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Transforming growth factor β1 and Fas ligand synergistically enhance immune tolerance in dendritic cells in liver transplantation

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ABSTRACT

Background: Long-term survival of patients following liver transplantation can be achieved by application of genetically modified, immune tolerogenic immature dendritic cells (imDCs) to overcome allograft-induced acute cellular rejection, a major cause of death. In this study, using a rat model of liver transplantation, we determined whether cotransfection of transforming growth factor β1 (TGF-β1) and Fas ligand (FasL) in imDCs synergistically enhances immune tolerance.

Materials and methods: We first determined the immune tolerogenic effects of TGF-β1 and FasL independently or together in imDCs by measuring the levels of CD86 and CD80 and by assessing T-cell proliferation using mixed lymphocyte reaction tests. Next, a rat model of liver transplantation, in which dark agouti and Lewis rats treated with DCs exogenously expressing TGF-β1 and/or FasL served as donors and recipients, respectively, was used to examine TGF-β1/FasL-induced immune tolerance. Specifically, we assessed the Banff rejection activity index (RAI), liver functions (alanine transaminase and total bilirubin levels), serum levels of interleukin (IL)-1, IL-10, and IL-12, apoptosis by TUNEL, and posttransplant survival.

Results: TGF-β1/FasL cotransfection of imDCs resulted in greater reduction of CD85 and CD80 expression and T-cell proliferation than a monotransfection. Cotransfected imDCs also showed reduced RAI scores, decreased plasma alanine transaminase and total bilirubin, altered cytokine levels, increased apoptosis, and prolonged survival than monotransfected imDCs in liver-allografted rats.

Conclusions: By enhancing immune tolerance, reducing liver damage, and achieving long-term postsurgery survival, TGF-β1/FasL cotransfection of imDCs may prove more beneficial for patients undergoing liver transplantation.

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Introduction

Liver transplantation is currently the most effective way to treat patients with an end-stage liver disease. However, acute cellular rejection (AR) remains to be a major complication, hampering the long-term survival of the patients receiving allografts.[1-3] Although immunosuppressive therapy has been developed to inhibit the posttransplantation AR, it is often associated with strong side effects that limit its clinical application.[4] In contrast, enhancement of immune tolerance is more achievable and less toxic.[5-7] Dendritic cells (DCs) can both promote and inhibit immune responses depending on their activation state and developmental stages. In general, mature dendritic cells (mDCs) expressing elevated levels of MHC and costimulatory molecules are potent antigen-presenting cells (APCs), whereas immature dendritic cells (imDCs) expressing undetectable to low levels of costimulatory molecules promote immune tolerance through the mechanisms of anergy, apoptosis, default Th2 differentiation of T cells, and increased regulatory T cells (Tregs).[1-3,8-10] These tolerogenic imDCs can be generated *in vitro* from hematopoietic stem cells or more immediate DC precursors for clinical applications including solid organ transplantations and treatment of autoimmune diseases.[1-3]

However, imDCs may differentiate into mDCs over time and acquire the ability to stimulate effect T cells, consequently initiating immune responses that lead to graft rejection.[2] Thus, for a long-term tolerance, it is important to maintain a steady state of imDCs. To this end, imDCs prepared *in vitro* can be manipulated by genetic modifications or exposure to immunosuppressive or anti-inflammatory agents.[2]

Transforming growth factor β1 (TGF-β1), a secreted cytokine, plays a crucial role in immune tolerance by controlling the proliferation, differentiation, survival, and apoptosis of T cells, B cells, and macrophages.[11,12] It is also essential for Th1 cell differentiation and inhibition of allogeneic lymphocyte proliferation, thereby conferring immunosuppressive capacity on various cells of the immune system.[13] It has been shown that TGF-β1 expression leads to reduced expression of MHC and costimulatory molecules CD80 and CD86 in imDCs, inhibiting T-cell proliferation and immune responses.[14] Immune responses can also be suppressed by Fas ligand (FasL), a member of tumor necrosis factor (TNF) family that mediates apoptosis upon binding to TNF receptors of targeting cells.[15] In this study, we hypothesized that cotransfection of TGF-β1 and FasL in imDCs could synergistically enhance immune tolerance. To this end, we cotransfected imDCs with both TGF-β1 and FasL and assessed the effects of co-transfection *versus* monotransfection on immune tolerance. Specifically, we examined the expression levels of CD80 and CD86 and imDC-mediated T-cell proliferation using mixed lymphocyte reaction (MLR) assay. We then validated our *in vitro* findings by comparing the immune tolerance between cotransfected and monotransfected imDCs in a rat model of heterotopic liver transplantation.[10]

Materials and methods

Animals

Male dark agouti (DA) and Lewis rats (8-10 weeks, weight 253.7 ± 21.4 g) were purchased from the Experimental Animal Center at the Second Affiliated Hospital of Harbin Medical University and Shanghai SLAC Laboratory Animal Co Ltd, respectively. The rats were housed in microisolator cages in the barrier facility of the Fujian Medical University. The use of animals and experimental procedures were approved by the Institutional Animal Care and Use Committee of Fujian Medical University, which were in accordance with the Animal Research: Reporting *In Vivo* Experiments guidelines.

Isolation and culture of rat DCs and T cells

Bone marrow cells from the rat femur and tibia were flashed out with bone marrow flushing fluid with a 10 mL syringe under aseptic conditions. After adding an equal amount of lymphocyte separation solution, the mixture was centrifuged at 2000 rpm for 20 min. The top layer of fluffy part in the pellet was collected into RPMI 1640 medium for DC culture, whereas the middle layer was harvested for T-cell preparation. To prepare DCs, the top fluffy tissues were transferred to a new centrifuge tube with 5 mL RPMI 1640 media and broken up by gentle pipetting. After centrifugation (1500 rpm, 5 min), washing with RPMI 1640 media, and another centrifugation, the cells were resuspended in RPMI 1640 media containing 15% fetal bovine serum (Gibco) and plated on 6-well plate (2×10^6 per mL per well). Two hours after plating, the media containing suspending cells were aspirated away and replaced with 2 mL of fresh media. The cells were maintained in 2 mL RPMI 1640 media plus 15% fetal bovine serum per well at 37°C with 5% CO_2. The media were changed every 2 days. imDCs were induced in the media containing 20 ng/mL rat recombinant granulocyte–macrophage colony-stimulating factor (rrGM-CSF) and 10 ng/mL rr interleukin (IL)-4 (Peprotech). For mDCs, the media were supplied with 20 ng/mL rrGM-CSF, and 10 ng/mL rrTNF-α was added from day 5.

For preparation of T cells, the middle layer of fluff tissues pelleted in lymphocyte separation solution were transferred to a new centrifuge tube with 5 mL RPMI 1640 media and broken up by gentle pipetting. Following the same procedures as for DC preparation, T cells were maintained in 25 cm^2 culture flask at 37°C, 5% CO_2.

Cultured cells were examined every day under inverted microscope for morphologic changes. After 8 days in culture, DCs were stripped with 0.25% trypsin and fixed with 4% Glutaraldehyde for the confirmation of cell type with transmission electron microscopy and scanning electron microscopy (Zeiss, Germany). For H&E staining, cells were fixed with 10% buffered formalin for 30 min.

Immunophenotyping and immunohistochemistry (IHC) of cultured rat DCs

Immunophenotyping was performed on DCs cultured for 14 days. For flow cytometry, cell density was adjusted to

1×10^6/mL. Cells were centrifuged at 1000 rpm for 3 min on a bench top centrifuge and then resuspended in PBS. After three washes, cells were resuspended in 100 μL PBS. Fluorescein isothiocyanate-tagged primary antibodies (2 μg/10^6 cells) against OX62, CD80, or CD86 (all from PeproTech, USA) were then added, and cells were incubated in the dark for 30 min. Fluorescence-activated cell sorting (FACS) were performed using FACScan flow cytometry (Becton-Dickinson Immunocytometry Systems, San Jose, CA). For IHC, cultured mDCs and imDCs were stripped and collected with centrifugation at 1500 rpm for 15 min. The cell pellets were fixed with 10% formalin for 30 min and preserved in formalin-fixed, paraffin-embedded (FFPE) blocks. FFPE blocks were sliced at 3 to 4 μm thickness for IHC using antibodies against OX62 and MHC-II (PeproTech).

