

## Isolation and Phenotyping of Normal Mouse Liver Dendritic Cells by an Improved Method

\*<sup>1</sup>Ghasem Mosayebi, <sup>2</sup>Sayyed Mohammad Moazzeni

### Abstract

#### Introduction

Dendritic cells (DCs) are bone marrow-derived cells, which migrate to lymphoid and non-lymphoid organs via blood. Liver DCs are believed to play an important role in the regulation of hepatic allograft acceptance. However, because of inherent difficulties in isolating adequate numbers of DCs from liver, limited information is available on the phenotype and functions of liver DCs. To address this issue, we isolated DCs from normal C57BL/6 mouse liver using a modified procedure and described their immunophenotypic characteristics.

#### Materials and Methods

Non-parenchymal cells (NPCs) were obtained by collagenase digestion of perfused liver fragments and density gradient centrifugation (14.5% nycodenz column). After overnight (18 hr) incubation of the NPCs, enrichment for transiently adherent, low- density cells on 13% nycodenz gradients permitted the recovery of low numbers of cells (approximately  $1.2-1.5 \times 10^5$  per liver), many of which displayed distinct DCs morphology (abundant cytoplasm with prominent projections and irregularly shaped nuclei).

#### Results

Flowcytometric analysis revealed that most of these cells were recognized by anti-CD11c (60-70%). The results obtained from double staining with PE and FITC conjugated monoclonal antibodies indicated that these cells were CD11c<sup>+</sup>/MHC-II<sup>+</sup> (53%), CD11c<sup>+</sup>/CD86<sup>+</sup> (53.5%), CD11c<sup>+</sup>/CD8α<sup>+</sup> (36%) and CD11c<sup>+</sup>/CD11b<sup>+</sup> (45%).

#### Conclusion

These findings indicate that the purity of DCs isolated by nycodenz gradient is higher than other reported methods. Considering the similar ratio of lymphoid (CD11c<sup>+</sup>/CD8α<sup>+</sup>) and myeloid (CD11c<sup>+</sup>/CD11b<sup>+</sup>) DCs in the liver, and the known role of lymphoid DCs in tolerance induction, it seems that this subpopulation of DCs is not the main reason of liver tolerogenicity. Therefore, other factors such as the immaturity of liver DCs or the effect of liver microenvironment on these cells, etc. may explain the acceptance of hepatic allograft.

**Keywords:** Dendritic cells, Liver, Mice, Phenotype

1- Molecular and Medicine Research Center, School of Medicine, Arak University of Medical Sciences, Arak, Iran

\*Corresponding author: Tel: +98-861- 4173502; Fax: +98-861- 4173521; email: gmosayebi@yahoo.com

2- Department of Immunology, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran

## Introduction

Dendritic cells (DCs) are distributed ubiquitously throughout the body and play an important role in inducing immunity and/or tolerance (1, 2). Several DCs subgroups with different biological roles have been identified (3, 4). Myeloid DCs usually induce immune response and tolerance and usually are induced by lymphoid DCs (5, 6). Difference in frequency and ratio of DCs subtypes appears to be one of the reasons for the differences in immune response in various tissues (7). Donor liver dendritic cells have been identified within various lymphoid and non-lymphoid tissues of organ allograft recipients, including immunosuppressor non-treated mice transplanted with and permanently accepting major histocompatibility complex (MHC)-incompatible hepatic allograft (8-10). Freshly isolated DCs from lymphoid organs are potent stimulators of primary MLR (11). Whereas, freshly isolated DCs of liver are unable to initiate primary T cell responses (12). Therefore, DCs may play an important role in modulation of the immunological responses and tolerance induction in liver transplantation (13). It is thought that the DCs resident within non-lymphoid tissues may be functionally immature and/or more heterogeneous than the potent stimulators of T cell activation that can be isolated from lymphoid organs (14, 15). However, several questions have risen about the basis of the tolerogenicity of the liver, in particular, about the phenotype of liver DCs.

The phenotype and properties of liver DCs have been described both in the rat and man (16, 17). However, because of inherent difficulties in isolating adequate number of mouse liver DCs, there are few studies on phenotype and function of mouse liver DCs (11, 18). Furthermore, there are some reports regarding the properties of liver-derived DCs from GM-CSF or Flt3-L treated mice (19, 20), but little is known about the properties and phenotype of DCs present within normal mouse liver. However, DCs isolated from liver mouse in presence of GM-CSF and Flt3-L or DCs derived from CD34<sup>+</sup> progenitor cells may affect phenotype and function of DCs.

