Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Rapamycin-treated mature dendritic cells have a unique cytokine secretion profile and impaired allostimulatory capacity

Guo-Ying Wang,* Gui-Hua Chen,* Hua Li, Yong Huang, Gen-Shu Wang, Nan Jiang and Bin-Sheng Fu

Institute of Organ Transplantation, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

Keywords
apoptosis, cytokines, dendritic cells, immunosuppression, sirolimus.

Correspondence
Dr Gui-Hua Chen, Institute of Organ Transplantation, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China. Tel.: +86 20 85252276; fax: +86 20 85252276; e-mail: chgh1955@263.net

*Both authors contributed equally to this work.

Received: 14 December 2008
Revision requested: 21 January 2009
Accepted: 6 May 2009

doi:10.1111/j.1432-2277.2009.00901.x

Summary

Rapamycin (RAPA, sirolimus) is a recently introduced immunosuppressive agent. Its effect on the differentiation and antigen uptake of immature dendritic cells (iDCs) has been studied. However, whether it can also modulate the function of mature DCs (mDCs) is unknown. We investigated the effects of RAPA on rat bone marrow-derived DCs at different stages of maturation. RAPA affected maturation, increased apoptosis and reduced lipopolysaccharide (LPS)-induced IL-12 and IL-10 production in iDCs. However, mDCs were resistant to RAPA-induced apoptosis. RAPA-mDCs produced significantly less IL-10 and TNF-α when compared with mature DCs but similar amounts of IL-12. RAPA did not affect constitutive NF-κB activity, but inhibited allostimulatory activity in mature DCs. In conclusion, mDCs treated with RAPA are reprogrammed to produce a unique cytokine secretion profile and exhibit low allostimulatory capacity, which may play an important role in rapamycin-based immunomodulation.

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and play critical roles in the initiation and regulation of immune responses [1]. DCs can not only induce immunogenic reactions but can also exhibit tolerogenic properties [2]. Recent *in vitro* studies have reported that interfering with the process of DC maturation can profoundly modify the outcome of the immune response, and manipulation of DCs may provide a novel approach for preventing acute and chronic allograft rejection [3–6]. Nevertheless, it seems that DC maturation by itself is not the sole discriminating feature that separates their immunogenic from their tolerogenic functions. Several reports have proposed that mature DCs (mDCs) also possess an immune regulatory function [7,8]. However, maturation is more of a continuous process rather than a dichotomous process (wherein cells can only be either 'mature' or 'not mature'). There is a 'semi-mature' state in which DCs are phenotypically mature but remain poor producers of pro-inflammatory cytokines, and this state has been linked to tolerogenic function [6,9].

The T-cell response induced by DCs is determined, to some extent, by the cytokines secreted by the DCs. Upon activation, DCs produce a variety of cytokines including pro-inflammatory cytokines such as IL-12 and TNF-α [1] and anti-inflammatory cytokines such as IL-10 [7,8]. Phenotypic characteristics, such as high-level expression of major histocompatibility complex (MHC) and B7 co-stimulatory molecules on mDCs, are important for activating T cells, and cytokines originating from DCs during DC–T-cell interaction are also important for directing T-cell differentiation [1,10,11].

Rapamycin (RAPA, sirolimus) is a macrolide antibiotic with potent immunosuppressive activity and has been

introduced in recent years as an anti-rejection therapy in organ transplantation in both clinical [12,13] and animal model studies [14–16]. Although several studies have shown that RAPA affects endocytosis and antigen presentation of DCs [17–19], there have been conflicting reports regarding the effects of RAPA on DC phenotypic maturation and apoptosis [17,19–24]. Although it has been suggested that mDCs have different cytokine production profiles [25] and altered intracellular signalling networks [26] when compared with immature DCs (iDCs), the role of RAPA in mDC activity remains obscure.

In this study, we investigated the influence of RAPA on rat bone marrow DCs (BM-DCs), with respect to cell survival and function. The results indicate that RAPA interferes with DC function at various levels, impairing immune reactivity at both the immature and mature stages. Moreover, mDCs following RAPA treatment exert some immunosuppressive effects on allogeneic T lymphocytes. These novel findings shed some light on the molecular mechanisms of rapamycin-based immunomodulation, and have implications for clinically applicable strategies for immunomodulation using RAPA-treated DCs for therapy of graft rejection.

Materials and methods

Animals

Eight- to 10-week-old Lewis (LEW, $RT1^l$, 200 ± 250 g) and Brown Norway (BN, $RT1^n$, 200 ± 250 g) rats were purchased from Vital River Laboratory Animal Technology Co. Ltd, Beijing, China. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

Generation of bone marrow-derived dendritic cells

Bone marrow cells removed from rat femurs, tibias, and humeri were depleted of red blood cells with hypotonic buffered tris-ammonium chloride (0.83%, pH 7.21). The cells were washed twice in complete medium consisting of RPMI 1640 (Gibco Lab, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10 mM HEPES buffer (Gibco Lab). Two million of the resulting cells were then cultured in 1 ml complete medium containing 20 ng/ml recombinant rat granulocyte-macrophage-colony stimulating factor (rrGM-CSF; Peprotech, Rocky Hill, NJ, USA) and 10 ng/ml recombinant rat interleukin 4 (rrIL-4, Peprotech) in one well of 24-well plate. On day 2, non adherent cells were removed; cultures were fed every second day by exchanging half medium for fresh GM-CSF and IL-4 containing medium. On day 6, 100 ng/ml of LPS (*Escherichia coli*; Sigma, St. Louis, MO, USA) was added and the cells left for 48 h, to create mature DCs. Cells were harvested, and supernatant fluid was collected and stored frozen at −20 °C until testing. To assess the effects of RAPA on DCs, RAPA (1–100 ng/ml) (Fermentek, Jerusalem, Israel) was added to the culture media on day 6 (unless otherwise described) simultaneously with LPS (referred to subsequently as RAPA-iDCs). The DC preparations used in this study contained 85–91% $OX62^+$ cells.

Flow cytometric analysis

The directly fluorochrome-conjugated rat antibodies for surface staining, including fluorescein isothiocyanate (FITC)-conjugated anti-OX6 (Serotec, Oxford, UK), anti-CD40 (eBioscience, San Diego, CA, USA), Alexa Fluor® 647-conjugated anti CD86 (BioLegend, San Diego, CA, USA), and phycoerythrin (PE)-conjugated anti-rat OX62 (Serotec), were used according to the manufacturer's descriptions. Briefly, Immunofluorescence staining was performed after washing the cells twice with Phosphate buffer solution (PBS) containing 3% FCS. Cells were incubated for 30 min at 4 °C with each fluorochrome-conjugated antibody diluted to the optimal concentration for immunostaining. Cells were then washed with cold PBS twice and analysed by flow cytometry (FACSCalibur with CellQuest software; BD PharMingen, San Diego, CA, USA). Matched isotypes (IgG1, Serotec) were used as the negative control. Results are expressed as mean fluorescence intensity (MFI) over all cells of the sample.

Detection of DC apoptosis

Phosphatidylserine exposure was determined using an annexin V-FITC Kit (Bender MedSystems, Vienna, Austria) in combination with propidium iodide (PI). Cells (5×10^5) were washed twice in PBS, labelled with annexin V-FITC and PI according to the manufacturer's descriptions. Flow cytometry analysis was performed immediately after incubation.