MLR assay

MLR assay was performed on cultured mDCs and imDCs (stimulator cells, 8 days in culture) with or without DNA plasmid transfection and cultured T cells (responder cells, 8 days in culture). In each of a 96-well plate, 50 μL stimulator DCs (2×10^4 cells) and 50 μL responder T cells (2×10^5 cells) were mixed. Same number of stimulators or responders alone as well as RPMI 1640 media with no cells served as controls. Each preparation was quadruplicated. The mixed cells were maintained at 37°C and 5% CO_2 for 3 days. At day 4, 15 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (5 mg/mL) was added in each well and cultured for additional 4 hours. Acidified isopropanol (165 μL per well) was then added, and the plate was oscillated for 15 min on a horizontal oscillator. Finally, optical density (OD) as a measure of cell densities was determined at 570 nm on a microplate reader.

Plasmids and cell transfection

Plasmids encoding pIRES2-EGFP, pIRES2-EGFP-hTGF-β1, and pIRES2-EGFP-hFasL (generous gifts from Dr Huang G, Fujian Medical University, China) were purified using HiSpeed Plasmid Midi Kit (Qiagen). hTGF-β1 and hFasL sequences were verified by polymerase chain reaction (PCR) using the following primers hTGF-β1 (339 bp PCR product): forward primer 5′GCCCTGGACACCAACTATTGCT3′, reverse primer 5′TCAGCTGCACTTGCAGGAGCGC3′ and hFasL (846 bp): forward 5′ATGCAGCAGCCCTTCAATTACCCATATC3′, reverse 5′TTAGAGCTTATATAAGCCGAAAAACGTC3′. The PCR was run under the following condition: 94°C for 5 min; 30 cycles of 94°C 30s, 55°C 30s, 72°C 1 min, and 72°C 7 min.

imDCs cultured for 6 days were transfected with plasmids encoding EGFP alone, EGFP-hTGF-β1, or pIRES2-EGFP-hFasL or cotransfected with the last two plasmids using Lipofectamine 2000 (Invitrogen, USA). For each transfection, cells in each well of a 6-well plate were incubated for 6 hours in 2 mL serum-free RPMI 1640 media containing 2 to 4 μg plasmids and 10 μL Lipofectamine 2000. Cells were then cultured in complete media for an additional 24-48 hours. Transfection efficiency was evaluated using fluorescent microscope.

Western blot and ELISA

mDCs and imDCs (8 days in culture) transfected with plasmids expressing EGFP-hTGF-β1, EGFP-hFasL, or both plasmids together as well as those transfected with EGFP control were used to prepare protein lysates in ice-cold Tris buffer (20 mmol/L; pH 7.5) supplemented with 137 mmol/L of NaCl, 2 mmol/L of EDTA, 1% Triton X, 10% glycerol, 50 mmol/L of NaF, 1 mmol/L of DTT, and protease inhibitors (Sigma). The protein lysates were then subjected to electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Amersham). After blocking with 5% bovine serum albumin in TBS/0.1%Tween 20, the membrane blots were incubated with primary antibodies at 4°C for 1 h, followed by horseradish peroxidase-tagged secondary antibodies under the same condition. ECL Western Blotting Detection Kit (Amersham) was used to visualize immunosignals of hTGF-β1, hFasL, and the reference protein β-actin. The following primary antibodies were used: mouse monoclonal anti-β-actin (C4), rabbit polyclonal anti-TGF-β1, and rabbit polyclonal anti-hFasL (all from Santa Cruz).

Cell supernatants were also collected from the mDCs and imDCs (8 days in culture). The levels of TGF-β1 and FasL were then determined with TGF-β1 Human ELISA Kit (ab100647, Abcam, Shanghai, China) and FAS Ligand (CD95 L) Human ELISA Kit (ab45892, Abcam), respectively, following the manufacture's protocols.

Rat model of AR following heterotopic liver transplantation

We first injected 20 Lewis rats intravenous (IV) and 20 intraperitoneal (IP) with 1 mL saline containing 2×10^6 in vitro-prepared EGFR—expressing imDCs to determine the distribution efficiency of injected imDCs in different organs (i.e., thymus, spleen, abdominal lymph nodes, liver, and kidney). We harvested organs from four rats on each of days 1, 2, 3, 5, and 7 after injection. Fluorescent cells were counted under a microscope. Next, we performed liver transplantations using DA rats as donors and Lewis rats as recipients, as described previously.[10] In brief, a total of 140 recipients (20 rats in each of the following experimental and control groups) first received an IP injection 5 days prior to heterotopic transplantation: 1 mL saline containing 2×10^6 in vitro-prepared non-transfected mDCs, nontransfected imDCs, imDCs transfected with empty vector, TGF-β1, FasL, or TGF-β1/FasL. A group of animals that received 1 mL saline containing no cells served as control. Recipient rats then underwent fasting for 12 h before heterotopic transplantation. In each group, 0-2 rats died due to the surgery procedure. Three, seven, and 10 days after transplantation, four rats from each postsurgery day were assessed for liver functions and then sacrificed for pathologic studies.[10] Survival analysis was performed with the remaining transplanted rats for up to 90 days.

Liver function assay

Three milliliters of blood samples were drawn from the inferior vena cava of each rat. Sera were immediately prepared by centrifugation at 2000 g for 10 minutes. Liver functions were assessed by measuring the serum levels of alanine

transaminase (ALT) and total bilirubin (TBIL) on days 3, 7, 10 after transplantation as described previously.[10]

Liver pathologic studies

Liver tissues preserved in FFPE sections were used for H&E staining. Pathologic changes of transplanted livers, including portal inflammation, bile duct injury, and venous endothelial inflammation, were evaluated using the rejection activity index (RAI) established by an international panel.[16] In this system, portal inflammation, bile duct injury, and venous endothelial inflammation are each graded from 0 (absent in damage) to 3 (severe) semi-quantitatively. The overall rejection score is the sum of these three scores and indicates the severity of rejection as follows: 0-2, no rejection; 3, borderline; 4-5, mild; 6-7, moderate; and 8-9, severe.

Terminal transferase–mediated UTP nick end-labeling (TUNEL) assay

TUNEL assays were performed on transplanted livers from recipient rats 7 days after transplantation as described previously.[10] In brief, liver tissues were formalin-fixed and embedded in FFPE. FFPE-persevered sections were cut at 4 μm, deparaffinized, and protease K-digested. Apoptotic cells were detected using an *in situ* apoptosis detection kit (Boehringer, Mannheim, Germany) according to vendor's instruction. Apoptotic cells were stained with 3,3′-diaminobenzidine for visualization and counter-stained with H&E.

Serum cytokine levels

Two milliliters of blood samples were taken from recipient rats 7 days after transplantation to measure the serum concentrations of interleukin (IL)-1, IL-10, and IL-12 by ELISA. In brief, after blood samples were centrifuged at 3000 rpm for 5 minutes, supernatants were collected and subjected to ELISA using Quantikine M kits (Jingmei Biotech). Cytokine levels were determined according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS13.0 software. Data were presented as mean ± standard error of the mean. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's or Fisher's least significant difference (LSD) multiple comparisons or by two-way ANOVA with Bonferroni's multiple comparison tests. Survival analysis was performed by Mantel–Cox log-rank test. $P < 0.05$ was considered statistically significant.

Results

Cultured cells show DC features

Cultured rat imDCs and mDCs were monitored every day with inverted microscope. By day 4, a small portion of cells had started to vary in size and presented small burr-like protrusions (data not shown). By day 8, cells had begun forming clusters and showing irregular cell body shapes with asymmetric nucleus and uneven dendritic projections on the cell surface (Fig. 1A). By day 12-14, typical DC morphology with irregular-shaped dendritic protrusions on the cell surface was apparent (Fig. 1B and C).

Cultured imDCs were examined with transmission electron microscope at day 8. Most cells displayed slender tree branch–like dendritic protrusions on the cell surface (Fig. 1D). These cells also showed large but irregular and asymmetric nucleus as well as abundant organelles, including mitochondria, rough and smooth endoplasmic reticulum.

