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Full Length Research Paper

Differential expression of *Survivin* in mouse marrow-derived dendritic cells of different differentiation stages

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S*urvivin*, a newly discovered member of the inhibitor of apoptosis protein (IAP) family, is over-expressed in tumor and embryonic tissues, but its expression and function in normally differentiated and maturated tissues is rarely reported. The differential expression of *survivin* in different differentiation stages of dendritic cells (DC), which play an important role in immune tolerance, was explored in the present study. Bone marrow cells were isolated and harvested as DC precursors. Scanning electron microscopy was adopted to observe DC morphology, flow cytometry to identify DC phenotype and mixed lymphocyte reaction (MLR) to compare the ability of DC stimulating allogeneic T cell proliferation. *Survivin* expression in different differentiation stages of DC was detected. Scanning electron microscopy displayed all different differentiation stages of cells showed typical DC morphology. Flow cytometry showed high expression of CD11c, a relatively specific marker of mouse marrow-derived DC(mDC); CD40, CD86 and MHC-II showed high expression on rmTNF-α-stimulated DC, and MLR revealed the ability of stimulating allogeneic T cell proliferation was also stronger. RT-PCR and Western-blotting assay showed that relative repressions of *survivin* mRNA and protein in DC precursors, imDC and mDC were 0.3654±0.0702 and 0.1228±0.0162, 0.5582±0.0213 and 0.1443±0.0139, 0.7537±0.0415 and 0.3911±0.0298, respectively (*P*<0.05). Under different induction conditions, *survivin* expression varied, and showed an ascending trend with the differentiation and maturation of DC. Inhibition of *survivn* expression in DC could induce immature dendritic cells (imDC) with immune tolerance.

Key words: Survivin, dendritic cells, immune tolerance, mouse marrow.

INTRODUCTION

Dendritic cells (DC), mainly from bone marrow $CD34^+$ multipotent hemopoietic stem cells, are the most powerful and effective professional antigen-presenting cells (APCs) to activate resting T cells. DCs have bidirectional immune regulation function including promoting immune response and inducing immune tolerance. The unique function of DC is currently considered to be related with the different development stages (Legge et al., 2002), mature dendritic cells (mDC) induce immune activation, while immature dendritic cells (imDC) induce immune tolerance. *Survivin*, the smallest member of the inhibitor of apoptosis protein (IAP) family, has powerful anti-apoptotic function and regulating function on cell proliferation (Altieri, 2006). For a long time, it has been considered that *survivin* is highly expressed in embryonic tissues and various tumor tissues, while it is rarely detected in normal mature tissues (Andersen et al., 2007). However, in recent years, with the development of detection techniques, it has been reported in many studies that *survivin* also expressed in maturated tissues and cells with terminal differentiation such as gastric mucosa (Chiou et al., 2003), endometrium (Konno

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et al., 2000), renal tubular epithelial cells (Lechler et al., 2007), activated T cells (Xing et al., 2004), megakaryocytes (Gurbuxani et al., 2005), etc. At present, differential expression of *survivin* in different differentiation stages of DC and whether *survivin* is involved in induction of immune tolerance in imDC were rarely reported. In our present study, we examined the differential expression of *survivin* in DC precursors, imDC and mDC, thus providing a theory basis for exploring the mechanism of *survivin* to induce differentiation and immune tolerance of imDC.

MATERIALS AND METHODS

Isolation and culture of DC precursor cells

A modified method was adopted based on the previously reported methods by Charbonnier et al. (2006) and Van et al. (2004), bilateral femurs and tibias were removed from healthy female 4 to 6 weeks old C57BL/6 mice purchased from the Animal Center of Chongqing Medical University and kept under SPF condition. Bone marrow cells were harvested through cavitas medullaris washing with serum-free RPMI-1640 culture fluid (Hyclone, USA). Bone marrow cells were centrifuged at 1500 $r \cdot min^{-1}$ for 10 min, and the supernatant was removed. Cells were re-suspended with 1 ml of Tris-NH_4Cl lysate.