Allogeneic mixed lymphocyte reaction

One-way mixed-lymphocyte reaction (MLR) culture was performed in triplicate in 96-well, round-bottom microculture tissue culture plates. Allogeneic T lymphocytes were purified from BN splenocytes using nylon wool columns and were used as responders. The stimulators were LEW rat bone marrow-derived DCs. To avoid carry-over of residue rapamycin, DCs were extensively

washed in fresh medium at least three times. Cells were re-suspended at 4×10^5 cells/ml in a medium containing 25 μg/ml mitomycin C (Sigma). DCs were incubated at 37 °C in the dark for 30 min, and then washed three times in Hanks' balanced salt solution. Various numbers (4×10^4, 2×10^4, 10^4, 5×10^3) of DCs were incubated with 2×10^5 allogeneic T cells in each well in 200 μl RPMI 1640 complete medium for 4 days in a humidified 5% CO_2 atmosphere. Proliferation was measured using a tetrazolium salt (WST-8)-based colorimetric assay in the Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) [27]. Briefly, after 4 days of cultivation, 10 μl of CCK-8 was added to each well. After incubation for 4 h under the above-mentioned conditions, the absorbance was determined by scanning with a microplate reader at 450 nm. The absorbance corresponds linearly to the number of viable cells. To correct for the presence of BM-DCs in the co-cultures, the absorbance obtained from DC cultures was subtracted (corrected absorbance 450 nm). In order to confirm that there is no residual rapamycin in the supernatant of the culture, DCs used for MLR were cultured without rapamycin for 6 h; the concentration of remnant rapamycin in culture supernatant was less than 0.05 ng/ml, as determined by high-performance liquid chromatography (HPLC) analysis. The lower limit of detection was 0.05 ng/ml.

ELISA assay

After 6 days of culturing with GM-CSF (20 ng/ml) and IL-4 (10 ng/ml), iDCs (2×10^6 cells) were stimulated with LPS for 48 h both in the presence and absence of rapamycin. The supernatants were then collected and stored at −20 °C until analysis. To assess the effect of RAPA on mDCs, RAPA (1–20 ng/ml) was added to 8-day mDCs after the DCs were extensively washed in fresh medium twice (referred to subsequently as RAPA-mDCs). After 24 h, the supernatants were collected. RAPA-mDCs (4×10^4) were cultured with the allogeneic T cells as described above for 48 h. The supernatants were harvested. Measurements of DC cytokine (IL-12p70, IL-10, TNF-α) and T-cell cytokine (IFN-γ, IL-4) levels were performed by ELISA according to the manufacturer's protocols (KeyGen, Nanjing, China). A standard curve using recombinant cytokine was generated for each assay.

Quantitative real-time polymerase chain reaction

Mature DCs treated with RAPA (10 ng/ml) for 4 h were harvested and washed with PBS. Mature DCs (4×10^4) treated with 10 ng/ml RAPA for 24 h were co-cultured with the allogeneic T cells as described above for 48 h. Cells were harvested and washed with PBS. Total RNA was extracted using the TaKaRa RNAiso Reagent (TaKaRa, Otsu, Japan) in accordance with the instructions of the manufacturer. RNA concentration was determined at OD_{260}. Subsequently, total RNA (1 μg) was reverse-transcribed into a single-stranded cDNA by using avian myeloblastosis virus reverse transcriptase as specified by the manufacturer (TaKaRa). Real-time quantitative polymerase chain reaction (RT-PCR) on the ABI Prism 7000 (Applied Biosystems, Tokyo, Japan) was performed in duplicate in a final mixture volume of 50 μl contained SYBR green using the TaKaRa Taq™ PCR kit. Relative Quantification was performed by the Light Cycler® Software. Amplification of β-actin mRNA was done for each sample as an endogenous control. Primer pairs specific for IL-12p40 (forward, GGAGCACTCCCCATTCCTACTT; reverse, GAACGCACCTTTCTGGTTACACT), IL-12p35 (forward, CTGAATCACAGCGGCGAGAC; reverse, GGAGCAGGATACAGAGCTTCATCT), IL-10 (forward, CCAGTCAGCCAGACCCACAT; reverse, CAACCCAAGT AACCCTTAAAGTCC), TNF-α (forward, GACAAGGC TGCCCCGACTAT; reverse, GAGGCTGACTTTCTCCTG GTATGA), interferon-γ (IFN-γ) (forward, GAACTG GCAAAAGGACGGTAAC; reverse, GATCAGGTGCGAT TCGATGAC), IL-4 (forward, ACGGATGTAACGACA GCCCTCT; reverse, TTTGCGAAGCACCCTGGAAG) and β-actin (forward, GGAGATTACTGCCCTGGCTCCTA; reverse, GACTCATCGTACTCCTGCTTGCTG) were used. All data are expressed as a ratio in relation to the β-actin level.

Nuclear extractions and electrophoretic mobility shift assay

To determine the NF-κB-binding activity in mDCs, cells upon RAPA treatment for 24 h were collected on ice before isolation of nuclear extracts by the protocol reported by Homaidan [28]. The detection of the activated NF-κB in the nuclei of untreated and treated cells was completed by using a biotin-labelled EMSA kit (Viagene, Ningbo, China), according to the manufacturer's instructions. Specificity of binding was determined by using an excess of unlabeled oligonucleotide. NF-κB consensus oligonucleotide sequence used in electrophoretic mobility shift assay (EMSA) was 5′-GTA AGT TGA GGG GAC TTT CCC AGG CCG T-3′.

Statistical analysis

The two-tailed Student *t*-test was used to analyse the results, and a *P* value of less than 0.05 was considered to be statistically significant.

Results

Effect of RAPA on MHC class II and co-stimulatory molecules expression on DCs

To investigate the effect of RAPA on DC maturation, iDCs were treated with LPS both in the presence and absence of different concentrations (1–100 ng/ml) of RAPA added on day 6. RAPA-iDCs displayed significantly decreased expression of MHC class II molecules and co-stimulatory molecules (such as CD86, and CD40) during DC maturation process when compared with mature DCs. Even iDCs exposed to a low-concentration of RAPA (1 ng/ml) had less up-regulation of maturation molecules in response to LPS (Fig. 1). To determine whether RAPA affects the expression of MHC II and co-stimulatory molecules on mDCs, DCs were stimulated with LPS on day 6. After 48 h, cells were incubated with increasing concentrations of RAPA for 24 h. As shown in Fig. 2, RAPA (20 and 100 ng/ml) slightly inhibits the expression of CD40 and MHC II molecules but not inhibit the expression of CD86 on rat DCs in the fully matured phase.

RAPA induces apoptosis in iDCs but not in mDCs

In order to detect whether RAPA induces apoptosis of iDCs during the maturation process *in vitro*, the apoptotic status of the iDCs was determined by quantification of phosphatidylserine externalization using Annexin V-FITC. After 24-h incubation with 10–100 ng/ml RAPA, RAPA-iDCs underwent considerable apoptosis (Fig. 3a and b).