Scanning electron microscope presented cultured DCs at day 8 with burr-like protrusions and varying body sizes in clusters (Fig. 1E). A higher magnification showed cell surface being fully assembled with sail-like protrusions (Fig. 1F), a typical feature of imDCs.

We next performed H&E staining. More than 90% of cells had distinctive cell morphology, characterized by an asymmetric nucleus and a significant amount of membrane protrusions with a burr- or veil-like shape (Fig. 1G and H). Together, these microscopic and histological observations indicate that cultured imDCs and mDCs have acquired typical DC morphologies.

Cultured mDCs and imDCs show characteristic DC immunophenotypes

We further characterized cultured DCs by immunophenotyping with flow cytometry. The results demonstrated that the DC marker OX62 expression was high on both mDCs and imDCs at days 6 and 10 (Fig. 2A). However, the expression of costimulatory molecule CD86 on imDC was significantly lower than that on mDC at day 10 (Fig. 2A).

Immunocytochemistry examinations further demonstrated that OX62 was expressed on the cell bodies and dendritic protrusions in both imDCs and mDCs (Fig. 2B and C). In contrast, MHC-II molecules normally found only on APCs such as DCs were expressed highly in mDCs but barely detectable in imDCs (Fig. 2D and E). These findings are consistent with the notion that imDC has lower T cell stimulating capacity.[2]

imDCs show reduced T cell stimulating capacity compared to mDCs

To verify whether cultured imDCs indeed had lower T cell stimulating capacity, we performed MLR assay. To this end, reactive T cells were cocultured with imDCs or mDCs, and the proliferation of T cells was then assessed. MLR assay showed that T-cell proliferation was significantly lower ($P < 0.0005$, one-way ANOVA with Tukey's multiple comparisons) in the presence of imDCs than of mDCs (Fig. 3). These results suggest that imDCs have a significantly lower capability to stimulate allogeneic T cells than mDCs.

The effect of TGF-β1 and FasL overexpression on surface antigen expression

To investigate whether TGF-β1 and FasL synergize to improve imDCs' immune tolerance capacity, we transfected cultured cells with DNA plasmids expressing hTGF-β1 (hTGF-β1-IRES2-
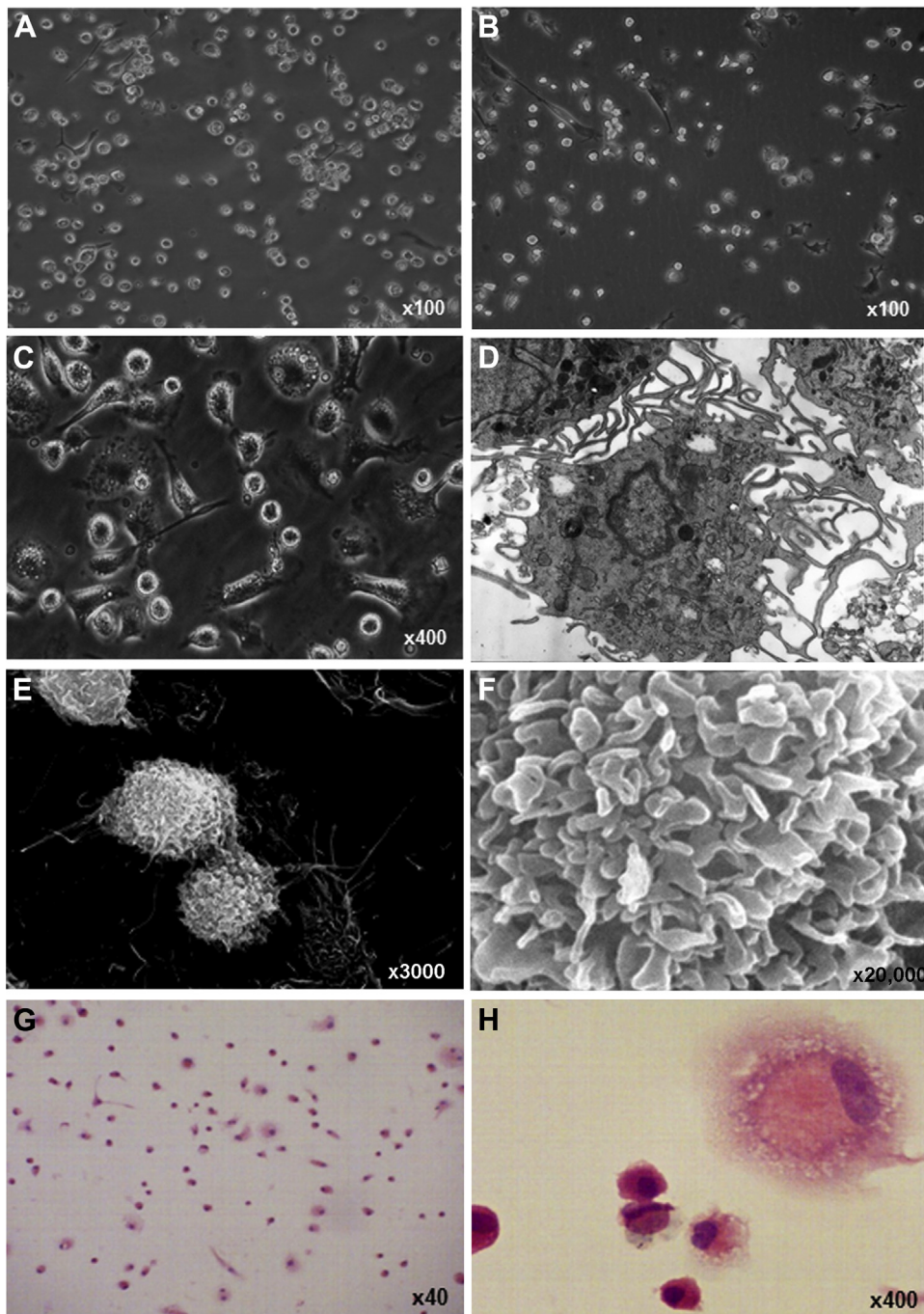
Fig. 1 — Morphological features of cultured DCs. (A-C) Microscopic images showing morphological changes of DCs after 8 days in culture (A) and 12-14 days in culture (B and C). (D) An image by transmission electron microscopy showing DCs after 8 days in culture. (E and F) Images by scanning electron microscopy showing morphological characteristics of imDCs after 8 days in culture. (G and H) H&E staining showing distinct cell morphologies of DCs after 8 days in culture. Image magnifications are indicated. (Color version of figure is available online.)

EGFP) and hFasL (hFasL-IRES2-EGFP). EGFP was detected 48 hours after transfection in imDCs expressing hTGF-β1, hFasL, or both (Fig. 4A-D). The expression of hTGF-β1 and hFasL was confirmed by Western blot (Fig. 4E). The level of secreted hTGF-β1 in the culture media, as determined by ELISA, was significantly higher (P < 0.001 for both, one-way ANOVA with Tukey's multiple comparison tests) in cells transfected with hTGF-β1 or cotransfected with hTGF-β1 and

hFasL than in other cells (Fig. 5A). Similarly, the level of hFasL in culture media was significantly higher (P < 0.05 for both, one-way ANOVA with Tukey's multiple comparison tests) in cells transfected with hFasL or cotransfected with both hTGF-β1 and hFasL than in other cells (Fig. 5B).

