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Effect of culture and maturation on human monocyte-derived dendritic cell surface markers, necrosis and antigen binding

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Abstract

Dendritic cells (DC) are antigen-presenting cells (APC) that are important for innate and acquired immune responses. Owing to their involvement in autoinflammation, autoimmunity and cancer, DC are useful cellular models for biomedical research. Appropriate DC production in vitro could aid the study of DC in many human diseases. We used fluorochrome-based flow cytometry assays to analyze the effects of culture period and maturation of monocyte-derived DC (MoDC) on their viability and necrosis, purity, CD11c expression and phagocytic capacity. The morphological changes that occur as purified monocytes become DC were assessed at 24 and 72 h, and 6 and 9 days in culture. The dynamics of certain cell surface markers of monocytes and mature MoDC (mMoDC) also were assessed using fluorescence-based assays. We found that day 6 of culture yielded the most functional immature MoDC (iMoDC) with maximal viability, purity, CD11c expression and appropriate phagocytic capacity. Mass production of viable MoDC could be useful for immunotherapy.

Key words: antigen-presenting cells, CD11c, CD14, cell culture, dendritic cells, flow cytometry, fluorescence, monocyte-derived dendritic cells, necrosis, phagocytosis

Dendritic cells (DC) are important antigen-presenting cells (APC) that participate in innate and acquired immunity (Pasare and Medzhitov 2005, Iwasaki and Medzhitov 2010). DC initiate immune responses that control and eliminate pathogens (Joffre et al. 2009). Although DC constitute only about 1% of mononuclear leukocytes in peripheral blood (Sato and Fujita 2007, Mann et al. 2012), they are localized in other peripheral tissues where they act as immune sentinels that patrol and sample environmental antigens continuously (Joffre et al. 2009). DC express pattern recognition receptors (PRR) that recognize pathogen/damage-associated molecular patterns (P/DAMPs) of microorganisms (Bianchi 2007). DC play a role in immunobiology, immunotolerance and pathogenesis of diseases including cancer.

DC also may be used for bioengineering, immunomodulation and transplantation (Schott 2006, Sumpter et al. 2009). For example, a patient's own immune cells, especially autogenic DC, have been used to treat a wide range of cancers (Steinman and Banchereau 2007, Kirschner 2006). Owing to their prophylactic and therapeutic potential for combating cancer (Kirschner 2006), mass production of pure, viable DC from blood and tissues is important for further analysis and application. Freshly

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produced DC develop small dendrites, but acquire their typical morphology spontaneously after cultivation in vitro (Mehrzad et al. 2014, 2015, Mohammadi et al. 2014).

In humans, monocytes cultured in the presence of GM-CSF and IL-4 acquire some properties of DC (Mann et al. 2012). These monocyte-derived DC (MoDC) efficiently bind and present antigen to naive T-cells by up-regulating MHC-II and co-stimulatory molecules. Furthermore, the cytokine secretion pattern of MoDC causes differentiation of naive CD4-positive T-cells into T-helper (TH)-1, TH2 or TH17 effector or regulatory cells, which in turn drives the ensuing adaptive immune responses (Huang et al. 2001, Joffre et al. 2009, Devriendt et al. 2010, Mehrzad et al. 2014, 2015).

We investigated the most appropriate culture period for producing human effector MoDC including immature MoDC (iMoDC) and mature MoDC (mMoDC) using key cytokines. We assessed our methods using characteristics of DC including viability, purity, necrosis and phagocytosis, to identify surface molecular phenotype, biomarkers of monocytes, iMoDC and mMoDC.