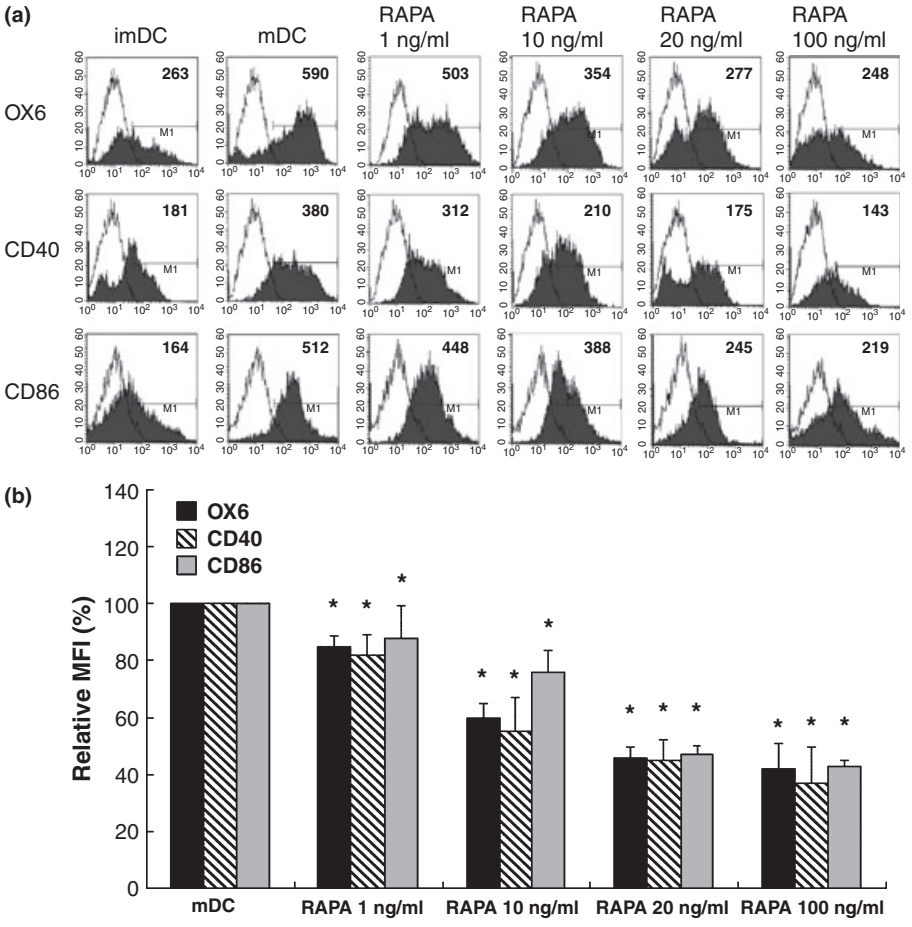

Figure 1 Rapamycin impairs the maturation of iDCs to mDCs. Bone marrow-derived DCs were stimulated with 100 ng/ml LPS and both with and without increasing concentrations of RAPA on day 6 for 48 h. Cells were harvested and analysed by FACS. (a) The level of MHC class II (OX6), CD40 and CD86 molecules are shown by gray histograms and isotype controls (IgG1) are shown by open histograms. Numbers indicate MFI for each condition. The results are representative of three independent experiments. (b) Results are expressed as percentage of variation compared with mDCs, calculated according to the formula (mean fluorescence intensity drug/mean fluorescence intensity mDC) × 100. Data shown are mean ± SD. $n = 3$, $^*P < 0.05$ vs. mDC.

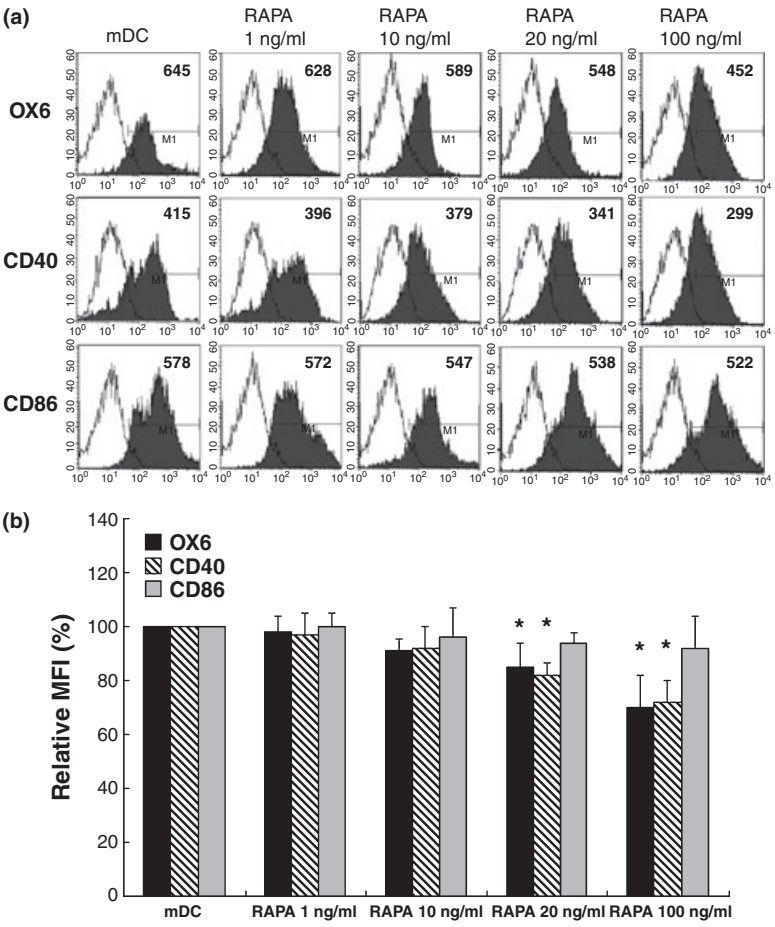

Figure 2 Effect of rapamycin on MHC class II and co-stimulatory molecules expression on mDCs. DCs were stimulated with 100 ng/ml LPS on day 6. After 48 h, cells were incubated with or without increasing concentrations of RAPA for 24 h. Cells were harvested and analysed by FACS. (a) The level of MHC class II, CD40 and CD86 molecules are shown by gray histograms and isotype controls (IgG1) are shown by open histograms. Numbers indicate MFI for each condition. The results are representative of three independent experiments. (b) Results are expressed as percentage of variation compared with mDCs, calculated according to the formula (mean fluorescence intensity drug/mean fluorescence intensity mDC) × 100. Data shown are mean ± SD. $n = 3$, $^*P < 0.05$ vs. mDC.

Although at 1 ng/ml concentration of RAPA, the number of early apoptosis cells (annexin V^+/PI^- cells) did not increase ($P > 0.05$ vs. mDCs), the percentage of all apoptotic or necrotic cells (annexin V^+ and PI^+ cells) already increased after 48 h ($P < 0.05$ vs. mDCs). The number of early apoptotic cells after 48 h did not increase compared with the number after 24 h, because the number of apoptotic cell deaths increased markedly. After 24-h or 48-h incubation with 10–100 ng/ml RAPA, the number of annexin V^+ cells strongly increased in a time- and dose-dependent manner (Fig. 3c). These findings indicate that RAPA induces apoptosis of immature DCs.

To investigate whether RAPA also increases apoptosis of mDCs, DCs were stimulated with LPS on day 6. After 48 h, cells were incubated with increasing concentrations of RAPA for 24 or 48 h. The percentage of apoptotic cells was low and was similar in control mDCs and mDCs treated with clinically relevant concentrations of RAPA (1–20 ng/ml) [29]. However, mDCs exposed to 100 ng/ml RAPA displayed significantly increased apoptosis when compared with control cells ($P < 0.05$ vs. control DCs) (Fig. 4).