We then investigated whether expression of exogenous TGF-β1 and/or FasL affected the levels of CD86 and CD80 on imDCs by flow cytometry. The expression of both markers was
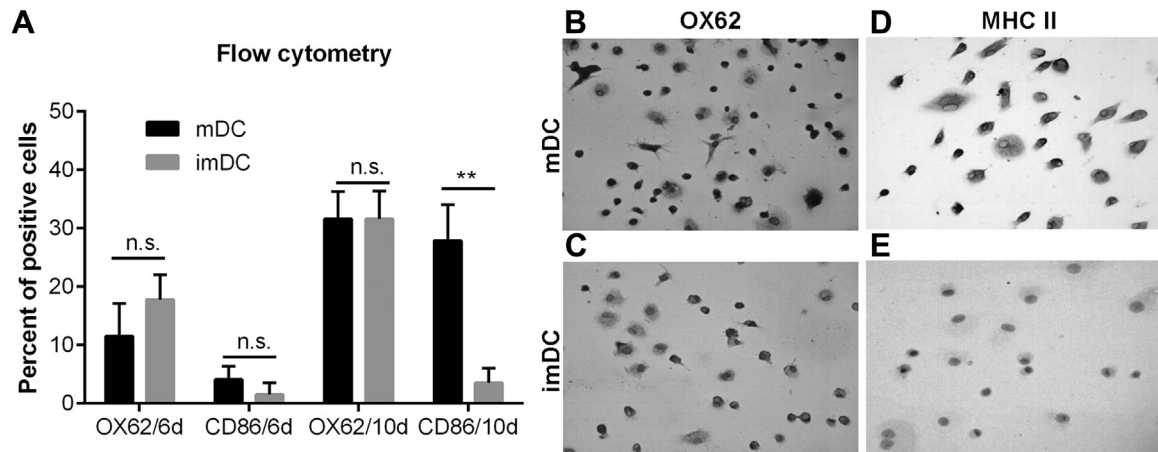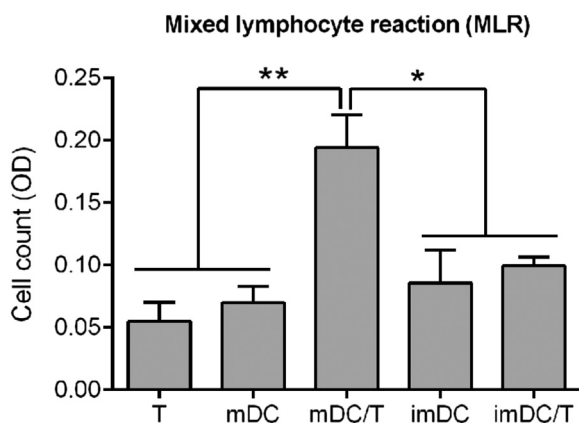
Fig. 2 – Cultured mDC and imDC showed characteristic DC immunophenotyping. (A) Flow cytometry showing that the surface expression of CD86 but not OX62 is lower in imDCs than that in mDCs at 10 days; experiment was repeated three times. Values are mean ± standard error of the mean. $n = 3$, n.s. = not significant, **$P < 0.01$ determined by two-way ANOVA with Bonferroni's multiple comparisons test. (B-E) Immunohistochemistry images (×200) showing the expression levels of cell surface markers OX62 and MHC-II in cultured DCs. OX62 levels were not significantly different between mDC (B) and imDC (C). MHC-II levels were significantly lower in imDC (E) than those in mDC (D).

significantly higher in mDCs than that in imDCs (Fig. 6A). Among imDCs, CD86 was decreased in TGF-β1—transfected cells, but not FasL-transfected or co-transfected cells (Fig. 6A), suggesting that it is TGF-β1 that downregulates costimulatory molecules, whereas FasL abrogates Fas-expressing cells. We found no difference in CD80 level among all imDCs (Fig. 6A).

Overexpression of TGF-β1 and/or FasL reduces imDC-induced T-cell proliferation

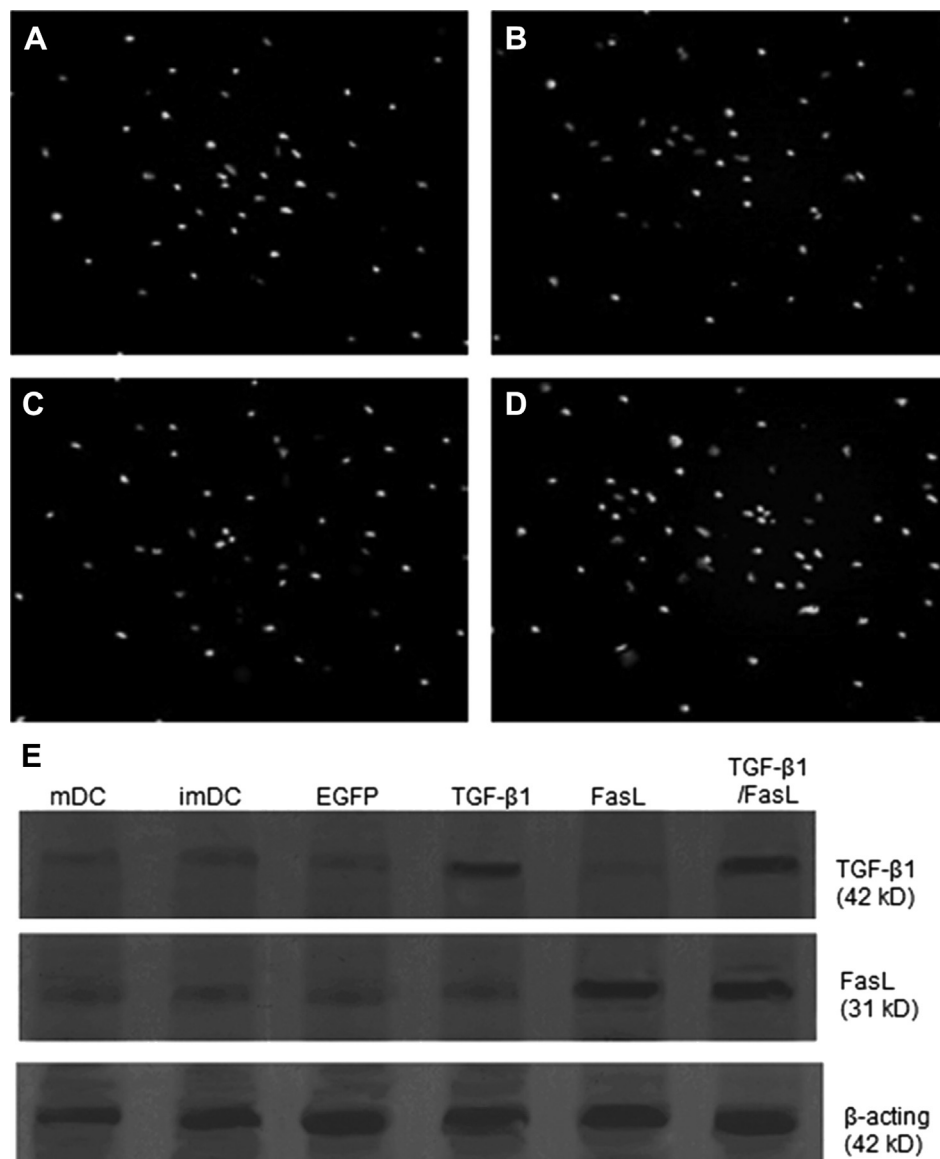To determine whether overexpression of TGF-β1 and FasL, alone or together, in imDCs affected on their T-cell activating capacity, we performed MLR assay. As shown in Figure 6B, T-cell proliferation, represented by OD value, was significantly lower when they were cocultured with imDCs than with mDCs, consistent with the notion that imDCs is less immunoreactive than mDCs.[2] T-cell proliferation was significantly reduced by overexpression of either TGF-β1 or FasL compared to the control imDCs (untransfected or transfected with control vectors; Fig. 6B). Interestingly, TGF-β1 and FasL transfected together further decreased T-cell proliferation (Fig. 6B; $P < 0.01$ in both cases, one-way ANOVA with Tukey's multiple comparison tests). These results suggest that TGF-β1 and FasL have a synergistic effect on imDC-induced T-cell proliferation.

Distribution of injected imDCs differs between IP and IV injections

To validate our *in vitro* findings, we aimed to determine the benefits of imDCs overexpressing both TGF-β1 and FasL *versus* those expressing either protein alone, using a rat model of liver transplantation. First, we analyzed the distribution of EGFP-expressing imDCs in different organs (i.e., the thymus, the spleen, the abdominal lymph nodes, the liver, and the kidney). EGFP allowed visualization of imDCs delivered by IP or IV injection, as shown in representative images in Figure 7A and B. In IV-injected animals, the level of imDCs was the highest in the liver among all the organs examined 1 day after injection (Fig. 7C). The number of imDCs then decreased steadily during the next 6 days (Fig. 7C). Although fewer imDCs were detected in the thymus, the spleen, and the abdominal lymph nodes, the number of imDCs remained relatively constant throughout 7 days after injection. Although kidney was also examined for the presence of injected imDCs, it was excluded from the analysis as imDCs were barely detectable in both IV and IP groups. In IP-injected rats, the number of imDCs was the highest in abdominal lymph nodes 24-48 h after injection (Fig. 7D). In contrast, the

Fig. 3 – Mixed lymphocyte reaction showing reduced T-cell stimulating capacity in imDCs compared to mDCs. OD at 570 nm was measured to determine cell proliferation rates in culture of T cells alone (T), mDCs alone (mDC), the mixture of mDCs and T cells (mDC/T), imDCs alone (imDC), and the mixture of imDCs and T cells (imDC/T); experiment was repeated three times. Values are mean ± standard error of the mean. $n = 3$. *$P < 0.05$, **$P < 0.01$ determined by one-way ANOVA with Tukey's multiple comparisons test.

