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## ORIGINAL ARTICLE

### Mechanism of Immune Hyporesponsiveness Induced by Recipient-derived Immature Dendritic Cells in Liver Transplantation Rat<sup>△</sup>

Li Li\*, Sheng-ning Zhang, Jiang-hua Ran, Jing Liu, Zhu Li, and Lai-bang Li

Department of Hepatobiliary Pancreatic Surgery, First People's Hospital of Kunming,  
Kunming Medical College, Liver Transplantation Center of Organ Transplantation  
Institute of Yunnan Province, Kunming 650011, China

**Key words:** rat; liver transplantation; rejection; immature dendritic cell; immune hyporesponsiveness

**Objective** To investigate the mechanism of immune hyporesponsiveness induced by donor-antigen-unloaded recipient-derived immature dendritic cell (imDC) of liver grafts in rats.

**Methods** Forty Sprague-Dawley rats (donor) and forty male Wistar rats (recipient) were randomly divided into 4 groups: control, cyclosporine A (CsA), mature DC (mDC), and imDC groups respectively, with 10 donor rats and 10 recipient rats in each group. Recipient rats in CsA group were treated with 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> CsA starting day 2 after the transplantation. Recipients in the mDC or imDC groups were given Wistar rat derived mDCs (1×10<sup>6</sup>/rat) or imDCs (1×10<sup>6</sup>/rat) *via* dorsal vein of the penis respectively 1 day before the transplantation. In each group, 5 recipients were kept for determination of survival time and the other 5 rats were executed at day 10 after transplantation. Blood samples were collected for the measurement of serum alanine aminotransferase (ALT), total bilirubin (TBIL), interleukin 2 (IL-2), interferon gamma (IFN-γ), IL-4, and IL-10 levels. Liver tissue was harvested for HE staining and acute rejection evaluation. Expression levels of Fas-L/Fas in the grafts were detected by immunohistochemical staining; and Western blot was used to detect the expression level of Scurfin.

**Results** The survival time of CsA and imDC groups was significantly longer than that of control and mDC groups (all  $P < 0.05$ ). The levels of serum ALT and TBIL in the control group (2072.20±217.93 IU/L and 147.42±22.02 μmol/L) and mDC group (2117.00±285.13 IU/L and 141.58±20.82 μmol/L) were significantly higher than those in the CsA group (59.68±13.48 IU/L and 15.40±2.13 μmol/L) or imDC group (50.80±9.63 IU/L and 14.44±3.49 μmol/L) (all  $P < 0.05$ ). In the CsA and imDC groups, the levels of IL-2 (22.52±3.75 pg/mL and 22.12±3.90 pg/mL) and IFN-γ (309.20±25.19 pg/mL and 321.00±21.64 pg/mL) were significantly lower, but the levels of IL-4 (297.60±25.07 pg/mL and 277.00±22.47 pg/mL) and IL-10 (1226.00±140.49 pg/mL and 1423.00±106.39 pg/mL) were higher than those of the control (IL-2: 147.78±12.80 pg/mL, IFN-γ: 1758.60±106.22 pg/mL, IL-4: 17.40±4.77 pg/mL, IL-10: 81.00±9.47 pg/mL) and mDC groups (IL-2: 142.34±9.29 pg/mL, IFN-γ: 1835.00±82.63 pg/mL, IL-4: 15.60±3.96 pg/mL, IL-10: 68.80±11.23 pg/mL) (all  $P < 0.01$ ). The expression level of Scurfin protein on CD4<sup>+</sup>

CD25<sup>+</sup> T cells of the imDC group ( $1.34 \pm 0.29$ ) was significantly higher than that in the control ( $0.72 \pm 0.13$ ), CsA ( $0.37 \pm 0.11$ ), and mDC groups ( $0.78 \pm 0.17$ ) (all  $P < 0.05$ ).