Effect of RAPA on LPS-induced cytokine expression in DCs

To determine whether RAPA affects LPS-induced DC cytokine production, kinetic studies were performed for iDCs, mDCs and iDCs incubated with LPS (100 ng/ml) and RAPA (1–100 ng/ml) for 48 h. Mature DCs produced higher IL-10 and IL-12 levels than iDCs did. However, RAPA-iDCs produced progressively less IL-10 and IL-12 than mDCs (Fig. 5a and b).

To find out whether RAPA-mDCs have a tolerogenic phenotype in terms of cytokine production, RAPA (1–20 ng/ml) was added to 8-day mDCs after DCs were extensively washed twice. After 24 h, the synthesis of cytokines was assessed. As shown in Fig. 5c and d, mDCs produced lower LPS-induced IL-10 and TNF-α levels in the presence of RAPA. In contrast, IL-12 production was slightly increased in the presence of RAPA (10–20 ng/ml) when compared with mDCs, although no statistically significant differences were found. (Fig. 5e). These results indicate that the effect of RAPA on cytokine production is associated with DC maturation. RAPA-mDCs are reprogrammed with a distinct cytokine-producing profile.

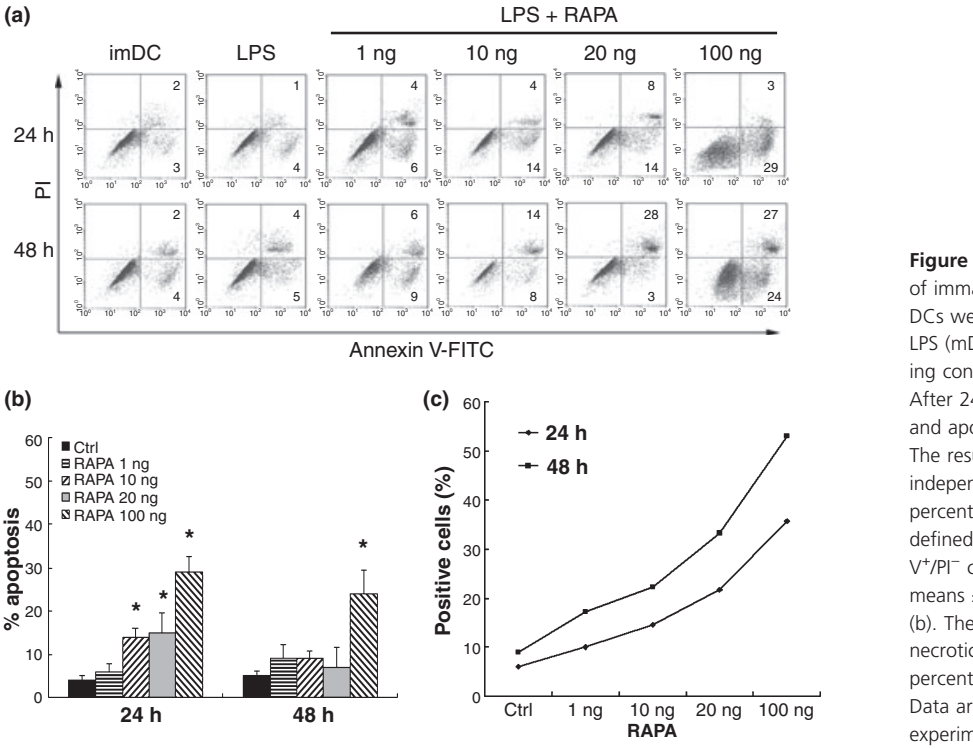

Figure 3 Rapamycin induces apoptosis of immature DCs. Bone marrow-derived DCs were stimulated with 100 ng/ml LPS (mDCs) and with or without increasing concentrations of RAPA on day 6. After 24 or 48 h, cells were harvested and apoptosis was detected by FACS. The results are representative of six independent experiments (a). The percentage of early apoptosis cells was defined by the percentage of annexin V^+/PI^- cells. Ctrl, mature DCs. Values are means ± SD. $n = 6$, $^*P < 0.05$ vs. mDC (b). The percentage of all apoptotic or necrotic cells was defined by the percentage of annexin V^+ and PI^+ cells. Data are mean of six separate experiments (c).

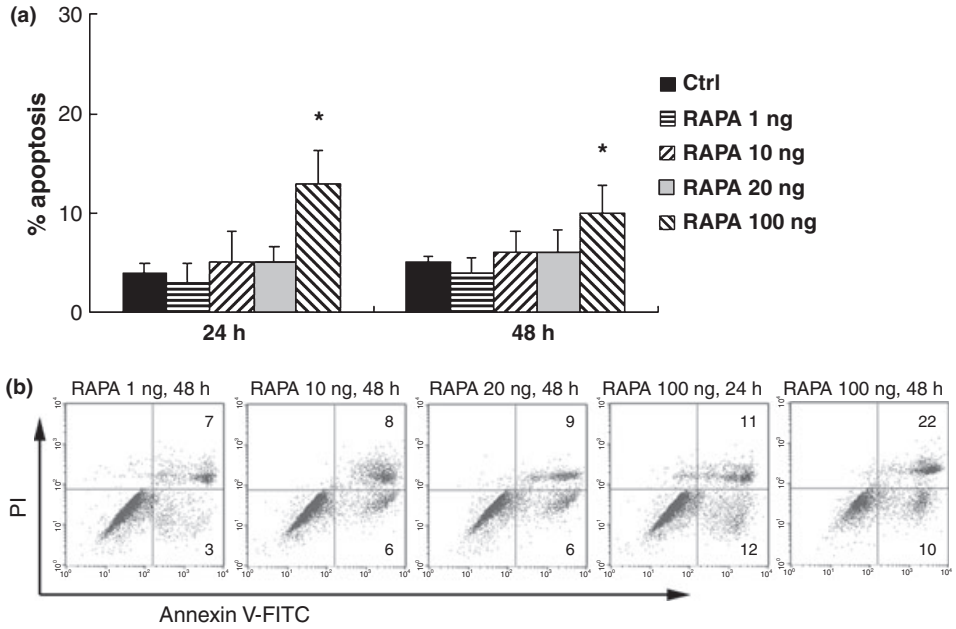

Figure 4 Fully mature DCs are resistant to rapamycin-induced apoptosis. BM-DCs were stimulated with 100 ng/ml LPS on day 6. After 48 h, cells were incubated with or without (Control DCs) increasing concentrations of RAPA. After 24-h or 48-h incubation, cells were harvested and apoptosis was detected by FACS (a). The percentage of early apoptosis cells was defined by the percentage of annexin V^+/PI^- cells (b). Ctrl, Control DCs. Values are means ± SD. The results are representative of six independent experiments. $^*P < 0.05$ vs. Control DCs.

