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Original Article

Induction of immune tolerance to rat liver allograft by sTNFRI-IgGFc and CCR7 gene modified immature dendritic cells

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Abstract: Background: Dendritic cells (DC) are the most powerful antigen presenting cells, which play key roles in recognition and presentation of antigens, initiation of immune response, initiation of immune rejection and induction of immune tolerance. DC is a heterogeneous group of cells, the immature dendritic cells (imDC) mainly induce immune tolerance, while mature dendritic cells (mDC) can initiate inflammation or immune rejection. Soluble tumor necrosis factor receptor I (sTNFRI) can competitively bind with TNF-α, block the biological function of TNF-α, as a result to negatively regulate the imDC activation induced by TNF-α. Recent studies suggest that imDC which migrate to the secondary lymphoid organs plays an important role in the induction of immune tolerance. The interaction of chemokine and chemokine receptor 7 (CCR7) on DC surface is the main driving force and pathway for the migration of peripheral DC to lymphoid tissues. Studies have indicated that the enhanced CCR7 expression may promote the imDC migration to lymphoid organs, thereby inducing immune tolerance. Based on the establishment of a rat model of liver transplantation rejection, this study aims to investigate the role of sTNFRI-IgGFc fusion gene and CCR7 gene co-modified imDC in liver graft immune tolerance. Methods: Bone marrow cells from rats were isolated, obtained imDC by vitro induction or mDC by treatment with TNF-α. The imDC infected by empty vector virus, carrying sTNFRI-IgGFc, CCR7 gene of the virus alone or together. After TNF-α induction, the expression of CD80 and CD86 was detected, the chemotaxis ability was detected by Transwell. To establish a rat liver transplantation rejection model, and 7 days before surgery, intravenous infusion PBS, empty vector virus, imDC carrying sTNFRI-IgGFc, CCR7 gene virus alone or co-infected to mice, the levels of alanine transaminase (ALT) and total bilirubin (Tbil) in the serum were measured; the acute rejection score was compared; the survival time of each group was compared; the levels of IL-2, IFN-γ, IL-4 and IL-10 in serum were measured by Elisa method. Results: TNF-α could significantly increase the expressions of CD86 and CD80 on the surface of imDC. STNFRI-IgGFc gene modification can significantly weaken the effect of TNF-α on the up-regulation of the expressions of CD80 and CD86 on the surface of imDC, so as to block the maturation and differentiation of imDC. CCR7 gene modification can significantly up-regulate the expression of CCR7 protein in imDC cells, and enhance its ability of chemotactic migration. Intravenous infusion of sTNFRI-IgGFc or CCR7 genetically modified imDC to recipient rats before liver transplantation can significantly reduce the post operative serum ALT and Tbile contents, decrease the acute liver rejection score, and prolong the survival time of the recipient rats. Furthermore, infusion of sTNFRI-IgGFc and CCR7 gene co-modified imDC can more improve the liver function, reduce the acute rejection of rats, and prolong the survival time after operation. Intravenous infusion of sTNFRI-IgGFc and (or) CCR7 genetically modified imDC can significantly reduce the contents of inflammatory cytokines IL-2 and IFN- in the recipient rats, and increase the contents of anti-inflammatory cytokines IL-4 and IL-10 simultaneously. Conclusion: sTNFRI-IgGFc and CCR7 gene co-modified imDC can induce immune tolerance in liver transplantation recipient rats, reduce the graft rejection, and prolong the survival time effectively.

Keywords: Dendritic cells, sTNFRI-IgGFc, CCR7, liver transplantation, immune tolerance

Introduction

Dendritic cell (DC) is the known antigen-presenting cell (APC) with the strongest function and effect in the body, and it is also the only antigen-presenting cell that can activate the naive T lymphocyte in the body, and only when DC migrates from periphery to the cellular

region of lymphoid tissue, could DC that carrying antigen information implement the function of antigen presenting and naive T lymphocyte activation [1]. DC plays key roles in the recognition and presentation of antigen, initiation of immune response, initiation of immune rejection and induction of immune tolerance [2, 3]. DC is a kind of heterogeneous cell populations, different subsets and different stages of DC with different functional states, immature DC (imDC) can induce immune tolerance and mature DC (mDC) can initiate inflammation or immune rejection reactions [3].

Tumor necrosis factor-α (TNF-α) is one of the most key cytokines to promote DC maturation and activation, it can induce DC development and maturation and activate the function of DC, and plays key roles in initiating the immune response, triggering the inflammation or immune rejection reactions [4]. Soluble tumor necrosis factor receptor I (sTNFRI) is a natural inhibitor of TNF-α in the body formed by the dropping and lysis of TNF-α receptor on the surface of cell membrane, which could competitively bind with TNF-α, but could not pass TNF-α signal pathway, however, it can block the combination of TNF-α and TNF-α receptor (mTNFR) and block the biological function of TNF-α, as a result to play a negative regulatory role in inflammatory response and rejection response induced by TNF-α [5].

