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Original Article

BMSCs-induced CD45RB$^+$ dendritic cells suppress T cell activity

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Abstract: Mesenchymal stem cells (MSCs) exhibit strong immunoregulatory capabilities, however the mechanism by which MSCs regulate T cell function via modulating dendritic cell (DC) activity is poorly understood. Rat MSCs and DCs were isolated from bone marrow, and CD4$^+$ T cells were isolated from the spleen. DCs were co-cultured with Lipopolysaccharide and MSCs, and after 5 days, DC cell surface markers were assessed by flow cytometry. CD45RB$^+$ DCs were then isolated and expression of costimulatory molecules, Foxp3, and antigen uptake capability was assessed. We also incubated CD4$^+$ T cells in the presence or absence of conA, CD45RB$^+$ DCs, immature DCs (imDCs) and mature DCs (mDCs) and then measured T cell proliferation, T-bet, GATA-3, RORγt, and Foxp3 mRNA content by qPCR, and supernatant cytokine content by cytometric bead array. MSC-DC co-culture induced CD45RB and ILT4 expression, and reduced CD86 and MHC-II expression. These purified CD45RB$^+$ DCs exhibit surface marker expression patterns, antigen uptake ability, Foxp3 expression typical of imDCs. Proliferation of T cells incubated with imDCs and CD45RB$^+$ DCs was significantly slower than those incubated with mDCs. T cells co-cultured with imDCs and CD45RB$^+$ DCs expressed significantly higher levels of GATA-3 and Foxp3 mRNA and lower levels of T-bet and RORγ mRNA, and released higher levels of IL-4 and lower levels of IL-2, IFN-γ, IL-17A, IL-6 and TNF-α than those co-cultured with mDCs. Rat bone marrow derived MSC induce CD45RB$^+$ DC differentiation, therefore promoting differentiation of T cells to Th2 and CD4$^+$Foxp3$^+$ T cells.

Keywords: Mesenchymal stem cells, dendritic cells, CD45RB, CD4$^+$ T cells, immunosuppression

Introduction

The capacity of mesenchymal stem cells (MSCs) to differentiate into mesenchymal lineages and exert immune-regulatory effect suggests that MSC transplantation may confer clinical benefits to a range of conditions [1-6]. Specifically, MSCs can regulate innate and acquired immune responses by directly modifying T and B cell responses, or indirectly affecting differentiation of T and/or B cells, or myeloid-derived cells (e.g. dendritic cells, DCs) and macrophages [7, 8]. These extraordinary capabilities suggest that transplantation of MSCs may represent a clinically useful treatment for graft versus host disease (GVHD) [7]. Although the mechanisms by which MSC modulate the behaviors of T cells, B cells and macrophages have been well studied, little is known about the mechanism by which MSCs regulate DC activity.

As the most important antigen presenting cells in the body, DCs process and present antigens mainly to naive and memory T cells. However, in order to initiate an immune response, DCs must mature; immature DCs are not only incapable of adequately activating T cells but can also induce tolerance [9]. MSCs can regulate the recruitment, maturation, and function of DCs, and have been reported to significantly reduce monocyte differentiation into DCs, downregulateCD40, CD80, CD86, and MHC II [10-12]. MSCs also inhibit DC secretion of IL-12, the deficiency of which may induce T cell anergy and tolerance [13]. Recently, Sadeghi*et al.* discovered that bone marrow derived MSCs (BMMSCs) can inhibit the maturation of splenic DCs, and thus drive responding T cells toward Th2 cytokine responses [14]. However, the precise mechanism by which MSCs regulate T cells though DCs modification is not yet clear.

DCs represent a heterogeneous population, expressing a range of different types and levels of surface markers [15]. For example, DCs expressing high levels of CD11c and no CD45RB are termed $CD11c^{high}CD45RB^{-}$ DCs, and are found to mainly secrete IL-12. In contrast, $CD11c^{low}CD45RB^{+}$ DCs are found to mainly secrete IL-10 [16-18]. $CD11c^{low}CD45RB^{+}$ have been reported to suppress immune responses by inducing tolerance and differentiation of regulatory T cells (Tregs) *in vivo* [19]. Based on these observations, we postulated that co-incubation of DCs with MSCs may induce maturation of $CD11c^{low}CD45RB^{+}$ DCs, which can subsequently regulate the behavior of T cells and exert an immunosuppressive effect. In this study, we demonstrated that co-incubation of DCs with MSCs induced maturation of DCs expressing higher levels of CD45RB and ILT4, and lower levels of CD86 and MHC-II. These $CD45RB^{+}$ DCs exhibit surface marker expression patterns and antigen uptake capability more similar to immature DCs. Isolated $CD45RB^{+}$ DCs inhibited the proliferation of $CD4^{+}$ T cells, induced the differentiation of $CD4^{+}$ T cells to Th2 and Tregs.

Methods and materials

Rats

Wistar rats (weight: 200-250 g) were provided by the Laboratory Animal Center of Soochow University (Suzhou, China). Animals were maintained under specific pathogen free and standard conditions. All experimental procedures involving animals were approved by the animal ethical committee of Soochow University.

Isolation and culture of cells

MSCs were isolated from rat bone marrow as previously described [20, 21]. Briefly, bone marrow cells were isolated from femurs and tibias of Wistar rats. Isolated cells were cultured in flasks in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in a CO_2 incubator at 37°C. After 3 days, non-adherent cells were removed. Adherent cells were grown to 85% confluency, then trypsinized and passaged. At passage 3, osteogenic and adipogenic differentiation was assessed by measurement of MSC surface expression of CD90, hematopoietic markers CD45 and monocyte cell marker CD11b/c by flow cytometry, as previously described [22].