Material and methods

Generation of human MoDC

The blood of healthy males (23 ± 2 years old) was used as a source of peripheral blood mononuclear cells (PBMC) for producing DC. The donors were free of medications. The CBC and total number of circulating WBC were determined using a Coulter counter (MEK-6450K, Nihon Kohden, Tokyo, Japan). Leukocytes were identified microscopically in blood smears (Mehrzad et al. 2001, 2011, Mohammadi et al. 2014). The PBMC were isolated from the blood using Ficoll density gradient centrifugation (Mehrzad and Zhao 2008, Mehrzad et al. 2014, 2015, Mohammadi et al. 2014). Briefly, each blood sample was diluted 1:4 in Dulbecco phosphate-buffered saline (DPBS, without Mg^{+2} and Ca^{+2}), layered on a 15 ml Ficoll-Paque PLUS Lympholyte (Zierikzee, the Netherlands), then centrifuged at $1100 \times g$ at 20° C for 40 min. The PBMC layer was collected and the purified PBMC were washed once with culture medium containing RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin,100 µg/ml streptomycin, then centrifuged at $450 \times g$ at 4° C for 10 min. The PBMC pellets twice were re-suspended, washed as above, then centrifuged at $180 \times g$ at 4° C for 5 min; this procedure yielded >98% viable PBMC. The isolated PBMC from each sample were used to produce MoDC.

Pure monocytes were isolated from PBMC by plastic adherence. Briefly, pure PBMC were dispersed in 3 ml volumes (10^7 PBMC/ml culture medium) into 3 cm diameter culture plates and incubated for 2 h in a 37° C chamber with 95% humidity and 5% CO_2. Non-adherent cells were removed by washing with warm culture medium. To obtain DC, the adherent cell fractions then were incubated at 37° C, 5% CO_2, 95% humidity in DC medium consisting of RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 800 U/ml (72 ng/ml) GM-CSF (R & D Systems, Minneapolis, MN) 1000 U/ml (100 ng/ml) IL-4 (R & D Systems) and 0.05 M 2-ME as described earlier (Mehrzad et al. 2014, 2015, Mohammadi et al. 2014). To obtain DC, the adherent cell fractions were placed in petri dish tissue culture plates at a concentration of 2.5 x 10^6 cells/ml, then incubated in a 95% humidity and 5% CO_2 environment at 37° C for 4, 6 and 9 days with DC medium. iMoDC were harvested on days 4, 6 and 9 of incubation. We had optimized the cultivation time for producing both iMoDC and mMoDC in our report on human (Mohammadi et al. 2014) and porcine (Mehrzad et al. 2014, 2015) models. To produce mMoDC, iMoDC were cultured for an additional $24-48$ h with the following maturation stimuli: 100 ng/ml LPS (Sigma-Aldrich Chemical, Taufkirchen, Germany).

Evaluation of monocytes and MoDC during DC generation

Differential cell counts and confirmation of the quality of PBMC, monocytes, iMoDC and mMoDC were performed by microscopy of eosin-Giemsa stained smears (Mehrzad et al. 2001, Mohammadi et al. 2014). To prepare eosin-Giemsa stained smears, 25 µl human serum and 25 µl of the cell suspension described above were mixed on a coverslip, then spread homogenously and dried completely using cytocentrifugation at 113 x g for 20 sec at room temperature. The coverslip then was mounted on a glass slide for staining and microscopic viewing. Briefly, the smears were stained sequentially using a rapid eosin-Giemsa as follows: 5 sec in methanol for fixation, 5 sec in eosin and 7 sec in Giemsa (Hemacolor®, Merck Diagnostics, Darmstadt, Germany) followed by washing with tap water, then double-distilled water; between applications of stains the slides were drained briefly using tissue paper. After air drying, the smears were used for examination and imaging of the cells using light microscopy at x 1000. The viability and number of each cell type also were confirmed using nigrosin solution and a Neubauer chamber (Mehrzad et al. 2014, 2015,

Mohammadi et al. 2014). Briefly, 0.2 g of water soluble nigrosin powder (Sigma-Aldrich) was dissolved in 100 ml of 0.85 % sodium chloride (dark-bluish solution). A mixture of 10 µl DC suspension and 90 µl of nigrosin solution was placed in microtubes to which an additional 10-fold nigrosin solution was added and used immediately for microscopic cell counting and viability assessment using a hemocytometer. Viable and necrotic cells appeared bright and dark-blue in the mixture, respectively.

