Article

Phenotype and Function of Monocyte-Derived Dendritic Cells from Chinese Rhesus Macaques

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Dendritic cells (DCs) play a pivotal role in linking the innate immunity and acquired immunity in responses to pathogen. Non-human primates such as Chinese Rhesus Macaque (CRM) are the favorable models for preclinical study of potential therapeutic drugs, vaccines and mechanisms of human diseases. However, the phenotypical characterization of monocyte-derived dendritic cells (MDDCs) from CRM has not been elucidated. Monocytes from CRM were cultured with GM-CSF and IL-4 in RPMI-1640. Six days later, these cells were differentiated with typical dendritical morphology. CD11c and DC-SIGN were highly expressed. The immature MDDCs expressed the low levels of CD25, CD80, CD83, moderate CD40, CD86, and high MHC. After stimulation, the mature MDDCs increased expression of mature molecules CD25 and CD83, co-stimulatory molecules such as CD80, CD86 and CD40, and kept a high level of MHC. The capacity of endocytosis decreased with maturation. The mature MDDCs have strong ability of inducing allogeneic T cell proliferation and producing IL-12. In conclusion, we have characterized the phenotype and ultimate function of MDDCs from CRM for the first time. *Cellular & Molecular Immunology*. 2009;6(3):159-165.

Key Words: monocyte-derived dendritic cell, animal model, Chinese Rhesus Macaque

Introduction

Dendritic cells (DCs) are generated from bone marrow and undergo four main stages of development: (*a*) bone marrow progenitors; (*b*) precursor DCs; (*c*) tissue-residing immature DCs; and (*d*) mature DCs (1). After encountering microbial or inflammatory stimulus, precursor DCs can differentiate to immature DCs (2). The immature DCs reside in the peripheral tissue to patrol the surrounding and monitor for pathogens. Once the pathogens are recognized, it will be processed to peptides and loaded on the MHC molecules. Meanwhile, immature DCs migrate to lymphoid organs and gradually get mature. Mature DCs will up-express the co-stimulatory molecules, namely CD80 and CD86, and decrease their phagocytic capacity. Mature DCs interact with T lymphocytes through peptide-MHC complex and co-stimulatory molecules, and induce them proliferation and polarization. The polarized T lymphocytes could induce strong antigen-special immune response (1).

Monocytes are often used as substitution for DC research *ex vivo*. $CD14^+$ monocytes in the peripheral blood are differentiated into immature monocyte-derived DCs (MDDCs) after 6 day culture in the presence of IL-4 and GM-CSF (3). These immature MDDCs can be converted into mature MDDCs in the presence of LPS or CD40L, or the mixture of pro-inflammatory factor, including IL-1β, IL-6, TNF-α, PGE2 (4).

Non-human primates such as Chinese Rhesus Macaque (CRM) are favorable models for pre-clinical study of potential therapeutic drugs, vaccines and mechanisms of human diseases. It is therefore important to understand the primate immune systems. The first detailed research for non-human primate MDDCs was from Chimpanzee (5). Later, the origin of MDDCs extended to Indian rhesus macaques (6), Cynomolgus monkeys (7), and African green monkeys (8). Chinese rhesus macaques (CRM) are different from Indian rhesus macaques in genetic background. Intravenous inoculation with SIVmac239, steady state plasma viral loads were found to be significantly lower in Chinese-origin monkeys than in Indian-origin monkeys, and more closely paralleled those found in untreated HIV-infected human (9, 10). Lu et al. first used AT-2-inactived SIV loaded MDDCs to immunize CRM infected with

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SIVmac251 and led to considerable viral suppression without any antiviral therapy (11). Here, we first cultured and characterized MDDCs from CRM and compared their phenotype and function to human MDDCs. We also identified suitable mAbs to cross-react with CRM MDDC molecules.

Materials and Methods

Animal

Twelve healthy SIV-negatives CRMs are from the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). They are 10-12 years old and all are male. Housing, maintenance and care of the animals are performed under the regulations and recommendations of the Animal Care Committee of KIZ, CAS.