Since liver is the most tolerogenic of all transplanted organs and its graft can be accepted across MHC barriers in mice without the need for immunosuppressive therapy (21), studies on mouse liver DCs may provide important clues to understand mechanisms underlying tolerance induction. In this study, we have isolated DC-enriched cell populations from normal mouse liver with a modified separation method and described their immunophenotype.

## Materials and Methods

### Mice

Adult 6-8 weeks old female C57BL/6 mice were purchased from the Razi vaccine and serum research institute of Iran.

### Isolation of NPCs from liver

Mice were anesthetized with ketamine, swabbed with 70% ethanol and an abdominal mid-line incision was performed. NPCs were isolated from the liver of mice, as Rao *et al* have described previously (22), with the following modifications (Figure 1). Briefly, the liver was perfused for 3 min *in situ* via the inferior vena cava, using 20-30 ml ice-cold phosphate buffer saline (PBS) and a 22 G intravenous catheter. 2 ml collagenase solution (Roche, Mannheim, Germany; 1mg/ml) in RPMI-1640 (Gibco, BRL) was then injected. The liver was excised immediately, diced into small pieces and digested in collagenase solution (5 ml/liver) for 30 min at 37 °C, with constant stirring. The digested tissue was then filtered through a 0.1 mm sterile wire mesh. Cells from three to four livers were pooled. The cell suspension was washed twice with PBS or RPMI-1640 medium by centrifugation at 400 g for 5 min at 4 °C. The final pellet was re-suspended in 2-3 ml RPMI medium and overlaid on nycodenz (Pharma AS, Oslo, Norway) gradient 14.5% (w/v) and centrifuged at 600 g for 15 min at 4 °C. The recovered low density (LD) cells from the interface were collected using a Pasteur pipet and washed as mentioned above.

**Enrichment of dendritic cells**

To enrich the DCs, NPCs were suspended in complete tissue culture medium (RPMI-1640 containing 10% FCS) and incubated overnight (18 hr) at 37 °C, 5% CO<sub>2</sub> in tissue culture petridishes. At the end of incubation time, non-adherent cells were recovered and layered on a 3 ml column of 13% (w/v) nycodenz and centrifuged at 600 g for 15 min at 4 °C. Low-density cells separated carefully from interface using a Pasteur pipette. These cells referred to as “DCs-enriched cells”, were washed and used for flowcytometric analysis.

**Flow cytometric analysis**

DC-enriched cells ( $1 \times 10^5$  cells/tube) in flow cytometric buffer (PBS with 2% FCS and 0.01% sodium azide; Sigma) were stained by indirect double staining immunofluorescence technique. Briefly, the cells surface was first blocked with 5% normal mouse serum for 10 min on ice and then stained with primary

monoclonal antibodies (mAbs): hamster anti-CD11c (clone HL3), rat anti-MHC class II (clone 2G9), rat anti-CD8 $\alpha$  (clone 53-6.7), rat anti-CD11b (clone M1/70), and rat anti-CD86 (clone GL1). All of mAbs were purchased from PharMingen Company. Optimal concentration of hamster anti-mouse CD11c was added to all tubes (except the tube of isotype control) and then rat anti-mouse CD86, MHC-II, CD11b and CD8 $\alpha$  were added to each tube, separately. The species and isotype-matched irrelevant antibodies were used as negative controls. The cells were then incubated on ice for 30 min and washed with flow cytometric buffer. Phycoerythrin (PE)-conjugated mouse anti-hamster IgG (clone G70-204) and fluorescein isothiocyanate, (FITC)-conjugated mouse anti-rat IgG2a (clone RG7/1.30), and IgG2b (clone RG7/11.1) were used as secondary antibodies. After staining, the cells were fixed in 1% paraformaldehyde and flow cytometric