Flow cytometry-based staining of cell surface markers

Flow cytometry (FACS; Beckton Dickinson, Franklin Lake, NJ) analysis was performed using antibodies against human cell surface markers such as fluorescein isothiocyanate (FITC)-CD11c, phycoerythrin (PE)-CD40, PE-CD80, PE-CD83, PE-CD86 and PE-HLA-DR (eBioscience, San Diego, CA) to ascertain production of monocytes, iMoDC and mMoDC. Because in our generated MoDC population CD11c was the key surface marker and an indicator of DC, we focused further evaluation mainly on this surface DC marker. The cultured iMoDC assayed by flow cytometry using FITC-labeled α-CD11c mAb were gated on forward scatter-side scatter (FSC-SSC) dot plot. The selected FSC/SSC-gated DC, here called CD11c, were used for detection, analysis and calculation of $CD11c^+$ DC. To account for any nonspecific antibody binding, staining of parallel samples of $CD11c^+$ DC were treated with fluorescein-labeled mouse IgG1 (isotype control) as a control for flow cytometric analysis of iMoDC. A mouse IgG isotype (Immunotech, Quebec, Canada) followed by FITC-labeled goat anti-mouse-Ig (Immunotech) was used as a control for mMoDC. This control normally stains isotype-matched irrelevant mAb to assess nonspecific binding of the cell marker, CD11c, by excluding nonspecific binding in the analysis.

Effect of culture duration on viability/necrosis, purity and CD11c expression of MoDC

The total number, purity, viability/necrosis and CD11c expression of culture grown DC were determined on days 4, 6 and 9 in duplicate using FITC-labeled α-CD11c mAb flow cytometry and propidium iodide (PI) exclusion methods (Mehrzad et al. 2001, Mohammadi et al. 2014). Briefly, after addition of 10 µl of PI (5 µg final concentration) to 490 µl diluted DC (10^6 cells/ml DPBS, final concentration), the red fluorescence of DC selected in the FSC-SSC dot plot was determined as the percentage of necrotic cells, from which the percentage of viable cells could be calculated. In this way, viable and necrotic cells were analyzed using one-dimensional (one fluorochrome/monocolor, i.e., PI) plotting of flow cytometric analysis. MoDC were harvested on ice using gentle flushing by pipetting and washed twice in PBS before subsequent flow cytometric examination.

Effect of duration of culture on MoDC phagocytosis

To determine the effect of culture period on functional activity of MoDC, 10^7 FITC-loaded polystyrene microparticles (1.0 µm, 10 beads/DC; Sigma-Aldrich) were added to 10^6 MoDC on days 4, 6 and 9 of culture and incubated for 3 h at 37° C, 5% CO_2, 95% humidity. MoDC then were harvested on ice, washed with ice-cold PBS and the internalization of the microparticles was assessed by flow cytometry with a minimum event count of 10,000. The results of this assay were recorded as the mean fluorescence intensity, i.e., the intensity of phagocytized microparticles; the number of FITC positive MoDC, which reflected the number of MoDC that could phagocytize microbeads, indicated the functional ability of MoDC.

Statistical methods

The flow cytometry results for viability, purity, percent CD11c and phagocytosis are presented as means ± SEM. The statistical analyses were performed using one-way ANOVA and compared further using Bonferroni test for multiple comparisons. A p value ≤ 0.05 was considered significant.

Results

Microscopy of cells during MoDC production

We obtained PBMC from the blood of healthy males; >98% were viable. The time between the collection of blood and the establishment of DC cultures was less than 3 h. The CBC and hematological characteristics of the collected blood was normal (data not shown). Figure 1 shows examples of eosin-Giemsa staining of PBMC, monocytes and MoDC after 24 h, 72 h, 6 days and 9 days of cultivation.

