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Role of programmed death ligand 1 and Kupffer cell in immune regulation after orthotopic liver transplantation in rats

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ABSTRACT

Introduction: Role of programmed death ligand 1 (PD-L1) and Kupffer cells (KCs) in liver transplantation immune regulation was unclear.

Methods: Lewis and Brown-Norway (BN) rats were assigned to LEW-BN group (Lewis-to-BN liver transplantation) and BN-BN group (BN-to-BN). Receipts were sacrificed for histology and assessment of cytokines and PD-L1 production. Effect of PD-L1 and KCs on T cells (TCs) was monitored by co-culture of ^{3}H-Thymidine TCs. KCs transfected with PD-L1-shRNA interference plasmids were co-cultured with TCs, PD-L1 expression and cytokines production were measured respectively.

Results: Recipients in BN-BN group survived a long time while acute rejection was found in LEW-BN group. ELISA showed plasma levels of IL-2, IFN-γ and TNF-α in BN-BN group were significantly lower and levels of IL-10 were significantly higher than that in LEW-BN group on day 7 after transplantation ($P < 0.05$). PD-L1 expression of KCs in BN-BN group was significantly higher than that in the LEW-BN group ($P < 0.05$). Proliferation rate of TCs in KCs + TCs group was significantly lower and its apoptosis rate was significantly higher than that in TCs group ($P < 0.05$). IL-2, TNF-α and INF-γ levels were remarkably higher and IL-10 levels were lower in KCs + TCs group than that in TCs group ($P < 0.05$). Levels of IL-2, IFN-γ and TNF-α in transfection group were significantly higher and that of IL-10 was notably lower than that in the un-transfected group ($P < 0.05$).

Conclusion: KCs with high expression of PD-L1 could significantly suppress the proliferation and function of TCs. Silencing the expression of PD-L1 in KCs *in vivo* could restore the function of TCs.

1. Introduction

Liver transplantation has been established as the preferred treatment for patients with irreversible end-stage liver diseases. Owing to efficient immunosuppressives, preservation solutions, modern anesthetic and surgical techniques, recipients' survival rate has increased significantly. However, acute rejection and adverse reaction of immunosuppressive agents remain a critical problem in clinic. Inducement of donor-specific transplant tolerance has been suggested as one of the potential solutions to prevent immune rejection after transplantation. Liver grafts are spontaneously accepted in several animal combinations [1,2]. The exact mechanism of the induction of this unique tolerance remains unclear. Some studies suggest that the development of tolerance was associated with higher apoptosis rates of T cells (TCs) in the portal area [3,4]. Further investigations suggest that a subset of cells in donor graft might induce the apoptosis of recipient TCs, thus preventing graft rejection [5,6]. The mediating cell population as well as its mechanism has not been well elucidated.

Kupffer cells (KCs) are the resident macrophages of the liver. Its role in immune response has been highlighted in early studies [7], yet its role in liver transplantation tolerance has been rarely reported [8]. KCs are known to express co-stimulator molecules (CD80, CD86), Fas ligand (FasL), and have strong antigen presentation capability [9,10]. It is not only directly recognized and attacked by host TCs, but also has interaction with the host TCs that migrate into the graft. Our previous study has demonstrated that graft KCs could induce TCs depletion *via* Fas/FasL pathway resulting in liver transplant tolerance [11]. This effect could not be completely eliminated by the rising concentration

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gradient of FasL antibody, indicating that some other signaling pathway may take part in the inhibition effects of KCs against TCs.

PD-L1 (also B7-H1), ligands for programmed death-1 (PD-1), is a member of the CD28/B7 superfamily of co-stimulatory molecules and expressed on KCs, other monocyte-derived cells, as well as epithelial, endothelial, and tumor cells [12–14]. Several reports demonstrated that PD-L1 plays an important role in regulating peripheral tolerance, autoimmune, and cancer immunity [15,16]; however, there are no reports about immune tolerance in liver transplantation. Thus, we hypothesized that the expression of PD-L1 on KCs modulates the proliferation and function of TCs affecting the immune regulation after liver transplantation leading to self-tolerance.

2. Materials and methods

2.1. Animals and liver transplantation

Adult male Lewis and Brown-Norway (BN) rats (200–250 g weight and 8–10 weeks old) were housed in a specific pathogen-free animal facility with free access to tap water and pelleted chow. They were randomly assigned to two groups. Orthotopic liver transplantation was performed using the Kamada two-cuff method. For LEW → BN group, Lewis-to-BN liver transplantation was performed. For BN → BN group, BN-to-BN liver transplantation was performed. No immunosuppressant drugs were given to recipients in this study. Recipients were sacrificed and graft tissue samples were collected at days 1, 3, 5 and 7 post-transplantation. Eight recipients in each group were set aside for survival observation. All sacrificed rats were subjected to necropsy. The study was carried out according to the National Research Council's guide for the care and use of laboratory animals and approved by the Ethics Committee of Chongqing Medical University.

2.2. Morphological changes of liver grafts

Tissue samples were immersion-fixed in 4% buffered formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin/eosin according to the standard protocol. Morphological changes were observed under light microscope (Olympus BX51, Japan). Allograft rejection was scored by rejection activate index (RAI) of Banff criteria [17,18]. RAI scoring: no rejection (0–2), borderline rejection (3), mild rejection (4–5), moderate rejection (6–7) and severe rejection (8–9).