Chemokine receptor 7 (CCR7) is one member of the chemokine receptor superfamily, which is mainly expressed on the immune cell surface such as DC, naive T lymphocytes, naive B lymphocytes, regulatory T lymphocyte and other immune cells; it plays a key role in DC chemotactic migration from peripheral tissues to the secondary lymphoid organs through binding with its chemokine C ligand 19 (CCL19) and CCL21 [6]. At present, it is believed that the interaction between the chemokine and CCR7 on DC surface is the most important driving force and pathway of the migration of peripheral DC to lymphoid tissue. Recent studies suggest that imDC that migrate to the secondary lymphoid organs plays an important role in the induction of immune tolerance [8]. In the process of imDC differentiating into mDC, the CCR7 expressed on the surface of imDC can chemotactic migrate imDC to lymph nodes, which will help the body to produce immune tolerance [9]. Studies have indicated that the enhancement of CCR7 expression may promote imDC migrating to lymphoid organs, thus helping to induce immune tolerance. Based on the establishment of rat liver transplantation rejection model, this study aims to explore the function and the possible mechanism of the co-modified imDC that transfected with sTNFRI-IgGFc fusion gene adenovirus and CCR7 gene adenovirus in liver graft immune tolerance, in order to provide solution and experimental basis for clinical prevention and treatment of transplant rejection.

Materials and methods

Major reagents and materials

Admax Kit D recombinant adenovirus vector was purchased from Microbix (Canada); adenovirus Ad-CCR7-EGFP carrying CCR7 and adenovirus Ad-EGFP carrying enhanced green fluorescent protein were purchased from Guangzhou Saiye Biological Technology Co. Ltd; restriction endonuclease and DNA ligase were purchased from New England Biolabs; Plasmid Extraction Kit was purchased from Promega (USA); Agarose gel extraction kit was purchased from QIAGEN (USA); DH5 α competent bacteria was purchased from Shanghai Fanke Biological Technology Co. Ltd; Recombinant rat GM-CSF, IL-4, TNF-α were purchased from Peprotech (USA); PE labeled CD80, CD86 flow cytometric antibody were purchased from eBioscience (USA); Rat anti CCR7 was purchased from Santa Cruz (USA); Rabbit anti TNFRI was purchased from Abcam (USA); RPMI1640 medium, FBS, penicillin and streptomycin were purchased from Gibco (USA); Liposome transfection reagent Lipofectamine 2000 was purchased from Invitrogen (USA).

Laboratory animals

SPF grade male SD rats were used as recipients, and SPF grade male Wistar rats were used as donors, both were 6~8 weeks old, weighing 220±25 g, provided by the experimental animal center of Second Military Medical University.

Construction of Ad-sTNFRI-IgGFc fusion gene of recombinant adenovirus

The sequence of the upstream primer was designed as follows: 5'-ATGAGCTCATGGGCCTC-

TCCACCGTGCCTG-3', with SacI restriction endonuclease site was introduced into it, the sequence of the downstream primer was designed as follows: 5'-ATGTCGACTCATTTAC-CCGGAGACAGGGAGAGG-3', with SacI restriction endonuclease site was introduced into it, using plasmid pBluescrip/TNFRI-IgGFc as a template, sTNFRI-IgGFc gene was amplified by PCR. The PCR product digested by SacI and SalI endonucleases was inserted into pGEM-T vector; and then the vector was transformed into DH5 α competent bacteria, plasmid DNA was extracted, and verified by sequencing, then it was digested by SacI and SalI endonucleases, and the target fragment was recovered by gel electrophoresis, and then inserted into the same site of shuttle vector pDC316, the product was used to transform DH5 α competent bacteria, and then the plasmid were extracted and identified by enzyme digestion. The shuttle plasmid pDC316-sTNFRI-IgGFc and helper plasmid pBHGloxΔE1, 3Cre and Lipofectamine 2000 were mixed, and the mixtures were transfected into HEK293 cells. The virus supernatant was collected and centrifuged by CSCL density gradient, then the CSCL was removed by dialysis and the purified virus solutions were obtained, which was the recombinant adenovirus with Ad-sTNFRI-IgGFc-EGFP fusion gene.

Culture of rat bone marrow-derived DC

Wistar rats were sacrificed by cervical dislocation, and immersed in ethanol with 75% of volume fraction for 5 minutes, the femur and tibias on both sides were removal off under aseptic conditions, and the two ends of bones were cut off, the bone marrow cavity was washed with PBS repeatedly till it turned white, the bone marrow suspension was diluted with equal volume of PBS, then the dilution was slowly dropped onto the lymphocyte separation liquid along the centrifugal tube wall and centrifuged at 300 g for 30 minutes, then the buffy coat was transferred to another centrifugal tube, and diluted with PBS of more than three times of the volume, the dilution was centrifuged at 1200 rpm for 5 minutes, and the washing centrifugation was repeated once. The harvested cells were cultured in RPMI 1640 medium containing 10% FBS, 1% mycillin and placed at an incubator with 5% CO_2 at 37°C, after culturing for 24 hours, the culture medium was abandoned, and the non adherent cells were removal off; then re-cultured with RPMI 1640 medium containing 10% FBS, GM-CSF, 20 ng/mL, 1% mycillin, 10 ng/mL IL-4, and the culture medium was replaced with half of the volume at an interval of two days, the imDCs were obtained on the seventh day of culturing or the mDCs were obtained on the seventh day after stimulating with 20 ng/mL of TNF-α for 48 hours, which were added when the culture medium was replaced on the fifth day.

sTNFRI-IgGFc and CCR7 gene co-modified imDC

During the process of imDC culturing, the concentrated virus particles were added at the concentration of MOI = 200 on the fifth day, and the polybrene with the final concentration of 10 mg/L was added, the culture medium was exchanged after transfection for 6 hours, and continue to cultivate to the seventh day. ImDC cells were divided into 6 groups: untreated control group, empty vector Ad-EGFP1 transfection group (Ad-EGFP1 was the empty vector control of Ad-sTNFRI-IgGFc-EGFP) and empty vector Ad-EGFP2 transfection group (Ad-EGFP2 was the empty vector control of Ad-CCR7-EGFP), Ad-sTNFRI-IgGFc-EGFP transfection group, Ad-CCR7-EGFP transfection group, the co-modified group were transfected with Ad-sTNFRI-IgGFc-EGFP and Ad-CCR7-EGFP two kinds of virus particles to modified imDC.