After mDCs were extensively washed twice on day 8, RAPA (10 ng/ml) was added to culture medium for 4 h. The effects of RAPA on LPS-induced cytokine mRNA expression, including IL-12p40, IL-12p35, IL-10 and TNF-α, were further examined by real-time RT-PCR. Consistent with the earlier protein assay results, no

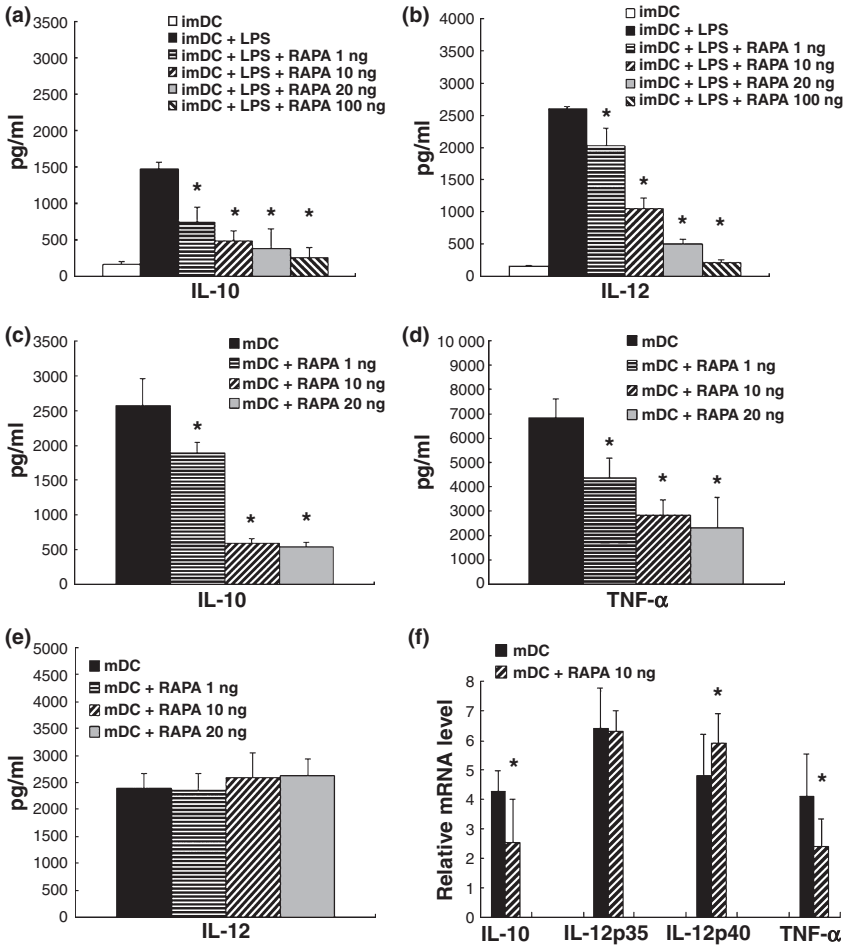

Figure 5 Effect of rapamycin on LPS-induced cytokine expression in DCs. (a and b) BM-DCs were incubated with 100 ng/ml LPS and with or without RAPA (1–100 ng/ml) on day 6. After 48-h incubation, IL-12 and IL-10 levels in the culture supernatant were determined by ELISA. (c–e) RAPA (1–20 ng/ml) was added to 8-day mDCs after DCs were extensively washed in fresh medium twice. After 24-h incubation, levels of IL-12, IL-10 and TNF-α in culture supernatant were determined by ELISA. (f) RAPA (10 ng/ml) was added to 8-day mDCs for 4 h. IL-12p40, IL-12p35, IL-10 and TNF-α mRNA levels were assessed by real-time PCR using β-actin mRNA as a reference. Data are mean ± SD of three separate experiments carried out in duplicate. *$P < 0.05$ vs. mDC.

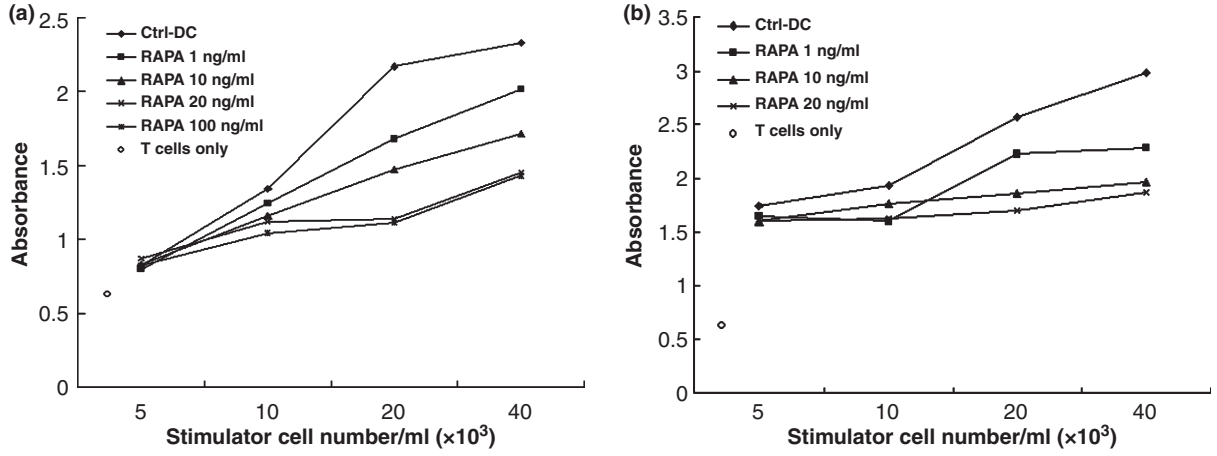

Figure 6 Rapamycin inhibits the allostimulatory activity of both immature and mature DCs. T lymphocytes were purified from BN splenocytes. Various numbers of BM-DCs from LEW rats were incubated with 2×10^5 allogeneic T cells for 4 days. T-cell proliferation was assessed by WST-8 assay. (a) LPS (100 ng/ml) and RAPA (1–100 ng/ml) or the vehicle (Ctrl-DCs) was added to 6-day iDCs for 48 h. DCs were harvested for MLR. (b) RAPA (1–20 ng/ml) or the vehicle (Ctrl-DCs) was added to 8-day mDCs for 24 h. Cells were harvested for MLR. Data shown are expressed as mean of triplicates and representative of three separate experiments.

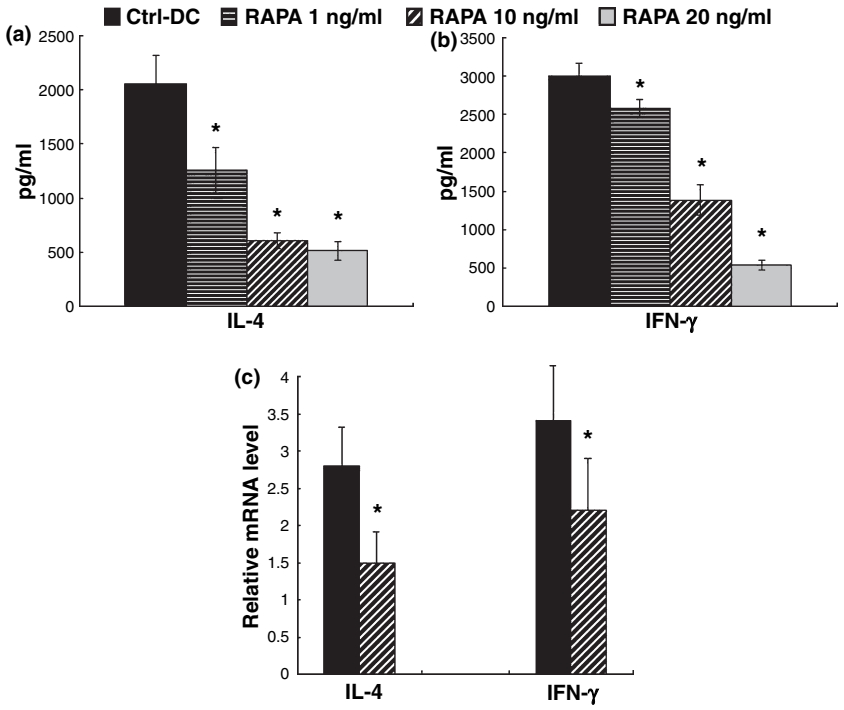

Figure 7 Allogeneic T cells produce less IFN-γ and IL-4 in response to mDCs exposed to rapamycin. (a and b) RAPA (1–20 ng/ml) or the vehicle (Ctrl-DCs) was added to 8-day mDCs from LEW rats for 24 h. DCs (4×10^4) were incubated with 2×10^5 allogeneic T cells for 48 h. IFN-γ and IL-4 levels in the culture supernatant were assessed by ELISA. (c) Mature DCs (4×10^4) treated with 10 ng/ml RAPA were cultured with the allogeneic T cells as described above for 48 h. Cells were harvested. IFN-γ and IL-4 mRNA levels were assessed by real-time PCR using β-actin mRNA as a reference. Data are mean ± SD of three separate experiments performed in duplicate. $^*P < 0.05$ vs. Ctrl-DCs.