**Conclusion** Donor-antigen-unloaded recipient-derived imDC is an effective treatment in inducing immune hyporesponsiveness through induction of T cell apoptosis, shift in Th1/Th2 balance, and proliferation of regulatory T cell.

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LIVER transplantation is an effective strategy in treating end-stage liver diseases, still post-transplantation rejection reaction, among other issues, hinders the development of liver transplantation. Currently, the use of immature dendritic cells (imDC) has become a promising approach to induce immune tolerance or immune hyporesponsiveness.<sup>1</sup> imDC used in these approaches were donor-derived DC, which induces immune tolerance or immune hyporesponsiveness by targeting direct recognition. However, donor-derived imDC need to be harvested for a few days and transfused into the recipient 5-10 days before transplantation, which is practically impossible in a clinical setting where donor organs are mainly harvested from cadavers. Moreover, donor-derived imDC might be cleared by allogeneic reaction offsetting induced immune tolerance or immune hyporesponsiveness. The use of recipient-derived imDC, should avoid all these unfavorable factors. In our study we further explored the underlying mechanism of immune hyporesponsiveness induced by donor-antigen-unloaded recipient-derived imDC by transfusing these imDC into rats 1 day before liver transplantation.

## MATERIALS AND METHODS

### Experimental animal

Forty male Wistar rats, weighing 250-280 g and serving as recipient of liver transplantation, were purchased from Vital River Laboratory Animal Technology Co. Ltd., Beijing. Forty Sprague-Dawley (SD) rats weighing 220-250 g, were purchased from Laboratory Animal Center of Kunming Medical University, and served as the donors. All experimental procedures described below were approved by our institutional animal research committees and were in accordance with nationally approved guidelines for the treatment of laboratory animals.

### Cell preparations and flow cytometry analysis

Recipient (Wistar rats)-derived imDC and mature DC (mDC) were obtained by treating DC with inhibitory cytokine interleukin 10 (IL-10) based on a modified version of the method described by Chen-Woan *et al.*<sup>2</sup> The phenotypes of mDCs and imDCs derived from bone marrow cells were

assayed by cytofluorometry day 9 after culture. Cells ( $5 \times 10^6$ ) were stained with FITC-conjugated anti-rat MHC class II and OX62 antibodies and PE-conjugated anti-rat CD80 and CD86 antibodies (Pharmingen, CA, USA).

### Assignments of model animal

The SD-Wistar rat liver transplantation model was established with a modified two-cuff technique.<sup>3-5</sup> All rats were divided into 4 groups, with 10 donor rats and 10 recipient rats in each group. Rats in these groups were given corresponding treatment before or after transplantation. As for control group, rats were not given any treatment. In the cyclosporine A (CsA) group, recipient rats were treated with CsA ( $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) starting day 2 after the transplanation. In the mDC group, recipients were given Wistar rat derived mDCs ( $1 \times 10^6/\text{rat}$ ) *via* dorsal vein of the penis, 1 day before the transplantation. For the imDC group, Wistar rat derived imDCs ( $1 \times 10^6/\text{rat}$ ) were given *via* dorsal vein of the penis, 1 day before the transplantation. The dosages of mDC and imDC were determined with reference to related literature.<sup>6,7</sup> Rats in mDC and imDC groups underwent two-cuff orthotopic liver transplantation 1 day after cell transfusion.

### Determination of post-transplantational survival time

For each group, 5 recipient rats were kept for determination of post-transplantation survival. If animals of any group died at varied time points due to errors during surgery or infection, a replacement animal would be added accordingly.

### Blood tests

At day 10 after transplantation, 5 randomly selected rats were executed and blood samples were obtained *via* the peritoneal vein. Blood sample (2-3 mL) was centrifuged for 10 minutes at 4°C (1500 g). Supernatant (600  $\mu\text{L}$ ) was obtained and preserved at -70°C until all the samples were ready for measurement of alanine aminotransferase (ALT), total bilirubin (TBIL), IL-2, interferon gamma (IFN- $\gamma$ ), IL-4, and IL-10 using ELISA reagent kits (Biosource, CA, USA).