Determination of transfection efficiency

Cells were digested by 0.25% trypsin and harvested. The cells were washed and centrifuged with PBS containing 2% FBS for twice, and then were re-suspended with PBS containing 2% FBS, the positive rate of EGFP expression was detected by flow cytometry on channel one.

Detection of the expression of sTNFRI-IgGFc in the supernatant of culture medium of imDC transfected with adenovirus by ELISA

The concentration of TNFRI was determined by ELISA method, and the brief steps were as follows: 100 μL standard products or 100 μL transfection supernatant were added into the 96-well plate, and incubated at 37°C for 60 minutes, the wells were washed with wash buffer for three times, and then 100 μL biotinylated anti-TNFRI antibody were added, and incubated at 37°C for 60 minutes, the wells were washed with wash buffer for three times, and

then 100 μL HRP coupled antibody were added, and incubated at 37°C for 30 minutes, the wells were washed with wash buffer for three times, and then 100 μL TMB developing liquids were added, and incubated in dark for 20 minutes, and then the stop solutions were added, the OD value at 450 nm was determined, the sample concentration was calculated according to the standard curve and the OD value of the sample.

Detection of CCR7 protein expression by western blot

The cellular protein was extracted by the RIPA lysates, after quantification, 50 μg protein was sampled and run in 10% SDS-PAGE for 2.5 hours, then the gel was electric transferred to PVDF membrane for 1.5 hours, the membrane was blocked with 5% skim milk at room temperature for 60 minutes, and incubated with the first antibody at 4°C overnight (the dilution ratio of CCR7 and β-actin were 1:300, 1:500, respectively), rinsed the membrane with PBST for 3 times × 5 minutes, and incubated with HRP labeled secondary antibody (ratio dilution was 1:15000) at room temperature for 60 minutes, rinsed the membrane with PBST for 3 times × 5 minutes, and then ECL chemiluminescence liquid was added and incubated at room temperature for 2~3 minutes, exposure, developing and fixing were performed in darkroom, and the data were analyzed after scanning.

Detection of CD80 and CD86 expressions on DC surface by flow cytometry

DC cells of each group were digested by 0.25% trypsin and collected, the cells were washed with PBS containing 2% FBS and centrifuged for twice, the cells were re-suspended with 100 μL PBS containing 2% FBS, and then 5 μ CD80, CD86 flow cytometry antibodies were added, and incubated in dark at 4°C for 30 minutes, the cells were washed with PBS containing 2% FBS and centrifuged for twice, then the cells were re-suspended with 500 μL PBS containing 2% FBS and detected immediately.

Transwell assay for chemotaxis of cells

DCs in each group were collected, and the cell concentration was adjusted to 1×10^6/mL by the complete culture solution. 800 μL of complete medium containing 100 ng/mL of CCL19 were added in each well of the 24-well plate, then the Transwell chamber was placed in the 24-well plate, 100 μL cell suspension were added in the upper chamber of the Transwell, and cultured for 24 hours, the migration rate was calculated according to the number of cells in the lower chamber: the number of cells in the lower chamber/the original total cell numbers in the upper chamber × 100%.

Establishment of liver transplantation model

Liver donor Wistar rats were anesthetized by isoflurane, the liver was exposed after the abdominal cavity was opened, and then the liver was freed after mutilation of the ligament and ligation of blood vessels, and lactated Ringer's solutions were continuously infused in the liver, mutilations of the suprahepatic inferior vena cava, portal vein, arteriae hepatica communis, right renal vein and right adrenal vein were performed, respectively, then the isolated liver was preserved in the lactated Ringer's solutions at 0~4°C, and cuffs were installed at the suprahepatic inferior vena cava and portal vein. The SD recipient rats were anesthetized, the abdominal cavity was opened and the original liver was removal off, the donor liver was placed at the liver bed of the recipient rat, the suprahepatic inferior vena cava and portal vein were sutured with vascular cuff, and then donor bile duct support tube was placed in the bile duct and was ligated and fixed, the abdomen was closed when no bleeding was observed.

Grouping of laboratory animals

Seven days before liver transplantation, PBS or DC derived from donor rats were infused into the recipient rats via intravenous infusion. According to the different materials infused, the recipient rats were randomly divided into 8 groups, and 15 rats were enrolled into each group: the control group, 500 μL PBS were infused into the recipient rats before operation; the imDC group: 5×10^6 of imDC without transfection were infused into the recipient rats before operation; the mDC group: preoperative infusion of 5×10^6 of mDC derived from donor rats; the Ad-EGFP1 group: preoperative infusion of 5×10^6 of imDC transfected with Ad-EGFP1; the Ad-EGFP2 group: preoperative infusion of 5×10^6 of imDC transfected with