Fig. 4 − Overexpression of EGFP-TGF-β1 and EGFP-FasL in cultured DCs. (A-D) Fluorescent images (100×) showing EGFP expression in cultured DCs transfected with plasmids encoding EGFP (A), EGFP-FasL (B), EGFP-TGF-β1 (C), and both EGFP-FasL and EGFP-TGF-β1 (D). (E) Western blot showing the expression of TGF-β1 and FasL in cultured mDCs (lane 1) or imDCs (lane 2) without transfection, and in imDCs transfected with EGFP alone (lane 3), TGF-β1 (lane 4), FasL (lane 5), or TGF-β1/FasL together (lane 6).

number of imDCs in other organs examined was lower but remained steady throughout 7 days after injection (Fig. 7D).

Rats receiving FasL/TGF-β1−expressing imDCs show less liver damage

In our liver transplantation model, Lewis and DA rats were organ recipients and donors, respectively. The Lewis rats were divided into seven groups according to whether and what types of DCs were injected (IP) prior to liver transplantation: no injection (control), mDCs, imDC, vector, FasL, TGF-β1, and FasL/TGF-β1. Compared to normal livers, all transplanted livers started to show damages (represented as RAI scores) at day 3 (Fig. 8). These damages became progressively worse at days 7 and 10 (Fig. 8). Nevertheless, at days 7 and 10 after

transplantation, liver damages were significantly smaller in rats injected with FasL/TGF-β1−expressing imDCs than in those with other imDCs (Fig. 8G). Thus, FasL/TGF-β1−expressing imDCs may be more effective in preventing organ rejection.

FasL induces apoptosis of targeting cells

To determine whether FasL can mediate apoptosis of targeting cells in our animal model of acute transplanted liver rejection, we conducted TUNEL assay with transplanted livers in rats. There were significantly more apoptotic cells, as evidenced by condensed brown staining of nuclei, in transplanted livers of rats injected with FasL- or FasL/TGF-β1−expressing imDCs (Fig. 9C and D) than in those with
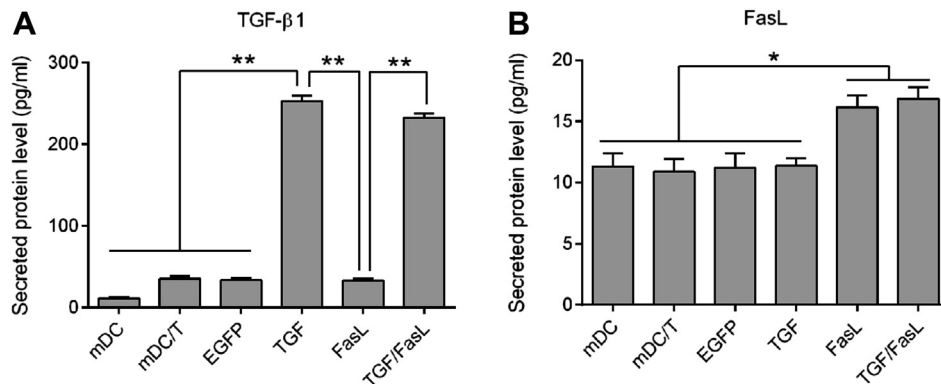
Fig. 5 — ELISA analysis of TGF-β1 and FasL expression in supernatants of cultured DCs. (A) Secreted TGF-β1 level in culture supernatant was significantly higher in imDCs transfected with TGF-β1 or TGF-β1/FasL than mDCs/imDCs without transfection or transfected with FasL alone. (B) Secreted FasL level in culture supernatant was significantly higher in imDCs transfected with FasL or TGF-β1/FasL than that in mDCs/imDCs without transfection or transfected with TGF-β1 alone. Experiments were repeated three times. Values are mean ± standard error of the mean. $n = 3$. *$P < 0.05$, **$P < 0.01$ determined by one-way ANOVA with Tukey's multiple comparisons test.

non–FasL-expressing imDCs (Fig. 9A and B). The difference in the number of apoptotic cells was significant ($P < 0.01$ by one-way ANOVA with Fisher's LSD multiple comparison) between recipients of FasL- or FasL/TGF-β1–expressing imDCs and those of TGF-β1–expressing imDCs or other types of DCs (Fig. 9E). We also performed TUNEL assay in spleens and abdominal lymph nodes in recipient rats and found that the apoptotic cells were significantly higher ($P < 0.05$ or 0.01 by one-way ANOVA with Fisher's LSD multiple comparison) in animals injected with FasL- or FasL/TGF-β1–expressing imDCs than those with DCs without FasL overexpression (Fig. 9E). In all three types of tissues examined, the difference was not significant between FasL- and FasL/TGF-β1–expressing imDCs (Fig. 9E). These data suggest that FasL induces apoptosis of targeting cells.

