Cell. Mol. Life Sci. 64 (2007) 104–111
1420-682X/07/010104-8
DOI 10.1007/s00018-006-6407-x
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Cellular and Molecular Life Sciences

Research Article

Macrophages derived from bone marrow modulate differentiation of myeloid dendritic cells

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Received 12 September 2006; received after revision 20 October 2006; accepted 13 November 2006
Online First 14 December 2006

Abstract. Dendritic cells (DC) are specialized antigen-presenting cells. Bone marrow monocytes have been widely used to generate murine myeloid DC. We found that mouse macrophages derived from bone marrow $CD11b^+$ monocytes influenced the differentiation of these precursors into DC. Modulation of differentiation was demonstrated by the down-regulation of CD11c, CD40, and CD86 expression and by IL-12 production. DC differentiated in the presence of conditioned medium from bone marrow-derived macrophage culture (MCM) had impaired ability to stimulate proliferation of, and IFN-γ production by, allogeneic $CD4^+$ T cells. This inhibition of DC differentiation was mainly mediated by secretory products from macrophages but not by cell-cell contact. MCM contained higher concentrations of macrophage-colony-stimulating factor (M-CSF), IL-10, and TGF-β1, whereas IL-6 remained unchanged compared with conditioned medium from fresh monocytes. M-CSF may be the major mediator in MCM inhibiting DC differentiation. This study demonstrates an important influence of bone marrow-derived macrophages on DC precursors during DC differentiation.

Keywords. Macrophage, dendritic cell, differentiation, interaction, bone marrow.

Introduction

Dendritic cells (DC) are major antigen-presenting cells (APC) capable of stimulating a response in naïve T cells. They are more potent than other APC, including monocytes, macrophages (Mϕ) and B cells [1]. Several factors modulate the differentiation and maturation of DC [2], such as 1α,25-dihydroxyvitamin D3 [3], phosphatidylserine [4], dexamethasone [5], and cyclic nucleotides [6].

It is known that monocytes and Mϕ can differentiate into immature myeloid DC under stimulation by granulocyte Mϕ-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). Immature DC can be further triggered to develop into mature DC by various stimuli, including lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), CD40 ligation, or a combination of cytokines [7–10]. Various cells have been shown to interact with and alter the characteristics of DC under normal or pathological conditions. Zhang et al. [11] reported that endothelial-like splenic stromal cells drove mature DC to differentiate into regulatory DC. *Plasmodium falciparum*-infected

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erythrocytes inhibit the maturation of DC, and reduce their capacity to stimulate T cells [12]. Human mesenchymal stem cells suppress monocyte differentiation into DC and impair the allostimulatory effect of mature DC on T cells [13]. Neutrophils interact with immature DC and induce DC maturation to trigger T cell proliferation [14]. Clearly, it is important to understand cell-cell interaction between DC and other types of cells.

In living tissues, bone marrow monocytes differentiate into Mϕ and DC in response to various growth and differentiation factors [15]. The Mϕ, in multiple forms, are present extensively in many kinds of tissues. Takahashi et al. [16] reported that immature DC reduce the production of Mϕ-inflammatory protein from Mϕ and enhance their phagocytosis ability. Yamaguchi [17] demonstrated that FcR^+ adherent Mϕ promote DC maturation even in the presence of TGF-β1. However, while bone marrow-derived Mϕ co-exist *in vivo* with DC precursors, the influence of those Mϕ during DC differentiation has not been fully investigated.

In this study, we investigated the effect of bone marrow-derived Mϕ on the differentiation and allostimulatory activity of myeloid DC.

Materials and methods

Isolation of bone marrow $CD11b^+$ and spleen cells to generate Mϕ, and preparation of Mϕ-conditioned medium (MCM). Bone marrow cells flushed from BALB/c mice tibia and femur were resuspended in RPMI 1640 (Gibco BRL, NY) containing 10% fetal calf serum (FCS) and then plated in six-well plates. After incubation for 2 h at 37 °C, nonadherent cells were removed and adherent cells were collected. Subsequently, $CD11b^+$ (also known as Mac-1) cells were purified by high-gradient magnetic sorting using the miniMACS system with anti-CD11b microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To characterize these $CD11b^+$ cells, the expression of surface murine Mϕ marker F4/80 was examined by using flow cytometry as described below.