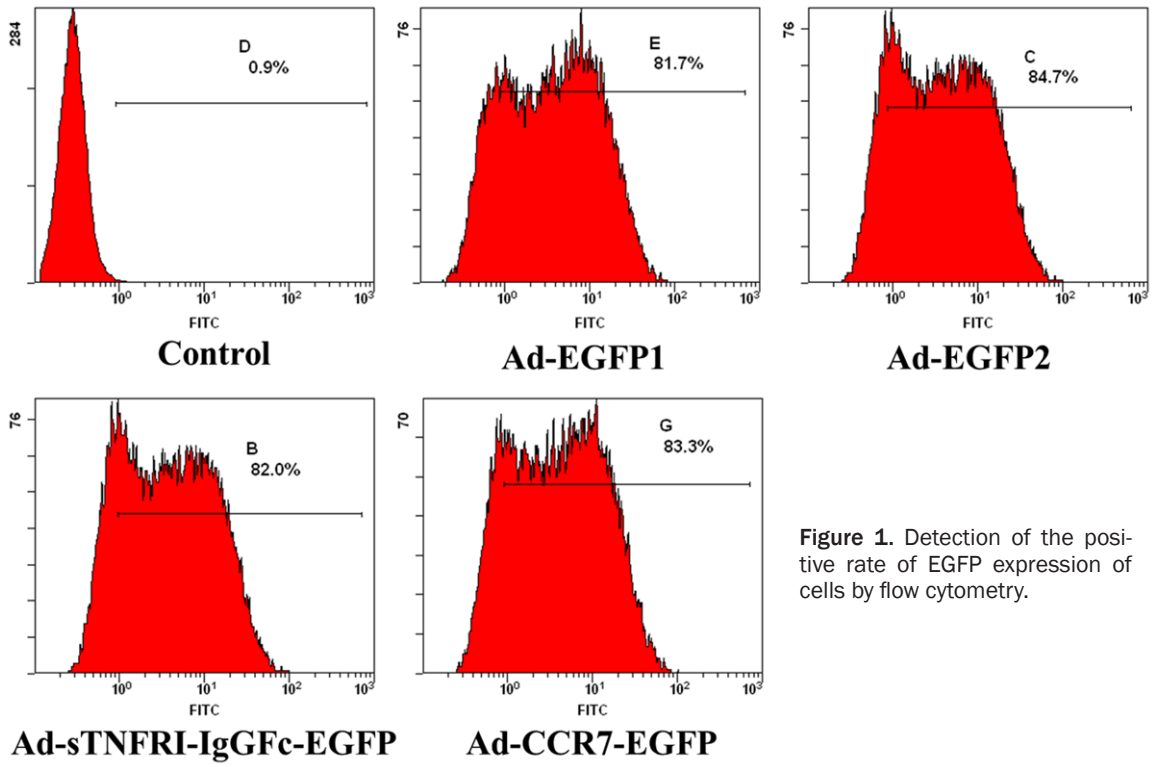

Figure 1. Detection of the positive rate of EGFP expression of cells by flow cytometry.

Ad-EGFP2; the sTNFRI-IgGFc modified group: preoperative infusion of 5 × 10^6 of imDC transfected with Ad-sTNFRI-IgGFc-EGFP; the CCR7 modified group: preoperative infusion of 5 × 10^6 of imDC transfected with Ad-CCR7-EGFP; co-modified group: preoperative infusion of 5 × 10^6 of imDC co-transfected with Ad-sTNFRI-IgGFc-EGFP and Ad-CCR7-EGFP.

Detection index

Liver function test: the peripheral venous bloods were sampled from recipient rats of each group on postoperative day 7 and day 14. The serum was separated by centrifuging at 2000 g for 5 minutes, and stored at -20°C. The contents of ALT and TBil were detected by OLYMPUS AU 2700 biochemical analyzer.

Acute rejection scoring: acute rejection grading of recipient rat liver of each group (n = 5): 0~2 points were classified for non rejection; 3 points were classified for the borderline rejection; which is suspected with mild acute rejection, but not up to the standard of diagnosis with acute rejection, and ischemic injury cannot be excluded; 4~5 points were classified for mild acute rejection; 6~7 points were classified for moderate acute rejection; and 8~9 points were classified for severe acute rejection.

Detection of inflammatory factors: the peripheral venous bloods were sampled from each group on postoperative day 7. The serum was separated by centrifuging at 2000 g for five minutes. The concentrations of IL-2, IFN-γ, IL-4 and IL-10 in serum were detected by ELISA.

Observation of postoperative survival time of the recipient rats: 5 rats were set aside in each group, and the postoperative survival time was recorded.

Statistical analysis

SPSS 18.0 was used for statistical analysis of data, the measurement data were expressed as mean ± standard deviation, and *t* test was used in the comparison of the measurement data between groups, $P<0.05$, represented for the difference was statistically significant.

Results

Detection of transfection efficiency

The results of flow cytometry revealed that the positive rate of EGFP in imDC cells transfected with empty vector virus, virus carrying sTNFRI-IgGFc or CCR7 gene were more than 80% (**Figure 1**), suggesting that the efficiency of the