After mixing for 1 min, 5 ml of serum-free medium was added to stop the reaction. The cells were centrifuged at 1500 $r \cdot min^{-1}$ for 10 min to remove the lysed erythrocytes. The supernatant was removed, and the cells were the DC precursor cells on day 0. The precursor cells were re-suspended under the same condition with RPMI-1640 complete medium including 10 ng/ml rmGM- CSF, 5 ng/ml rmIL-4 (Purchased from Peprotech, UK), 10% fetal bovine serum (GIBCO, USA), 50 µmol/L β-mercaptoethanol (Invitrongen, Germany), 12.5 mmol/L Hepes (Amresco, USA), 100 U/ml penicillin and 100 µg/ml Streptomycin. Cells were mixed and then seeded into a 6-well plate, with 4 ml/well. Cells were incubated at 37°C in 5% CO_2 for 48 h. The suspension cells were removed by washing. The adherent cells were cultured continuously in the equal complete medium. Half of medium was changed every other day. The floating and loosely adherent cells were harvested on the 5th day for the next experiment. In another group, 50 ng/ml rmTNF-α (Peprotech, UK) was added into cells on the 5th day, and floating and loosely adherent cells were harvested after continuous culturing for 48 h. The study and animal use were approved by the ethics committee of our hospital.

Scanning electron microscopy

The cells on the 5th and 7th day were collected into a centrifuge tube respectively and washed with phosphate buffer solution (PBS) for three times. The concentration of cells was adjusted to 1.0×10^6 ml. The sections were conventionally prepared and observed under scanning electron microscope.

Identification of precursor cells, imDC and mDC

The harvested cells on the 5th and 7th day were re-suspended with PBA (PBS, 2% of bovine serum albumin plus 0.1% of sodium azide), respectively. Then cells were incubated with phycoerythrin-Cy5 (PE-Cy5)-labeld hamster anti-mouse CD11c, phycoerythrin (PE)-labeled rat anti-mouse CD40, CD86, MHC-II (I-A/I-E) fluorescent antibodies and homeotype control antibody (eBioscience Inc., USA), at 4°C for 25 min. After centrifuged, the supernatant was removed, and cells were washed twice with PBA, and then detected with FACScan flow cytometry. The results were analyzed with CELLQuest software.

Allogeneic mixed lymphocyte reaction

Allogeneic T cells which served as the responder cells, were obtained from freshly isolated BALB/c mouse splenocytes and further to be purified using a nylon wool columns filter after being dissolved erythrocytes with Tris-NH_4Cl. T cells were added into a 96-well plate at 10^5 cells/well. Two groups of DC treated with a final concentration of 50 µg/ml of mitomycin C served as stimulating cells. DC and T cells were added into a 96-well plate for mixed culture with the ratios (stimulating cell: responder cell) of 1:10, 1:20, 1:50 as well as 1:100, respectively, and the final volume was 200 µl. Three repeated wells were set up in each group, and single T cell group and blank control group were also set up simultaneously. Cells were cultured for 4 days at 37°C in 5% CO_2, and 1 µCi/well [^{3}H] –thymidine (Nuclear Medicine Institute, Southwest Hospital, Third Military Medical University) was added into wells for the last 18 h of incubation. Cells were conventionally collected after culture. Counts per minute (cpm) were recorded with a liquid scintillation counter. Results were expressed as the means of three wells.

