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• BASIC RESEARCH •

Functional changes of dendritic cells derived from allogeneic partial liver graft undergoing acute rejection in rats

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Abstract

AIM: To investigate functional change of dendritic cells (DCs) derived from allogeneic partial liver graft undergoing acute rejection in rats.

METHODS: Allogeneic (SD rat to LEW rat) whole and 50 % partial liver transplantation were performed. DCs from liver grafts 0 hr and 4 days after transplantation were isolated and propagated in the presence of GM-CSF *in vitro*. Morphological characteristics of DCs propagated for 4 days and 10 days were observed by electron microscopy. Phenotypical features of DCs propagated for 10 days were analyzed by flow cytometry. Expression of IL-12 protein and IL-12 receptor mRNA in DCs propagated for 10 days was also measured by Western blotting and semiquantitative RT-PCR, respectively. Histological grading of rejection were determined.

RESULTS: Allogeneic whole liver grafts showed no features of rejection at day 4 after transplantation. In contrast, allogeneic partial liver grafts demonstrated moderate to severe rejection at day 4 after transplantation. DCs derived from allogeneic partial liver graft 4 days after transplantation exhibited typical morphological characteristics of DC after 4 days' culture in the presence of GM-CSF. DCs from allogeneic whole liver graft 0 hr and 4 days after transplantation did not exhibit typical morphological characteristics of DC until after 10 days' culture in the presence of GM-CSF. After 10 days' propagation *in vitro*, DCs derived from allogeneic whole liver graft exhibited features of immature DC, with absence of CD40, CD80 and CD86 surface expression, and low levels of IL-12 proteins (IL-12 p35 and IL-12 p40) and IL-12 receptor (IL-12Rβ_1 and IL-12Rβ_2) mRNA, whereas DCs from allogeneic partial liver graft 4 days after transplantation displayed features of mature DC, with high levels of CD40, CD80 and CD86 surface expression, and as a consequence, higher expression of IL-12 proteins (IL-12 p35 and IL-12 p40) and IL-12 receptors (IL-12Rβ_1 and IL-12Rβ_2) mRNA than those of DCs both from partial liver graft 0 hr and whole liver graft 4 days after transplantation (P<0.001) was observed.

CONCLUSION: DCs derived from allogeneic partial liver graft undergoing acute rejection display features of mature DC.

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INTRODUCTION

The shortage of donor organs remains a major obstacle to the widespread application of liver transplantation in patients with end-stage liver disease. Although split grafting was used to increase the number of donor livers, the accelerated rejection induced by liver regeneration[1,2] can interfere with the outcome of these liver grafts. Therefore, It is important to investigate the mechanism responsible for the accelerated rejection of split liver grafting.

After organ transplantation, interstitial donor dendritic cells (DCs) migrate to recipient lymphoid tissue. In the case of experimental skin, heart, or kidney allografts, these cells have been implicated as the principal instigators of rejection. Despite similar patterns of donor DCs migration, liver grafts are accepted without immunosuppressive therapy between MHC-mismatched mouse, and certain rat strains, and induce donor-specific tolerance. These phenomena and the persistence of donor hematopoietic cells, including DCs, in successful, long-term graft recipients, have raised important questions about the possible role of donor DCs in liver transplant tolerance. The capacity of DCs to initiate immune responses is determined by their surface expression of MHC gene products and costimulatory molecules (CD40, CD80 and CD86), and the secretion of the immune regulator, interleukin (IL)-12[3-12]. Immature DCs resident in nonlymphoid tissues such as normal liver are deficient at antigen capture and progressing[3,13], whereas mature DCs, resident in secondary lymphoid tissues, are potent antigen-presenting cells, which can induce naive T-cell activation and proliferation[3-7]. Immature DCs that express surface MHC class II, but that are deficient in surface costimulatory molecules, can induce T-cell hyporesponsiveness[13-15] and inhibit immune reactivity[16,17]. It has been observed that liver-derived[13,18] or bone marrow-derived immature DCs[17], propagated *in vitro* and lacking surface costimulatory molecules, can prolong heart or pancreatic islet allograft survival. Whereas, marked augmentation of DCs numbers and maturation of DCs in liver allografts by donor treatment with the hematopoietic growth factor fms-like tyrosine kinase 3 (Flt3) ligand (FL) results in acute liver graft rejection[19, 20].