For preparation of splenic Mϕ, the spleens were removed from BALB/c mice, washed with PBS, homogenized into single cells, dispersed into suspension using a 1-mm metal sieve, and cultured in 10% FCS-containing RPMI 1640 medium at 37 °C in a fully humidified atmosphere of 5% CO_2. The culture medium was changed completely every 3 days for 7 days or longer. The bone marrow-derived and spleen-derived Mϕ were designated BMDM and SDM, respectively. To prepare Mϕ-conditioned medium (MCM), serum-free medium was used in the Mϕ cell culture for 3 days. It was then collected and passed through a Millipore filter with 0.45-µm pores and stored at –80 °C until used.

Direct co-culture and transwell co-culture model. For direct co-culture, freshly purified $CD11b^+$ monocytes were seeded onto a terminally differentiated Mϕ monolayer at a density of 1×10^5/ml in six-well plates. Differentiated Mϕ, either from bone marrow or spleen, were pre-stained with 1 µM PKH67 (Sigma, St. Louis, MO) to distinguish DC by flow cytometry. For transwell co-culture, $CD11b^+$ cells and differentiated Mϕ were cultured in the lower and upper chamber, respectively, of a transwell insert (Millicell-CM, 0.4-µm mesh, 30-mm diameter; Millipore, Bedford, MA).

Generation of bone marrow-derived DC and isolation of allogeneic $CD4^+$ T lymphocytes. Immature DC were generated from $CD11b^+$ monocytes by culture in RPMI 1640 medium supplemented with 10% FCS, 10 ng/ml GM-CSF (R & D Systems, Minneapolis, MN), and 10 ng/ml IL-4 (R & D Systems) every 3 days for 6 days. To trigger maturation, immature DC were incubated with 5 µg/ml LPS (Sigma-Aldrich, St. Louis, MO) for a further 24 h. The recovery rate of DC was estimated by dividing the number of DC by the total number of sorted $CD11b^+$ monocytes. To purify allogeneic $CD4^+$ T cells, mononuclear cells were isolated from spleen cells of C57BL/6 mice by density gradient centrifugation with Histopaque (Amersham Pharmacia Biotech, Piscataway, NJ). After lysis of erythrocytes by incubating in 0.9% ammonium chloride for 3 min at 37 °C, $CD4^+$ T cells were enriched by a $CD4^+$ T cell isolation kit (Miltenyi Biotec) using a negative selection technique. The purity of isolated $CD11b^+$ monocytes and $CD4^+$ T cells was greater than 94% by flow cytometric analysis.

Assessment of morphology. Cells from the original $CD11b^+$ culture and DC generated in the presence or absence of the MCM prepared from BMDM were harvested and cytocentrifuged onto a microscope slide using a Cytospin 2R (Shandon Southern Instrument Inc.) and then stained with Wright's stain. Light micrographs were taken under an Olympus microscope at a magnification of 1000×.

Viability of bone marrow-derived DC. To identify dead cells with damaged plasma membranes, we used flow cytometry to measure the uptake of 7-aminoactinomycin D (7-AAD). A total of 10^6 DC were incubated with 5 µg/ml 7-AAD (Sigma) for 10 min before analysis of viability.

Analysis of cell surface marker expression. Two-color immunolabeling was performed using fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies. For analysis of CD11c expression, cells were incubated with saturating concentrations of primary hamster anti-mouse CD11c mAb followed by $F(ab')_2$ goat anti-hamster IgG-FITC at 4 °C for 30 min. The rat

anti-mouse IgG conjugated with PE (Serotec, Oxford, UK) used for the other markers were anti-CD40-PE, anti-CD86-PE, and anti-MHC class II (I-A)-PE. Anti-F4/80-PE (Serotec) was used for characterization of Mϕ. Isotype controls were purchased from Serotec. After washing twice with PBS, 1×10^6 cells were placed in a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA). Thus, CD11c was stained indirectly, whereas the other markers were stained directly. The expression by CD11c$^+$ cells of CD40, CD86, and MHC class II was measured. According to the manufacturer's product information, the secondary reagent has been adsorbed against both mouse and rat immunoglobulins to minimize cross-reactivity. Data were collected and analyzed using CellQuest software (BD Biosciences).