Survivin mRNA expression in different differentiation stages of DC

Primers were designed according to *survivin* sequence (NM-009689) published in GenBank. GAPDH served as the internal control. The primers were shown as follows: Survivin: forward: 5′-CTGGGAACCCGATGACAACCC-3′; reverse: 5′-GCAGCCAGCTGCTCAATTGACT-3′. GAPDH: forward: 5′-GTGCTGAGTATGTCGTGGAGTCT-3′, reverse: 5′-GAGTGGGAGTTGCTGTTGAAGT-3′. The primers of *survivin* and GAPDH were 221 bp and 603 bp, respectively. Total RNA was extracted with Trizol (Invitrongen, Germany). The amplification was performed with RT-PCR according to the instruction (TaKaRa, Japan). The PCR product was separated with 1.5% agarose gel electrophoresis and identified with EB staining. The gray scale was analyzed with ChemiImager 5500 image analysis system. The ratio of *survivin*/GAPDH served as the relative amount of gene expression.

Survivin protein expression in different differentiation stages of DC

Total protein of three groups was extracted, respectively. After quantitation with BCA method, the protein was separated by SDS-PAGE with 15% separating gel and 6% concentrated gel. Target protein was transferred onto 0.22 µm pore size-PVDF membrane with wet trans-membrane method. The membrane was blocked with 5% skimmed milk at 4°C overnight. Then the membrane was incubated with rabbit anti-mouse *survivin* antibody (1:1000, Cell Signaling, USA) and HRP-labeled goat anti- rabbit antibody (1:500, Beijing Biosynthesis Biotechnology Co., LTD, China) for 2 h at room temperature, respectively. Then the membrane was washed with TBS-T for 10 min for four times. The band was displayed with ECL chemiluminescent reagent, and the gray scale was analyzed with Labworks 4.6 software.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical

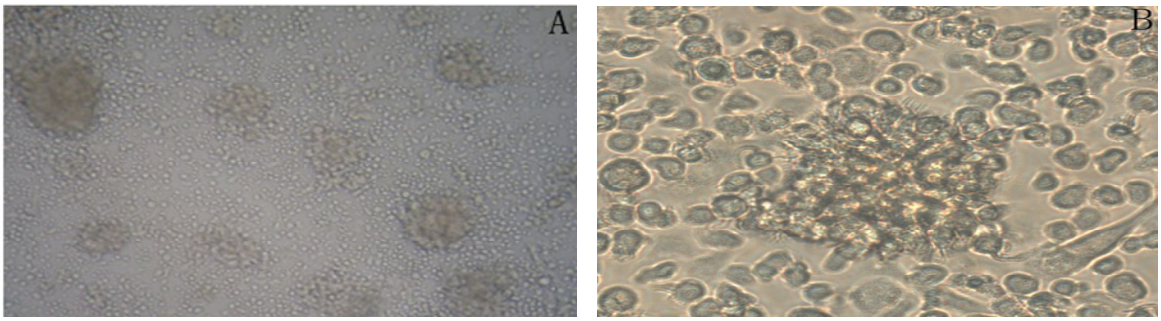

Figure 1. DC morphology under light microscope (A) 2 days, ×200. (B) 4 days, ×400).

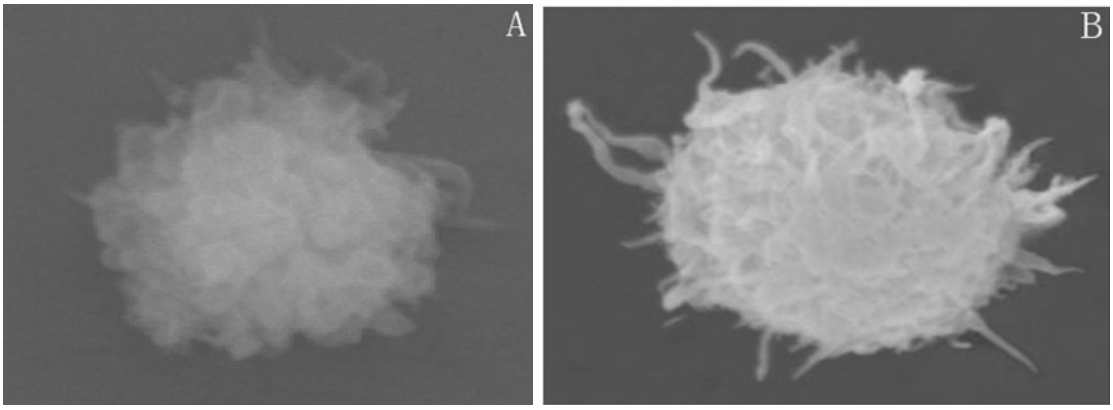

Figure 2. DC under scanning electron microscope (A) imDC, ×4000. (B) mDC, ×4000).