Medium and cytokines

The cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum together with 100 U/ml penicillin, 100 μg/L streptomycin and glutamine. Incubation was done at 37ºC in 5% CO_2. Recombinant human IL-4 and GM-CSF were kindly donated from Prof. Xuetao Cao of the Second Military Medical University, China. TNF-α, LPS, PGE2 and IL-1β were purchased from Sigma. 7-Aminoactinomycin D (7AAD) was from Jingmei Biotech Company (China).

Generation and stimulation of CRM MDDCs

The simian peripheral blood mononuclear cells (PBMCs) were isolated by EZ-Sep™ Monkey 9× (Dakewe, China). The whole blood was mixed with 1/8 volume EZ-Sep™ Monkey 9× solution. Then about 200 μl RPMI-1640 were laid on the mixture carefully. PBMCs were collected into the PBS after centrifugated the mixture at 800 g for 30 min at room temperature.

Monocytes were selected by EasySep human CD14 positive selection cocktail (Stemcell Corp). CRM PBMCs were washed twice and then adjusted the cells at a concentration of 1×10^8 cells/ml. PBMCs were mixed with EasySep Positive Selection Cocktail at 100 μl/ml cells, and incubated at room temperature for 15 minutes. Then EasySep Magnetic Nanoparticles were added at 50 μl/ml cells, mixed well and incubated at room temperature for 10 min. The cell suspension was added recommended buffer to a total volume of 2.5 ml in a Falcon Polystyrene Round-Bottom tube (Becton Dickinson). The tube was placed into the magnet (Stemcell) for 5 min and the supernatant fraction was poured off carefully. Monocytes were held by the magnetic filed and remained inside the tube. Selected monocytes were seeded (10^6 per 1.5 ml) into 12-well culture plates (Costar). GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) were supplemented into complete culture medium. Cell cultures were replenished every 2 days with fresh medium and cytokines at the same final concentrations. Six days later, the cells were collected and assessed by FACS analysis. MDDCs were stimulated with a mixture of cytokine cocktail consisting of IL-1β (10 ng/ml), LPS (100 ng/ml), PGE2 (10 ng/ml) and TNF-α (100 U/ml) for 2 days.

Table 1. The antibodies used in study

Antigen	Source	Clone	Isotype
CD3ε-FITC	Becton Dickinson	SP34	IgG_3
CD4-FITC	Miltenyi Biotec	MT-466	IgG_1
CD8-FITC	Miltenyi Biotec	BW/135/80	IgG_{2a}
CD11c-PE	eBioscience	3.9	IgG_1
CD14-FITC	Miltenyi Biotec	TÜK4	IgG_{2a}
CD25-PE	Miltenyi Biotec	4E3	IgG_{2b}
CD40-FITC	Becton Dickinson	5C3	IgG_1
CD80-FITC	Becton Dickinson	L307.4	IgG_1
CD83-PE	Becton Dickinson	HB15e	IgG_1
CD86-PE	Becton Dickinson	FUN-1	IgG_1
DC-SIGN-FITC	R&D	120507	IgG_{2b}
HLA-ABC-PE	Becton Dickinson	G46-2.6	IgG_1
HLA-DR-FITC	Becton Dickinson	G46-6	IgG_1

Morphologic analysis

The photomicrographs of immature and mature MDDCs were performed using digital camera (Nikon, Coolpix 4500) at ×400 magnifications on TS100-F microscope (Nikon).

Flow cytometry

The mouse anti-human mAb clones were described in Table 1, and 2×10^5 MDDCs were stained with antibody at room temperature for 30 minutes, washed and resuspended in PBS containing 1% paraformaldehyde. Appropriate isotype-matched controls Abs were used in all labeling experiments. Stain cells were acquired and analyzed by FACSCalibur using CellQuest software (BD).

FITC-dextran uptake assay

FITC-dextran (Sigma) was used to detect the uptake ability of MDDCs. Cells (2×10^5) were incubated with 1 mg/ml FITC-dextran for 30 min at 37ºC or at 4ºC as a control for background passive uptake. Cells were washed twice with cold PBS and then re-suspended in PBS, 1% paraformaldehyde. FITC-dextran uptake was calculated, and percentage determined by flow cytometry minus background.