### Assessment of rejection reaction in recipient rats

Two liver samples were harvested from each Wistar rat at execution. One of the samples was fixed in 10% neutral

formalin solution and embedded with paraffin. Sections were stained with haematoxylin and eosin (HE). The other sample was wrapped in tinfoil and preserved in liquid nitrogen until Western blot assay. The acute rejection were observed and scored by the staff of Pathological Teaching and Research Unit of the Kunming Medical University, and graded according to Banff classification.<sup>8-10</sup> For Grade 0, no definite evidence of rejection reaction is seen, with borderline inflammatory cell infiltration in the hepatic artery, portal vein, and bile duct of the portal area. Grade I rejection is described as mild rejection; inflammatory cell infiltration can be found in bile ducts and blood vessels among some of the portal area, with a few inflammatory cells confined to a few portal areas. A Grade II reaction is described as moderate rejection; inflammatory cell infiltration can be seen in bile ducts and blood vessels among most portal areas. A Grade III reaction is defined as severe rejection; inflammatory cell infiltration further spreads into peri-portal area, and moderate to severe phlebitis can be found in portal area as well as hepatic parenchyma, with hepatic cellular necrosis around affected veins.

### Immunohistochemical staining

The formalin-fixed, paraffin-embedded liver tissue was used to determine the expression level of Fas/FasL with streptavidin-peroxidase conjugate (SP) method as described in the protocol of the reagent kit (Maixin Bio., Fujian, China). FAS and FAS-ligand (product identification serial number RAB-1090 and RAB-002 respectively) were purchased from Fuzhou Maxim Biotech Inc.

All slices were examined under light microscopy at a magnification of 400. Fas/FasL-positive hepatic cells were with brownish nuclei and/or cytoplasm. Ten visual fields in each slice were randomly selected to calculate the Fas/FasL-positive hepatic cells number (N1) and total hepatic cell number (N). The positive rate of Fas/FasL expression was calculated by  $N1/N \times 100\%$ .

### Protein extraction from liver tissue

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer according to the manufacturer's protocol (Pierce, IL, USA). Total 50 mg of liver tissue was harvested and subsequently homogenized in 0.5 mL RIPA buffer containing 1.5×protease inhibitor cocktail on ice for 30 minutes. After centrifugation for 15 minutes at 4°C (13 500 *g*), the supernatant was obtained and the protein concentration was determined by BCA Protein Assay kit (Pierce, USA).

### Western blot assay

Proteins (30 µg) were separated on sodium dodecyl sul-

fate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a nitrocellulose membrane. And the membrane was blocked with TBST [Tris-hydroxymethyl aminomethane (Tris) solution with 5% bovine serum albumin and 0.05% Tween-20]. The membrane was cultured with rabbit anti-rat Foxp3 monoclonal antibody at the dilution of 1:500 (e-Bioscience, CA, USA) and rabbit anti-rat β-actin monoclonal antibody at the dilution of 1:50 000 (Sigma, St. Louis, MO, USA) respectively at 4°C overnight. After washing, the membrane was incubated with HRP labeled anti-rabbit IgG (Pierce, IL, USA) at the dilution of 1:2500 for 40 minutes at room temperature. Finally, the bands were visualized using the enhanced chemiluminescence detection system as recommended by the manufacturer. Quantity one 4.1 software system (Bio-Rad, CA, USA) was used to calculate gray scale intensity. β-actin was served as an internal control.

### Statistical analysis

SPSS 13.0 software package was used in the analysis. Data were shown as  $\bar{x} \pm s$ . Analysis of variance was used to determine whether the differences in means between the groups were statistically significant. The means of Banff scores between different groups were compared with rank sum test. Differences in survival time were tested with Log-rank method, with test level  $\alpha=0.05$ .