### TGF-β1 overexpression in imDCs upregulates IL-10 and downregulates IL-1 and IL-12 in liver-transplanted rats

Serum levels of the cytokines IL-1, IL-10, and IL-12 were analyzed in heterotopic liver-transplanted rats to determine whether TGF-β1 in FasL/TGF-β1–expressing imDCs still maintained the ability to regulate immune responses by modulating the expression of those cytokines.[17-19] The level of IL-10, an important molecule in inhibiting immune and inflammation responses, was significantly higher ($P < 0.01$ by one-way ANOVA with Fisher's LSD multiple comparison) in both TGF-β1–expressing groups than in other groups (Fig. 10). In contrast, the levels of IL-1 and IL-12, potent immunoregulatory cytokines, were significantly lower ($P < 0.01$ by one-way ANOVA with Fisher's LSD multiple comparison) in TGF-
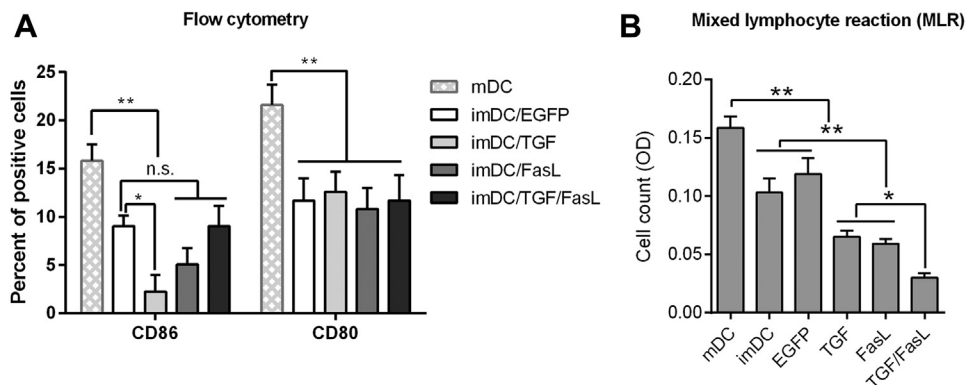
Fig. 6 — Effect of TGF-β1 and FasL transfection on surface antigen expression and imDC-induced T-cell proliferation. (A) Flow cytometry showing surface expressions of CD86 and CD80 in cultured mDCs or imDCs with or without the overexpression of TGF-β1 and FasL; experiments were repeated three times. Values are mean ± standard error of the mean. $n = 3$, n.s. = not significant, *$P < 0.05$, **$P < 0.01$ determined by two-way ANOVA with Tukey's multiple comparisons test. (B) Mixed lymphocyte reaction shows the effect of cotransfection of TGF-β1/FasL on T cell stimulating capacity in imDCs. T cells were cultured with mDCs or imDCs without transfection or imDCs transfected with EGFP, TGF-β1 (TGF), FasL, or TGF-β1/FasL (TGF/FasL); experiments were repeated six times. Values are mean ± standard error of the mean. $n = 6$. *$P < 0.05$, **$P < 0.01$ determined by one-way ANOVA with Tukey's multiple comparisons test.
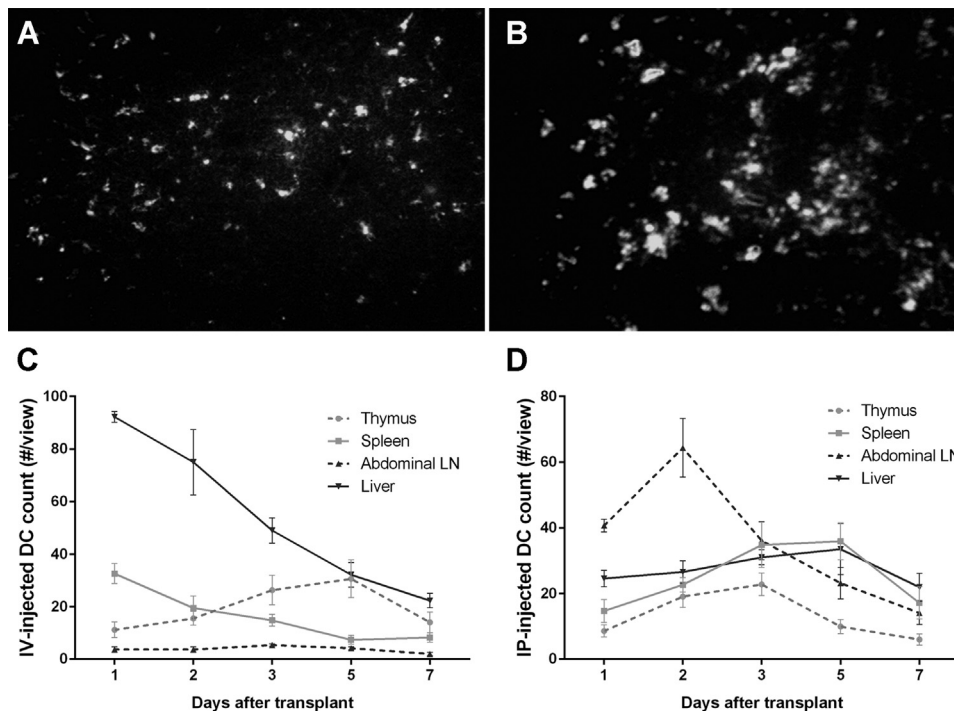
Fig. 7 − Distribution of EGFP-expressing imDCs in rats via either IP or IV injections. (A and B) Representative images showing EGFP-expressing imDCs in liver 1 day after IV injection (A) and in abdominal lymph nodes 2 days after IP injection (B). (C and D) Summary graphs showing the counts of imDCs in organs indicated on days 1, 2, 3, 5, and 7 post-IV injection (C) or -IP injection (D). Values are mean ± standard error of the mean. $n = 4$.

β1−expressing groups than in other groups (Fig. 10). Interestingly, we observed the opposite trend with FasL group; IL-10 was downregulated, whereas IL-1 and IL-12 were upregulated in FasL group compared to vector group ($P < 0.01$ by one-way ANOVA with Fisher's LSD multiple comparison, Fig. 10). These results suggest that TGF-β1 overexpression in imDCs, alone or together with FasL, can promote expression of the immunosuppressive IL-10 and inhibit the expression of the immunostimulatory IL-1 and IL-12.

imDCs expressing FasL and TGF-β1 provides a faster recovery of liver function

Transplant-induced liver damages were also determined by measuring the serum levels of ALT and TBIL. The serum levels of both ALT and TBIL were not significantly different among different groups of animals 3 days after transplantation (Fig. 11A and B). However, 7 days after transplantation, serum ALT was significantly lower ($P < 005$ or 0.01 by one-way ANOVA with Fisher's LSD multiple comparison) in imDC groups compared to the control or mDC groups (Fig. 11A). Importantly, it was significantly lower in FasL/TGF-β1 group than in all other groups (Fig. 11A). The difference between other imDC groups was not significant (Fig. 11A). Serum TBIL was significantly lower in imDC groups compared to the control or mDC groups (Fig. 11B). Moreover, as with ALT, we observed that TBIL was the lowest in FasL/TGF-β1 group ($P < 0.05$ or 0.01 by one-way ANOVA with Fisher's LSD multiple comparison). (Fig. 11B). Ten days after

surgery, the levels of both ALT and TBIL were significantly lower ($P < 0.05$ or 0.01 by one-way ANOVA with Fisher's LSD multiple comparison) in FasL/TGF-β1 group than in all other groups (Fig. 11A and B). In summary, our results show that, although imDC-injection provides a better recovery of liver function than mDC- or no DC-injection, imDCs expressing FasL and TGF-β1 are the most efficient in this capacity.

TGF-β1 expressing imDCs endow longer survival after liver transplantation

Survival analysis was completed in 47 eligible rats for up to 90 days. The median survival was 9, 6, 24, 25, 49.5, 24.5, and not reached for the control ($n = 6$), mDC ($n = 7$), imDC ($n = 6$), vector ($n = 7$), TGF-β1 ($n = 6$), FasL ($n = 8$), and TGF-β1/FasL ($n = 7$) groups, respectively (Fig. 12). Overall survival was longer ($P < 0.01$ by Mantel-Cox log-rank test) in all imDC rats than in mDC or the control rats. Among the imDC groups, TGF-β1/FasL rats survived the longest (Fig. 12). TGF-β1 rats also showed significantly longer survival compared to non-TGF-β1 imDC rats (Fig. 12). These results suggest that the expression of both TGF-β1 and FasL improves overall survival in the liver-transplanted rats injected with imDCs.

Discussion

DCs can induce and maintain self-tolerance through a variety of mechanisms, which have been exploited for creating
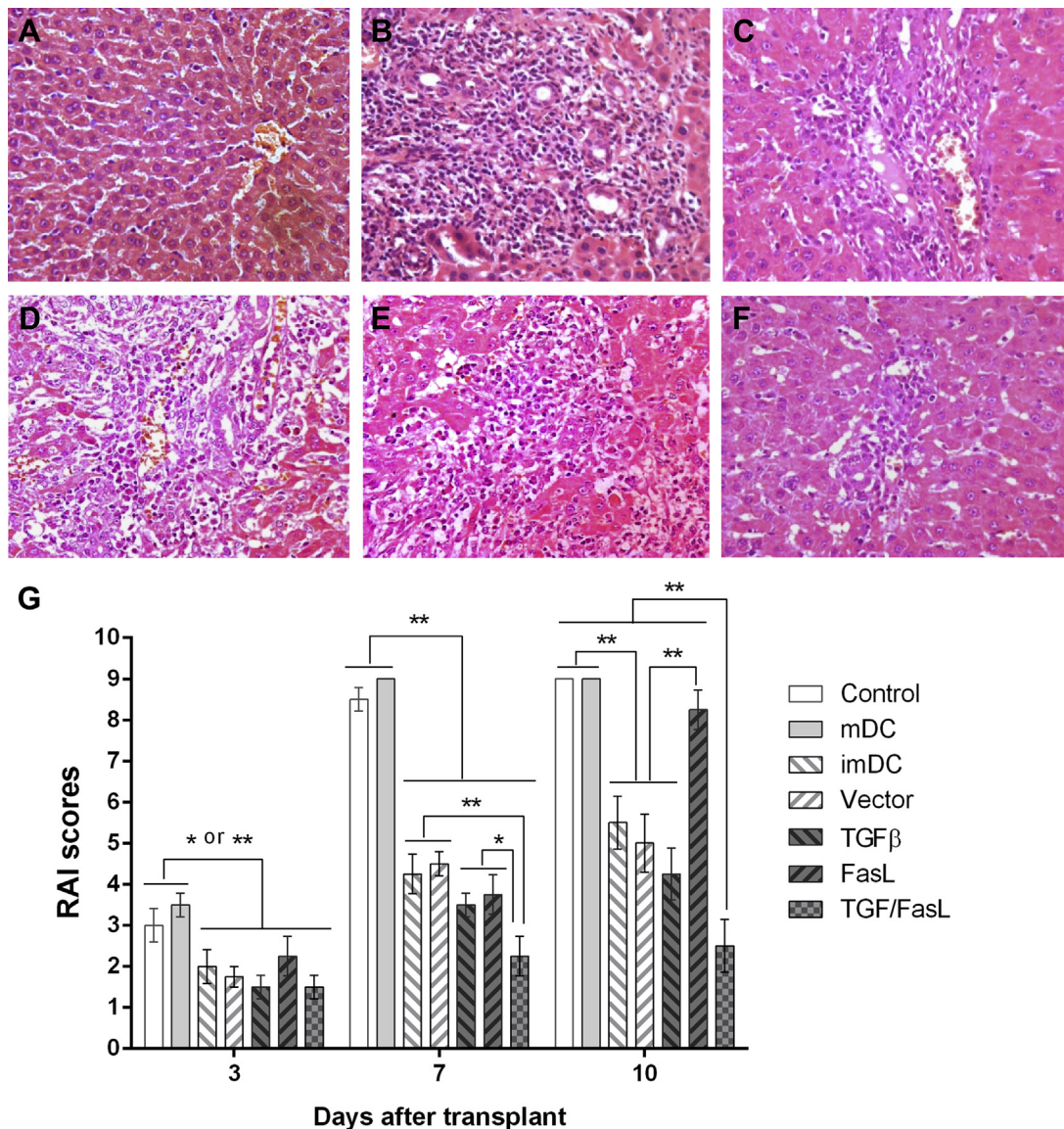
Fig. 8 — Pathological evaluation using the RAI in heterotopic transplanted livers. (A-F) Representative H&E staining in normal liver (A), or in the heterotopic transplanted livers without DC-injection (B), or with nontransfected (C), FasL-expressing (D), TGF-β1 (E), or TGF-β1/FasL-co-transfected (F) imDCs. (G) Summary graph showing RAI scores in control- or DC-injected rats with heterotopic liver transplantation on days 3, 7, 10 after transplantation. Values are mean ± standard error of the mean. $n = 4$. *$P < 0.05$, **$P < 0.01$ determined by two-way ANOVA with Fisher's LSD multiple comparisons test. (Color version of figure is available online.)