Mixed lymphocyte reaction (MLR)

Allogeneic PBMCs were first labeled with PKH26 according the user manual (Sigma). Then, labeled PBMCs were seeded at 2×10^5/200 μl in triplicate in RPMI-1640 medium with 10% FCS in 96-well flat bottom microtiter plates (Costar). Stimulated MDDCs were added at a DC/PBMC ratio of 1/10, 1/20 and 1/40 for 6 days. PBMCs alone were as a negative control. PKH26 fluorescence was calculated for $CD3^+$ T cells by flow cytometry. $CD4^+$ and $CD8^+$ T cells were also calculated at a ratio of 1/10. The results are expressed as division index (DI) calculated as follows: percentage of positive proliferating cells/total positive cell population.

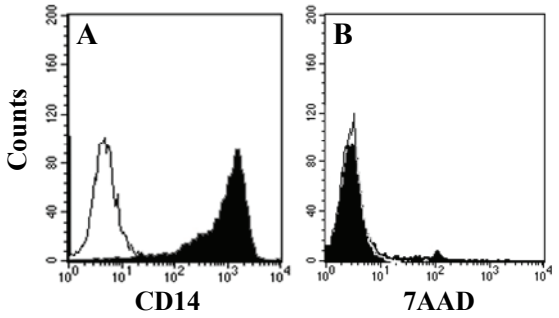

Figure 1. The purity and viability of monocytes from CRM PBMCs. (A) The purity of CRM monocytes. (B) The viability of CRM monocytes. The positive cells of 7AAD indicated dead cells. Open histograms correspond to isotype controls and filled histograms to CD14 antibody or 7AAD staining.

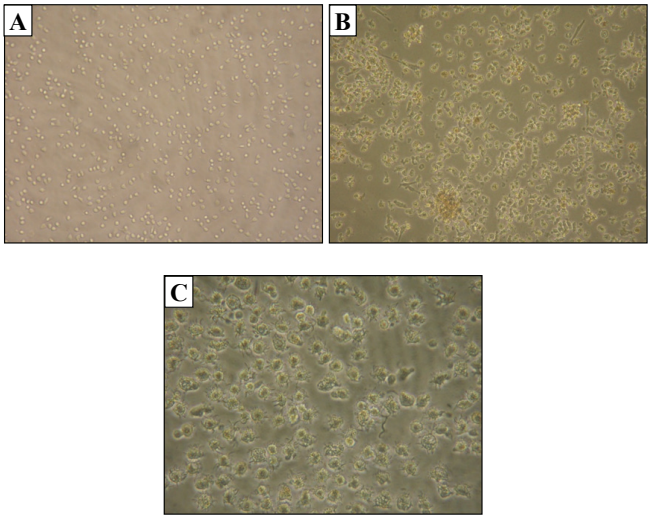

Figure 2. Morphology of cultured MDDCs. (A) Monocytes after isolation (magnification, ×100). (B) MDDCs cultured with GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) after 6 days (magnification, ×200). (C) Mature MDDCs stimulation with IL-1β (10 ng/ml), LPS (100 ng/ml), PGE2 (10 ng/ml) and TNF-α (100 U/ml) after 2 days (magnification, ×400).

Quantification of cytokine secretion

Mature MDDCs and PBMCs were cultured together or alone for 6 days. Supernatants were collected and kept at -20ºC. IL-12 was measured using Monkey IL-12 Total ELISA Kit (Bender MedSystems). The plates were read on a Bio-Tek ELX800 ELISA reader at 450/630 nm.

Statistical analysis

Results are expressed as the mean ± SD. The results were analyzed by Student's *t*-test for statistical significance using SPSS.

Results

Purity and viability of CRM monocytes

Unlike using Ficoll-Hypaque, CRM blood can be directly mixed with EZ-Sep^{TM} Monkey 9× solution without dilution and overlay. The dye 7AAD takes place of PI to distinguish viable cells from dead cells. The results showed the percentage of monocytes in PBMCs was about 7%, which was lower than that of Ficoll-Hypaque method (about 14%), but the viability could increase to 99% (data not shown). After magnetic selection, the purity of monocytes could reach from 88% to 92%, and the viability was more than 97% (Figure 1).