Allogeneic T lymphocyte reaction. CD11b$^+$ monocyte-derived DC were harvested and irradiated (3000 cGy, 4.0 Gy/min in a single fraction) at 6 MeV generated by a linear accelerator (Clinac® 1800, Varian Associates, Inc., CA). Full electron equilibrium was ensured for each fraction by a parallel plate PR-60C ionization chamber (CAPINTEL, Inc., Ramsey, NJ). After incubating the irradiated DC with 1×10^6 T cells at ratios of 1 : 10, 1 : 20 or 1 : 40 for 5 days, bromodeoxyuridine (BrdU) was added to cultures of allogeneic CD4$^+$ T cells for a 30-min incubation at 37 °C. The cells were collected, fixed with cold 75% ethanol for 30 min, permeabilized by Triton X-100 for 30 min, stained with anti-BrdU-FITC (BD Biosciences), and then the amount of incorporated BrdU was detected using flow cytometry. Compared with isotype control, the percentage of positive stained cells was estimated using CellQuest software (BD Biosciences).

Quantification of cytokines. The levels of IL-12p70 in the DC supernatant, interferon-γ (IFN-γ) in the allogeneic T lymphocyte reaction supernatant, and IL-10, transforming growth factor-β1 (TGF-β1), IL-6 and M-CSF in MCM were measured using enzyme-linked immunosorbent assay (ELISA) kits purchased from R & D Systems according to the manufacturer's instructions.

Antibody neutralization. Neutralizing anti-TGF-β1 (1–5 µg/ml), anti-IL-10 mAb (0.1–0.2 µg/ml), and anti-M-CSF (10 ng/ml), purchased from R & D Systems, were added alone or together to 10% MCM for 90 min at day 0 of DC culture.

Statistics. The results were expressed as mean ± standard error of mean (SEM). The results of each experiment were compared using a paired Student's *t*-test. A p value of less than 0.05 was considered to be statistically significant. SPSS 10.0 software (SPSS Inc, Chicago) was used for data analysis.

Results

Morphological changes and recovery of DC. The isolated CD11b$^+$ BMDM expressed surface F4/80 extensively (83.2 ± 6.8% on day 1 and 93.7 ± 5.3% on day 7) and had features typical of Mϕ (Fig. 1a). Immature DC collected at day 6 before cytokine triggering had enlarged contours without evident dendrites, an appearance that was similar in both the absence (Fig. 1b) and presence (Fig. 1c) of 10% MCM. Cytokine-triggered DC at day 7 possessed morphological characteristics typical of mature DC, including being nonadherent and having multiple cytoplasmic projections and abundant cytoplasm (Fig. 1d). The number of these cells was markedly lower in the presence of 10% MCM added at the beginning of CD11b$^+$ cell culture. The majority of MCM-treated CD11b$^+$ cell-derived DC manifested fewer and shorter cell projections (Fig. 1e). No characteristic features of Mϕ, such as pseudopods or abundant cytoplasmic vacuoles, were seen (Fig. 1e). Mature DC with typical features accounted for 41.6 ± 4.6% (average 0.83×10^6/ml) of the cells in the original CD11b$^+$ culture (2×10^6/ml) when cultured in the absence of and 20.6 ± 2.3% when cultured in the presence of 10% MCM ($p < 0.05$), indicating that MCM inhibited the generation of DC. The percentage of

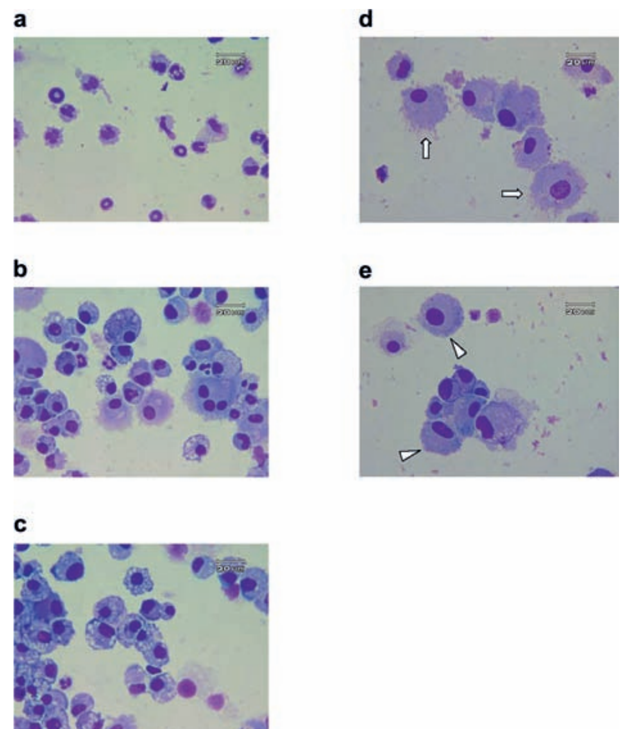

Figure 1. Morphology of initial CD11b$^+$ cells in culture (*a*), immature DC differentiated in the absence (*b*) or presence (*c*) of 10% MCM, and differentiated DC matured in the absence (*d*) or presence (*e*) of 10% MCM. Magnification 1000×. The arrows indicate representative typical mature DC and the arrowheads indicate cells without typical characteristics of mature DC.