## RESULTS

### Phenotype of mDC and imDC

At least 88% of mDC and imDC were OX62-positive, which was the characteristic marker for DCs. imDC expressed lower level of MHC-II and co-stimulatory molecules (CD86, CD80) compared with mDC (Fig. 1).

### Post-transplantational survival time of Wistar rats

The survival time of both the CsA and imDC groups was significantly longer than that of the control group as well as the mDC group (all  $P<0.05$ ). However, no significant differences were seen between the imDC and CsA groups ( $P>0.05$ ). Additionally, no significant differences were detected when comparing the control group with mDC group ( $P>0.05$ ) (Table 1).

### Post-transplantational liver function and cytokine level

ALT ( $P<0.05$ ), TBIL ( $P<0.05$ ), IL-2 ( $P<0.01$ ), and IFN-γ ( $P<0.01$ ) levels in the control and mDC groups were significantly higher than those in the CsA and imDC groups, while IL-4 and IL-10 levels in the control and mDC groups were significantly lower than those in the CsA and imDC groups (all  $P<0.01$ ) (Table 2).

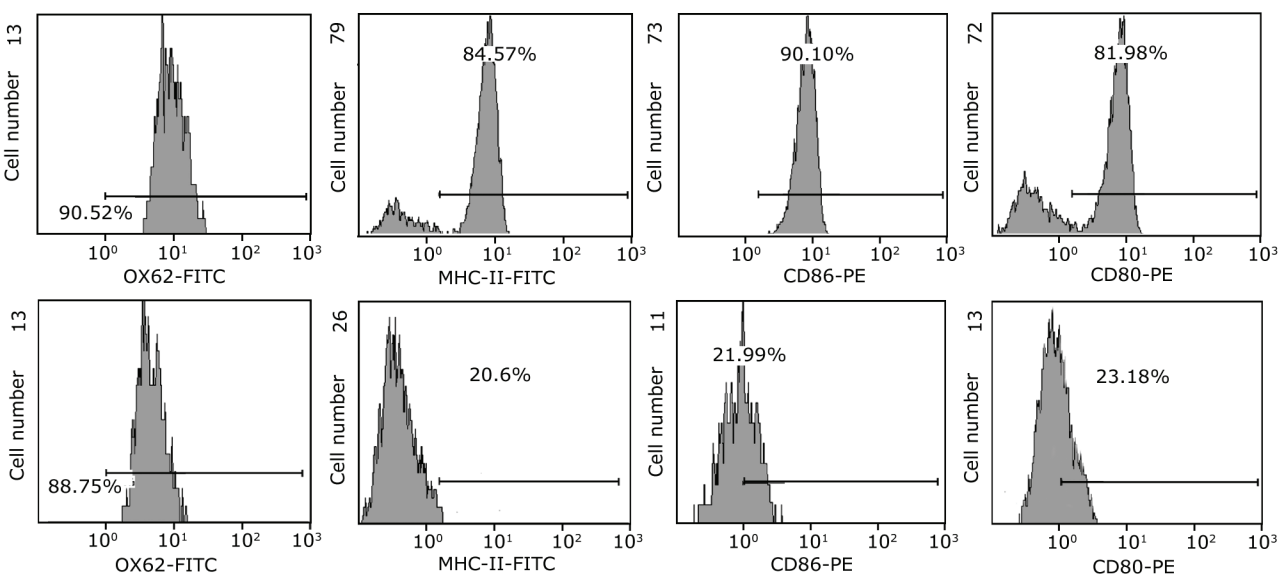


**Figure 1.** Flow cytometry analysis of the phenotype of bone marrow-derived mature dendritic cells (mDC) and immature DC (imDC). The phenotype of mDCs and imDCs derived from bone marrow were analyzed by cytofluorometry on day 9 of culture. Cells ( $5\times10^6$ ) were stained with FITC-conjugated anti-rat MHC class II and OX62 antibodies and PE-conjugated anti-rat CD86 and CD80 antibodies respectively. Results were similar in five independent experiments. Upper row: mDC; lower row: imDC.