Recent findings have revealed increased immune responses to regenerating allogeneic partial liver graft in rats[1, 2], but little is known about the mechanism responsible for the accelerated rejection. The purpose of this study was to investigate the property of DCs isolated from allogeneic partial liver graft in comparison with DCs isolated from allogeneic whole liver graft, in an attempt to elucidate the possible mechanism responsible for the accelerated rejection of allogeneic partial liver graft. Given mature DC can induce naive T-cell activation and proliferation[3-7], and consequently induce acute rejection[19,20], we suspected that maturation of DCs derived from liver graft would be a key inducer of the accelerated rejection of allogeneic partial liver graft. In the present study, we first demonstrated that DCs derived from partial liver graft undergoing acute rejection displayed features of mature DC, including positive expression of costimulatory molecules, higher level expression of IL-12 protein and IL-12 receptor mRNA in these mature DCs.

MATERIALS AND METHODS

Animals

One hundred male LEW rats and one hundred male SD rats weighing 220-300 g were used in all the experiments. Allogeneic whole and 50 % partial liver transplantation were performed using a combination of SD rats to LEW rats. The animals were purchased from Chinese Academy of Science and Sichuan University. They were maintained with a 12-hr light/dark cycle in a conventional animal facility with water and commercial chow provided ad libitum, with no fasting before the transplantation.

Liver transplantation

All operation were performed under ether anesthesia in clean but not sterile conditions. All surgical procedures were performed from 8 a.m. to 5 p.m. Donors and recipients of similar weight (±10 g) were chosen. Liver reduction was achieved by removing the left lateral lobe and the two caudate lobes, which resulted in a 50 % reduction of the liver mass. Whole liver transplantation (WLT) and partial liver transplantation (PLT) were performed with the two-cuff method described by Kammada and Calne[21], Knoop *et al*[22] and Uchiyama *et al*[23]. Briefly, the whole and partial liver were perfused with 20 ml of chilled lactated Ringer's solution containing 200 U of heparin through the aorta. The liver was removed and immersed in chilled University of Wisconsin solution. Immediately after cuffs were placed on the portal vein and the infrahepatic vena cava, the liver graft was perfused with 8 ml of chilled University of Wisconsin solution through the portal vein and 2 ml of the same solution through the hepatic artery, and then the hepatic artery was ligated. Cold preservation time was approximately 1 hr in all experiments. After a total hepatectomy was performed in the recipient, the suprahepatic vena cava was anastomosed in a continuous fashion with 8-0 sutures. The portal vein and infrahepatic vena cava were then connected by a 6-FG and 8-FG polyethylene tube cuff, respectively. The bile duct was internally stented with a 22-gauge i.v. catheter. The portal vein was clamped for <18 min in all animals. In each group, the survival rate after grafting was >89 % at 24 hr after surgery. Deaths that occurred within this period were defined as resulting from technical failure. Penicillin was given perioperatively.

Histology

Part of liver graft tissues 4 days after transplantation were sectioned and preserved in 10 % Formalin, embedded in paraffin, cut with microtome, and stained with hematoxylin and eosin.

Propagation and purification of liver graft-derived DC populations

DCs from liver graft 0 hr and 4 days after transplantation were propagated in GM-CSF from nonparenchymal cells (NPC) isolated from collagenase-digested liver graft tissue, as described by Lu[18]. Nonadherent cells, released spontaneously from proliferating cell clusters, were collected after 4 days' and 10 days' culture, and purified by centrifugation 500×g, 10 min at room temperature on a 16 % w/v metrizamide gradient (DC purity 80-85 %).