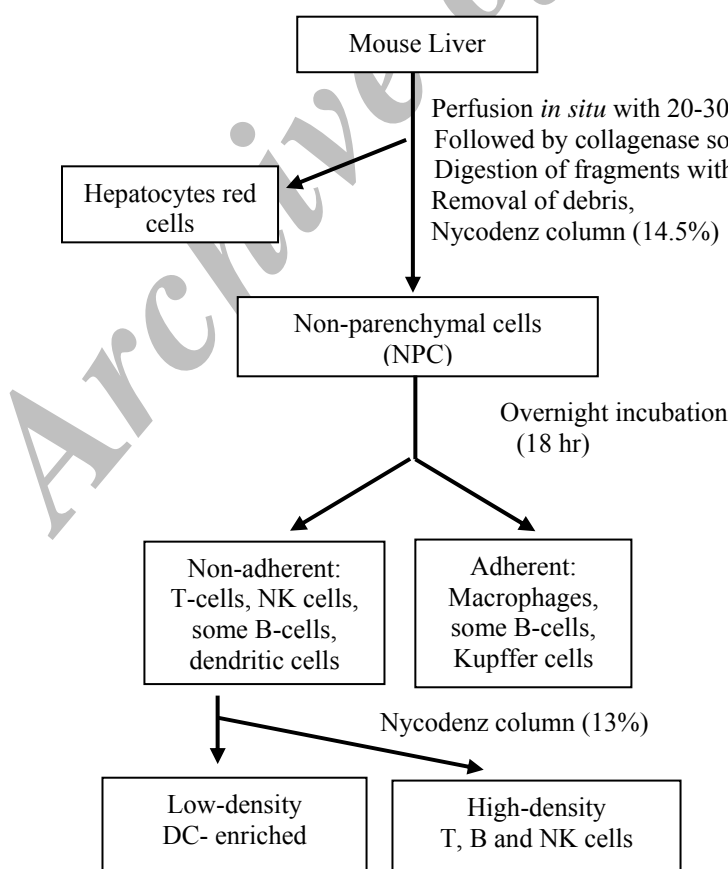


Figure 1. Flow plan for the isolation of non-parenchymal and DCs-enriched cells from normal mouse liver

analysis was performed using FACScan (Culter).

## Results

### Isolation of hepatic NPCs

Liver tissues were digested with collagenase and NPCs were separated on a 14.5% nycodenz column. The number of NPCs isolated from a normal mouse liver was approximately  $4 \times 10^6$  cells, with less than 5% hepatocyte contamination on microscopic examination (Figure 2A). DC population ( $CD11c^+$  cells) constituted  $\leq 1.0\%$  of the normal nonparenchymal cells.

To enrich the DCs, freshly isolated NPCs were cultured overnight at  $37^\circ\text{C}$  and  $5\% \text{CO}_2$ . Non-adherent cells were recovered and layered on a nycodenz column (13% w/v). Low-density fraction was separated and referred to as DC-enriched cells. DC population was enriched 20-30% after overnight culture and 13% nycodenz gradient centrifugation.  $1.2\text{--}1.5 \times 10^5$  DC-enriched cells were isolated from each mouse liver. This population consisted of mononuclear cells with distinct DC morphology. Giemsa-stained preparations exhibited cells with irregularly shaped eccentric nuclei and abundant cytoplasmic projections or veils (Figure 2B). Double indirect immunostaining was used to detect co-expression of MHC class-II, CD86, CD8 $\alpha$  and CD11b molecules with CD11c on DC-enriched cell populations. The harvested low-density cell fraction was incubated with primary monoclonal antibodies then stained by PE and FITC-conjugated monoclonal antibodies.

The obtained results showed that, approximately 65% of the DC-enriched cells possess CD11c marker. The levels of MHC-II and co-stimulatory molecule (CD86) expression on CD11c positive cells were 53% and 53.5%, respectively. 45% and 36% of  $CD11c^+$ -cells express the myeloid marker (CD11b) and lymphoid marker (CD8 $\alpha$ ) respectively (Figure 3).