Morphological features of CRM MDDCs

The monocytes were cultured in the presence of GM-CSF and IL-4. They adhered on the plate tightly after two-hour culture (Figure 2A). During the six-day culture period, cells got together to form various clusters, and detached from plates gradually. On the sixth day, most of clustered cells were non-adherent with typically long multiple dendritic projections and a few cells suspended alone (Figure 2B). After one or two days further stimulated culture, more cells suspended independent and the dendritic projections were more obviously (Figure 2C).

Phenotype of immature MDDCs and mature MDDCs

Flow cytometric analysis was used to identify MDDCs' surface markers. The CD11c and DC-SIGN were chosen as a DC standard. After 6 days culture, either CD11c or DC-SIGN expressing cells were over 95%. For further understanding the characteristic of the MDDCs, the important surface protein expressions of CRM MDDCs were detected and compared with that of human MDDCs. The percentage of HLA-DR was not significantly different between CRM and human MDDCs, and the CRM was a little low (Figure 3), whereas the percentage of HLA-ABC showed the contrary results. CRM MDDCs expressed obviously lower CD80 than that of human whether it was unstimulated or stimulated MDDCs. The expressions of CD25, CD40 and CD86 were increased after stimulated in both CRM and human MDDCs while DC-SIGN were decreased. As a mature MDDCs marker, CD83 showed a sharp raise, but the expression of CRM stimulated MDDCs was lower than the human, which might be because of the cross-reactive ability of the CD83 antibody.

Phagocytic capacity of CRM MDDCs

The FITC-dextran was used to detect the phagocytic capacity of CRM MDDC. As shown in Figure 4, the unstimulated CRM MDDCs were able to take up FITC-dextran the same extend as human MDDCs. After stimulation, CRM MDDCs showed a significantly declined capacity of antigen uptake, which in line with further maturation. However, the mature human MDDCs still kept a high uptake capacity.

Effects of CRM MDDCs on T cell proliferation

PKH26 is a red fluorescent cell linker and often chosen as a marker of cell proliferation. After PKH26-labled cells