Flow cytometry (FACS) analyses of monocyes, iMoDC and mMoDC

Monocytes cultured for 48 h in DC medium containing GM-CSF and IL-4 displayed the

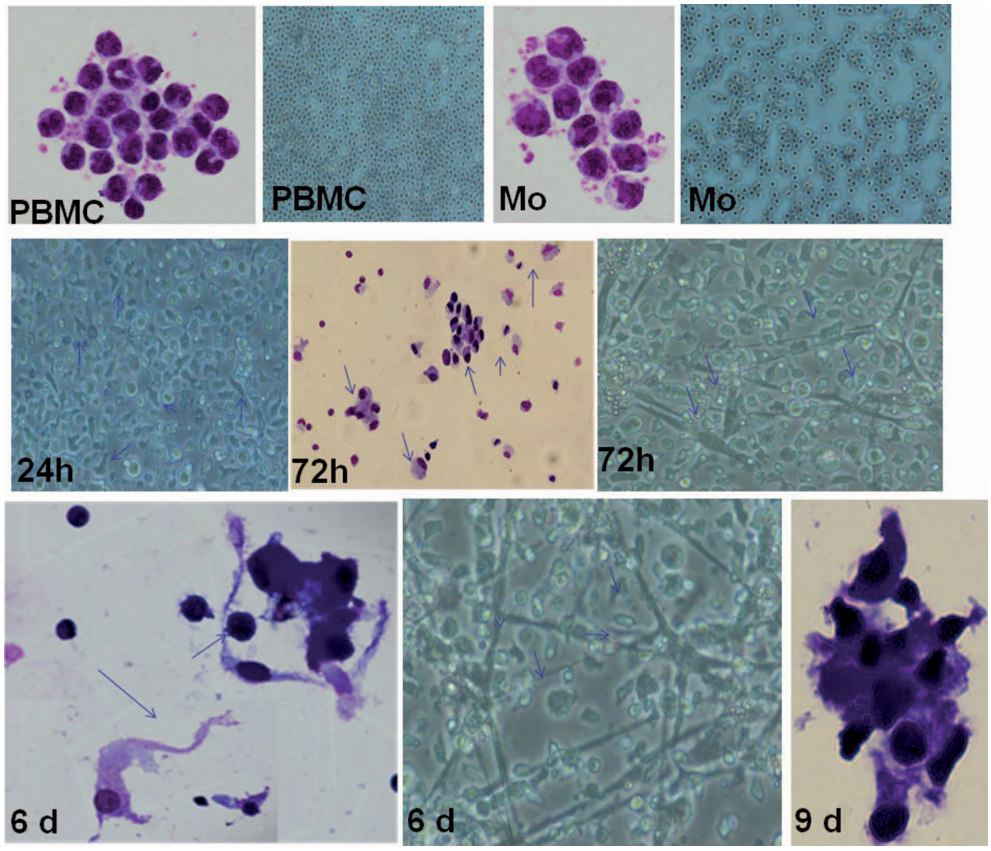

Fig. 1. Isolated PBMC, highly purified monocytes after 2 h culture adhered to the bottom of culture dish, pure monocytes after cultivation for 24 h in which the first step of iDC formation, elongation of some cells, iDC of unusual shape and many long membrane protrusions or dendrites after 72 h cultivation, and iDC after cultivation for 6 and 9 days with some long processes and dendrites. Eosin-Giemsa staining. ×1000. Inverted microscope. ×100.

morphology of monocytes and expressed CD14, CD86 and HLA-DR as analyzed by flow cytometry. Also, flow cytometry was performed to detect surface markers including CD11c, CD40, CD80, CD83, CD86, HLA-DR on monocytes, iMoDC and mMoDC (Fig. 2). Some surface markers specifically identified monocytes ($CD11c^{+/low}$, $HLA\text{-}DR^{-}$, $CD40^{-}$, $CD80/86^{-}$), iMoDC ($CD11c^{+/high}$, $HLA\text{-}DR^{-}$, $CD40^{-/low}$, $CD80/86^{-/low}$) and mMoDC ($CD11c^{-/low}$, $HLA\text{-}DR^{+/high}$ $CD40^{+/high}$, $CD80/86^{+/high}$) (superscripts $^{+}$, $^{-}$, ^{high} and ^{low} mean yes, no, high and slight expressions of the examined cell surface marker(s), respectively) (Fig. 2). There was a significant shift to the right in the expression of CD40, CD80, CD83, CD86, HLA-DR in monocytes, iMoDC and mMoDC during maturation. The CD11c marker was expressed clearly in monocytes and iMoDC (Fig. 2). Stained cultivated DC without and with FITC-labeled α-CD11c mAb revealed a noticeable shift upward and to the right was observed for DC stained with FITC-CD11c (Fig. 2).

