model showed that EXOs given intravenously after transplantation can significantly extend the survival time of allograft model rats. Although there were species difference between exosomes (mouse) and liver transplant model (rat), we still obtained positive results, suggesting that different species share the same regulation mechanism, which might be a universal finding across studies.

Conclusions

In summary, in our study, we purified and amplified *in vitro* $CD4^+CD25^+$ Treg cells. Afterwards, EXOs were extracted from $CD4^+CD25^+$ Treg cells cell culture supernatants and verified by various methods. The effect of $CD4^+CD25^+$ Treg cells on $CD8^+$ CTL proliferation, cell cycle, and protein expression was investigated. We proved that natural $CD4^+CD25^+$ Treg cell-released EXOs can prolong liver allograft survival. EXOs derived from $CD4^+CD25^+$ Treg cells might become an alternative tool for manipulating the immune system to discover novel underlying immunomodulatory mechanisms.

Conflict of interests

None.

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