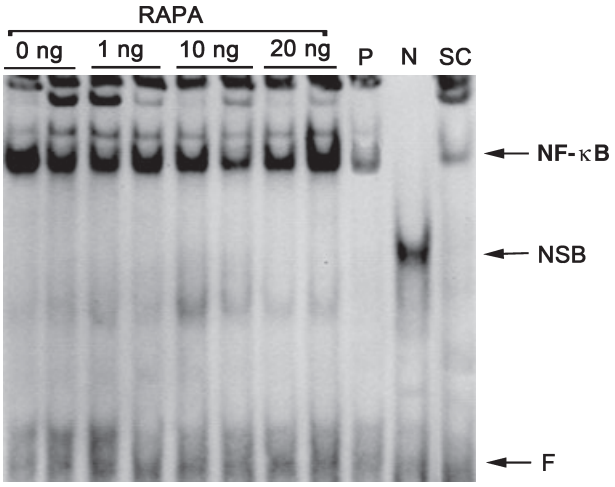

Figure 8 RAPA treatment can not decrease NF-κB DNA-binding activity in mDCs, BM-DCs from LEW rats were stimulated with 100 ng/ml LPS on day 6. After 48 h, cells were incubated with or without increasing concentrations of RAPA for 24 h. Cells were harvested. Nuclear proteins were extracted and NF-κB DNA binding activity was determined by EMSA as described in Materials and methods. Specificity of binding was determined by using an excess of unlabeled oligonucleotide. F, free probe; N, negative control; P, positive control; SC, specific competition; NSB, nonspecific band. Rapamycin showed no inhibitory effect on NF-κB binding capacity in rat fully mature DCs. Data are representative of three separate experiments.

significant change was found in the IL-12p35 mRNA levels in RAPA-mDCs when compared with mDCs. However, the expression of IL-12p40 mRNA was slightly enhanced ($P < 0.05$). In contrast, IL-10 and TNF-α mRNA expression in mDCs were suppressed in the presence of RAPA (Fig. 5f). These results collectively confirm that cytokine production may be regulated through regulation of gene transcription.

Allogeneic T cells were hyporesponsive to RAPA-mDC stimulation and produced less IFN-γ and IL-4

To evaluate the effect of RAPA on the immunostimulatory function of DCs, RAPA was added during DC maturation from LEW cells. RAPA added at 1–100 ng/ml on day 6 of culture significantly suppressed the allostimulatory activity of DCs (Fig. 6a). In subsequent studies, RAPA (1–20 ng/ml) was added on day 8 of DC culture 48 h after iDCs were stimulated to mature with LPS. Fig. 6b shows that the proliferative response of allogeneic BN cells to RAPA-mDCs was low when compared with the response to normal DCs in an MLR assay. BN T cells stimulated by allogeneic LEW mDCs that were exposed to RAPA also displayed notably lower IFN-γ and IL-4 production in the culture supernatant (Fig. 7a and b). This was associated with suppressed IFN-γ and IL-4 mRNA levels (Fig. 7c). These results indicate that RAPA can inhibit the allostimulatory activity of both immature and mature DCs. The suppressed allostimulatory effect of RAPA-DCs was unlikely to be the result of any residual rapamycin, carried over from DC cultures. Less than 0.05 ng/ml of rapamycin was detected in the MLR

supernatant in which RAPA-DCs were used as stimulators, and the supernatant did not have any inhibitory effects on T-cell proliferation (data not shown).

RAPA treatment can not decrease NF-κB DNA-binding activity in mDCs

Activation of NF-κB is essential for LPS-induced IL-12p40 gene expression in DCs and responsible for DC maturation and survival. In order to understand the mechanism of mDCs resistant to RAPA-induced apoptosis and LPS-induced IL-12 production in mDCs treated with RAPA, we went on to investigate the effect of rapamycin on NF-κB in mDCs. Nuclear proteins extracted from mDCs were assessed for the DNA-binding activity of NF-κB by EMSA using a biotin-labelled NF-κB consensus sequence-specific DNA probe. We observed NF-κB is strongly activated in mDCs stimulated by LPS. However, NF-κB binding capacity in fully mature DCs was not inhibited by treatment with rapamycin (Fig. 8), suggesting that rapamycin does not affect the NF-κB pathway in rat fully mature DCs.

Discussion

We first investigated the effects of RAPA on the maturation and activation of rat bone marrow-derived DCs. We observed that the up-regulation of co-stimulatory molecules (CD86, CD40) and MHC II molecules normally seen during the course of DC maturation in response to LPS was impaired when DCs were treated with RAPA. The blocking of DC co-stimulation properties may offer a means of inducing graft tolerance. Our findings accord with some previous studies in mouse [16,17,22] and human [19] models. Previous studies have demonstrated the robust resistance of murine myeloid RAPA-DCs to maturation following exposure to LPS or IL-4 [16,17,22]. Our findings show the profound resistance of rat myeloid DCs to maturation induced by LPS when DCs were exposed to RAPA. However, no significant effects on DC maturation were observed in other studies [20,23,24]. Interestingly, a recent study shows that rapamycin increases CD86 expression on human monocytes and peripheral myeloid DCs [30]. This large discrepancy could be explained by our treating DCs only during the maturation process and not throughout differentiation or the fully mature phase. Our further study also shows that RAPA can slightly affect the expression of MHC II and co-stimulatory molecules on rat DCs in the post maturation stage after stimulation with LPS.

Although the reduced up-regulation of co-stimulatory molecules on DCs induced by immunosuppressants such as mycophenolic acid is not necessarily related with cell apoptosis [31], our findings show RAPA-induced apoptosis during DC maturation, which is consistent with recent studies [20,21]. Cell apoptosis is frequently associated with cells in the G_1 phase of the cell cycle, and arrest in late G_1 or S phase can accelerate or induce apoptosis. RAPA causes a G_1-phase cell cycle arrest in T lymphocytes [32] and induces apoptosis on murine T cells [33]. Our finding that RAPA induces apoptosis of immature DCs seems to be independent of cell cycle regulation because myeloid DCs are non proliferating cells. However, upon maturation, DCs exposed to clinically relevant concentrations of RAPA (1–20 ng/ml) were resistant to apoptosis. This finding further underlines the specificity of RAPA's effect on DC apoptosis. Induction and maintenance of efficient immune tolerance through regulation of DC function requires, to some extent, DC maturation and survival in the host [7,8]. It should be noted that we examined the effects of RAPA on rat DCs responding to LPS. Recent studies show that the TLR4 ligand LPS, which is an effective activator of DCs, reduces apoptosis and prolongs survival of DCs [34,35]. LPS–TLR4 interactions in rat DCs cause NF-κB activation, which controls DC maturation, survival and cytokine production [34,36,37].