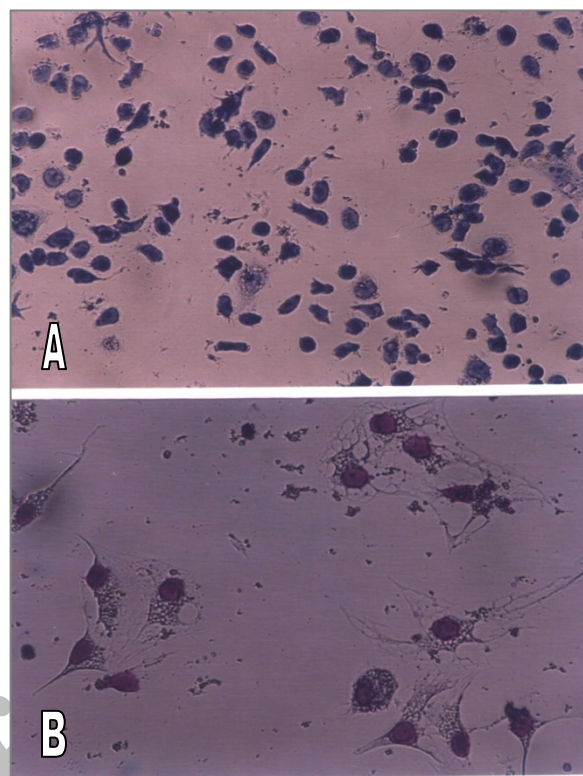


Figure 2. Giemsa-stained cytocentrifuge preparations of freshly isolated Non-parenchymal cells (A) and low-density DC enriched cells recovered from nycodenz gradients after overnight culture of NPCs from normal C57BL/6 mouse liver (B). The cells are agranular, with variable degrees of cytoplasmic vacuolation and irregularly shaped distinct cytoplasmic projections.

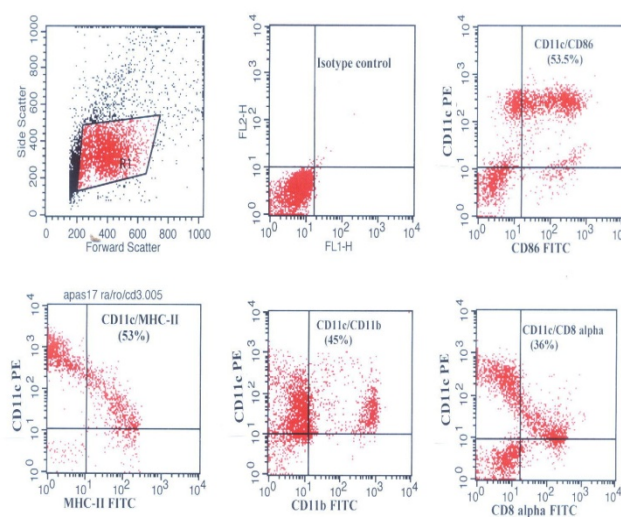


Figure 3. Flow cytometric analysis of the cell surface markers on the DC-enriched cells.

Non-adherent LD cells were harvested from nycodenz gradients after overnight culture of NPCs from normal C57BL/6 mouse liver. The cells were stained by indirect immunofluorescence. The results are representative of 5 separate experiments.

## Discussion

Dendritic cells (DCs) are a minor population of bone marrow-derived leukocytes that are distributed ubiquitously throughout the body (23). DCs are resident in the interstitial connective tissue of non-lymphoid organs, such as the liver, kidney or heart. They are believed to be important passenger leukocytes that migrate to T dependent areas of host lymphoid tissue following organ transplantation (24). The participation of liver DCs in development of tolerance to grafted tissue has been suggested in previous studies (13). It has been suggested that the chimeric cells, which are observed in various organs of recipients of liver or other allografts consist predominantly of DC lineage cells (21, 25). However, the study of DCs located in the liver has been restricted by their relative scarcity and difficulty of their isolation and consequently limited information is available on characteristics of these cells. Some studies have showed that injection of Flt3-Ligand to mouse or culture of DCs in the presence of GM-CSF increases the number of liver DCs dramatically (19, 26). However, these cytokines probably affect the function and/or phenotype of DCs. In the present study, we used a modified method (Nycodenz gradient) for isolating of DCs from normal mouse liver without using cytokines or coarse manipulation. We isolated  $4 \times 10^6$  non-parenchymal cells from each liver. After overnight-culture of NPCs and separating low-density cells by nycodenz gradient, approximately  $1.2\text{--}1.5 \times 10^5$  cells/liver had DC-like morphology. This degree of enrichment is similar to that reported by others (11, 27, 28). Following flow cytometric analysis, it was found that the great majority (60-70%) of these cells possess DC-related marker (CD11c<sup>+</sup>).

Some previous studies have suggested that freshly-isolated DCs from non-lymphoid tissues, such as liver, are functionally immature and are more heterogeneous than the lymphoid organs (15, 29). In contrast, the results of this study showed that the majority of isolated DCs possess phenotype of mature

DCs with high expression of MHC class-II and co stimulatory molecule, CD86 on their surface. Abe *et al* have reported that the expression of MHC class-II and CD86 on GM-CSF treated liver derived DCs were 30% and 20%, respectively (28). Other investigators showed that DCs isolated from mice treated with Flt3-L are phenotypically immature (CD11c<sup>+</sup>, CD86<sup>Low</sup>, MHC-II<sup>Low</sup>) (26). However, the phenotypic changes after overnight culture have also been reported for DCs isolated from other mouse non-lymphoid organs (27). Therefore, some of these variations are probably because of culturing.