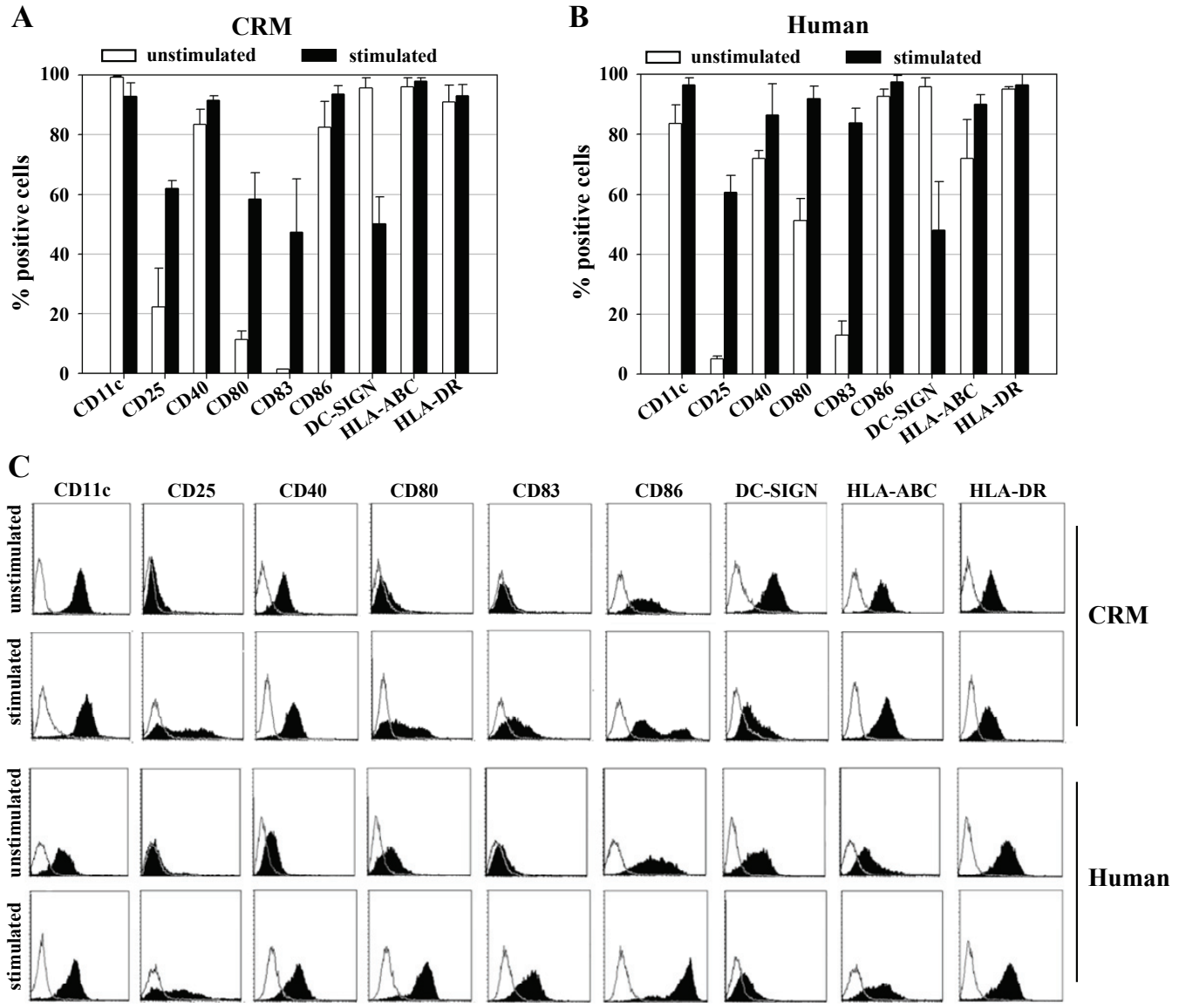

Figure 3. Comparative phenotypic analysis of immature CRM and human immature (unstimulated) and (stimulated) mature MDDCs. (A) Phenotypic analysis of CRM MDDC. Unstimulated MDDC and stimulated MDDC of six CRM blood were analyzed. Results are expressed as percentage means ± SD. (B) Phenotypic analysis of Human MDDC. Human MDDC of three distinct donors were studied. Results are expressed as percentage means ± SD. (C) A representative experiment for CRM and human MDDCs is depicted. Open histograms correspond to isotype controls and filled histograms to specific mAb staining.

proliferated, the fluorescence became weaker. Allogeneic PBMCs were first labeled with PKH26, and then co-cultured with either un-stimulated or stimulated MDDCs at three varying MDDC/PBMC ratio (1/10, 1/20, 1/40). After six-day co-culture, the T cell proliferation was detected by using the PKH26 and CD3 fluorescent antibody. As expected, stimulated MDDCs showed a much stronger immuno-stimulatory capacity than that of unstimulated MDDCs, and when the MDDC/PBMC ratio achieved 1/20, the effect of stimulation of mature MDDCs was the best (Figure 5A). Unstimulated MDDCs showed low capacity to stimulate both allogeneic $CD4^+$ and $CD8^+$ T cell proliferation at the 1/10 ratio, while the stimulated MDDCs showed a higher immuno-stimulatory capacity (Figure 5B). Altogether, these results corroborate phenotypic data, showing the stimulated CRM MDDCs acquired a mature state.

IL-12 production of mature CRM MDDCs

When DCs induces T cell polarization, some proinflammatory cytokines are produced by DCs, like IL-12 (12). The production of IL-12 was measured in the supernatants of mature CRM MDDCs, PBMCs and allogeneic MLR. The results showed that the concentration of IL-12 was higher at a higher MDDC/PBMC ratio than PBMCs or MDDCs alone