**Table 1.** Post-operational comparison of survival time of four groups (n=5)

| Group         | Survival time (d)   | Median survival time (d) | 95% CI      |
|---------------|---------------------|--------------------------|-------------|
| Control group | 7, 10, 10, 12, 13   | 10                       | 8.38-12.42  |
| CsA group     | 33, 48, 61, 83, 102 | 61*                      | 41.31-89.49 |
| mDC group     | 5, 7, 9, 10, 14     | 9                        | 6.03-11.97  |
| imDC group    | 32, 44, 57, 84, 90  | 57*                      | 39.42-83.38 |

CsA: cyclosporine A.  
\* $P<0.05$  compared with control group and mDC group respectively.

**Table 2.** Post-operational comparison of liver function and cytokine level of four groups<sup>§</sup> (n=5)

| Group         | ALT (IU/L)     | TBIL (μmol/L) | IL-2 (pg/mL)  | IFN-γ (pg/mL)   | IL-4 (pg/mL)   | IL-10 (pg/mL)    |
|---------------|----------------|---------------|---------------|-----------------|----------------|------------------|
| Control group | 2072.20±217.93 | 147.42±22.02  | 147.78±12.80  | 1758.60±106.22  | 17.40± 4.77    | 81.00± 9.49      |
| CsA group     | 59.68± 13.48*  | 15.40± 2.13*  | 22.52± 3.75** | 309.20± 25.19** | 297.60±25.07** | 1226.00±140.49** |
| mDC group     | 2117.00±285.13 | 141.58±20.82  | 142.34± 9.29  | 1835.00± 82.63  | 15.60± 3.97    | 68.80± 11.23     |
| imDC group    | 50.80± 9.63*   | 14.44± 3.49*  | 22.12± 3.90** | 321.00± 21.64** | 277.00±22.47** | 1423.00±106.39** |

§: Plus-minus values are means±SD.  
ALT: alanine aminotransferase; TBIL: total bilirubin; IL: interleukin; IFN-γ: interferon gamma.  
\* $P<0.05$ , \*\* $P<0.01$  compared with control group and mDC group respectively.

**Histopathological features of recipient liver tissue after transplantation**

Grade III rejection, as evaluated with Banff classification, was observed in the control and mDC groups, with massive monocyte infiltration in the portal area. Both bile ducts and blood vessels were affected and the structure of the hepatic lobule was destroyed. Focal hemorrhage and necrosis were also found. As for the CsA and imDC groups, a few monocytes could be found in the portal area, but the hepatic parenchyma, bile ducts, and blood

vessels were not affected. Borderline inflammatory cell infiltration, which was rated as Grade 0 rejection by Banff classification, could be seen in hepatic arteries, portal veins, and bile ducts of the portal area (Fig. 2). Banff scores at day 10 after transplantation for each group are shown in Table 3.

**Fas/FasL expression in recipient liver tissue at day 10 after transplantation**

The rate of Fas-positive expression in liver cells at day 10

after transplantation for the control, mDC, CsA, and imDC groups were  $7.66 \pm 0.89$ ,  $6.29 \pm 0.47$ ,  $2.54 \pm 0.44$ , and  $2.16 \pm 0.39$ , respectively. FasL expression rates in liver cells in CsA group and imDC group were  $5.14 \pm 0.86$  and  $5.22 \pm 0.69$ , respectively. FasL expression was also found in the inflammatory cells in portal area. Low level of FasL expression was found in either the control or mDC groups. Both the positive rates of Fas and FasL expression in CsA and imDC groups were significantly lower than those in the other two groups (all  $P < 0.01$ ) (Figs. 3 and 4).