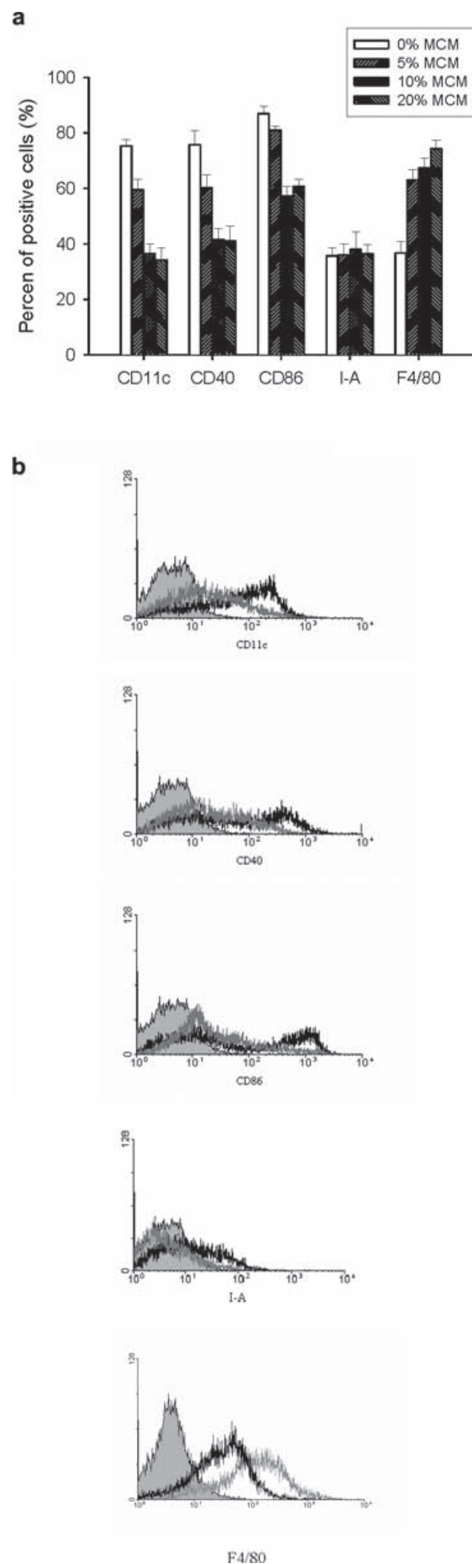

Figure 2. Expression of surface molecules on DC differentiated in the presence of MCM. (*a*) Dose-dependent effect of MCM. (*b*) Flow cytometric analysis of surface molecules (shaded histograms, isotype control antibodies; black line, DC differentiated in the absence of MCM; gray line, DC differentiated in the presence of 20% MCM). Data from five to seven separate experiments are expressed as mean + SEM.

dead DC in the absence and presence of 10% MCM did not differ significantly (14.6 ± 3.6% *versus* 11.6 ± 2.5%, $p > 0.05$), indicating that the decreased number of DC was not due to a cytotoxic effect of MCM. To exclude the effect of LPS on cell viability, we also compared the percentage of dead immature DC in culture with or without LPS. A similar percentage of dead cells (13.3 ± 1.5%) was found in the absence of LPS, so that LPS also was unlikely to have had a cytotoxic effect.

Modulation of DC differentiation by MCM and Mϕ co-culture. As shown in Table 1, 10% MCM inhibited the recovery of DC and the expression of CD11c, CD40, and CD86 by these cells. We further used transwell co-culture and direct co-culture to see if this inhibitory effect is mediated solely by secretory factors or through a combination of secretory factors and cell-cell contact. BMDM, regardless of whether in transwell or direct co-culture, had a similar inhibitory effect on these DC markers. This suggests that BMDM inhibits DC differentiation mainly by secreted soluble factors, because the effect was not enhanced by direct contact between cells.