tolerogenic DCs *in vitro* for therapeutic purposes.[2] Tolerogenic DCs are generally immature, mature resistant, expressing reduced levels of costimulatory or immune inhibitory signals, and deficient in producing T cell–driving cytokines.[1-3] To enhance tolerogenic ability, *in vitro*–propagated DCs for use in organ transplantation to reduce immune tolerance have been exposed to a range of immunosuppressive reagents and anti-inflammation factors, including but not limited to cytokine IL-10 and TGF-β1,[10,14,20,21] tumor necrosis factor FasL,[15] vitamin D3 metabolite,[22] and the clinically approved immunosuppressive drug rapamycin.[23,24] In this study, we demonstrated that better immune tolerance was accomplished by coexpressing in imDCs both TGF-β1 and FasL than by single

gene transduction with either TGF-β1 or FasL alone. More importantly, such cotransfected imDCs injected in rats with heterotopic liver transplantation showed significantly better immune tolerance and longer survival than single gene–transfected or nontransfected imDCs/mDCs.

In vitro–propagated DCs can be enhanced in their tolerogenic potential through genetic modification, including ectopically expressing immunosuppressive molecules, such as IL-10, TGF-β11, or CTLA4, which inhibit the expression of cell surface costimulatory molecules.[10,14,25] Genetic manipulation of propagated DCs *in vitro* can induce and maintain T-cell anergy by overexpressing PDL1, prevent the proliferation of allogeneic T cells through the expression of IDO, inhibit
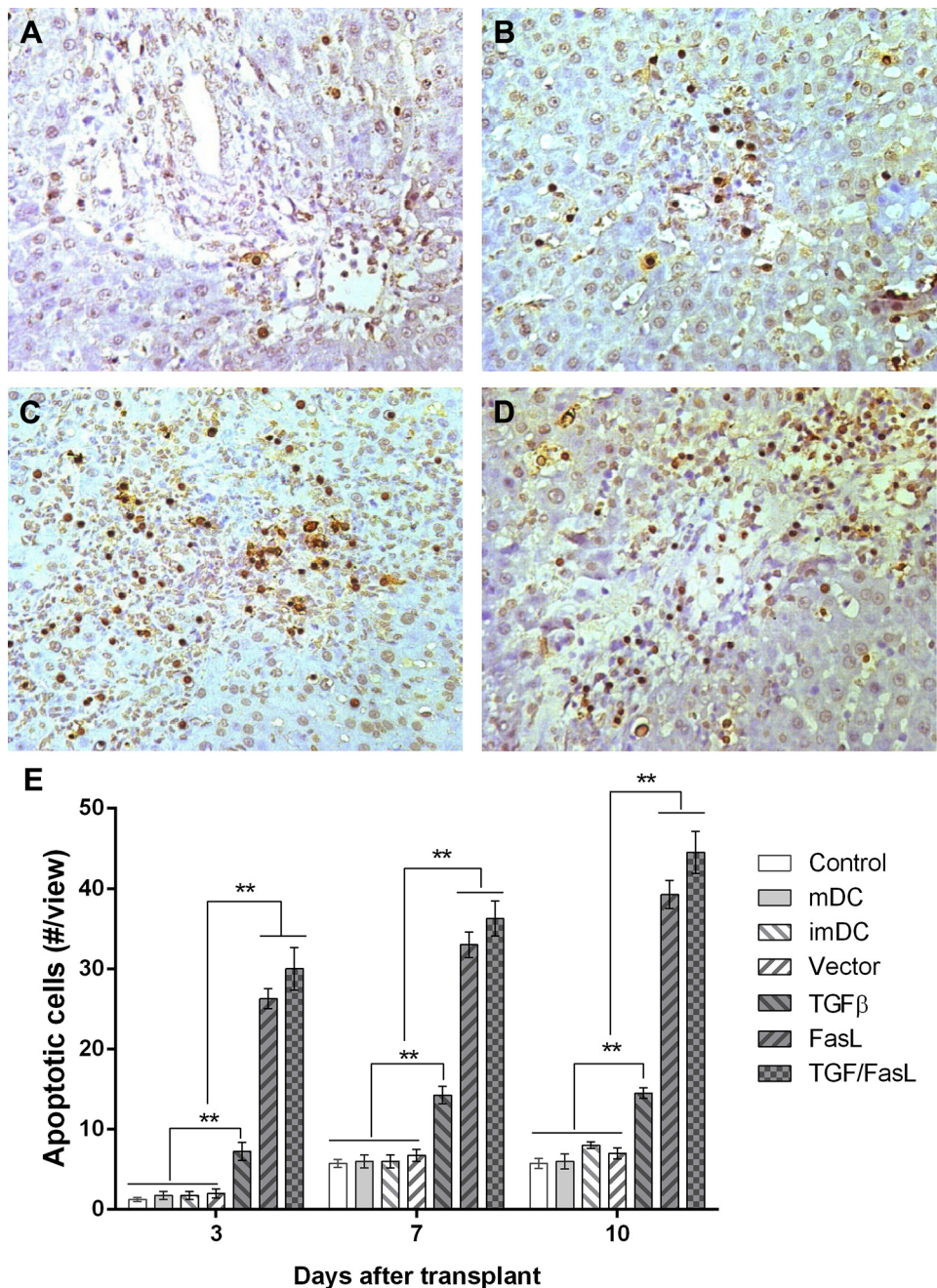
Fig. 9 − Apoptosis by TUNEL in allografted livers after transplantation. (A-D) Representative images (400×) showing apoptotic cells (brown staining) 7 days after transplantation in grafted livers in recipient rats injected with imDCs transfected with nothing (A), TGF-β1 (B), FasL (C), and TGF-β1/FasL (D). (E) Summary graph showing the percent of apoptotic cells in the grafted livers. Values are mean ± standard error of the mean. $n = 4$. *$P < 0.05$, **$P < 0.01$ determined by two-way ANOVA with Fisher's LSD multiple comparisons test. (Color version of figure is available online.)