### Expression of Scurfin protein encoded by Foxp3 in recipient liver tissue at day 10 after transplantation

Scurfin is encoded by a specific transcription factor Foxp3 and expressed on the surface of  $CD4^+CD25^+$  regulatory T cell. On day 10 after transplantation, expression level of Scurfin in imDC group ( $1.34 \pm 0.29$ ) was significantly higher than that in the control ( $0.72 \pm 0.13$ ), CsA ( $0.37 \pm 0.11$ ), and mDC ( $0.78 \pm 0.17$ ) groups (all  $P < 0.05$ ). Scurfin expression level of CsA group was the lowest among the four groups (Fig. 5).

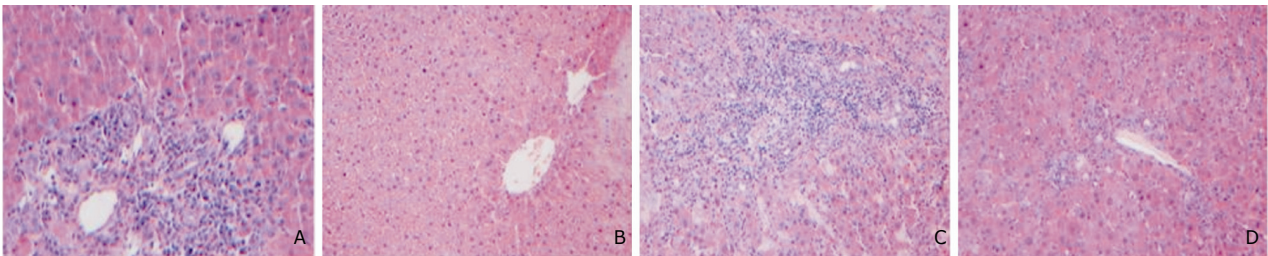


**Figure 2.** Histopathological features in recipient liver tissue at day 10 after transplantation. HE staining  $\times 400$   
A. control group; B. CsA group; C. mDC group; D. imDC group.

**Table 3.** Banff scores of recipient liver tissue at day 10 after transplantation in each group ( $n=5$ )

| Group         | Banff score     |                 |                  |                   |
|---------------|-----------------|-----------------|------------------|-------------------|
|               | Grade 0 ( $n$ ) | Grade I ( $n$ ) | Grade II ( $n$ ) | Grade III ( $n$ ) |
| Control group | 0               | 0               | 1                | 4                 |
| CsA group*    | 4               | 1               | 0                | 0                 |
| mDC group     | 0               | 0               | 0                | 5                 |
| imDC group*   | 3               | 2               | 0                | 0                 |

\* $P < 0.05$  compared with control group and mDC group respectively.

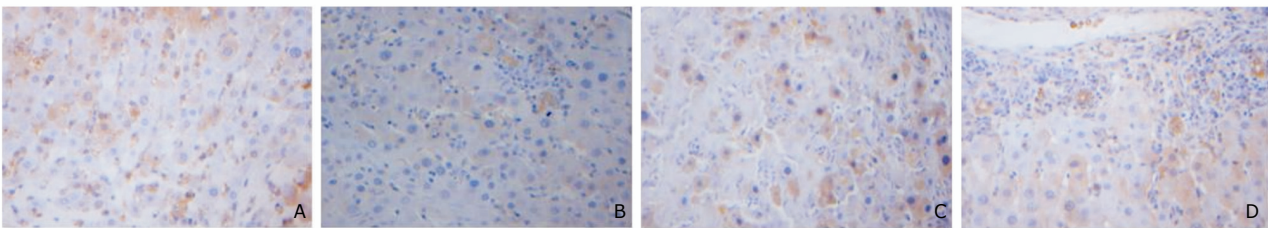


**Figure 3.** Immunohistochemical assay of Fas expression in recipient liver at day 10 after transplantation. SP  $\times 400$   
A. control group; B. CsA group; C. mDC group; D. imDC group.