Effect of MCM and transwell co-culture with BMDM on IL-12p70 secretion by DC. IL-12 p70 production was decreased when DC were generated in the presence of 10% MCM (94.5 ± 21.2 *versus* 32.3 ± 4.8 pg/ml, $p < 0.05$). Transwell co-culture with BMDM at the beginning of DC culture also inhibited IL-12p70 production by mature DC (23.3 ± 7.8 pg/ml).

Concentration-dependent modulation of surface marker expression of DC by MCM and the target cells of MCM. After culture in the presence of GM-CSF and IL-4 with or without MCM for 6 days plus LPS treatment for 1 more day, the expression of CD11c, CD40, and CD86 on $CD11b^+$ cell-derived DC was decreased in inverse proportion to the concentration of MCM (Fig. 2). MCM was effective in decreasing the expression of CD11c, CD40 and CD86 only when it was added at day 0 and day 3 during differentiation, demonstrating a time-dependent effect as well (Fig. 3). When 10% MCM was added together with LPS on day 6 to assess its effect on DC maturation, the expression of CD11c, CD40, and CD86 was not affected (Fig. 3). These results indicate that Mϕ affect DC at the stage of differentiation but not maturation. Mϕ co-culture and MCM had no significant effect on the expression of MHC class II (I-A) molecules (Table 1 and Fig. 2). MCM enhanced the expression of murine Mϕ marker, F4/80, an effect that was concentration dependent (Fig. 2a). Similarly, MCM affected expression of F4/80 during differentiation only when it was added at day 0 and day 3 but not at day 6, (Fig. 3).

Table 1. Direct and indirect influence of macrophages on DC differentiation.

	Recovery rate (%)	CD11c (%)	CD40 (%)	CD86 (%)	MHC II I-A (%)
Control	41.6 ± 4.6	78.5 ± 3.2	76.3 ± 3.2	82.7 ± 2.0	39.3 ± 2.0
10% MCM	20.6 ± 2.3	36.3 ± 3.5	39.0 ± 2.6	56.3 ± 2.4	38.0 ± 3.8
Transwell with BMDM	20.3 ± 2.0	35.7 ± 4.3	31.0 ± 3.5	53.7 ± 2.4	37.7 ± 4.6
Co-culture with BMDM	26.3 ± 2.6	ND	36.0 ± 5.1	48.3 ± 2.2	33.3 ± 5.5
Co-culture with SDM	18.0 ± 3.5	ND	46.3 ± 2.3	55.0 ± 1.7	35.0 ± 3.6

Control, DC differentiated by GM-CSF plus IL-4 and matured by LPS; MCM, macrophage-conditioned medium; BMDM, bone marrow-derived macrophage; SDM, spleen-derived macrophage.
ND, not done because the wavelengths for detection of CD11c-FITC and PKH67 were the same.

MCM effects on DC stimulation of allogeneic T cells. Because the co-stimulatory molecules CD40 and CD86 play an important role in DC stimulation of T cells, we examined the allostimulatory activity of MCM-triggered DC. DC generated in the presence of MCM had an impaired capacity to stimulate proliferation of allogeneic $CD4^+$ T cells (Fig. 4). IFN-γ production by these allogeneic $CD4^+$ T cells was also decreased (Fig. 5). Impaired stimulation of allogeneic T cells by MCM-treated DC is thus compatible with the decreased expression of co-stimulatory molecules.

Spleen-derived Mϕ effects on DC differentiation. To test whether Mϕ derived from tissues other than bone marrow also inhibit differentiation of DC, SDM were added to $CD11b^+$ cells at the beginning of DC differentiation. SDM, similar to BMDM, inhibited the recovery rate of DC and expression of CD40 and CD86 (Table 1).