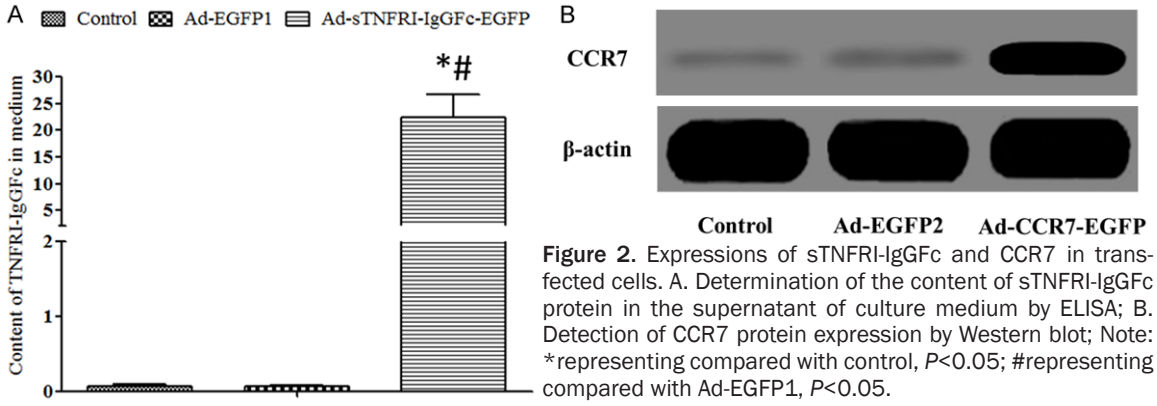

Figure 2. Expressions of sTNFRI-IgGFc and CCR7 in transfected cells. A. Determination of the content of sTNFRI-IgGFc protein in the supernatant of culture medium by ELISA; B. Detection of CCR7 protein expression by Western blot; Note: *representing compared with control, *P*<0.05; #representing compared with Ad-EGFP1, *P*<0.05.

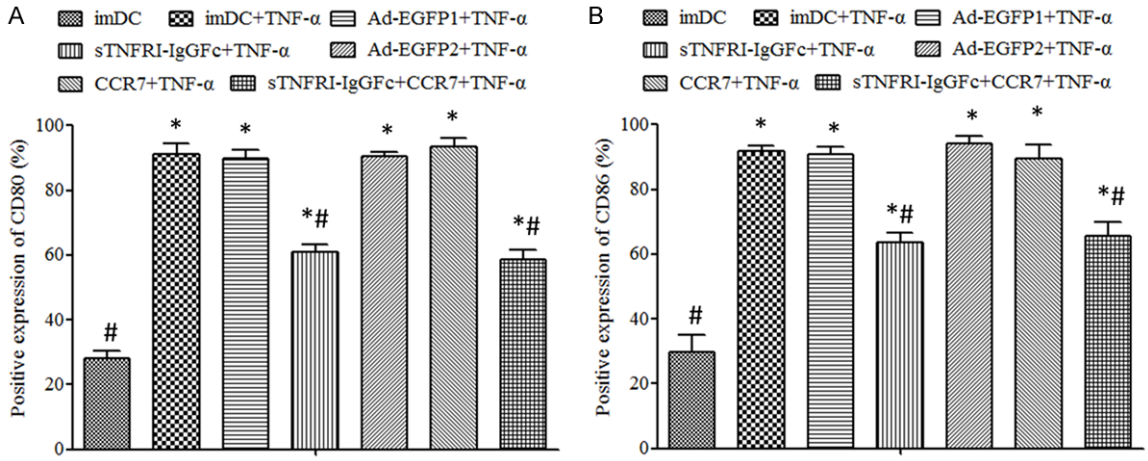

Figure 3. sTNFRI-IgGFc modification down-regulated the expressions of CD80 and CD86 on the surface of DC. A. Detection of CD80 expression by flow cytometry; B. Detection of CD86 expression by flow cytometry; Note: *representing comparison with imDC, *P*<0.05; #representing comparison with imDC+TNF-α, *P*<0.05.

virus transfection was high, which can meet the needs of the study.

Expressions of sTNFRI-IgGFc and CCR7 in the transfected cells

ELISA test results revealed that, 48 hours after transfection with Ad-sTNFRI-IgGFc-EGFP, the secretion of sTNFRI-IgGFc protein in the supernatant of the cultured imDC cells was 22.5±3.0 ng/mL, however, almost no sTNFRI-IgGFc protein expression and secretion were detected in the supernatant of cultured imDC cells transfected with the empty vector virus and untransfected imDC cells (**Figure 2A**), the former was significantly higher than that of the latter two, and *P*<0.01. The results of Western Blot revealed that the expression of CCR7 protein in imDC cells was significantly enhanced when the cells were transfected with adenovirus Ad-sTNFRI-IgGFc-EGFP, while there was no significant effect on the expression of CCR7 protein in imDC cells when the cells were transfected with empty vector virus (**Figure 2B**).

sTNFRI-IgGFc modification down regulate the expressions of CD80 and CD86 on the surface of DC

The expressions of co-stimulatory molecules CD80 and CD86 were low on imDC cell surface, the expressions of CD80 (**Figure 3A**) and CD86 (**Figure 3B**) on the surface of imDC were significantly up-regulated in the process of imDC differentiating into mDC induced by TNF-α, the positive rate of expression was up to 80%. There were no significant effects on the expression of CD80 and CD86 in imDC when it was transfected with empty vector virus; the expressions of CD80 and CD86 were significantly

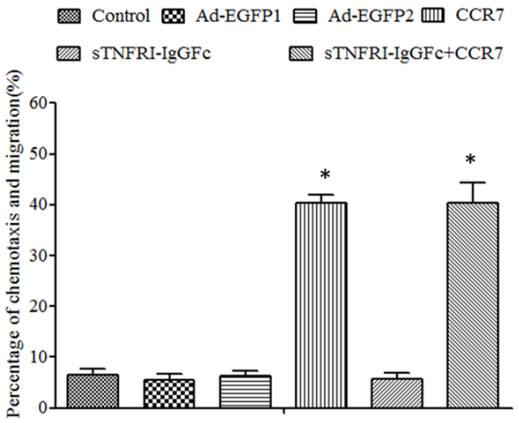

Figure 4. CCR7 modification enhanced the chemotacitic ability of imDC. Note: *representing compared with control, $P<0.05$.

down-regulated by sTNFRI-IgGFc gene modification in the process of imDC differentiating into mDC induced by TNF-α, however, CCR7 gene modification had no obvious effects on the expressions of CD80 and CD86 in the process of imDC differentiating into mDC; suggesting that sTNFRI-IgGFc gene modification can inhibit the differentiation and maturation of imDC.