Morphological and Phenotypical features of DCs

Morphological characteristics of DCs derived from liver graft were observed by electron microscopy. Expression of cell surface molecules was quantitated by flow cytometry as described by Mehling *et al*[4]. Aliquots of 2×10^5 DCs propagated for 10 days *in vitro* were incubated with the following primary mouse anti-rat mAbs against OX62, CD40, CD80, CD86 (Serotec, USA), or rat IgG as an isotype control for 60 min on ice (1 μg/ml diluted in PBS/1.0 % FCS). The cells were washed with PBS/1.0 % FCS and labeled with FITC-conjugated goat anti-mouse IgG, diluted 1/50 in PBS/1.0 % FCS for 30 min on ice. At the end of this incubation cells were washed, PBS were added, and the cells were subsequently analyzed in an FACS-4200 flow cytometer (Becton-Dikison, USA).

Semiquantitative RT-PCR for expression of IL-12R mRNA in DCs

Analysis of expression of IL-12Rβ_1 and IL-12Rβ_2 mRNA was determined by reverse transcription-PCR (RT-PCR) amplification in contrast with house-keeping gene GAPDH. Total RNA from 1×10^7 DCs propagated for 10 days *in vitro* was isolated using Tripure™ reagent (Promega, USA). First-strand cDNA was transcribed from 1 μg RNA using AMV and an Oligo (dT_{15}) primer. PCR was performed in a 25 μl reaction system containing 10 μl cDNA, 2 μl 10 mM dNTP, 2.5 μl 10× buffer, 2.5 μl 25 mmol·L^{-1} $MgCl_2$, 2 μl specific primer, 5 μl water and 1 μl Taq. IL-12Rβ_1 and IL-12Rβ_2 were amplified using specific primer for IL-12Rβ_1[24] and IL-12Rβ_2[25]. Specific primers for GAPDH[26] were also used for control. Thermal cycling of IL-12Rβ_1 and IL-12Rβ_2 and GAPDH primers were performed as follows[25]: denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, and extention at 72 ℃ for 1 min, all cycling were performed for 35 cycles. The predicted PCR product size were 331 bp for IL-12Rβ_1, 1 200 bp for IL-12Rβ_2 and 576 bp for GAPDH. PCR products of each sample were subjected to electrophoresis in a 15g·L^{-1} agarose gel containing 0.5 mg·L^{-1} ethidium bromide. Densitometrical analysis using NIH image software was performed for semiquantification of PCR products, and the expression level of each sample were expressed by IL-12R mRNA/GAPDH mRNA (%).

Western blotting for IL-12 protein expression in DCs

DCs cultured for 10 days *in vitro* were starved in serum-free medium for 4hr at 37 ℃. These cells were washed twice in cold PBS, resuspended in 100 μl lysis buffer (1 % Nonidet P-40, 20mM Tris-HCl, pH8.0, 137mM NaCl, 10 % glycerol, 2mM EDTA,10 μg/ml leupeptin, 10 μg/ml aprotinin, 1mM PMSF, and 1 mM sodium orthovanadate), and total cell lysates were obtained. The homogenates were centrifuged at 10 000×g for 10 min at 4 ℃. Cell lysates (20 μg) were electrophoresed on SDS-PAGE gels, and transferred to PVDC membranes for Western blot analysis. Briefly, PVDC membranes were incubated in a blocking buffer for 1 hr at room temperature, then incubated for 2 hr with Abs raised against IL-12 p35 (M-19, goat anti-rat, Santa Cruz,CA), IL-12p40 (H-306, rabbit anti -rat, Santa Cruz, CA). The membranes were washed and incubated for 1 hr with HRP-labeled horse anti-goat or HRP-labeled goat anti-rabbit IgG. Immunoreactive bands were visualized by ECL detection reagent.

Statistics

Statistic analysis of data was performed using the Student's *t*-test; $P<0.05$ was considered statistically significant.

RESULTS

Histological rejection

Histology features of allografted livers were compared between the whole and partial groups on Day 4 after transplantation, allogeneic whole liver grafts demonstrated no rejection. In contrast, partial liver grafts demonstrated moderate to severe rejection, including inflammatory cellular infiltration in the portal tract, andothelialitis and bile duct damage.