Immature DCs were also derived from the femur and tibia bone marrow of Wistar rats. Briefly, bone marrow was collected from femurs and passed through a 100-μm pore size mesh to remove fibrous tissue. Red blood cells were lysed, and the remaining cells were cultured at 1×10^6 cells/ml in RPMI 1640 medium (Hyclone, USA) supplemented with cytokines (10 ng/ml each of mouse GM-CSF and IL-4; R&D, USA). After 5 days, cells were harvested and the immature DCs (imDCs) phenotypewas analyzed by flow cytometry (Becton Dickson, USA) [23].

Naive $CD4^{+}$ T cells were isolated from the rat spleen as previously described [24]. Briefly, spleen cells were labeled with CD11b, TER-119, Gr-1, I-A/I-E, CD8α, B220, and Gr-1 directed mAbs (BD Pharmingen), and opsonized cells were removed by Dynabeads (Dynal Biotech). Purity of the remaining $CD4^{+}$ cells was consistently 90-95%.

Co-incubation of MSCs or T cells with DCs

To investigate the effect of MSCs on DC maturation, 10^6 freshly harvested imDCs were plated in 6-well plates with the same number of MSCs at a 1:1 ratio. MSCs were not irradiated before co-culture. The mixed cells were incubated in 2 ml of RPMI 1640 medium supplemented with 10% FBS, stimulated with lipopolysaccharide (LPS) (200 ng/mL final concentration, Sigma, USA) and in presence or absence of MSC for 5 days. Expression of PDL1, ILT4, CD1d, CD14, CD45RA, CD45RB, CD40, CD80, CD86 and MHC-II was then assessed by flow cytometry, and imDCs, MSCs+LPS-induced DCs ($CD45RB^{+}$ DCs) and LPS-induced DCs (mature DC) were isolated. Immature DC and mature DCs were separated according to CD86/CD80 expression level, and $CD45RB^{+}$ DCs were separated according to CD45RB expression, via fluorescence-activated cell sorting.

To explore the effects of imDCs, mDCs and $CD45RB^{+}$ DCs on $CD4^{+}$ T cells, DCs were co-incubated with $CD4^{+}$ T cells at a 1:10 ratio in RPMI 1640 medium supplemented with 10% FBS and conA (10 ng/ml final concentration, Sigma, USA) in 6-well plates for 3 days. $CD4^{+}$ T cells were then harvested by fluorescence-activated cell sorting, and the percentage of $CD4^{+}$ $Foxp3^{+}$ Treg was determined by flow cytometry. The supernatant was collected for cytometric bead array (CBA).

Table 1. Primers used in qPCR

Primers	Sequences (5'-3')
Foxp3-F	ACACCCAGGAAAGACAG
Foxp3-R	GGCAGTFCTTFAFAAAC
T-bet-F	GTTCCCATTCCTGTCCTTC
T-bet-R	CCTTGTTGTTGGTGAGCTT
GATA3-F	TTTACCCTCCGGCTTCATCCTCCT
GATA3-R	TGCACCTGATACTTGAGGCACTCT
RORγt-F	GGAGCTCTGCCAGAATGAGC
RORγt-R	CAAGGCTCGAAACAGCTCCAC
β-actin-F	GGTGTGATGGTGGGAATGGG
β-actin-R	ACGGTTGGCCTTAGGGTTCAG

DC antigen uptake assay

A total of 1.0×10^6 imDCs, CD45RB$^+$ DCs or mDCs were incubated with latex beads/FITC-OVA (DC/Bead at ratio of 1:20000) at 37°C. After 1, 3, 6 or 24 h, cells were harvested and antigen uptake was assessed. After quenching the unincorporated FITC signal with 0.4% trypan blue, uptake was quantified as mean cell-associated fluorescence intensity measured by flow cytometry.

Real-time qRT-PCR

The FOXP3 mRNA content of imDCs, CD45RB$^+$ DCs or mDCs and the T-bet, GATA-3, RORγt, Foxp3 mRNA content of T cells co-incubated with or without these DCs were determined by qPCR using Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA) and SYBR Green Realtime PCR Master Mix (Life, USA) following the manufacturer's instructions using the primers listed in **Table 1**.

Western blot

FOXP3 expression in imDCs, CD45RB$^+$ DCs or mDCs was determined by western blot (ebioscience), as previously described [25, 26].

Proliferation test

The effect of DCs on T cell proliferation was determined by CCK-8 assay. Briefly, imDCs, CD45RB$^+$ DCs and mDCs were inactivated by co-incubation with mitomycin (25 ug/mL, Sigma, USA) for 45 min at 37°C in 5% CO_2. Then mitomycin was removed and cells were washed twice with PBS. Then DCs were plated in 96-well plates at density of 1×10^5/well and co-incubated with or without T cells (1×10^5/well) and conA (10 ng/ml) at 37°C in 5% CO_2 for 5 days. CCK-8 (10 μl/well) was added and the plates were incubated for additional 4 h. The OD values at 450 nm were determined by a microplate reader (CliniBio128C, Austria).

Detection of cytokines

The cytokine content of co-culture supernatant was assessed by CBA using the human Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer instructions. We measured IL-2, IL-10, IL-4, IL-6, IFN-γ, TNF-α, and IL-17A.