analysis was performed with one way analysis of variance and *t* test by using SPSS version 13.0 statistical software. A *P* value less than 0.05 was considered statistically significant.

RESULTS

In vitro culture of DC

Under inverted phase contrast microscope, precursor cells showed small round cell body and suspension growth in the medium. 48 h after rmGM-CSF+rmIL-4-induced differentiation, cell volume increased, and cell morphology changed from a round shape to a oval shape, and single scattered cells aggregated to small colonies of adherent growth (Figure 1a). With the extension of culture time, the colonies gradually increased from adherent growth to suspension growth, with the sentus-like ecphymas in the edge (Figure 1b). 48 h after rmTNF-α treatment, a large number of typical dendritic cells were detached from DC colonies, with larger volume, irregular cell morphology and many slender surface ecphymas.

Scanning electron microscopy result of DC

Under scanning electron microscope, DC showed irregular shape and rough surface. imDC showed laminated folds and small short sentus-like ecphymas (Figure 2a). mDC showed rich and forky dendritic processes (Figure 2b).

Identification of DC phenotype

DC surface markers were detected by flow cytometry with double fluorescent antibody staining. The results showed that the CD11c positive rate reached 80.2%. MHC-II (I-A/I-E), CD40 and CD86 were lower expressed in imDC, and the expression rates were 45.1, 30.5 and 34.2%, respectively. However, MHC-II(I-A/I-E), CD40 and CD86 were highly expressed in rmTNF-α-stimulated mDC, and the expression rates were 96.7, 78.7 and 88.3%, respectively (Figure 3).

Allogeneic mixed lymphocyte reaction (MLR)

Experimental data showed that mDC which were stimulated by rmTNF-α for 48 h had stronger ability to stimulate the activation and proliferation of allogeneic T cells, while imDC could not. There was significant difference between these two groups ($P<0.05$) (Figure 4).