Viability/necrosis, purity and CD11c expression of iMoDC on days 4, 6 and 9 of culture

Figure 3 shows determination and confirmation of the quality of culture-grown iMoDC with the key surface iMoDC marker, CD11c. A high proportion of $CD11c^{+}$ iMoDC showed a shift to the right on day 6 of monocyte cultivation to generate iMoDC (Fig. 3). The DC produced from pure monocytes by 6 days of culture yielded >92% $CD11c^{+}$ DC with a viability of >93% (Fig. 4). Compared to the CD11c on day 6, the iMoDC expressed less CD11c on day 4, which meant that the cells needed more time to differentiate (Fig. 3). After culture for 9 days for DC differentiation, the DC lost much of their viability, purity (Fig. 4) and phagocytic capacity (Fig. 5).

The greatest viability, purity, and CD11c expression were observed on day 6, but a reduction in all parameters was observed by day 9 (Fig. 4). Viability and purity differed little between days 4 and 6 of culture, but we observed significant reductions

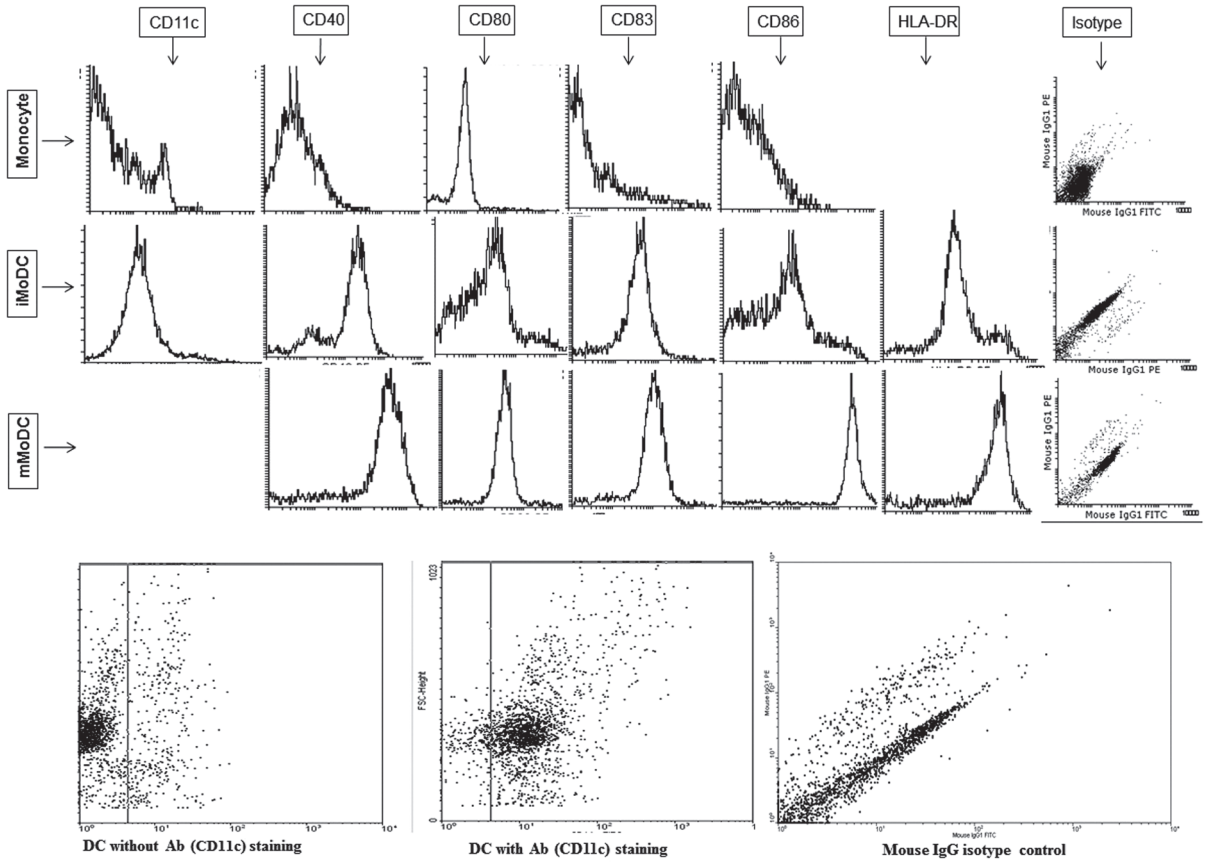

Fig. 2. Confirmation of monocytes, iMoDC, mMoDC using fluorochrome conjugated cell markers (CD11c, CD40, CD80, CD83, CD86 and HLA-DR). By increasing the differentiation of cells from monocytes toward DC, expression of these markers on cell surface increased (upper panel). $CD11c^+$ DC on the representative forward scatter (FSC) and side scatter (SSC) gate and dot plots for both DC with and without α-CD11c-FITC; a homogenous $CD11c^+$ cell population was obtained (lower panel).

after 6 days. Further, expression of CD11c on day 4 was approximately 60%; by contrast, on day 6 it approached 95%.