Statistical analysis

Data was presented as Mean ± SD, and analyzed using ANOVA post hoc LSD test with SPSS 18.0 and Graphpad Prism 5. $P<0.05$ was considered statistically significant.

Results

Characterization of rat BM-MSCs

Isolated bone BM-MSCs were initially observed to be round (**Figure 1A** and **1B**). After 3 passages, cells attached well and became strip-like in appearance (**Figure 1C** and **1D**). Expression of CD90 and lack of CD45 and CD11b/cexpressed in these cells indicated that they were MSCs (**Figure 1E**). Successful adipocyte or bone differentiation of these cells, induced by incubation with the corresponding mediums, was validated by Oil Red O and Alizarin Red S staining (**Figure 1F**), confirming that these cells were capable of adipocyte and bone differentiation.

Co-incubation with BM-MSCs induces DCs expression of CD45RB and ILT4, and reduces DC expression of CD86 and MHC-II

Cells (precursor cells of imDCs) isolated from bone marrow were incubated with GM-CSF and IL-4 for 7 days, and successful differentiation to imDCs was confirmed by flow cytometry (**Figure 2A-C**). These imDCs were then incubated with LPS in the presence or absence of BM-MSCs. Incubation with LPS alone induced imDC maturation to mDCs. However, when imDCs were co-incubated with BM-MSCs and LPS, cell surface markers of these DCs differed from mDCs. As shown in **Figure 2D**, co-incubation of imDCs with MSCs significantly increased the fraction of DCs expressing CD45RB and ILT4, and reduced the fraction of DCs expressing CD86 and MHC-II (**Figure 2D**). Expression of PLD-1, CD45RA, CD1D and CD14 did not differ significantly between imDCs and mDCs.

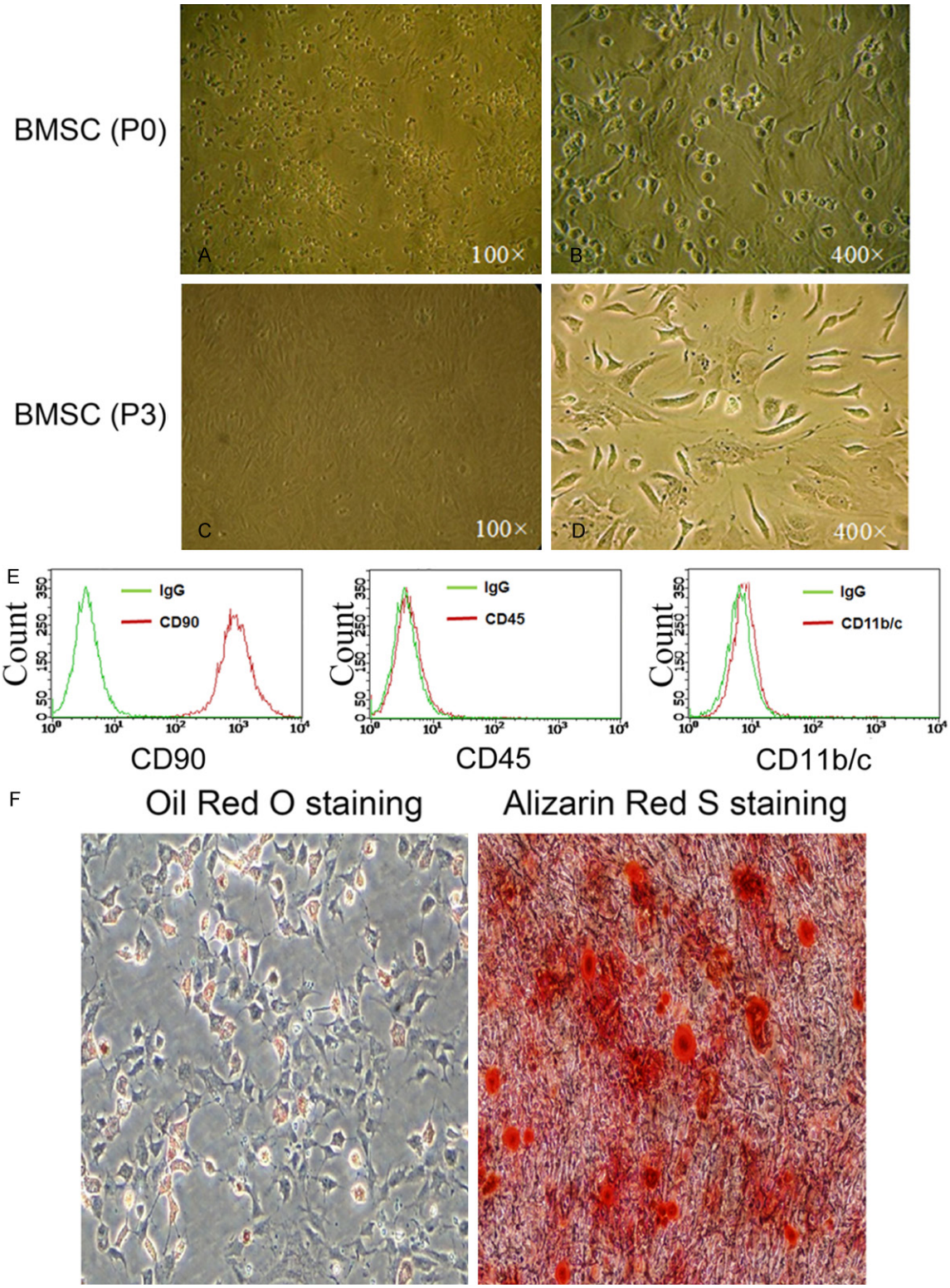


Figure 1. Isolation and characterization of rat BM-MSCs. A and B. Morphology of MSCs at passage 0. C and D. Morphology of MSCs at passage 3. E. Cell surface makers of MSCs, assessed by flow cytometry at passage 3. F. Differentiated MSCs strained by Oil Red O (left) or Alizarin Red S (right). Adipocyte differentiation was induced and cells were strained by Oil Red O. Bone differentiation of MSCs was induced and cells were strained by Alizarin Red S (200×).