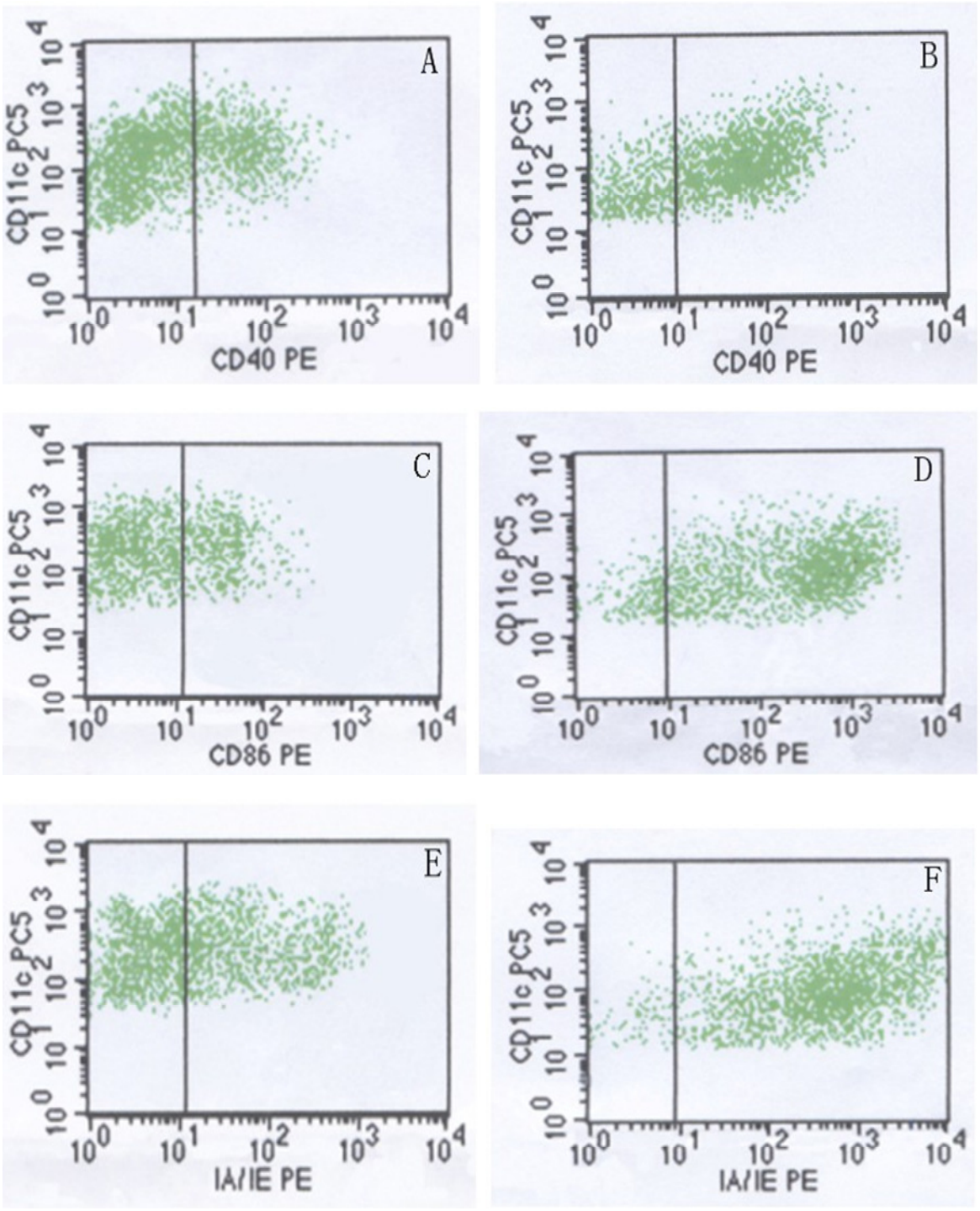

Figure 3. Analysis of DC phenotype (A, C, E: imDC; B, D, F: mDC).

Survivin mRNA expression detected by RT-PCR

Gel imaging scanning results (Figure 5) displayed that the bands around 200 bp and 600 bp were consistent with the fragments of *survivin* (221 bp) and GAPDH (603 bp). The gray scale analysis showed that *survivin* mRNA was expressed at a low level in precursor cells, and the relative expression was 0.3654±0.0702. *Survivin* mRNA was expressed at a middle level in imDC, and the relative expression was 0.5582±0.0213. *Survivin* mRNA was expressed at a high level in mDC, and the relative expression was 0.7537±0.0415.