Phagocytosis of $CD11c^+$ iMoDC

We also investigated the effect of culture period on DC-mediated phagocytosis of microbeads. We observed a significant decrease in phagocytosis by iMoDC during culture on days 4, 6 and 9, but especially on day 9 (Fig. 5). Elongation of MoDC in culture increased their antigen presentation, but decreased their phagocytosis. The results of our functional assay were consistent with the results of our study of viability, necrosis and purity of MoDC on days 4, 6 and 9.

Discussion

DC play important immunobiological roles in both innate and acquired immune responses against infectious diseases (Yamanaka et al. 2005, Schott 2006) and for DC-based immunotherapy for cancer (Steinman and Banchereau 2007, Kirschner 2006). The number of DC in the peripheral blood normally is low and they are difficult to separate from other peripheral blood leukocytes (Nicolette et al. 2007, Mann et al. 2012). We used monocytes to produce mature DC in vitro ultimately to supplement DC in peripheral blood.

The iDC are characterized by intermediate surface expression of MHC-II and low levels of co-stimulatory molecules including CD80/CD86 and CD40. The iDC can engulf antigens derived from pathogens and dead or dying cells, and can respond to indirect inflammatory signals (Stift et al. 2004, Münz et al. 2005). iDC process antigens efficiently, but they activate naïve T cells less efficiently. By contrast, mDC show poor phagocytic activity against foreign proteins and antigens, but their ability to present processed antigens to activate T-cells is more efficient (Winzler et al. 1997). We observed a similar pattern in our DC culture system.