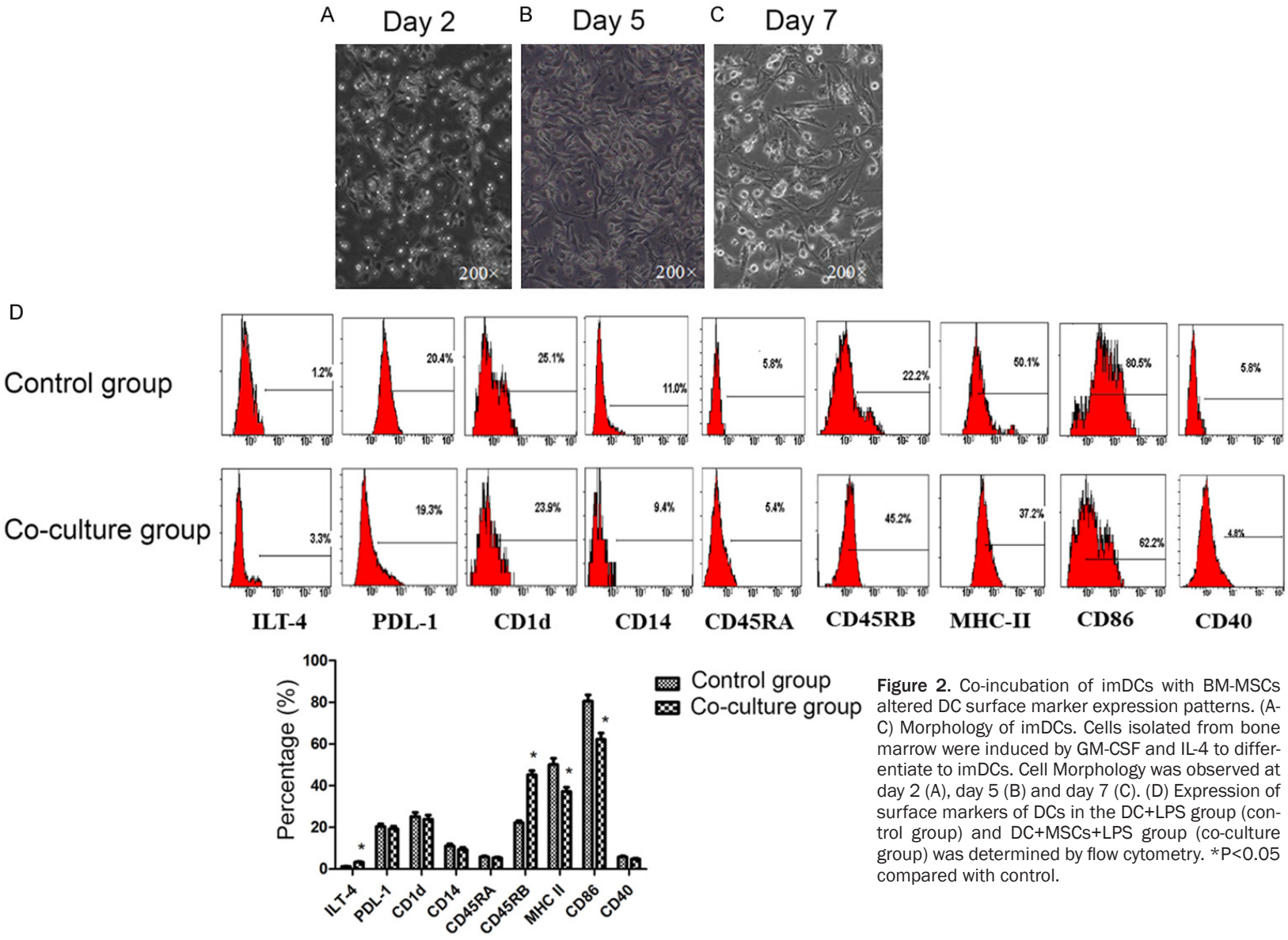


Figure 2. Co-incubation of imDCs with BM-MSCs altered DC surface marker expression patterns. (A-C) Morphology of imDCs. Cells isolated from bone marrow were induced by GM-CSF and IL-4 to differentiate to imDCs. Cell Morphology was observed at day 2 (A), day 5 (B) and day 7 (C). (D) Expression of surface markers of DCs in the DC+LPS group (control group) and DC+MSCs+LPS group (co-culture group) was determined by flow cytometry. *P<0.05 compared with control.