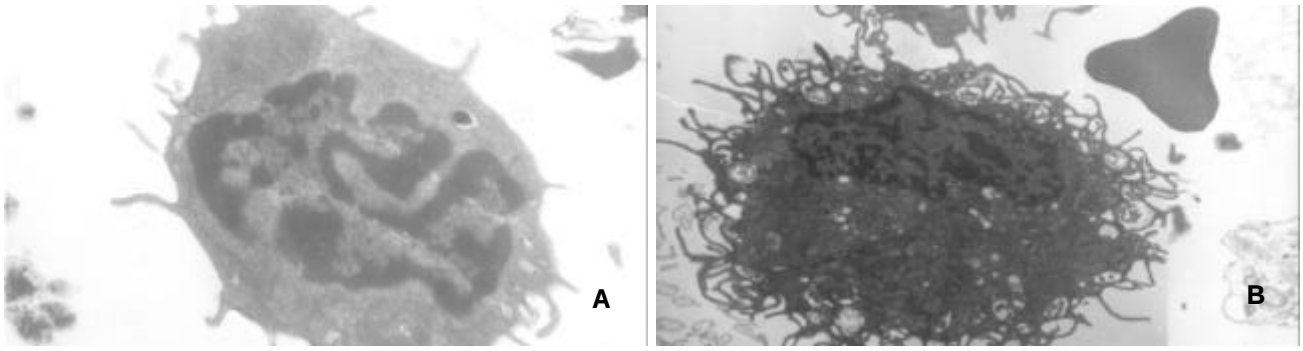

Figure 1 Morphological characteristics of liver graft-derived DCs propagated for 4 days in the presence of GM-CSF. **A**: DC from whole liver graft 4 days after transplantation (×1 200 b). **B**: DC from partial liver graft 4 days after transplantation (×600b).