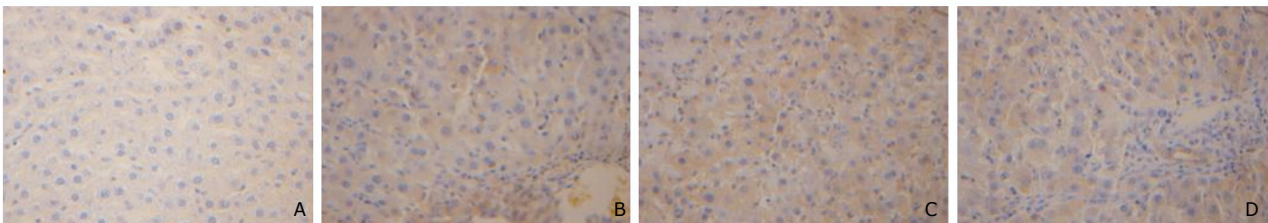


**Figure 4.** Immunohistochemical assay of Fas-L expression in recipient liver tissue at day 10 after transplantation. SP  $\times 400$   
A. control group; B. CsA group; C. mDC group; D. imDC group.

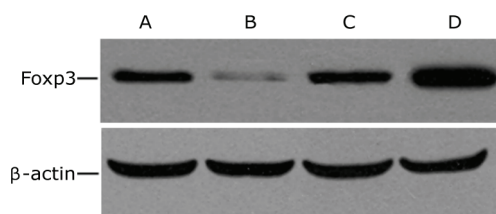


**Figure 5.** Western blot analysis of Scurfin expression encoded by Foxp3 at day 10 after transplantation.

A. control group; B. CsA group; C. mDC group; D. imDC group.

## DISCUSSION

Being an immunologically privileged organ, liver transplantation provokes much less rejection reaction as compared with other organs (such as heart, lung, pancreas, and kidney); however, rejection reaction after transplantation is a pending issue in current clinical practice.<sup>11</sup> Two signal pathways are involved in rejection reaction of allogeneic graft: direct and indirect recognition. Given the important role of direct and indirect recognition in graft rejection, numerous studies have sought to induce immune hyporesponsiveness or immune tolerance by targeting direct and indirect recognition.<sup>12-14</sup> Previously, it was suggested that direct recognition was the predominant pathway in the acute rejection, while indirect recognition mainly occurred in the chronic rejection reaction. However, Benham *et al*'s studies have revealed that indirect recognition was equally important in acute rejection.<sup>15</sup> Currently, few studies have investigated the role of indirect recognition in acute rejection reaction.

In our study, we used immature dendritic cells, which express low level of MHC and co-stimulatory molecules, to interfere with indirect recognition and thus block the activation of T cells and significantly protect allogeneic liver graft in rats. The protection was seen similar to that of CsA. Results of the study indicated that liver function in the imDC and CsA groups was continuously being restored after transplantation, which was significantly different from that in the control or mDC groups. Microscopic observation also showed that imDC markedly inhibited acute rejection, while mDC established no inhibition. imDC might have induced immune hyporesponsiveness of non-antigen-specific T cell by the following pathways.

### Induction of apoptosis in T cells

Immunohistochemical assay revealed elevated expression of Fas protein in the control and mDC groups at day 10 after transplantation, while little Fas expression was seen

in either the CsA or imDC groups. This suggested that the binding of FasL on the surface of T cells to Fas protein on surface of hepatic cells induced necrosis in the hepatic cells, and thus led to acute rejection reaction in liver graft. Few necrotic cells were found in either the imDC or CsA groups, with no marked rejection reaction. These findings suggest that imDC significantly inhibits rejection reaction. On the contrary, expression levels of FasL were higher in imDC group and CsA group than those in mDC group and control group. The binding of FasL on the surface of hepatic cells to the Fas protein on T cells induced apoptosis of T cells and therefore inhibited their immune response. Hence, imDC might have promoted T cell apoptosis and suppressed immune response by increasing FasL expression in hepatic cells.