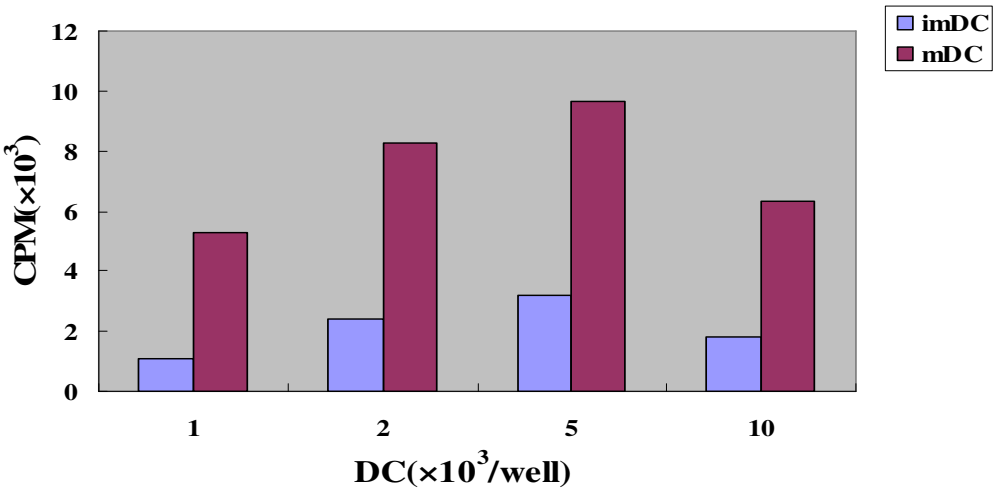

Figure 4. Comparison of the proliferation ability of two DC-stimulated allogeneic T cells.

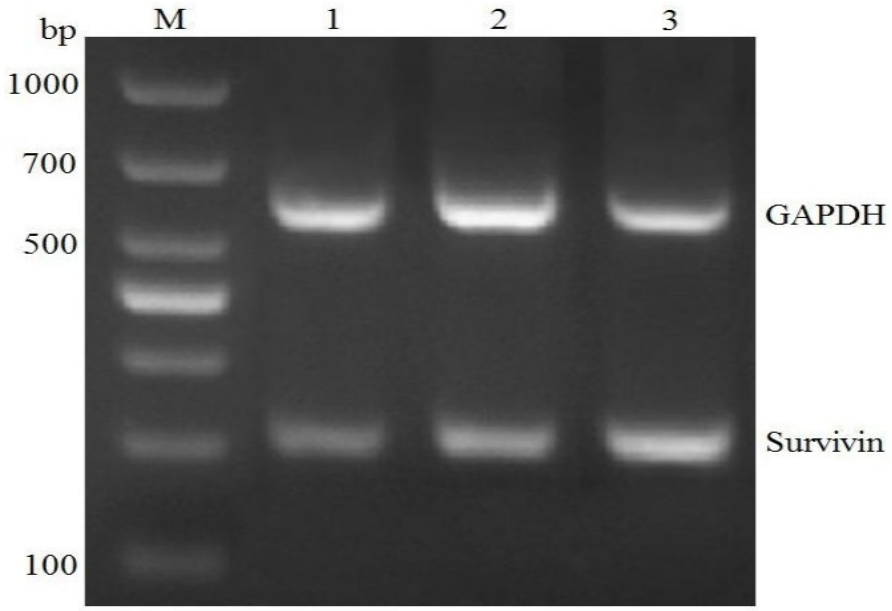

Figure 5. Survivin mRNA expression in different differentiation stages of DC (M: molecular weight standard,1: DC precursor, 2: imDC, 3: mDC).

There was significant difference among these three groups
(P<0.01).

Survivin protein expression

Western blot results showed that survivin protein band was gradually increased with the differentiation of DC from precursors to maturation. The gray scale was analyzed with Labworks 4.6 software. The ratio of the target protein/inner control was calculated (precursor cells: 0.1228±0.0162, imDC: 0.1443±0.0139, mDC: 0.3911 ± 0.0298). There was significant difference among these three groups by single-factor analysis of variance (P<0.05) (Figure 6).

DISCUSSION

Survivin, the smallest member of IAP family, was cloned by Ambrosini et al. (1997) with cDNA of effector cell protease receptor-1. *Survivin* gene codes a 142-amino acid protein which has a powerful anti-apoptotic function and regulating ability on cell proliferation, with 84.3% man/mouse homology and 16.5 KD relative molecular