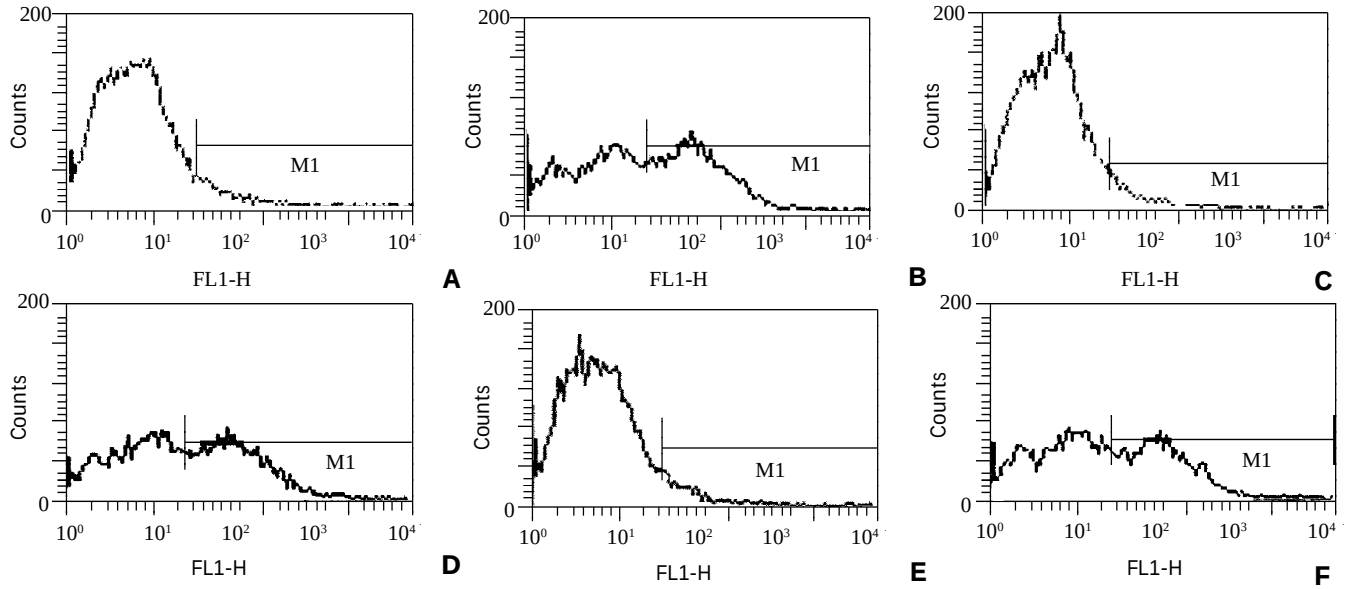

Figure 2 Expression of costimulatory molecules in DCs from liver grafts 4 days after transplantation by FCM. **A:** Expression of CD40 in whole liver graft–derived DCs; **B:** Expression of CD40 in partial liver graft –derived DCs; **C:** Expression of CD80 in whole liver graft –derived DCs; **D:** Expression of CD80 in partial liver graft -derived DCs; **E:** Expression of CD86 in whole liver graft-derived DCs; **F:** Expression of CD86 in partial liver graft -derived DCs.

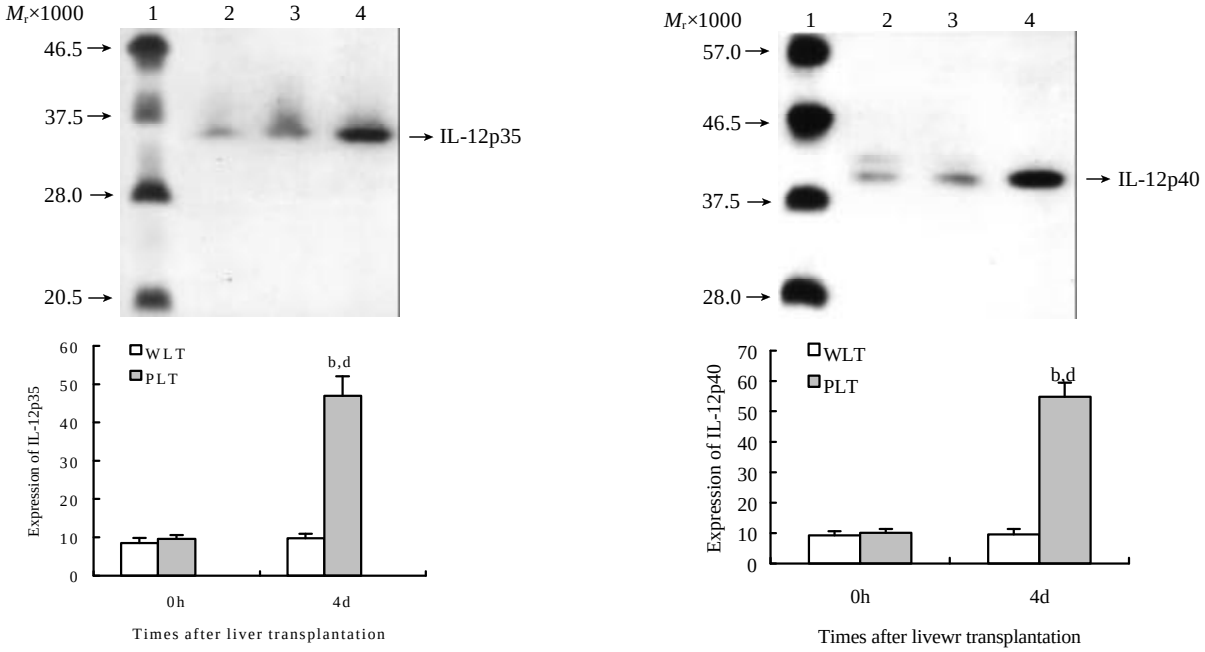

Figure 3 Detection of IL-12 proteins in liver graft-derived DCs by Western blotting. Lanes 1: protein marker. Lanes 2-4: extracts derived from DCs from liver graft 0 h after transplantation, whole liver graft (WLT) and partial liver graft (PLT) 4 days after transplantation, respectively. Compared with 4 d WLT group, $^{b}P<0.001$; Compared with 0 h PLT group,$^{d}P<0.001$.