Cytokines levels in MCM and effects of neutralizing antibodies. IL-10, TGF-β1, M-CSF, and IL-6 are known to inhibit DC differentiation. Using ELISA to compare their levels in MCM at week 3 with those on day 1, we found significantly higher concentrations of IL-10 (24.0 ± 1.8 *versus* 10.3 ± 2.3 pg/ml, $p < 0.05$), TGF-β1 (2851.3 ± 183.8 *versus* 1707.0 ± 69.9 pg/ml, $p < 0.05$) and M-CSF (124.0 ± 11.4 *versus* 60.3 ± 12.8 pg/ml, $p < 0.05$) at week 3. No significant change in the concentration of IL-6 was noted (112.3 ± 15.2 *versus* 106.7 ± 4.2 pg/ml, $p > 0.05$). It did not appear that IL-10 and TGF-β1 were responsible for the inhibition of DC differentiation by MCM, as neutralizing anti-TGF-β1 and anti-IL-10 antibodies had no significant influence on the MCM-mediated inhibition of DC differentiation (Table 2). To verify that the presence of IL-10 (24.0 ± 1.8 pg/ml) in 10% MCM is not a factor influencing DC differentiation, we added equal an amount of recombinant human IL-10 to DC culture at day 0 and found no inhibition of CD11c expression (79.4 ± 3.6% in control group *versus* 75.2 ± 4.5% in IL-10-treated group). Similar results were found for of TGF-β1 (79.4 ± 3.6% in control group *versus* 78.1 ± 3.3% in TGF-β1-treated group). However,

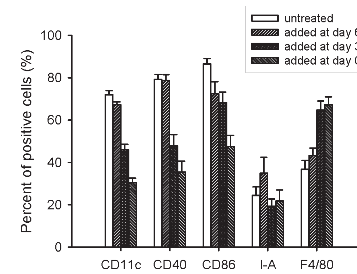

Figure 3. Flow cytometric analysis of surface molecule expression on DC differentiated in the presence of MCM added at various stages. Data from five to seven separate experiments are expressed as mean + SEM.

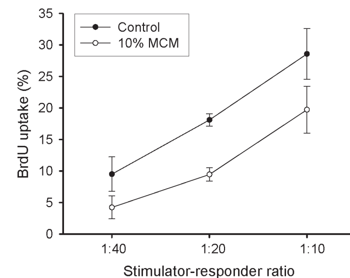

Figure 4. Allogeneic $CD4^+$ T cell proliferation stimulated by DC differentiated in the absence or presence of 10% MCM. The $CD4^+$ T cell proliferation stimulated by DC was measured by BrdU uptake. Data from three separate experiments are expressed as mean ± SEM.

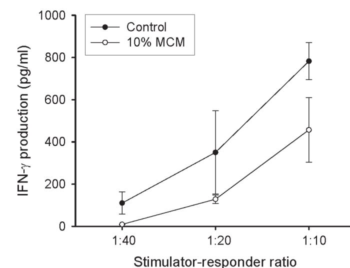

Figure 5. IFN-γ production in allogeneic $CD4^+$ T cell stimulated by DC differentiated in the absence or presence of 10% MCM. IFN-γ production by DC-stimulated $CD4^+$ T cells was measured by ELISA method. Data from three separate experiments are expressed as mean ± SEM.

Table 2. Effect of neutralizing monoclonal antibodies on inhibitory activity of MCM.

	CD11c (%)	CD40 (%)	CD86 (%)	F4/80 (%)
Control	73.2 ± 4.1	78.5 ± 3.7	80.3 ± 3.2	26.3 ± 3.0
10% MCM	39.2 ± 3.1	41.3 ± 4.3	53.5 ± 3.6	65.4 ± 4.1
10% MCM + anti-TGF-β1 (1 µg/ml)	43.3 ± 2.9	45.1 ± 3.5	48.6 ± 4.1	–
10% MCM + anti-TGF-β1 (5 µg/ml)	38.7 ± 4.3	44.5 ± 3.3	50.4 ± 4.3	–
10% MCM + anti-IL-10 (0.1 µg/ml)	46.2 ± 4.5	37.5 ± 4.2	54.3 ± 5.2	–
10% MCM + anti-IL-10 (0.2 µg/ml)	42.2 ± 3.4	41.7 ± 2.5	57.6 ± 3.9	–
10% MCM + anti-TGF-β1 (5 µg/ml) + anti-IL-10 (0.2 µg/ml)	40.6 ± 3.8	45.4 ± 4.1	56.3 ± 3.0	–
10% MCM + anti-M-CSF (10 ng/ml)	62.5 ± 5.0	59.3 ± 3.5	75.2 ± 5.4	43.7 ± 4.5

neutralizing M-CSF activity by specific antibody to M-CSF prevented MCM-mediated inhibition of DC differentiation (Table 2). Inhibition of DC differentiation, on the other hand, was accompanied by up-regulation of F4/80 expression, indicative of a switch towards Mϕ differentiation. This up-regulation by MCM was reduced by neutralizing anti-M-CSF antibody (Table 2).