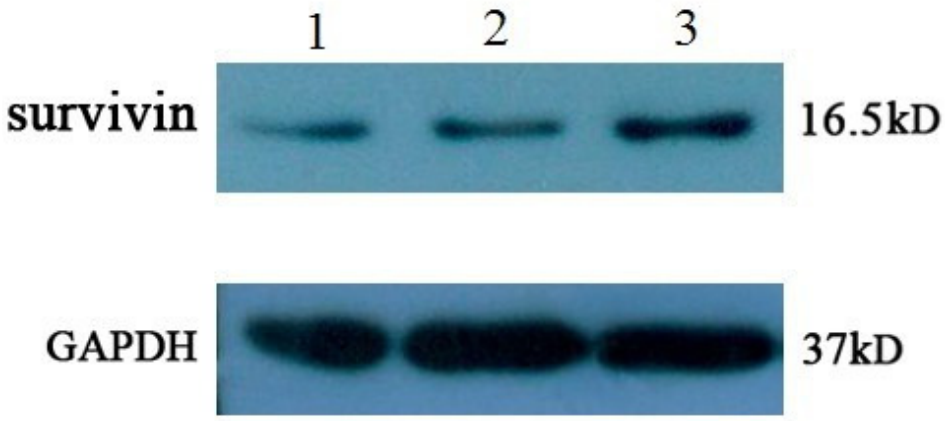

Figure 6. *Survivin* protein expression in different differentiation stages of DC (1: DC precursor, 2: imDC, 3: mDC).

weight (Altieri, 2006). *Survivin* plays an important role in cell differentiation and maturation, and imDC can induce immune tolerance, but it will inevitablely differentiate into mDC, resulting in the loss of function to induce immune tolerance. The main purpose of this study was to explore the effect of survivin on DC differentiation by inhibiting or enhancing its expression and to provide a scientific experimental basis for clinical organ transplantation.

For a long time, it has become a consensus that *survivin* is expressed in a variety of tissues with high-proliferative potential (such as embryos and tumors), but not in resting normal mature tissues (Andersen et al., 2007). However, in recent years, with the development of detection techniques, it was reported in many studies that *survivin* was detected in parts of normally differentiated and maturated tissues and cells (Chiou et al., 2003; Konno et al., 2000; Lechler et al., 2007; Xing et al., 2004; Gurbuxani et al., 2005). For different cell types, the anti-apoptotic mechanism of *survivin* has its own special emphasis. For instance, in tumor cells, *survivin* inhibits the various factors-induced apoptosis mainly through inhibiting activation of caspases (capase-3, capase-7 and capase-9, etc.) of mitochondrial-dependent pathway and blocking the apoptosis signal transduction by interaction with cyclin-dependent kinases Cdk4 and p34cdc2 (Mita et al., 2008; Liu et al., 2004). In normal haemopoietic stem cells, *survivin* regulates cell apoptosis mainly through p21WAF1/Cip1 pathway (Fukuda et al., 2004). While in activated T cells, the transcription factor p53 is up-regulated and activated after down-regulation of *survivin*, thus inducing cell apoptosis (Okada et al., 2004). In addition, Altieri et al. (2003) considered that *survivin* was not highly expressed in all tumor tissues, that was the low level in Ki-67-negative breast cancer MCF-7 cells. It has been indicated that *survivin* expression was not the direct result of cell proliferation, and also not special to tumor tissues. Li et al. (1998) detected endogenous *survivin* mRNA expression through blocking the cells in different mitosis phases with cell division inhibitor, and they found that *survivin* expression was related to cell cycle: it was highly expressed in G2/M phase, but weakly expressed in S phase and almost not expressed in G0/G1 phase. In further studies, it was found that T cells were arrested in G1-S phase and pluripotent hematopoietic stem cells were recovered in S phase after down-regulation of *surviving* (Song et al., 2005; Fukuda et al., 2002). These recoveries indicated that *survivin* regulated the transition of cells from G1 to S phase in some normal tissues.