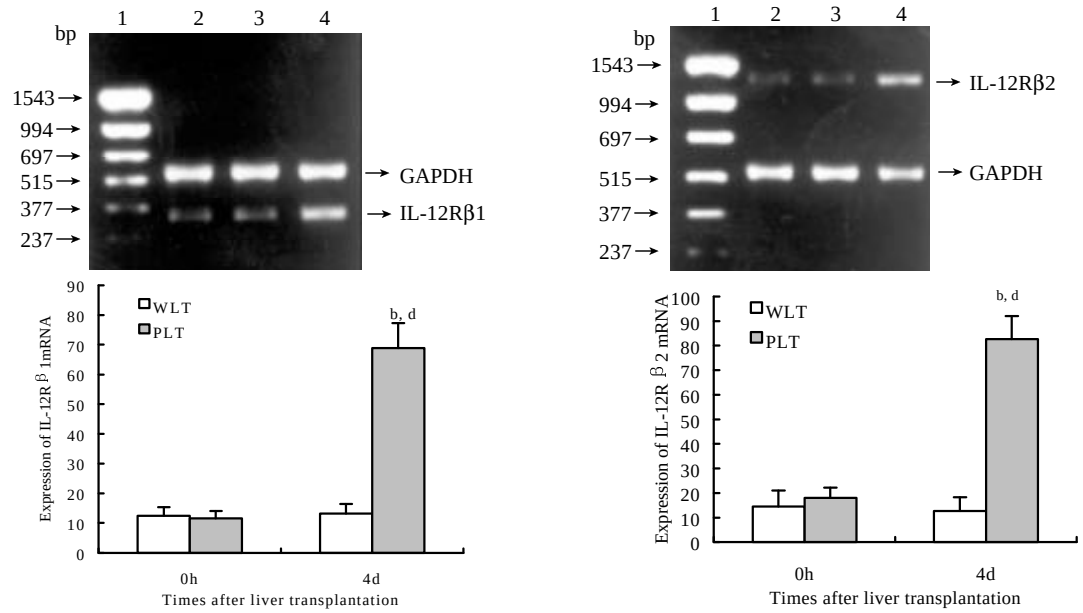

Figure 4 Detection of IL-12 R mRNA in liver graft-derived DCs by RT-PCR. Lanes 1: marker; Lanes 2- 4: extracts derived from DCs from liver graft 0 h after transplantation, whole liver graft (WLT) and partial liver graft (PLT) 4 days after transplantation, respectively. Compared with 4 d WLT group, $^{b}P<0.001$; Compared with 0 h PLT group, $^{d}P<0.001$.

Phenotypic characteristics of liver graft-derived DCs propagated in vitro

After 4 days' culture in the presence of GM-CSF, liver graft-derived DCs were observed with electron microscopy, DCs from whole liver grafts 0 hr and 4 days after transplantation exhibited round shape, smaller body, bigger nucleus, and a few shorter dendrites, whereas DCs derived from partial liver graft 4 days after transplantation displayed typical morphological features of DC including anomalous shape, bigger body, and numerous longer dendrites (as shown in Figure 1). Flow cytometry showed 80-85 % of DCs both from whole and partial liver grafts strongly expressed rat DC-specific OX62 antigen molecule (as shown in Table 1), which was suggested high purity DCs were obtained. After 10 day' culture in the presence of GM-CSF, although DCs both from whole and partial liver grafts exhibited typical morphological features of DC by electron microscopy, flow cytometric analysis showed (as shown in Figure 2 and Table 1) whole liver graft-derived DCs displayed low amounts of costimulatory molecules (CD40, CD80 and CD86), whereas DCs from partial liver graft 4 days after transplantation expressed moderate to high levels of these markers. These results suggested allogeneic partial liver transplant could promote maturation of DCs derived from partial liver graft.