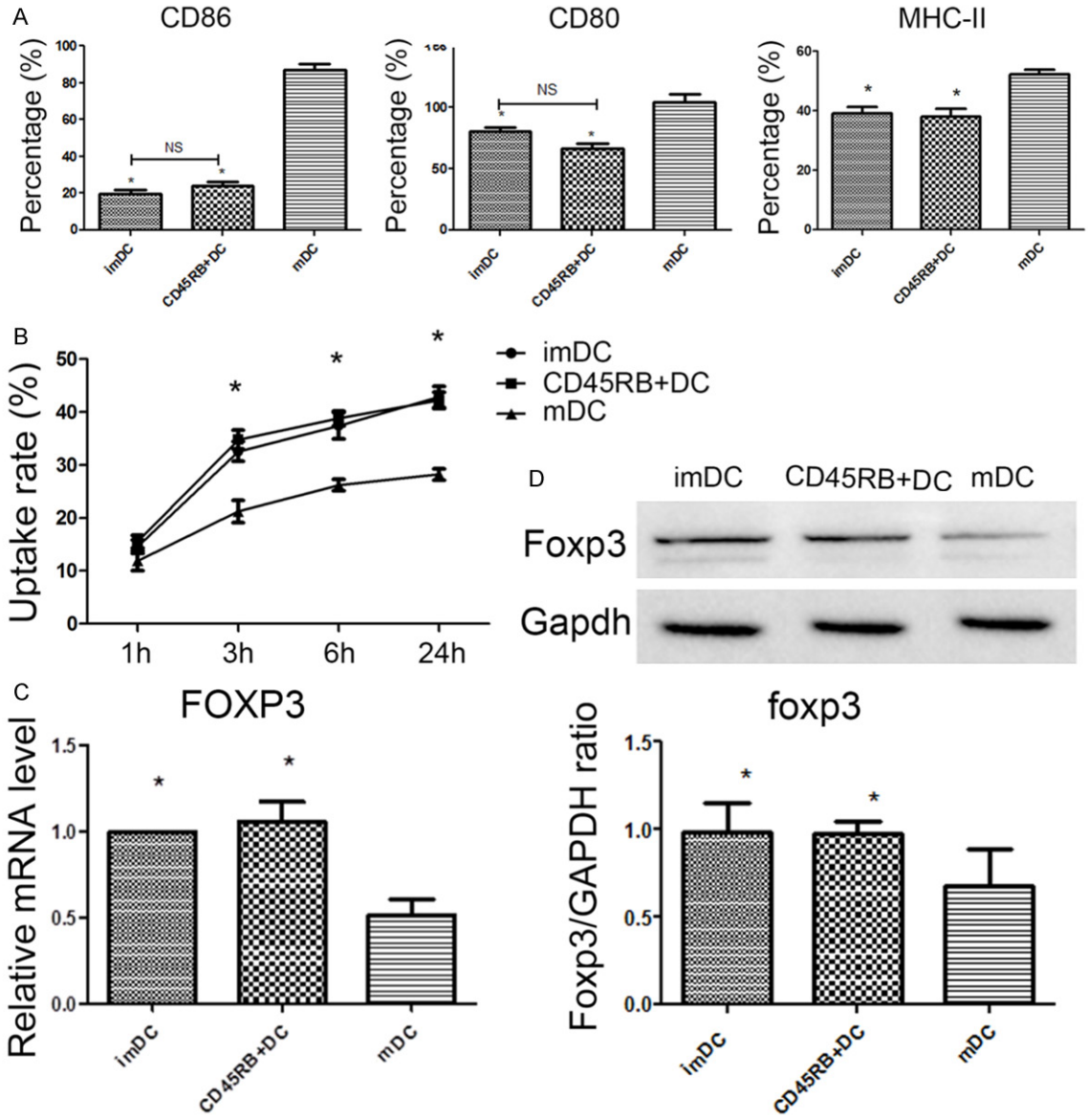


Figure 3. Surface markers and foxp3 expression in CD45RB$^+$ DCs. (A) CD80, CD86 and MHC-II expression in DCs was determined by flow cytometry. *P<0.01 compared with mDC group, NS, no significant difference (B) Antigen uptake of DCs. imDCs, CD45RB$^+$ DCs or mDCs were incubated with latex beads/FITC-OVA. Uptake was quantified by measuring cell-associated fluorescence intensity by flow cytometry. *P<0.05 compared with mDC group. (C) Foxp3 mRNA expression in different DCs was determined by q-PCR. *P<0.05 compared with mDC group. (D) Foxp3 protein expression in different DCs was measured by western blot. *P<0.05 compared with mDC group.

CD45RB$^+$ DCs shows similar characteristics to imDCs

The CD45RB$^+$ DCs induced by co-incubation with BM-MSCs and LPS were isolated using flow cytometry. Purified CD45RB$^+$ DCs exhibit similar characteristics to imDCs, including surface marker expression pattern (**Figure 3A**), antigen uptake ability (**Figure 3B**), Foxp3 mRNA content (**Figure 3C**) and Foxp3 protein content (**Figure 3D**). These features differ significantly from those of mDCs, suggesting that BM-MSCs may inhibit the LPS-induced differentiation of imDCs to regulatory DCs.