NF-κB activation through ectopic expression of a dominant negative form of IKK2, and promote the apoptosis of antigen-specific T cells through the expression of FasL (also known as CD95 L) or TRAIL.[2] Thus, gene transfer technology can be exploited to promote DC-mediated T-cell tolerance for potential therapeutic applications in organ transplantation.[1,2] In this study, we specifically chose to manipulate imDCs to enhance their immune tolerance through two distinct mechanisms: suppressing the expression of cell surface

costimulatory molecules by overexpressing TGF-β1[14] and promoting antigen-specific T-cell apoptosis by the expression of transfected FasL.[15]
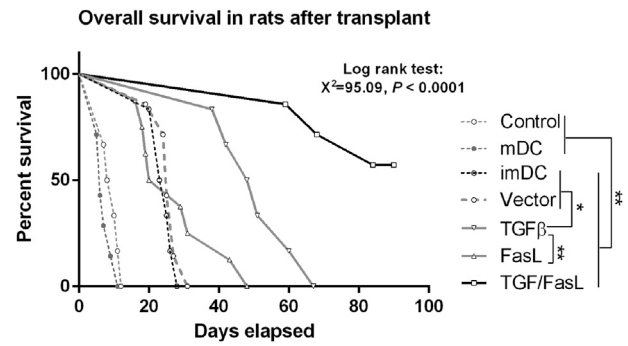
TGF-β1 is a pleiotropic cytokine important in multiple cellular functions including the control of cell proliferation and differentiation, immune regulation, and apoptosis.[11] It can either function as an immune suppressor to inhibit effector cell proliferation, differentiation, and activation, or promote a proinflammatory immune response.[26] It has been
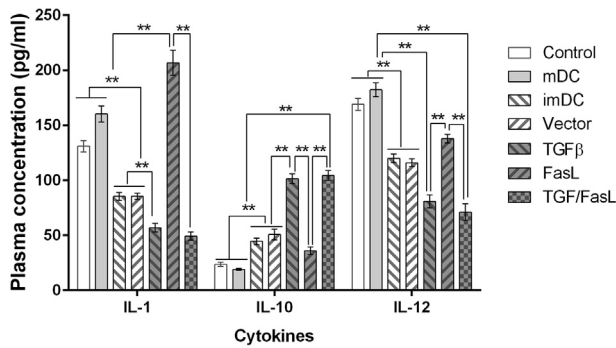
Fig. 10 — Cytokine levels in plasma. Summary graph showing the plasma levels of cytokines IL-1, IL-10, and IL-12 by ELISA in recipient rats with liver transplantation. Values are mean ± standard error of the mean. $n = 4$. **P < 0.01 determined by two-way ANOVA with Fisher's LSD multiple comparisons test.

Fig. 12 — Rat survival curves. Rats with heterotopic liver transplantation were injected with control and DC cells as indicated in the key and survival monitored. *P < 0.05, **P < 0.01 determined by Mantel-Cox log-rank test.

suggested that TGF-β1 is a key factor regulating Th differentiation by increasing Foxp3 expression in Th0 cells resulting in Foxp3+ Tregs, which plays a crucial role in immune tolerance.[27] TGF-β1 has been extensively studied and used for its potential in enhancing peripheral tolerance, including improving DC-mediated immune tolerance.[13] It was reported that TGF-β1–transduced imDCs induced higher percentages of Foxp3+ CD4 Tregs *in vitro*.[28] In contrast, FasL induces immune tolerance through a distinctively different mechanism, i.e., apoptosis mediated by FasL interaction with Fas receptor on targeted T cells.[15] It has been shown that FasL expressed in CD8+ DCs may interact with Fas on the surface of activated CD4+ T cells to induce apoptosis.[29] It has been suggested that the FasL-mediated immunosuppressive function is regulated through the ERK/β-catenin pathway.[30]

Consistent with their immunosuppressive roles, our study has shown that overexpression of either TGF-β1 or FasL alone in imDCs significantly downregulated proliferation of cocultured T cells in MLR experiments compared to control imDCs transfected with empty vectors or nothing. Moreover, we demonstrated that cotransfection of TGF-β1 and FasL together

further significantly reduced proliferation of cocultured T cells compared to individual complementary DNA transfection. Therefore, we reasoned that cotransfection of both genes would be much more effective at enhancing imDC-induced immune tolerance in T cells than single transfections. Our *in vitro* experiments showed that this was indeed the case. Moreover, our *in vivo* experiments demonstrated that this cotransfection in imDCs injected in liver-transplanted rats was much more effective at enhancing imDC-induced immune tolerance than single transfections. We showed this as reduced liver damage by pathological assessment, enhanced recovered liver function by ALT and TBIL tests, and longer survival. These *in vivo* experiments also showed that cotransfection of both constructs maintained the advantage of each gene in promoting immune tolerance in host animals. Thus, FasL still mediated apoptosis of lymphocytes, and TGF-β1 still mediated upregulation of immunosuppressant IL-10 and downregulation of immune-responding molecules IL-1 and IL-12. It is therefore probable that *in vitro*–propagated imDCs expressing both constructs will be more likely to enhance immune tolerance, preventing graft rejection and prolonging the survival of liver transplants and recipient patients. Intriguingly, FasL (or CD95 L) has been implicated in
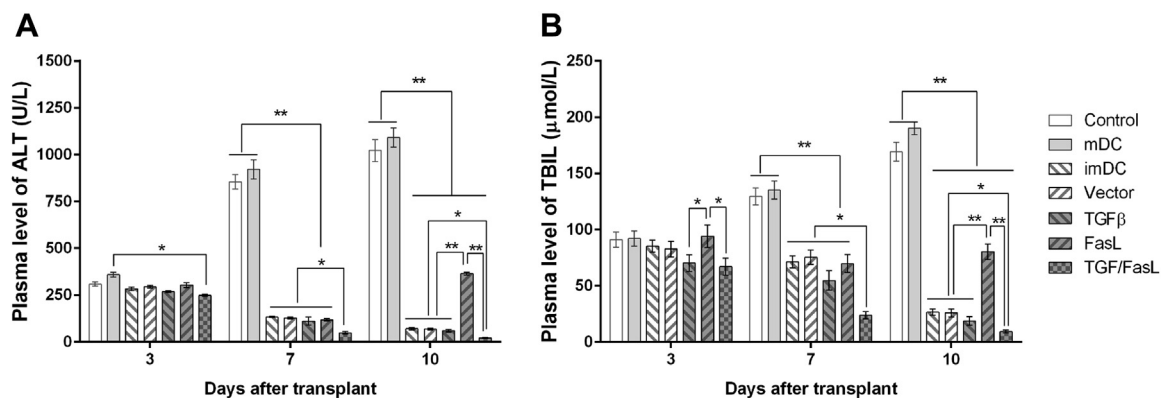
Fig. 11 — Liver function assays by ALT and TBIL tests in recipient rats with liver transplantation. Summary graphs showing the serum levels of ALT (A) and TBIL (B) in control- and DC-injected rats with heterotopic liver transplantation. Values are mean ± standard error of the mean. $n = 4$. *P < 0.05, **P < 0.01 determined by two-way ANOVA with Fisher's LSD multiple comparisons test.

cancer-relevant nonapoptotic and tumor-promoting activities,[31] characteristics that should be carefully examined for any application in cell transplantation to cancer patients.

In conclusion, we have demonstrated that imDCs overexpressing both TGF-β1 and FasL can be successfully generated *in vitro* for potential transplantation. Coexpression of both TGF-β1 and FasL is more efficient in promoting imDC-induced T cell immune tolerance than transfection of either TGF-β1 or FasL alone. Using an *in vivo* rat model of heterotopic liver transplantation, we validated the enhanced tolerogenic effect of imDCs coexpressing both TGF-β1 and FasL, including better liver recovery and longer survival of transplanted animals. Thus, the strategy of using imDCs overexpressing both TGF-β1 and FasL as immune suppressors may possess clinical potential and warrants further studies in liver transplantation.

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Authors' contributions: M.Q., L.C., J.Z., and J.L. contributed to the conception and design of the study. All authors participated in experiments. M.Q., Y.C., and L.C. analyzed data, interpreted results, and prepared figures. M.Q., Y.C., and L.C. wrote the manuscript. J.Z. and J.L. approved the final version of the manuscript.

Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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