Table 1 Comparison of expression of DC surface markers from whole liver graft and partial liver graft ($\bar{x}\pm s$, n=13)

Groups	OX62	CD40	CD80	CD86
Whole liver graft–DC	85.63±3.81	7.24±1.87	6.58±2.39	7.01±2.54
Partial liver graft–DC	88.42±5.75	50.18±3.26^{b}	45.31±3.67^{b}	43.29±2.03^{b}

$^{b}P<0.001$ *vs* whole liver graft-DC

IL-12 protein expression in liver graft-derived DCs

To investigate the functional change of DCs derived from liver grafts, we evaluated IL-12 protein expression in these DCs. As shown in Figure 3, DCs derived from both whole and liver grafts 0 hr after transplantation expressed detectable but low levels of IL-12 p35 and IL-12 p40, and expression levels of IL-12 p35 and IL-12 p40 in DCs from whole liver graft 4 days after transplantation were similar to those of DCs from whole liver graft 0 hr after transplantation ($P>0.05$). However, expression of IL-12 p35 and IL-12 p40 in DCs from partial liver graft 4 days after transplantation was markedly increased, and their expression levels were significantly higher than those of DCs both from partial liver graft 0 hr and whole liver graft 4 days after transplantation ($P<0.001$).

IL-12R mRNA expression in liver graft-derived DCs

As shown in Figure 4, semiquantitative RT-PCR analysis revealed detectable but low levels of IL-12Rβ_1 and IL-12Rβ_2 mRNA expression in DCs both from whole and partial liver grafts 0 hr after transplantation, expression levels of IL-12Rβ_1 and IL-12Rβ_2 mRNA in DCs from whole liver graft 4 days after transplantation were not markedly changed compared with those of DCs from whole liver graft 0 hr after transplantation ($P>0.05$). Whereas DCs from partial liver graft 4 days after transplantation expressed higher levels of IL-12Rβ_1 mRNA and IL-12Rβ_2 mRNA, and their expression levels were markedly higher than those of DCs both derived from partial liver graft 0 hr and whole liver graft 4 days after transplantation ($P<0.001$).

DISCUSSION

DCs play critical roles in the initiation and modulation of immune responses[27-34]. After vascularized organ transplantation, donor passenger leukocytes (mainly interstitial DCs) are mobilized out of the graft via peripheral blood to the recipient lymphoid and nonlymphoid tissues[35-39]. Maturation of donor DC from thyroid, pancreatic islet, skin, or kidney allografts in recipient lymphoid organs lead to the activation of naive, alloreactive Th0 lymphocytes, and thus provides the primary stimulus for acute allograft rejection. However, in mouse and certain rat strain combinations, fully MHC-mismatched liver allografts accepted without any of immune suppression, and fail to elicit an effective rejection response[38,39]. Moreover, in humans, the liver is considered the least immunogenic of transplanted whole organs. In a tolerant rat strain combination, depletion of interstitial leukocytes from liver by pretransplant donor radiation prevents the tolerogenic effect, and results in acute rejection[40]. On the other hand, it has been reported that

immature, costimulatory molecule-deficient DCs (such as normal liver or bone marrow-derived DCs) propagated *in vitro* can promote graft survival in allogeneic recipients[14,41,42], and posttransplant administration of donor leukocytes induces long-term acceptance of liver transplants[43].

Therefore, passenger leukocytes (most likely DCs) may have a dualistic role with potential to elicit T cell activation and graft rejection, or induce T cell tolerance and graft acceptance. The sustained release from the transplanted liver of immature DCs, may contribute to allogeneic liver graft tolerance induction. These liver-derived DCs migrate *in vivo* to T cells areas of secondary lymphoid tissue, where they persisted for weeks in allogeneic recipients[18, 44]. It is accepted that alloantigen-specific Th1 cells initiate allograft rejection, and that Th2 cells exert an inhibitory influence on the development of Th1 clones. It has been proposed that preferential induction of alloantigen-specific Th2 lymphocytes could suppress the development of Ag-specific Th1 cells, and as a consequence, inhibit allograft rejection. Liver-derived DCs might induce the proliferation of Th2 clones with capacity to inhibit Th1 responses[18]. Liver-derived DCs display an immature phenotype with absence of costimlatory molecules (CD40, CD80 and CD86) surface expression, low levels of MHC classIandII, and as a consequence, low stimulatory capacity for naive allogeneic T cells. Unlike mature DC, these liver-derived DCs do not induce detectable levels of intracytoplasmic IFN-γ in allogeneic $CD4^+$ cells in 72-h MLR, and elicited very low levels of CTLs in vitro[3,13,18]. In contrast, acute liver graft rejection would be induced by maturation of liver grafts derived-DCs[20]. These findings point to a pivotal role for donor immature or mature DCs in determining the outcome of liver transplantation. Mature DCs express high levels of costimulatory molecules such as CD40, CD80 and CD86. Activation of T cells by mature DCs has been shown to require direct contact between T cells and DCs through CD40-CD40L interaction[45], upon ligation of CD40L on T cells with CD40 on DCs, DCs are triggered to produce even high quantities of IL-12, thus consigning T cells to Th1 responses[46].