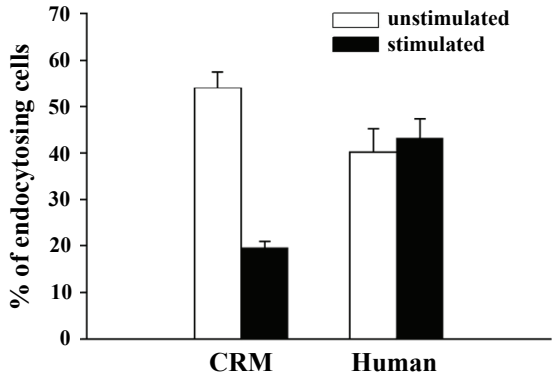

Figure 4. Comparison of phagocytic function of CRM and human immature (unstimulated) and (stimulated) mature MDDCs. The cells were incubated with FITC-dextran (1 mg/ml) as explained in Materials and Methods. Data were expressed as mean ± SD.

(Figure 6). These data suggested that mature CRM MDDCs owned a high immuno-stimulatory capacity.

Discussion

In this study, we successfully obtained CRM, human and cynomolgus monkeys' MDDCs by culturing blood monocytes in the presence of GM-CSF and IL-4 for 5-7 days (3, 6, 13, 14). First we chose EZ-Sep^{TM} Monkey 9× solution substitute for Ficoll-Hypaque to isolate PBMCs. This method increased the viability of PBMCs, but decreased the percentage of monocytes in PBMCs. We tried using the percoll density gradient, but the purity and viability of the obtained cells were worse. We washed the 2-hours incubated PBMCs to get adhesive monocytes, but this method used up a lot of PBMCs to get monocytes in high purity. Finally, we selected the magnetic beads to isolate the CD14 positive monocytes, and got higher purity and quantity monocytes in contrast with the traditional ways.

We identified several mAbs to cross-react specifically with homologous molecules expressed by CRM MDDCs. The cells derived from CRM blood monocytes also express most typical markers of mDCs, such as CD11c and DC-SIGN. There are two kinds of important molecules on the MDDCs surface: one is co-stimulation molecules, including CD40, CD80 and CD86, which are used for activating T cell response (15); the other is the MHC molecule with responsibility for presenting antigen to T cells. We detected these molecules and found the immature MDDCs expressed low CD80, but high CD40, CD86, and MHC molecules. CD83 is considered a relative special DC mature marker and up-regulation of CD25 has been implicated as a marker of complete DC maturation, correlating with the stable MHC-peptides required for potent T cell activation. After stimulation, expression of all the surface molecules of MDDCs increased more or less. In particular the CD25 and CD80 expression increased from a