### Selective activation of type 2 helper T cell by imDC

Mosmann *et al*<sup>16</sup> discovered that CD4<sup>+</sup> T cells in rats differentiated into Th1 and Th2 subsets when stimulated. They secrete different cytokines, such as IL-2 and IFN- $\gamma$  secreted by Th1 subset, and IL-4 and IL-10 secreted by Th2 subset. Th1 and Th2 cells cross-inhibit each other's differentiation and function. Elevated proliferation of Th2 down-regulates the development of Th1, and *vice versa*. In recent years, a handful of studies have indicated that Th1 had a major role in acute rejection reaction after liver transplantation.<sup>17-19</sup> Th1 cell is an important effector cell in mediating graft rejection; and large amounts of Th1 cells can be detected in graft organs when acute rejection reaction occurs. IL-2 and IFN- $\gamma$  produced by Th1 cell are critical cytokines in acute rejection. On the other hand, Th2 subset of helper T cells mediated immune hyporesponsiveness or tolerance. Wang *et al*<sup>20</sup> found that expression of IL-2 increased in peripheral blood during acute rejection after transplantation, while Gibelli *et al*<sup>21</sup> discovered that IL-2 receptor antagonist basiliximab significantly reduced incidence of acute rejection after transplantation.

Our study has shown that serum IL-4 and IL-10 levels were markedly higher in the CsA and imDC groups than those in the control and mDC groups, while IL-2 and IFN- $\gamma$  were significantly lower than those in control group and mDC group. These findings suggest that CsA and imDC might suppress the immune response by inducing differentiation from Th0 into Th2.<sup>22</sup> In the control and mDC groups, Th0 cells might have developed into Th1 and thereby lead to acute rejection.

### Induction of regulatory T cell (Tre)

Induction of Tre by imDC is another possible explanation to immune hyporesponsiveness induced by imDC in liver

transplantation rats. Tre has an important role in both autoimmune response and immune response against pathogens. It has been shown that positive feedback regulation exists between imDC and Tre,<sup>23</sup> suggesting that we could manipulate the immune response by targeting these cells, thereby achieving long-term graft survival.

Foxp3 is a new member of Forkhead/Winged Helix transcription factor family. It encodes Scurfin protein, which is mainly expressed on CD4<sup>+</sup>CD25<sup>+</sup> Tre cells. Proliferation and immune regulation of CD4<sup>+</sup>CD25<sup>+</sup> Tre cells were related to transcription factor Foxp3, which was regarded as a specific surface marker of these cells.<sup>24, 25</sup> It was also reported that immune regulation of CD4<sup>+</sup>CD25<sup>+</sup> Tre cells was related to expression level of Scurfin protein.<sup>26</sup>

In our study, Western blot assay revealed that expression level of Scurfin protein in imDC group was markedly higher than that in the other 3 groups. Expression of Scurfin protein was significantly lower in CsA group when comparing to other groups ( $P < 0.05$ ), which indicated CsA might be involved in decreased proliferation of CD4<sup>+</sup>CD25<sup>+</sup> Tre cells. CsA suppresses immune response by effectively inhibiting the transcription of IL-2, indicating that CsA might damage CD4<sup>+</sup>CD25<sup>+</sup> Tre cells.<sup>27</sup> These evidences were in line with the assumption that imDC might induce immune hyporesponsiveness of T cells by inducing proliferation of Tre.

In conclusion, donor-antigen-unloaded recipient-derived imDC significantly prolonged graft survival by interfering with indirect recognition. The following processes might be involved in immune hyporesponsiveness of liver graft induced by imDC: T cell apoptosis, selective activation of Th2 subset, induction of the shift of Th1/Th2 balance, and proliferation of regulatory T cells. However, as imDCs responsible for the induced hyporesponsiveness mature in human body, immune tolerance would eventually disappear. Therefore, more studies are needed on how to achieve long-term immune hyporesponsiveness, and how to induce specific immune tolerance.

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