CD45RB$^+$ DCs and imDCs inhibit the proliferation of CD4$^+$ T cells

Cell morphology indicted that proliferation of T cells cultured with imDCs+conA and CD45RB$^+$

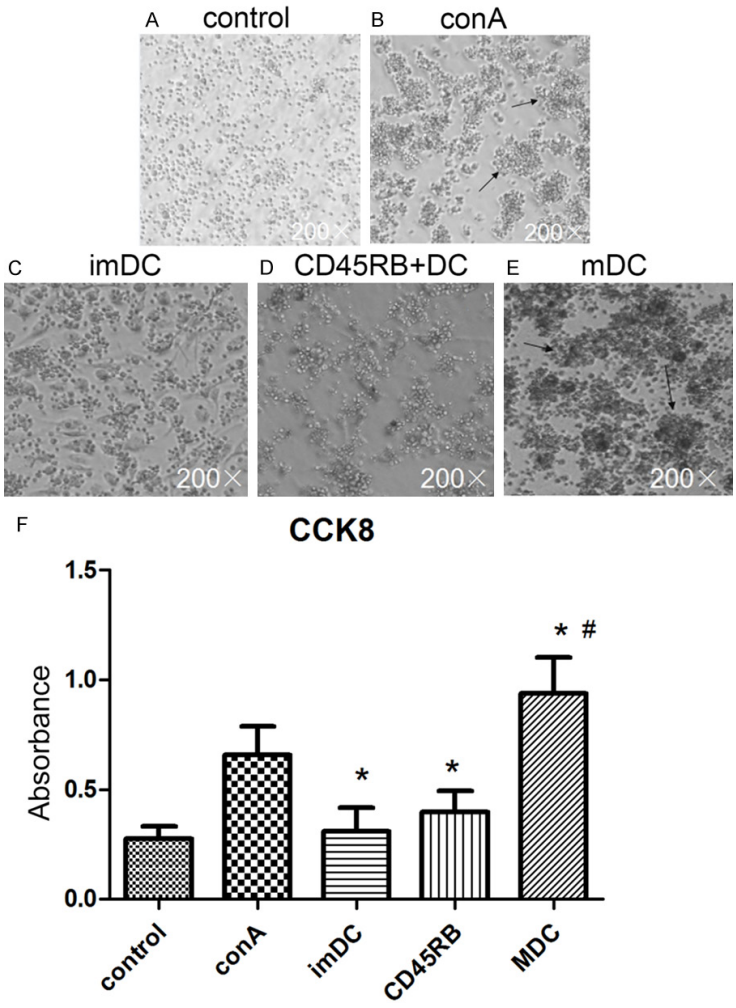


Figure 4. Effect of different DCs on proliferation of $CD4^+$ T cells. (A-E) T cell morphology. T cells were incubated alone (A) orintheor presence (B) of conA. Arrows indicate proiliferating T cell colonies. T cells were co-incubated with imDC+conA (C), $CD45RB^+$DC+conA (D) and mDC+conA (E). T cell morphology was captured by light microscope. (F) T cell proliferation was determined by CCK-8 assay. *P<0.05 compared with conA group. #P<0.05 compared with imDC+conA and $CD45RB^+$DC+conA groups.

DCs+conA was significantly lower than in cells incubated with conA (**Figure 4A-E**). CCK-8 assay confirmed that proliferation rate was significantly slower in T cells incubated with imDCs and $CD45RB^+$ DCs than those incubated with conA alone, but significantly faster in T cells incubated with mDCs+conA than those incubated with conA alone, which indicated that mDCs promoted the proliferation of T cells (**Figure 4F**).

imDCs and $CD45RB^+$ DCs promote differentiation of T cell to Th2 and $CD4^+Foxp3^+$ T cells