2.3. ELISA for changes of cytokines in plasma

Enzyme-linked immunosorbent assays (ELISA) were used to determine levels of IL-2, INF-γ, TNF-α and IL-10 in plasma. All reagents, samples and standards were prepared according to the manual. Microtiter plates were coated with the appropriate antigen solution (100 μl/well) and incubated at 4 °C for 24 h. After being washed with PBS containing 0.1% Tween 20, plates were incubated with biotinylated anti-IL-4, anti-TNF-α and anti-IFN-γ (0.1 ml/well) antibodies at 37 °C for 60 min and then streptavidin-labeled peroxidase was added (1 h at 37 °C) prior to the addition of developing solution (tetramethylbenzidine). All ELISAs were read on a plate reader at 450 nm.

2.4. Immunohistochemical staining for IL-2, INF-γ, IL-10, TNF-α and PD-L1 expression in liver graft

The expressions of IL-2, INF-γ, IL-10, TNF-α and PD-L1 in liver grafts were determined using a goat-anti-rabbit streptavidin peroxidase kit (Santa Cruz, USA), respectively. It was performed according to the manufacturer's instructions. Briefly, 4 μm paraffin sections were spread on slides and fixed in acetone, and endogenous peroxidase activity was quenched with 0.03% H_2O_2. Sections were incubated with rabbit anti-rat IL-2, INF-γ, IL-10, TNF-α and PD-L1 monoclonal antibodies (Santa Cruz, USA) and Biotin-labeled goat anti-rabbit secondary antibody (Santa Cruz, USA), respectively. Followed by incubation with the streptavidin-horseradish peroxidase complex. Bound antibody was detected with 3,3′-diaminobenzidine (Santa Cruz, USA). Cells with brown granules appearing in cytoplasm or cell membrane were considered positive for cytokine expression, respectively. The expression intensity was determined with HPIAS-1000 image analysis system under light microscopy.

2.5. Western blot analysis

Protein extracts were obtained by homogenizing samples in a cell lysis buffer containing 20 mmol HEPES, 25% glycerol, 0.42 mmol NaCl, 15 mmol MgCl2, 0.2 mmol EDTA, 0.5 mmol phenylmethylsulfonyl fluoride and 0.5 mmol dithiothreitol, and by two cycles of centrifugation at 12,000 × *g* for 15 min. Protein concentration was determined by Bradford Assay Kit (Bio-Rad, USA). Extracted protein was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. The membrane was washed with 0.1% Tween 20-PBS and incubated with 5% dry non-fat skimmed milk powder dissolved in 0.1% Tween 20-PBS for 1 h, then with rabbit anti-rat PD-L1 polyclonal antibody for 1 h and goat anti-rabbit IgG for 1 h. Finally, the membrane was developed with an Enhanced Chemiluminescence Detection Kit (Pierce, USA) and the membranes were then immediately exposed to auto radiographic film (Kodak, USA). The relative amount of PD-L1 protein was quantified from relative optical density of the band by image analysis system (Bio-Rad Gel Doc 2000, USA).

2.6. Flow cytometry for TCs apoptosis in liver graft and PD-L1 expression by KCs

TCs were isolated from liver grafts and sorted with anti-CD4 antibodies as previously described [19]. Flow cytometry was used to quantify TCs undergoing apoptosis. To analyze PD-L1 expression on KCs before and after transplantation cells from liver samples were processed and fixed for 1 h in IncellFP (IncellDx, Inc.) and washed with PBS. Cells were stained with PD-L1 PE (clone 29E.2A3, Biolegend, Inc.), and F4/80 PE/Cy7 (clone BM8, Biolegend, Inc.) in PBS + 2% bovine serum albumin (BSA) for 30 min at room temperature in the dark. Cells were washed twice with PBS and nuclei stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) before analysis. Gating was applied to the CD45 + population and PD-L1 expression above the normal cutoff was recorded.

2.7. KCs cytotoxicity against TCs

The ^{3}H-Thymidine release assay was performed as previously described [20] to confirm the cytotoxicity of KCs against TCs. Isolated TCs (1×10^6/ml) incorporated with ^{3}H-Thymidine and KCs (1×10^6/ml) were incubated with or without conA (10 μg/ml) as follows: group A (TCs + conA) and group B (TCs + KCs + conA). Cells were incubated for 24 h in the 96-well plates. After incubation, cells were harvested over fiberglass filter and washed three times with distilled water. The radioactivity was measured by a liquid scintillation spectrometer (LS-6500, Beckman, USA).

2.8. ELISA after cell culture

Isolated cells (KCs, 1×10^6/ml and TCs, 1×10^6/ml) were incubated with or without conA (10 μg/ml) as follows: group A (TCs + conA) and group B (TCs + KCs + conA). Cells were then incubated for 30 h in RMPI-1640 medium with 15% FBS. After centrifugation, the supernatant was used to determine levels of TNF-α, IFN-γ, IL-2 and IL-10 by ELISA as previously described.