Discussion

In this investigation, we have shown that Mϕ derived from bone marrow and spleen modulated the differentiation but not the maturation of DC. This effect occurred mainly through the activity of soluble factors, as the effect was no greater in cell co-cultures than in transwell experiments.

Activation of immune responses to pathogens must be controlled at an optimal level and then restored to a resting state upon recovery from the disease. The interaction between various immune cells and the surrounding microenvironment is crucial in the modulation of the immune response. For example, innate lymphocytes [18] such as natural killer cells [19], natural killer T cells [20], and $\gamma\delta$ T cells [21] trigger DC maturation. Regulatory DC, in contrast to their immunogenic counterparts, may contribute to the induction of $CD4^+CD25^+$ regulatory T cells and peripheral tolerance [22]. However, the regulatory activity of Mϕ on DC differentiation and maturation had not been previously investigated.

We found that Mϕ inhibited the differentiation of myeloid DC through soluble factors present in MCM but not via direct contact between these two kinds of cells. Mϕ secrete a diverse array of cytokines, chemokines, and bioactive molecules, including IL-1 [23], IL-6 [24], IL-8 [25], IL-10 [26], TGF-α [27], TGF-β [28], TNF-α [29], IFN-α [30], and IFN-β [31], *etc*. In this study, we showed an increase in the amount of TGF-β1, IL-10, and M-CSF in MCM, three well-known factors that affect DC and Mϕ differentiation [32, 33]. Chomarrat et al. [34] reported that IL-6 might switch the differentiation of monocytes from DC to Mϕ through up-regulation of M-CSF receptor expression. In our work, the concentration of IL-6 was not increased in MCM, suggesting that IL-6 was not involved in the effect of MCM on DC differentiation in this experimental model. Increased TGF-β1 and IL-10 concentrations also did not contribute to the inhibitory activity of Mϕ. Moreover, the results of M-CSF neutralization indicated that M-CSF was responsible for the inhibition by MCM of DC differentiation and a switch towards Mϕ.

We also investigated whether Mϕ inhibited DC differentiation by acting at the stage of initial commitment of monocytes to DC or during the maturation of immature DC. We demonstrated that, in contrast to differentiating DC precursors, already differentiated immature DC were resistant to the inhibitory effects of Mϕ. Furthermore, the earlier Mϕ were added to $CD11b^+$ monocytes during the DC differentiation process, the greater the inhibition, indicating that the target cells of Mϕ are more likely DC precursors at an early stage of DC differentiation. Recently, a clonogenic progenitor expressing both CX_3CR1 and CD117 and specific for Mϕ and DC has been found in mouse bone marrow [35]. Whether Mϕ derived from this progenitor can inhibit DC differentiation remains to be seen.

MCM enhanced the expression of the murine Mϕ marker, F4/80, by DC in a concentration-dependent fashion. However, MCM-treated DC did not possess morphological features of Mϕ, such as pseudopods and abundant cytoplasmic vacuoles. This makes it likely that MCM skews the differentiation of DC precursors towards atypical Mϕ and has no effect on the maturation of immature DC into mature DC.

DC develop and mature normally in the steady state in the presence of Mϕ *in vivo* but the effect of Mϕ on DC differentiation has not been reported. It may be that *in vitro* studies that isolate only a few factors fail to reflect the dynamic cell-cell interactions between various cell lineages in living tissues. We demonstrated the inhibitory effect of Mϕ from bone marrow on DC differentiation but the possible influence of DC on differentiation of Mϕ remains undetermined. Since SDM also inhibited DC dif-

ferentiation (Table 1), the role of Mϕ in the other tissues warrants further study. Moreover, MCM-modulated DC had the capacity to suppress $CD4^+$ T cell proliferation. The fate and function of the resulting $CD4^+$ T cells should be investigated to see whether Mϕ-modulated DC have a role in regulating unwanted immune responses.

In conclusion, our findings indicate that, during DC differentiation, an important interaction occurs between Mϕ and DC precursors, two distinct cell lineages derived from the same $CD11b^+$ monocytes from bone marrow.

Acknowledgements. The authors wish to thank Prof. Shie-Liang Hsieh for technical support and Dr. Mary Jeanne Buttrey for critical reading and correction of the manuscript. This study was supported by the grant NSC-93-2413-H-195-001, MMH-E-95006 and MMH-9578 from Mackay Memorial Hospital, Taipei, Taiwan.

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