CCR7 modification enhanced the chemotacitic ability of imDC

The result of in vitro migration assay revealed that the chemotactic migration rate of imDC cells in CCR7 genetically modified group was significantly higher than that of empty vector group and the unmodified group, while the sTNFRI-IgGFc gene modification had no effect on chemotactic migration ability of imDC cells (**Figure 4**).

Comparison of liver function of recipient rats in each group

Seven days after transplantation, the contents of ALT and Tbil of the recipient rats in the control group and the mDC group were the highest, and the contents continued to rise on day 14 post transplantation, indicating that the liver function continued to be deteriorated. The contents of ALT and Tbil in serum of the recipient rats could be decreased at a certain extent when the rats were infused of imDC without gene modification or infused of imDC transfected with empty vector virus. The contents of ALT and Tbil in serum of the recipient rats of imDC infusion group, in which imDC was genetically modified by sTNFRI-IgGFc or CCR7 were obviously lower than that of the imDC infusion group in which imDC was not genetically modified or transfected with empty vector virus; the contents of ALT (**Table 1**) and Tbil (**Table 2**) in serum of the recipient rats of the imDC infusion group, in which the imDC were co-modified by sTNFRI-IgGFc and CCR7 gene were the lowest. These results revealed that the liver function of the recipient rats infused with TNFRI-IgGFc or CCR7 genetically modified imDC could be significantly improved, and the co-modification of these two genes had the best effect on improving the liver function.

sTNFRI-IgGFc or CCR7 gene modification can reduce rejection

The acute liver rejection of recipient rats of imDC infusion group in which imDC were modified by sTNFRI-IgGFc or CCR7 genes were significantly weakened than those of imDC infusion group in which imDC were non genetically modified or transfected with empty virus vector, and the acute liver rejection of recipient rats of imDC infusion group in which imDC were co-modified by sTNFRI-IgGFc and CCR7 genes were the most weakened (**Table 3**). The results revealed that infusion of the sTNFRI-IgGFc or CCR7 genetically modified imDC could obviously alleviate the rejection, and the co-modification of these two genes had the best effect on reducing the rejection.

Effects of sTNFRI-IgGFc or CCR7 gene modification on the expression of inflammatory cytokines

ELISA test results revealed that compared with the control group, infusion of imDC before transplantation could down-regulate the contents of serum IL-2 and IFN-γ (**Figure 5A**), and up-regulate the contents of IL-4 and IL-10 (**Figure 5B**) at a certain extent, however, the infusion of mDC had the opposite effect. Furthermore, the contents of serum IL-2 and IFN-γ were more down-regulated and the contents of IL-4 and IL-10 were more up-regulated when imDC was modified by sTNFRI-IgGFc or CCR7 gene, and infusion of the co-modified imDC could induce the function shift of Th1/Th2 in a maximum extent (**Figure 5A** and **5B**).

sTNFRI-IgGFc or CCR7 gene modification can significantly prolong the survival time of rats

The result of Log-rank test revealed that there are obvious differences in survival time in each

Table 1. Comparison of ALT (IU/L, Mean ± SD) at different time points in rats of each group after transplantation

Grouping	7 days post operation	14 days post operation
Control	687.4±77.5#	900.4±88.2#
imDC	205.3±48.4*	115.7±31.5*
mDC	925.6±81.2*,#	1211.5±115.7*,#
Ad-EGFP1	213.4±51.6*	119.5±24.7*
Ad-EGFP2	209.1±45.9*	108.1±29.3*
sTNFRI-IgGFc	87.5±9.7*,#	55.7±8.2*,#
CCR7	80.4±10.5*,#	51.1±9.3*,#
sTNFRI-IgGFc+CCR7	59.7±6.7*,#	34.3±3.5*,#

Note: *representing compared with control, $P<0.05$; #representing compared with imDC, $P<0.05$.

Table 2. Comparison of Tbil (μmol/L, Mean ± SD) at different time points in rats of each group after transplantation

Grouping	7 days post operation	4 days post operation
Control	121.5±10.6#	142.7±11.9#
imDC	64.7±6.2*	31.3±3.8*
mDC	135.8±12.8*,#	164.3±15.9*,#
Ad-EGFP1	68.1±7.2*	35.4±4.1*
Ad-EGFP2	65.9±5.8*	29.8±3.7*
sTNFRI-IgGFc	33.5±4.2*,#	25.4±3.1*,#
CCR7	31.7±3.4*,#	22.9±3.3*,#
sTNFRI-IgGFc+CCR7	22.5±2.9*,#	15.7±1.8*,#

Note: *representing compared with control, $P<0.05$; #representing compared with imDC, $P<0.05$.

Table 3. Scoring result of acute liver rejection of rats in each group (point, Mean ± SD)

Grouping	7 days post operation
Control	7.1±0.4#
imDC	5.4±0.4*
mDC	7.8±0.5*,#
Ad-EGFP1	5.3±0.5*
Ad-EGFP2	5.5±0.6*
sTNFRI-IgGFc	3.1±0.2*,#
CCR7	3.3±0.3*,#
sTNFRI-IgGFc+CCR7	2.5±0.2*,#

Note: *representing compared with control, $P<0.05$; #representing compared with imDC, $P<0.05$.

group ($\chi^2 = 54.09$, $P<0.05$), infusion of mDC can decrease the survival time of the recipient rats at a certain degree, however, the infusion of imDC can significantly prolong the survival time of the recipient rats. Compared with the unmodified group, the survival time of the recipient rats of the modified imDC infusion group was significantly prolonged, in which the imDC was modified by sTNFRI-IgGFc or CCR7 genes. Co-modification of sTNFRI-IgGFc and CCR7 genes had the best effect on prolonging the survival time of the recipient rats, as shown in **Table 4** and **Figure 6**.