Although RAPA and FK506 share the same receptor (FK506-binding protein 12, FKBP12), the RAPA–FKBP12 complex binds to a distinct molecular target, known as the mammalian target of RAPA (mTOR). The RAPA–FKBP12-mTOR complex inhibits protein synthesis at the translational level. The same complex also blocks cytokine-mediated signal transduction pathways. NF-κB activation is responsible for DC maturation and survival. Recent studies have shown that RAPA inhibits DC Janus kinase 2/Stat4 activation but not the NF-κB pathway [23]. RAPA pretreatment of LPS-stimulated human monocytes can markedly modulate 290 genes by microarray analysis and genes containing NF-kB promoters are substantially overrepresented among the RAPA-upregulated genes [30]. We next examined the effect of rapamycin on NF-κB in mDCs. Our study shows NF-κB is strongly activated in mDCs stimulated by LPS. NF-κB binding capacity in fully mature DCs is not inhibited by treatment with rapamycin. Therefore, the mechanism of mDCs resistant to RAPA-induced apoptosis can probably be explained by LPS-induced NF-κB activation.

We further analysed the effects of RAPA on DC cytokine production and demonstrated that the production of IL-10 and IL-12 by iDCs in response to LPS was decreased in the presence of RAPA. Moreover, RAPA-iDCs had an inhibitory effect on T-cell proliferation, in accordance with their non mature phenotype. In this respect, our data confirm and extend previous observations [15,17,19,22,38]. Our results further demonstrate that mDCs have reduced IL-10 expression when exposed

to RAPA, while IL-12 production was unaffected. In this regard, our results are generally in agreement with some previous observations that rapamycin promotes LPS-induced IL-12 production but suppresses IL-10 production in mice BM-DCs [39] and human peripheral blood mononuclear cells (PBMCs) [30]. TNF-α is a significant pro-inflammatory cytokine and is involved in many pathologic processes. During DC–T-cell interaction, high amounts of TNF-α produced by mDCs may play an important role in regulating Th1 cell development [40]. Because IL-10 inhibits the LPS-induced production of pro-inflammatory cytokines, such as IL-12 and TNF-α, rapamycin may increase the production of TNF-α, as shown in human PBMCs [30]. Yet, our data shows rapamycin reduces LPS-induced TNF-α production in rat BM-DCs. The findings are also different from a previous study that rapamycin has no effect on LPS-induced TNF-α production in mice BM-DCs [39]. The discrepancy between our findings and others would be explained by different species or the difference in DC differentiation or maturation. This differential regulation of cytokine expression in fully mature DCs treated with rapamycin indicates that RAPA-mDCs induce intracellular signal transduction pathways and alter the transcription of factors. Two signaling pathways downstream of phosphoinositide 3-kinase (PI3K) – the mTOR and glycogen synthase kinase 3 (GSK3) pathways – regulate the Th1/Th2 balance by regulating IL-12 expression in DCs [39]. Thus, unlike calcineurin inhibitors, which primarily block TCR-dependent signaling pathways, RAPA appears to inhibit co-stimulatory signaling pathways and cytokine receptor-mediated signaling pathways [12].

The inhibitory effect of RAPA on IL-10 production may have a major impact on the adaptive immune response [41]. IL-10 is also an important growth and differentiation factor for B lymphocytes [42]. Therefore, its inhibition by RAPA could lead to a reduced level of B cell maturation and antibody production, which may be an important contributor to its immunosuppressive action *in vivo*. Interestingly, RAPA-mDCs maintained high-levels of IL-12 production. Our results have unravelled an unanticipated role of the mTOR signaling pathway in modulating pro-inflammatory cytokine production induced by LPS in mDCs. Our culture conditions may result in mDCs with a specific profile. This RAPA-mediated induction of IL-12 production may result from both indirect transcriptional enhancement of IL-12p40 and p35 secondary to the inhibition of autocrine IL-10 action upon treatment with LPS as well as direct transcriptional up-regulation of the IL-12p40 by mTOR inhibition in an IL-10-independent manner [24,30,39,43]. Investigation into the regulation of these opposing effects revealed a molecular switch in the IL-12 signaling networks that is a consequence of DC maturation. This shift of the IL-12/IL-10 balance toward IL-12 may promote Th1 responses and trigger the inflammatory process [1]. However, our study unexpectedly showed that allogeneic T cells were hyporesponsive to RAPA-treated mDC stimulation and produced both less Th1-type (IFN-γ) and Th2-type (IL-4) cytokines. In this regard, there are some opposite results [16,19,30,39]. A recent study shows that RAPA-DCs, which are markedly impaired in $Foxp3^-$ T-cell allostimulatory capacity, induce the proliferation of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) in MLR [16]. Thus, the ability of RAPA-DCs to allow the activation and proliferation of Treg while impairing effector T-cell proliferation may underlie this suppressed allostimulatory effect. Further studies should be conducted with regards to the proliferation of Treg associated to the suppressed allostimulatory effect of RAPA-mDCs. On the other hand, the suppressed allostimulatory effect of RAPA-mDCs was unlikely to be the result of RAPA-induced apoptosis because the percentage of apoptotic cells was similar in both mDCs and mDCs treated with 1–20 ng/ml of RAPA. Overall, although the precise molecular effects of RAPA on DCs remain unknown, the fact that RAPA inhibits the allostimulatory activity of mDCs *in vitro*, without inducing cell death, could provide valuable insights in cell therapy. Infusion of mDCs in combination with RAPA may eventually lead to more effective induction of allogeneic T-cell hyporesponse and treatment of transplant rejection, because RAPA is widely used in transplantation and this treatment might maintain a status of hyporesponsiveness, despite the pro-inflammatory context of the graft.

In conclusion, rapamycin affects the LPS-induced cytokine production and can inhibit allostimulatory activity in both immature and fully mature DCs. Furthermore, fully mature DCs treated with rapamycin induce intracellular signal transduction pathways and alter the expression levels of transcription factors. These findings have implications for the RAPA-based therapy of transplant rejection. Understanding the precise molecular mechanisms by which RAPA affects DCs will provide the opportunity to re-evaluate the timing of RAPA treatment and optimize immunosuppressive therapy.

Authorship

G-YW: wrote the paper and performed the study. G-HC: designed the study and revised the paper critically. HL: analysed data. YH, G-SW, NJ, B-SF: performed the study.

Funding sources

This study was supported by a grant from the Major State Basic Research Development Program of China (973

Program) (No.2009CB522404), the National Natural Science Foundation of China (No.30571769), the Major Special Project of Guangdong province (No.2007A032000001) and the Postdoctoral Science Foundation of China (No.20060390212). The authors have no conflicting financial interests.

Acknowledgement

The authors thank Dr Xin Liu from the University of Queensland School of Medicine for helpful comments on this manuscript. This study was supported by a grant from the Major State Basic Research Development Program of China (973 Program) (No.2009CB522404), the National Natural Science Foundation of China (No.30571769), the Major Special Project of Guangdong province (No.2007A032000001) and the Postdoctoral Science Foundation of China (No.20060390212). The authors have no conflicting financial interests.