Freshly isolated DCs from lymphoid organs are potent stimulators of primary MLR, whereas freshly isolated DCs from non-lymphoid organs are unable to initiate primary T cell responses (30, 31). These findings may reflect the heterogeneity of DC populations residing in different organs (32). The CD11c<sup>+</sup> DC population can be divided into at least two subsets on the basis of the expression of CD11b and CD8 $\alpha$ , which may represent the putative myeloid and lymphoid related subsets (33). Our results show that the frequency of DCs in freshly isolated normal liver NPCs is very low. However, CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> DCs were substantially enriched to 36 and 45 percent respectively by overnight culture and nycodenz density centrifugation. The obtained results also indicate that both subsets were present in normal mouse liver and in approximately equivalent numbers. O'Connel *et al* also showed that the frequency of lymphoid and myeloid DC subset is equal in the liver (27). However, we could not find an obvious difference between DCs isolated from the liver and the spleen (not shown data) of normal mouse.

## Conclusion

These findings indicate that purity of isolated DCs using nycodenz gradient is higher than other reports (34, 35). The similar ratio of lymphoid (CD11c<sup>+</sup>/CD8 $\alpha$ <sup>+</sup>) and myeloid (CD11c<sup>+</sup>/CD11b<sup>+</sup>) DCs in liver demonstrated that the difference in frequency of these

subpopulations of dendritic cells is not the main reason for the tolerance to liver transplants. However hepatic microenvironment may play an important role in tolerogenicity of DCs in this organ.

## Acknowledgment

We thank Dr Aghaie, Dr Vaeli and Mrs Tabatbaie the personnel of flow cytometry department of Iranian blood organization for their kind collaborations.

## References

1. Granucci F, Zanoni I, Ricciardi-Castagnoli P. Central role of dendritic cells in the regulation and deregulation of immune responses. *Cell Mol Life Sci* 2008; 65:1683-1697.
2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; 392:245-252.
3. Jacobs B, Wuttke M, Papewalis C, Seissler J, Schott M. Dendritic cell subtypes and *in vitro* generation of dendritic cells. *Horm Metab Res* 2008; 40:99-107.
4. Vremec D, Shortman K. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J Immunol* 1997; 159:565-573.
5. Steinman RM, Inaba K. Myeloid dendritic cells. *J Leukoc Biol* 1999; 66:205-208.
6. Coquerelle C, Moser M. DC subsets in positive and negative regulation of immunity. *Immunol Rev* 2010; 234:317-334.
7. Belz GT, Heath WR, Carbone FR. The role of dendritic cell subsets in selection between tolerance and immunity. *Immunol Cell Biol* 2002; 80:463-468.
8. Sumpter TL, Lunz JG 3rd, Castellaneta A, Matta B, Tokita D, Turnquist HR, *et al.* Dendritic cell immunobiology in relation to liver transplant outcome. *Front Biosci* 2009; 1:99-114.
9. Toyokawa H, Nakao A, Bailey RJ, Nalesnik MA, Kaizu T, Lemoine JL, *et al.* Relative contribution of direct and indirect allorecognition in developing tolerance after liver transplantation. *Liver Transpl* 2008; 14:346-357.
10. Bocrine O, Abdelouhab K, Martin L, Guignier F, Mousson C, Rifle G. Chimerism in lymphoid tissues and donor-specific antibody response after injection of allogeneic splenic dendritic cells from Fischer rats to Lewis recipients. *Transplant Proc* 2006; 38:2349-2351.
11. Woo J, Lu L, Rao AS, Li Y, Subbotin V, Starzl TE, *et al.* Isolation, phenotype, and allostimulatory activity of mouse liver dendritic cells. *Transplantation* 1994; 58:484-491.
12. Pillarisetty VG, Shah AB, Miller G, Bleier JL, DeMatteo RP. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. *J Immunol* 2004; 172:1009-1017.
13. Sumpter TL, Lunz JG, Demetris AJ, Thomson AW. Molecular regulation of hepatic dendritic cell function and its relation to liver transplant outcome. *Transplantation* 2009; 88:S40-44.
14. Helft J, Ginhoux F, Bogunovic M, Merad M. Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. *Immunol Rev* 2010; 234:55-75.
15. Lian ZX, Okada T, He XS, Kita H, Liu YJ, Ansari AA, *et al.* Heterogeneity of dendritic cells in the mouse liver: identification and characterization of four distinct populations. *J Immunol* 2003; 170:2323-2330.
16. Muench MO, Suskind DL, Barcena A. Isolation, growth and identification of colony-forming cells with erythroid, myeloid, dendritic cell and NK-cell potential from human fetal liver. *Biol Proced Online* 2002; 4:10-23.
17. Matsuno K, Kudo S, Ezaki T, Miyakawa K. Isolation of dendritic cells in the rat liver lymph. *Transplantation* 1995; 60:765-768.
18. Thomson AW, Lu L, Subbotin V, Li Y, Noyola H, Qian S, *et al.* Propagation of dendritic cell progenitors from mouse liver and their *in vivo* migration to T-dependent areas of allogeneic lymphoid tissue. *Transplant Proc* 1994; 26:3484-3486.
19. Lu L, Woo J, Rao AS, Li Y, Watkins SC, Qian S, *et al.* Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. *J Exp Med* 1994; 179:1823-1834.
20. Swanson KA, Zheng Y, Heidler KM, Zhang ZD, Webb TJ, Wilkes DS. Flt3-ligand, IL-4, GM-CSF, and adherence-mediated isolation of murine lung dendritic cells: assessment of isolation technique on phenotype and function. *J Immunol* 2004; 173:4875-4878.
21. Qian S, Demetris AJ, Murase N, Rao AS, Fung JJ, Starzl TE. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology (Baltimore, Md.)* 1994; 19:916-924.
22. Rao AS, Roake JA, Larsen CP, Hankins DF, Morris PJ, Austyn JM. Isolation of dendritic leukocytes from non-lymphoid organs. *Adv Exp Med Biol* 1993; 329:507-512.
23. Miloud T, Hammerling GJ, Garbi N. Review of murine dendritic cells: types, location, and development. *Methods Mol Biol* 2010; 595:21-42.