Discussion

The phenotypic characteristics and functions of DC can be changed greatly in the maturation process, imDC showed strong induction of immune tolerance function, and can mediate specific immune tolerance, while mDC showed strong immunogenicity and can induce immune response, initiate immune rejection or inflammation [10]. DC in different stages has different functions, CD80, CD86 and other co-stimulatory molecules are deficient in imDC, which make the second signal that is necessary for T lymphocyte activation unprovided to the T lymphocyte in antigen presenting, and as a result leading to T cell anergy or apoptosis, therefore, imDC tends to induce immune tolerance, and shows tolerogenic characteristic; However, the mDC has high expression level of co-stimulatory molecules such as major histocompatibility complex-II (MHC II), CD80, CD86 and others, and can effectively activate the naive T cells, and induce the immune response of the body [11].

A variety of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) [12] and interleukin-4 (IL-4) [13], tumor necrosis factor-α (TNF-α) [4] play a crucial role in promoting the development and maturation process of DC. TNF-α is mainly synthesized and secreted by the activated mononuclear macrophages, T lymphocytes, DCs and other immune cells, and participate in allograft rejection [14], acute and chronic inflammatory reactions [15] and other immune response processes. Studies have reported that [16], a great increasing expression and release of TNF-α were detected in the process of immune response of allograft rejection. The antagonizing effect of TNF-α can inhibit the maturation and activation of DC, and promote the proliferation and activation of regulatory T cells (Treg) [17] which mediate immune tolerance. The sTNFRI is formed by the shed-

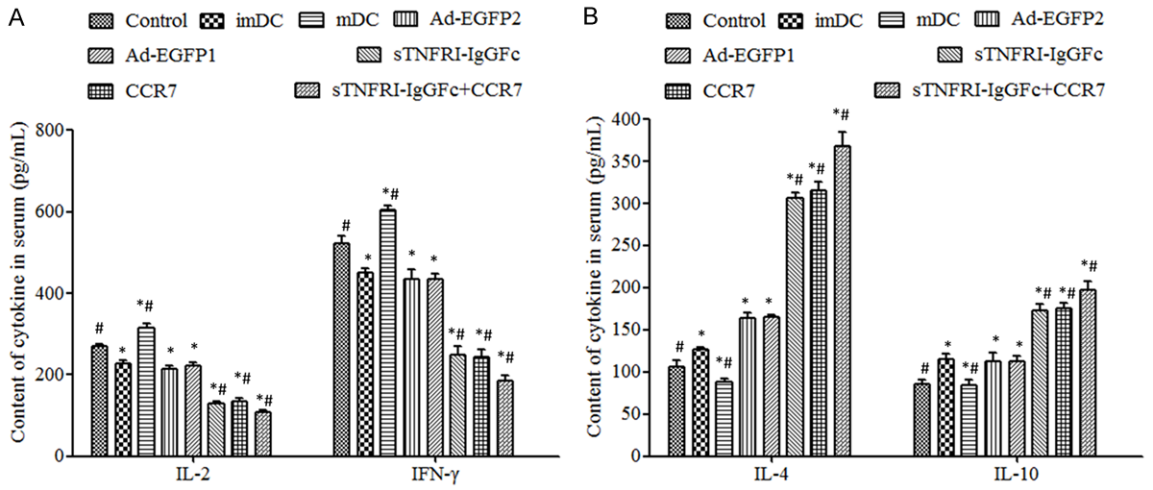

Figure 5. Effect of sTNFRI-IgGFc or CCR7 gene modification on the expression of inflammatory cytokines. A. Detection of the expression of Th1 type cytokines in rats of each group by ELISA; B. Detection of the expression of Th2 type cytokines in rats of each group by ELISA; Note: *representing compared with Control, $P<0.05$; #representing compared with imDC, $P<0.05$.

Table 4. Comparison of median survival time (day) of rats in each group

Grouping	Median survival time (day)
Control	15
imDC	21
mDC	11
Ad-EGFP1	19
Ad-EGFP2	20
sTNFRI-IgGFc	34
CCR7	30
sTNFRI-IgGFc+CCR7	47

ding and lysis of TNF-α receptor on the cell membrane; it can block the binding of TNF-α to its membrane receptor (mTNFR) through competitive binding to TNF-α, so as to play a negative regulatory role in inflammatory reaction and transplantation rejection and other immune responses induced by TNF-α [5].

In recent years, it is believed that the CCR7 expressed on the surface of imDC can chemotactic migrate imDC to lymph nodes in the process of differentiation and maturation of imDC to mDC, which will help the body to produce immune tolerance [9]. In addition to the low expressions of CD80 and CD86, CCR7 expression on the surface of imDC is also very low, and with low migration ability, so the induction of immune tolerance is limited [6]. However, CCR7 is highly expressed on mDC with a higher mobility; it can initiate the immune response effectively [6]. It was found in studies that [18], the capacity of DC (derived from CCR7 knockout (CCR7-/-) mice bone marrow) migrating to the lymph nodes was restricted. Studies have indicated that [9] enhancement of CCR7 expression is helpful in inducing immune tolerance by promoting imDC migrating to lymphoid organs.