References

1. Banchereau J, Briere F, Caux C, *et al.* Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; **18**: 767.
2. Lechler R, Ng WF, Steinman RM. Dendritic cells in transplantation – friend or foe? *Immunity* 2001; **14**: 357.
3. Jonuleit H, Schmitt E, Steinbrink K, Enk AH. Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol* 2001; **22**: 394.
4. McCurry KR, Colvin BL, Zahorchak AF, Thomson AW. Regulatory dendritic cell therapy in organ transplantation. *Transpl Int* 2006; **19**: 525.
5. Chaussabel D, Banchereau J. Dendritic cells, therapeutic vectors of immunity and tolerance. *Am J Transplant* 2005; **5**: 205.
6. Barratt-Boyes SM, Thomson AW. Dendritic cells: tools and targets for transplant tolerance. *Am J Transplant* 2005; **5**: 2807.
7. Albert ML, Jegathesan M, Darnell RB. Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. *Nat Immunol* 2001; **2**: 1010.
8. Nakamura I, Kajino K, Bamba H, Itoh F, Takikita M, Ogasawara K. Phenotypic stability of mature dendritic cells tuned by TLR or CD40 to control the efficiency of cytotoxic T cell priming. *Microbiol Immunol* 2004; **48**: 211.
9. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 2002; **23**: 445.
10. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. *Nat Immunol* 2000; **1**: 199.
11. McGuirk P, McCann C, Mills KH. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. *J Exp Med* 2002; **195**: 221.
12. Kahan BD, Camardo JS. Rapamycin: clinical results and future opportunities. *Transplantation* 2001; **72**: 1181.
13. Saunders RN, Metcalfe MS, Nicholson ML. Rapamycin in transplantation: a review of the evidence. *Kidney Int* 2001; **59**: 3.
14. Liu Y, Chen Y, Liu FQ, Lamb JR, Tam PK. Combined treatment with triptolide and rapamycin prolongs graft survival in a mouse model of cardiac transplantation. *Transpl Int* 2008; **21**: 483.
15. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. *Am J Transplant* 2005; **5**: 228.
16. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* 2007; **178**: 7018.
17. Hackstein H, Taner T, Logar AJ, Thomson AW. Rapamycin inhibits macropinocytosis and mannose receptor-mediated endocytosis by bone marrow-derived dendritic cells. *Blood* 2002; **100**: 1084.
18. Matsue H, Yang C, Matsue K, Edelbaum D, Mummert M, Takashima A. Contrasting impacts of immunosuppressive agents (rapamycin, FK506, cyclosporin A, and dexamethasone) on bidirectional dendritic cell-T cell interaction during antigen presentation. *J Immunol* 2002; **169**: 3555.
19. Monti P, Mercalli A, Leone BE, Valerio DC, Allavena P, Piemonti L. Rapamycin impairs antigen uptake of human dendritic cells. *Transplantation* 2003; **75**: 137.
20. Woltman AM, de Fijter JW, Kamerling SW, *et al.* Rapamycin induces apoptosis in monocyte- and CD34-derived dendritic cells but not in monocytes and macrophages. *Blood* 2001; **98**: 174.
21. Woltman AM, van der Kooij SW, Coffer PJ, Offringa R, Daha MR, van Kooten C. Rapamycin specifically interferes with GM-CSF signaling in human dendritic cells, leading to apoptosis via increased p27KIP1 expression. *Blood* 2003; **101**: 1439.
22. Hackstein H, Taner T, Zahorchak AF, *et al.* Rapamycin inhibits IL-4 – induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood* 2003; **101**: 4457.
23. Chiang PH, Wang L, Bonham CA, *et al.* Mechanistic insights into impaired dendritic cell function by rapamycin: inhibition of Jak2/Stat4 signaling pathway. *J Immunol* 2004; **172**: 1355.
24. Sordi V, Bianchi G, Buracchi C, *et al.* Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells:

selective upregulation by rapamycin. *Transplantation* 2006; **82**: 826.
25. Xia CQ, Peng R, Annamalai M, Clare-Salzler MJ. Dendritic cells post-maturation are reprogrammed with heightened IFN-gamma and IL-10. *Biochem Biophys Res Commun* 2007; **352**: 960.
26. Longman RS, Braun D, Pellegrini S, Rice CM, Darnell RB, Albert ML. Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of IFN-alpha/beta on antigen cross-presentation. *Blood* 2007; **109**: 1113.
27. Mauel S, Steinbach F, Ludwig H. Monocyte-derived dendritic cells from horses differ from dendritic cells of humans and mice. *Immunology* 2006; **117**: 463.
28. Homaidan FR, Chakroun I, El-Sabban ME. Regulation of nuclear factor-kappaB in intestinal epithelial cells in a cell model of inflammation. *Mediators Inflamm* 2003; **12**: 277.
29. MacDonald A, Scarola J, Burke JT, Zimmerman JJ. Clinical pharmacokinetics and therapeutic drug monitoring of sirolimus. *Clin Ther* 2000; **22**(Suppl. B): B101.
30. Weichhart T, Costantino G, Poglitsch M, *et al.* The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* 2008; **29**: 565.
31. Lagaraine C, Hoarau C, Chabot V, Velge-Roussel F, Lebranchu Y. Mycophenolic acid-treated human dendritic cells have a mature migratory phenotype and inhibit allogeneic responses via direct and indirect pathways. *Int Immunol* 2005; **17**: 351.
32. Bierer BE, Mattila PS, Standaert RF, *et al.* Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci U S A* 1990; **87**: 9231.
33. Shi Y, Frankel A, Radvanyi LG, Penn LZ, Miller RG, Mills GB. Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro. *Cancer Res* 1995; **55**: 1982.
34. Arimilli S, Johnson JB, Alexander-Miller MA, Parks GD. TLR-4 and -6 agonists reverse apoptosis and promote maturation of simian virus 5-infected human dendritic cells through NFkB-dependent pathways. *Virol* 2007; **365**: 1446.
35. Franchi L, Condo I, Tomassini B, Nicolo C, Testi R. A caspaselike activity is triggered by LPS and is required for survival of human dendritic cells. *Blood* 2003; **102**: 2910.
36. Ammon C, Mondal K, Andreesen R, Krause SW. Differential expression of the transcription factor NF-kappaB during human mononuclear phagocyte differentiation to macrophages and dendritic cells. *Biochem Biophys Res Commun* 2000; **268**: 99.
37. Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity* 2002; **16**: 257.
38. Horibe EK, Sacks J, Unadkat J, *et al.* Rapamycin-conditioned, alloantigen-pulsed dendritic cells promote indefinite survival of vascularized skin allografts in association with T regulatory cell expansion. *Transpl Immunol* 2008; **18**: 307.
39. Ohtani M, Nagai S, Kondo S, *et al.* Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. *Blood* 2008; **112**: 635.
40. Suryaprasad AG, Prindiville T. The biology of TNF blockade. *Autoimmun Rev.* 2003; **2**: 346.
41. Jorgensen PF, Wang JE, Almlof M, *et al.* Sirolimus interferes with the innate response to bacterial products in human whole blood by attenuation of IL-10 production. *Scand J Immunol* 2001; **53**: 1841.
42. Poussin MA, Goluszko E, Hughes TK, Duchicella SI, Christadoss P. Suppression of experimental autoimmune myasthenia gravis in IL-10 gene-disrupted mice is associated with reduced B cells and serum cytotoxicity on mouse cell line expressing AChR. *J Neuroimmunol* 2000; **111**: 152.
43. Aste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol* 1998; **160**: 5936.