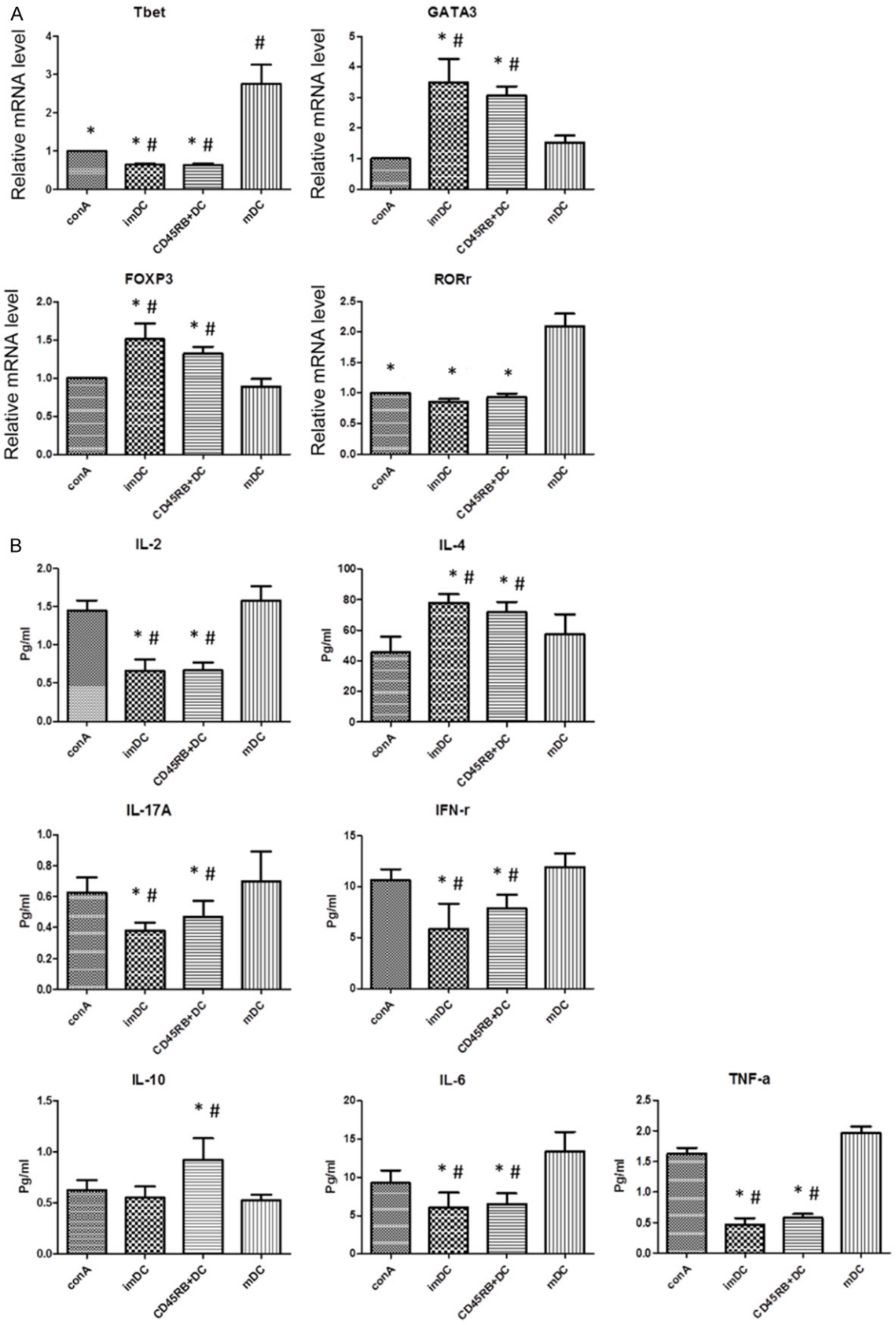
After co-incubation with different DCs for 3 days, T cells were isolated, and T-bet, GATA-3, RORγ, and Foxp3 mRNA content was measured by qPCR, indicating differentiation of T cells to Th1, Th2, Th17 or Treg, respectively. Compared with cells incubated with conA or mDCs+conA, T cells co-cultured with imDCs+conA and $CD45RB^+$ DCs+conA expressed significantly higher levels of GATA-3 and Foxp3 mRNA and lower levels of T-bet mRNA (**Figure 5A**). T cells co-cultured with mDCs+conA expressed significantly higher levels of T-bet and RORγ mRNA than those co-cultured with imDCs+conA or $CD45RB^+$DCs+conA (**Figure 5A**). T-bet, GATA-3, RORγ, and Foxp3 mRNA content did not differ significantly between T cells co-cultured with imDCs+conA and $CD45RB^+$DCs+conA.

Moreover, T cells co-cultured with imDCs+conA and $CD45RB^+$ DCs+conA release higher levels of IL-4 and lower levels of IL-2, IFN-γ, IL-17A, IL-6 and TNF-α (**Figure 5B**). Interestingly, IL-10 released from $CD45RB^+$ DCs+conA group was much high than from other group, IL-10 was the only cytokine expressed differently from imDCs+conA and $CD45RB^+$ DCs+conA group.

Lastly, the proportion of $CD4^+$ $Foxp3^+$ T *cells* in imDCs+conA and $CD45RB^+$ DCs+conA group was higher than that in mDC+conA group (**Figure 5C**).

Discussion

We postulated that co-incubation of DCs with MSCs may induce maturation of $CD11c^{low}CD45RB^+$ DCs, exert an immunosuppressive effect on T cells. To investigate this hypothesis we isolated rat BM-MSCs and prepared imDCs, and investigated the effect of MSCs on DC differentiation *in vitro*. Co-incubation with MSCs and LPS induced DC expression of CD45RB and ILT4, and down-regulated expression of CD86 and MHC-II. Isolation and characterization of


A
Tbet
GATA3
FOXP3
RORr
Relative mRNA level
B
IL-2
IL-4
IL-17A
IFN-r
IL-10
IL-6
TNF-a
Pg/ml
conA
imDC
CD45RB+DC
mDC