If the donor imDC can be modified by the factors that could inhibit the maturation and activation of DC, its immunogenicity would be reduced and the tolerant activation would be increased, and it would be maintained in the imDC state, which is the key to induce immune tolerance. Studies have found that [19], the number of intravenous infused exogenous DCs that reached to the secondary lymphoid organs was limited. If the imDC with high level expression of specific chemokine receptors could be constructed by gene engineering technology, it will promote the imDC migrating to lymph nodes, and enhance the effect of the induction of immune tolerance effectively. Based on the establishment of rat liver transplantation rejection model, this study aims to explore the roles and the possible mechanism of imDC co-modified by transfecting with adenovirus with sTNFRI-IgGFc fusion gene and CCR7 gene in liver graft immune tolerance, in order to provide experimental basis for the prevention and treatment of transplant rejection in clinic.

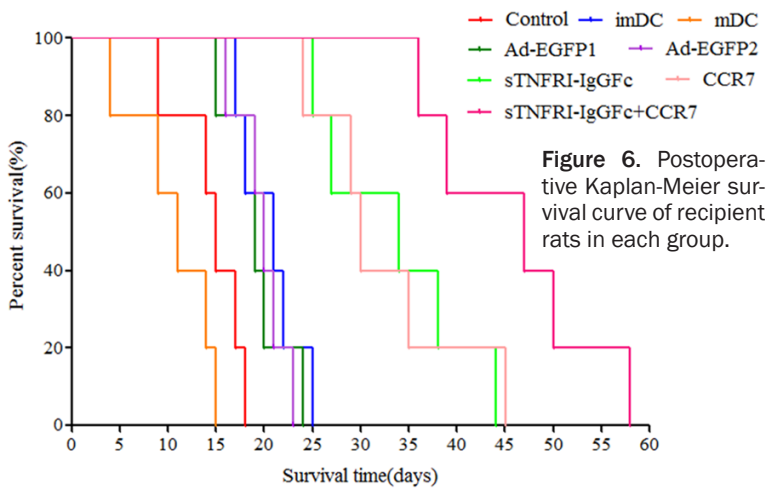

Figure 6. Postoperative Kaplan-Meier survival curve of recipient rats in each group.

The results of flow cytometry revealed that the positive rate of EGFP in the imDC cells transfected by virus was more than 80%, indicating that the efficiency of the virus infection was high, and could meet the needs of the research experiment; ELISA test results revealed that the amounts of sTNFRI-IgGFc protein in the supernatant of imDC cell culture were significantly increased by adenovirus transfection, indicating that the sTNFRI-IgGFc genetically modified imDC was successfully constructed; The results of Western blot revealed that CCR7 protein expression almost could not be detected in imDC, which are basically consistent with the research results of Pan and others [6]; Adenovirus transfection can significantly enhance the expression of CCR7 protein in imDC cells, indicating the successful construction of CCR7 genetically modified imDC; The results of flow cytometry revealed that TNF-α could significantly increase the expressions of CD80 and CD86 on the surface of imDC, which was the process of stimulating imDC to differentiate into mDC. Bjornberg and others [20] constructed the sTNFRI-IgGFc fusion protein with Fc segment of IgG antibody, and found that its affinity with TNF-α was 3~8 times as much as with the sTNFRI. What's more, Peppel and others also found [21] that the half-life of sTNFRI-IgGFc fusion protein was significantly longer than that of the sTNFRI, and it can play the role of antagonizing the biological effect of TNF-α at a greater extent. In the present study, the expressions of CD80 and CD86 on the surface of imDC up-regulated by TNF-α could be significantly reduced by sTNFRI-IgGFc gene, indicating that sTNFRI-IgGFc gene modification could block the maturation and differentiation of imDC, which was related to significant enhancement of blocking effect of sTNFRI-IgGFc fusion protein on TNF-α, which may has the common theoretical mechanism of research of Bjornberg, et al. [20] and Peppel et al. [21]. In the present study, imDC was transfected and modified by adenovirus vector carrying CCR7 gene according to the biological characteristics of CCR7 and imDC. It was found that the CCR7 gene modification could significantly induce imDC to express CCR7 protein, and the chemotactic migration ability of imDC was significantly enhanced simultaneously. The results of animal experiments revealed that intravenous infusion of imDC modified by sTNFRI-IgGFc or CCR7 genes to recipient rats before liver transplantation could significantly reduce the degree of liver injury, weaken the acute rejection, and prolong the survival time of recipient rats effectively post transplantation. However, more improvements described above would be achieved when sTNFRI-IgGFc and CCR7 gene co-modified imDC was infused. A large number of studies have shown that [22-24], the process of graft rejection is often accompanied by the increased expression of Th1 type cytokines (such as IFN-γ, IL-2) and the decreased expression of Th2 type cytokines (such as IL-4, IL-10), therefore, the correction of Th1/Th2 imbalance may induce immune tolerance and reduce rejection. It was found in the detection results of the present study that intravenous infusion of genetically modified imDC could significantly decrease the contents of inflammatory cytokines of LI-2 and IFN-γ, and increase the contents of anti-inflammatory factors of IL-4, IL-10 of recipient rats, so as to correct the imbalance of Th1/Th2 in liver transplantation, and reduce the graft rejection.

In summary, we believe that sTNFRI-IgGFc and CCR7 gene co-modified imDC could induce immune tolerance, alleviate the graft rejection effectively, and prolong the survival time of recipient rats in liver transplantation; ImDC immune therapy based on sTNFRI-IgGFc and

CCR7 gene co-modification has important significance in immune tolerance induction after organ transplantation, and it also has potential application value in clinic.

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Disclosure of conflict of interest

None.

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