IL-12 is an important immune regulator produced primarily by DCs and macrophages that drives the preferential induction of Th1 immune responses. IL-12 appears to be a central mediator of acute graft-vs-host disease in mice[47], whereas neutralization of bioactive IL-12 enhances allogeneic myoblast survival[48]. Moreover, exogenous IL-12 mediates liver allograft rejection[49], and IL-12 antagonism could promote liver graft tolerance[19]. IL-12 binds to a unique, high affinity receptor on activated Th cells and NK cells, enhances the expression of antiapoptotic factors (bcl_2 and bcl_{xl}), and facilitates activated T cell and NK-lymphokine activated killer cell expansion. IL-12 indirectly promotes Th1 and inhibits Th2 development by inducing the secretion of IFN-γ by Th1 and NK cells[19, 50-54]. Recent investigation showed IL-12 also induce autologus IL-12 production of DC by interaction with IL-12 receptor[25]. Previous studies have shown that IL-12R is detected in T cells and NK cells, and IL-12R plays a crucial role for IL-12 mediated activation of these cell types[25, 55-60]. IL-12Rβ$_1$ is the subunit primarily responsible for binding IL-12, and IL-12Rβ$_2$ plays an essential role in mediating the biological functions of IL-12. IL-12-induced phosphorylation of STAT4 and IFN-γ production are absent in Con A and anti-CD3-activated splenocytes from IL-12Rβ$_2^{-/-}$ mice[56]. Recent investigations suggested that DCs exhibited expression of IL-12Rβ$_1$ and IL-12Rβ$_2$[25,61-66], and mature DCs express high level of IL-12Rβ$_1$ and IL-12Rβ$_2$[25, 64].

Although Omura T *et al*[1] and Shiraishi M *et al*[2] reported that allogeneic partial liver grafts exhibited increased immune response compared with allogeneic whole liver grafts as early as 3 days after transplantation, little is known about the exact mechanism responsible for the accelerated rejection. In the present study, accelerated rejection was demonstrated in allogeneic partial liver graft 4 days after transplantation. Our results first demonstrated that DCs derived from allogeneic whole liver graft without acute rejection 4 days after transplantation exhibited an immature phenotype with absence of CD40, CD80 and CD86 surface expression, and low expression of IL-12 proteins (IL-12 p35 and IL-12 p40) and IL-12 receptor (IL-12Rβ$_1$ and IL-12Rβ$_2$) mRNA. In contrast with immature DCs derived from whole liver graft, DCs derived from partial liver graft undergoing acute rejection 4 days after transplantation displayed a mature phenotype with high level of CD40, CD80 and CD86 surface expression, and as a consequence, high level expression of IL-12 proteins (IL-12 p35 and IL-12 p40) and IL-12 receptors (IL-12Rβ$_1$ and IL-12Rβ$_2$) mRNA. Given immature DCs can induce T-cell hyporesponsiveness[13-15] and immune reactivity inhibition[16,17], whereas mature DCs can stimulate Th1 response and the development of alloantigen-specific CTLs[3-7, 27-34], together with IL-12 is an important inducer of liver graft rejection, we suggest that maturation of liver graft-derived DCs may be an important mechanism of the accelerated rejection of allogeneic partial liver graft, and inhibition of maturation of liver graft-derived DCs may suppress rejection of allogeneic partial liver graft.

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