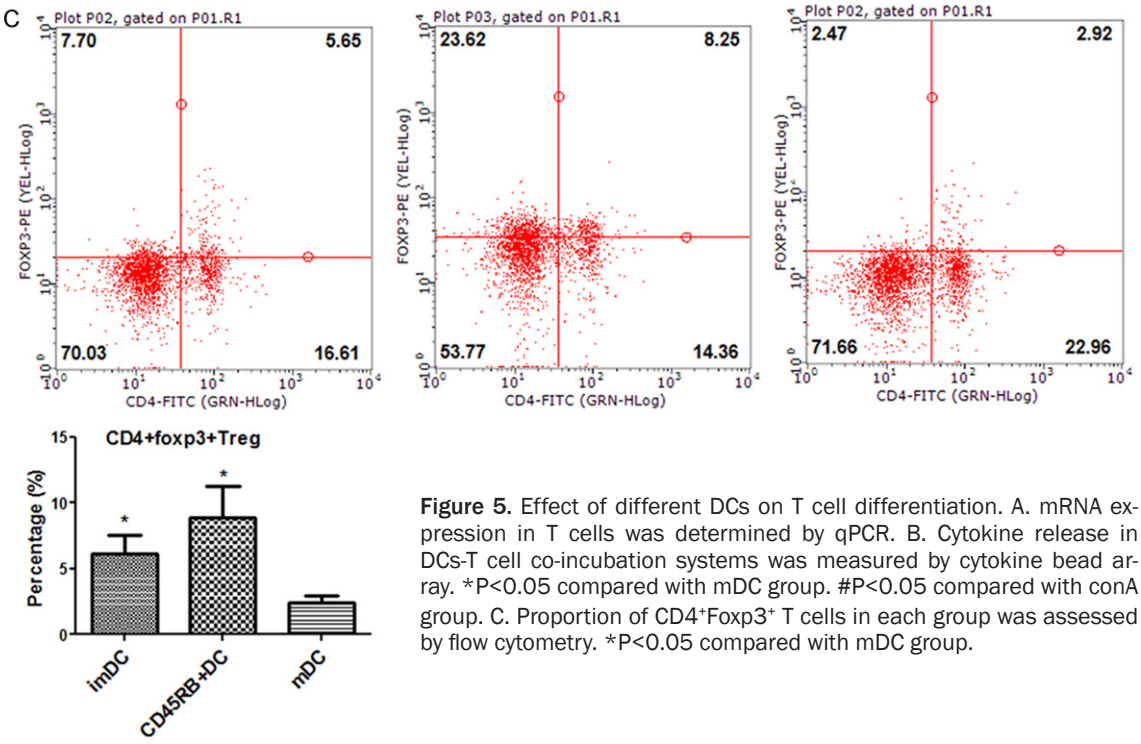


Figure 5. Effect of different DCs on T cell differentiation. A. mRNA expression in T cells was determined by qPCR. B. Cytokine release in DCs-T cell co-incubation systems was measured by cytokine bead array. *P<0.05 compared with mDC group. #P<0.05 compared with conA group. C. Proportion of $CD4^+Foxp3^+$ T cells in each group was assessed by flow cytometry. *P<0.05 compared with mDC group.

$CD45RB^+$ DCs indicated that MSCs-DC co-incubation inhibited DC maturation. $CD45RB^+$ DCs exhibited similar surface biomarker pattern, antigen uptake ability and Foxp3 expression to imDCs.

Proliferation of T cells incubated with imDCs and $CD45RB^+$ DCs was significantly slower than that of T cells incubated with mDCs. T cells co-cultured with imDCs and $CD45RB^+$ DCs expressed significantly higher levels of GATA-3 and Foxp3 and lower levels of T-bet and RORγ than T cells co-cultured with mDCs. Co-incubation of T cells with $CD45RB^+$ DCs enhanced release of IL-4 and reduced release of IL-2, IFN-γ, IL-17A, IL-6 and TNF-α, indicating promotion of T0 to Th2 differentiation, and production of Tregs.

MSCs were previously reported to exhibit immunosuppressive effects by directly modulating T cell survival, proliferation and differentiation [7], and MSCs have also been reported to modulate the behavior of T cells indirectly, specially via DCs. MSCs can inhibit differentiation of monocytes into imDCs [13] by blocking monocyte cell cycle at the G0 phase [27]. MSCs also inhibited maturation of imDCs and furthermore downregulated expression of CD86/CD80 and MHC-II [10], and reduce secretion of TNF-α and IL-12p70 from immature DCs, while increasing production of IL-10 [28, 29]. Our results confirmed that BM-MSCs downregulate DC expression of CD86 and MHC-II.

We found that imDCs-MSC co-incubation promoted up-regulated DC expression of $CD45RB^+$. Purified $CD45RB^+$ DCs exhibited similar surface marker expression, antigen uptake and Foxp3 expression to imDCs. These observations are consistent with previous reports that BM-MSCs inhibited maturation of imDCs into mDCs.

We also discovered that imDCs and $CD45RB^+$ DCs expressed higher levels of Foxp3 than mDCs. Foxp3 is a typical Treg biomarker and immunosuppressive factor, but Foxp3 expression in DCs was rarely evaluated. Our results suggest that in DCs Foxp3 may act as a specific biomarker distinguishing imDCs and $CD45RB^+$ DCs from mDCs.

Additionally, the expression of Foxp3 may indicate the mechanism by which this imDCs and $CD45RB^+$ DCs exert immunosuppressive functions. It is well understood that immature DCs

cannot activate T cells, and, in fact, induce immune tolerance. We found that both imDCs and CD45RB$^+$ DCs inhibited proliferation of T cells, prompting differentiation towards Th2, and increasing the proportion of CD4$^+$Foxp3$^+$ T cells. However DC45RB$^+$ DCs were not identical to imDCs. T cells incubated with CD45RB$^+$ DCs secreted more IL-10 than those incubated with imDCs or mDCs. This observation suggests that the mechanism by which CD45RB$^+$ DCs influence T cell responses differs, at least in part, from the effect of imDCs. The immunosuppressive effect of IL-10 has been previously reported [16, 19, 30], thus we conclude that MSCs may exert immunosuppressive effects by inducing CD45RB$^+$ DCs differentiation.

The mechanism by which of CD45RB$^+$ DCs differentiate is not well understood. Delgado *et al.* previously reported the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP) induced CD45RB$^+$ DCs by stimulating theVPAC1 receptor and protein kinase A [31]. In future studies, the pathway by which MSCs induce CD45RB$^+$ DC maturation will be further investigated.

Taken together, we conclude that co-incubation of MSCs and DCs inhibited maturation of imDCs into mDCs and induced differentiation of CD45RB$^+$ DCs. CD45RB$^+$ DCs further inhibited proliferation of CD4$^+$ T cells, and induced differentiation of CD4$^+$ T cells to Th2 and Tregs.

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Disclosure of conflict of interest

None.

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