DCs mainly come from bone marrow $CD34^+$ pluripotent hematopoietic stem cells. According to the current view, the differentiation and development process of DC is divided into four stages: 1) precursor phase, 2) non-maturation phase, 3) migration phase, 4) maturation phase. The process from precursor phase to mature phase is a procedure of increased expression of DC surface adhesion molecules and costimulatory molecules, decreased antigen uptake capacity, gradually enhanced antigen presenting capacity and stimulating ability to Tlymphocyte response. Mature DC have the stronger antigen presenting capacity and can activate immune response because of high surface expression of MHC-II, ahesion molecule CD40 and co-stimulatory molecules CD80 and CD86. In contrast, imDC could not effectively present the antigen due to low surface expression of CD40, CD80, CD86 and MHC-II molecules, which are able to induce the immune tolerance of the organism to autoantigens or foreign antigens through inducing the production of regulatory T cells (Marguti et al., 2009), secreting indoleamine 2, 3-dioxygenase (IDO) (Von Rango, 2008) and mediating the offset of Th1/Th2 immune response (Yang et al., 2006). However, under the stimulation of complex factors *in vivo*, imDC inevitably accept stimulation and rapidly maturate, thereby satisfactory effects of inducing immunological tolerance can not be obtained. In view of this, we investigated the differential expression of *survivin* gene in different differentiation stages to identify the key regulation

materials during DC differentiation, which will intended to be the intervention targets in the next experiment. S*urvivin* expression in imDC will be interfered by gene recombination and RNA interference (RNAi), and then scanning electron microscopy, flow cytometry and MLR assay will be performed to detect the changes in morphology, phenotype and function, so as to induce imDC with higher tolerance. Zhu et al. (2008) demonstrated that DC-based tumor vaccination targeted to *survivin* might be a useful strategy in developing immunotherapy of hematological malignancies.

In our study, rmGM-CSF, rmIL-4 and rmTNF-α were used to induce the differentiation and development of DC from precurosor to imDC and mDC. The results showed that a large number of DC could be harvested using the method *in vitro*. The cells in both groups had typical DC morphology. CD11c, a relatively specific marker of mouse marrow-derived DCs, was highly expressed on the cell surface. MHC-α (I-A/I-E), CD40 and CD86 were lowly expressed on the surface of imDC, and MLR assay revealed a weak ability of stimulating allogeneic T lymphocyte proliferation. However, MHC-α (I-A/I-E), CD40 and CD86 were highly expressed on the surface of mDC and had the stronger ability of stimulating allogeneic T lymphocyte proliferation compared to imDC. Therefore, the cells harvested had the immunological characteristics of DC in morphology, cell phenotype and functional identification (Charbonnier et al., 2006; Van et al., 2004).

The results of RT-PCR and Western-blotting showed that *survivin*, which was lowly expressed in DC precursors, showed a medium-level and high-level expression in the imDC and mDC, respectively, indicating that expression of *survivin* was gradually increased with the maturation of DC. Accordingly, we speculate that the expression of *survivin* may be related with the maturation extent of the DC. Highly-tolerant imDC can be induced through the inhibition of *survivin* expression in DC, which provides basis for research on imDC-induced immune tolerance after organ transplantation.

In addition, we also found that in experiments, the survival period of imDC *in vitro* was longer than that of the mDC which were further stimulated by rmTNF-α. Apoptosis began in the former on the 16th day, and on the 10th day in the latter, which had high expressions of TRANCE (tumor necrosis factor- related activation-induced cytokine) and RANK (receptor activator of NF-kB) due to imDC. It was previously concluded that the interaction between the two factors could extend survival period of imDC through upregulating the expression of Bcl-XL, while the shorten survival period of mDC *in vitro* due to lack of the two molecules (Cremer et al., 2002). Well, whether the differential expression of *survivin* in the process of DC differentiation is also related with survival time of DCs, whether it regulates the expression of marker molecule of DCs-differentiated immune function phenotype and whether it plays a related role in imDC-induced immune tolerance? Specific mechanisms still need to be further explored.

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