24. Martin-Fontecha A, Lanzavecchia A, Sallusto F. Dendritic cell migration to peripheral lymph nodes. *Handb Exp Pharmacol* 2009;18831-49.
25. Thomson AW, O'Connell PJ, Steptoe RJ, Lu L. Immunobiology of liver dendritic cells. *Immunol Cell Biol* 2002; 80:65-73.
26. Shurin MR, Pandharipande PP, Zorina TD, Haluszczak C, Subbotin VM, Hunter O, *et al*. FLT3 ligand induces the generation of functionally active dendritic cells in mice. *Cell Immunol* 1997; 179:174-184.
27. O'Connell PJ, Morelli AE, Logar AJ, Thomson AW. Phenotypic and functional characterization of mouse hepatic CD8 alpha+ lymphoid-related dendritic cells. *J Immunol* 2000; 165:795-803.
28. Abe M, Kajino K, Akbar SM, Yamamura K, Onji M, Hino O. Loss of immunogenicity of liver dendritic cells from mouse with chronic hepatitis. *Int J Mol Med* 2002; 9:71-76.
29. Morelli AE, O'Connell PJ, Khanna A, Logar AJ, Lu L, Thomson AW. Preferential induction of Th1 responses by functionally mature hepatic (CD8alpha- and CD8alpha+) dendritic cells: association with conversion from liver transplant tolerance to acute rejection. *Transplantation* 2000 ; 69:2647-2657.
30. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Nat Acad SciU S A* 1978; 75:5132-5136.
31. Austyn JM. New insights into the mobilization and phagocytic activity of dendritic cells. *J Eexp Med* 1996; 183:1287-1292.
32. Ardavin C. Dendritic cell heterogeneity: developmental plasticity and functional diversity. *Semin Immunol* 2005; 17:251-252.
33. O'Connell PJ, Li W, Takayama T, Logar AJ, Qian S, Thomson AW. CD8alpha(+) (lymphoid-related) and CD8alpha(-) (myeloid) dendritic cells differentially regulate vascularized organ allograft survival. *Transplant Proc* 2001; 33:94.
34. Lu L, Bonham CA, Liang X, Chen Z, Li W, Wang L, *et al*. Liver-derived DEC205+B220+CD19- dendritic cells regulate T cell responses. *J Immunol*. 2001; 166:7042-752.
35. Brasel K, De Smedt T, Smith JL, Maliszewski CR. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 2000; 96:3029-3039.

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