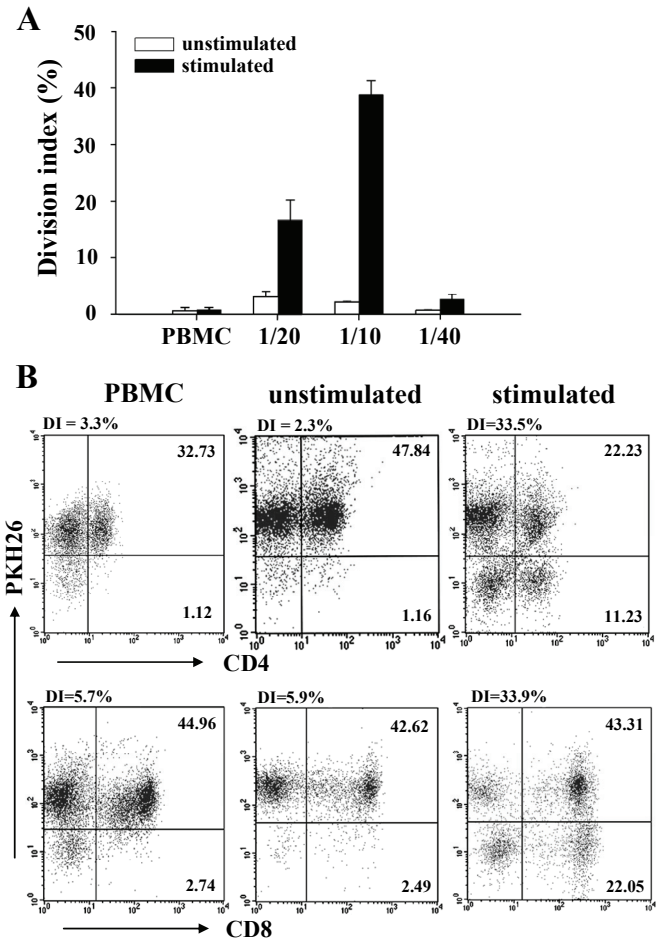

Figure 5. Mixed lymphocyte reaction. (A) The histograms represent the proliferative ability of $CD3^+$ T cells at various MDDC/PBMC ratio after co-culture. Data represent mean ± SD from three independent experiments. (B) Representive FACS profiles of proliferative ability of $CD4^+$ or $CD8^+$ T cells at the highest MDDC/PBMC ratio (1/10). The assays were conducted by co-culturing unstimulated or stimulated MDDCs with allogeneic PBMCs pre-stained with PKH26 fluorescent dye. Analyses were performed after 6 days of co-culture.

low to a moderate level.

Maturation is important for MDDCs to induce T cell response, but how maximally activate MDDCs hampered this work. Early studies have introduced the maturation of rhesus macaques by MCM (6, 14), CD40L (16). Erin Mehlhop et al. (17) compared the factors to induce human DC maturation, including MCM, CD40L, poly (I:C), LPS, PGE2/TNF-α, cocktail of PGE2/TNF-α/IL-1β/IL-6 (CKT), CKT lacking IL-6 (CKT-6) or CKT lacking IL-1β (CKT-1β), to assessed the resultant changes using phenotypic and functional analyses. They found the CKT was the most consistent induction of mature macaque MDDC phenotype and function, just like the previous studies reported on the effectiveness of cytokine combinations for human DC maturation in FCS-free conditions (6, 18). In the present study, we first chose LPS to

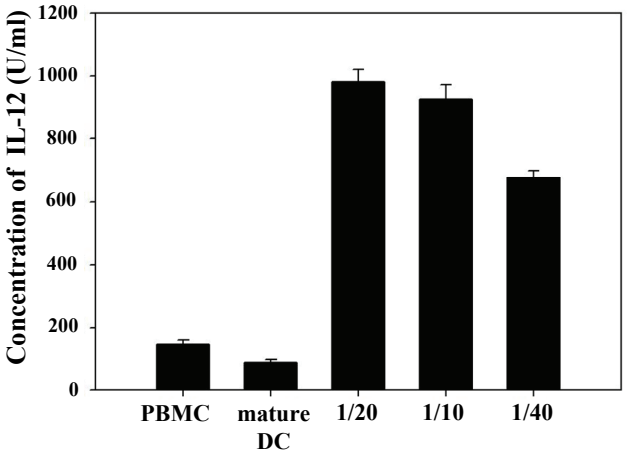

Figure 6. IL-12 levels in cell-free culture supernatants. Supernatants were collected at day 6 from cultures with PBMCs alone, mature MDDCs alone and co-cultures of mature MDDCs with allogeneic PBMCs at the ratios indicated. Results are expressed as mean ± SD.

stimulate the CRM MDDCs. LPS often induced appreciable weaker phenotypic differentiation, and the activated macaque MDDCs were less effective at stimulating T cell activation and IL-12 production in contrast to human MDDCs (19). So we turned our attention to the CKT, because LPS can stimulate APC to secrete IL-6 (20). The CKT-6 and LPS was put together to stimulate CRM MDDCs. Stimulated MDDCs highly promoted T cell proliferation and IL-12 production than that of unstimulated MDDCs.

The phagocytic capacity of CRM MDDCs dropped after stimulation. This may be related to the decrease of DC-SIGN after maturation. On the other hand, human MDDCs maintained high phagocytic ability, which might result from the culture condition or the mixed stimulator of maturation we provided.

In conclusion, we have successfully cultured the CRM MDDCs from blood monocytes, and characterized their phenotype and function for the first time. We detected all items before and after exposure to a mixed LPS and CKT-6. Just like the human and Indian rhesus macaque, but not the African green monkey, the MDDCs underwent a complete maturation and owned high immunostimulatory ability, which was associated with high T cell proliferation and IL-12 production.

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