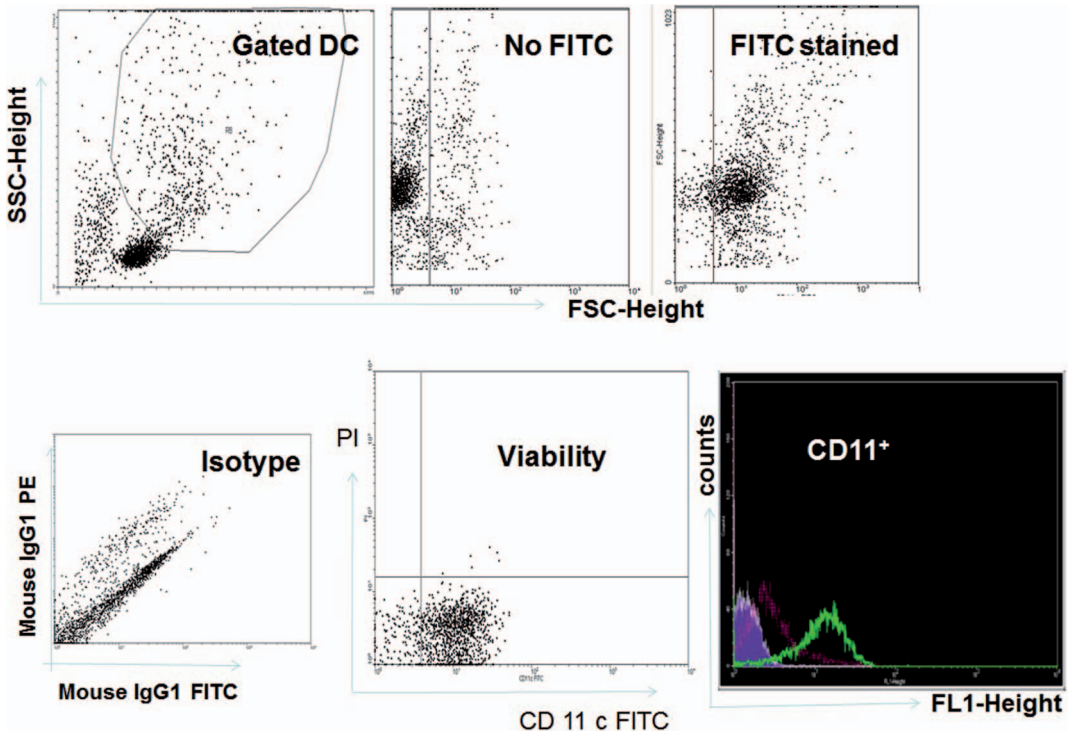

Fig. 3. Flow cytometry analysis of CD11c$^+$ using gated (FSC-SSC) dot plot on day 4 vs. day 6. DC stained without and with fluorescein isothiocyanate (FITC)-labeled α-CD11c mAb. Mouse IgG isotype with two-color, phycoerythrin (PE) and FITC, flow cytometry using CD11c$^+$ DC as control. Culture of DC from PBMC yielded > 92% CD11c$^+$ DC and viability > 93%. Lower right, flow cytometry results of CD11c$^+$iDC on day 4 (red) and day 6 (green) vs. control without FITC staining sample (purple). The high proportion of CD11c$^+$iDC (green) indicated a shift to the right on day 6 of monocyte culture to generate DC. On day 4 of DC generation (red), the DC expressed less CD11c.

Although there are some methods for isolating large numbers of DC for clinical and biomedical application (Berger et al. 2005), the preferred method is to produce large numbers of DC from isolated monocytes by adherence methods using IL-4 and GM-CSF followed by LPS (Mann et al. 2012), TNF-α or INF-γ to produce mDC. Sterility before and during culture of DC is vital. Preparation of fresh sterile aliquots without re-freezing is central to DC culture. It is important to prepare aliquots from the main stocks of solution, media, cytokines, reagents, antibodies and antibiotics etc. to avoid unnecessarily freezing and thawing; frequent freezing and thawing diminishes the quality of the biomolecules and reagents, which affects adversely the function of DC both in vitro and in vivo.

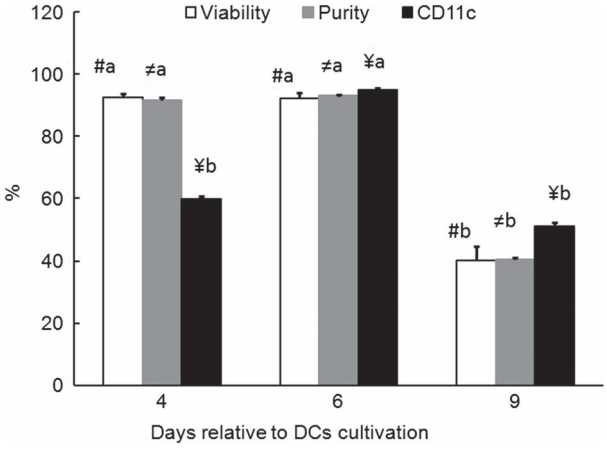

Fig. 4. Overall results of viability, purity and CD11c expression of monocyte-derived DC (MoDC) with flow cytometric analyses of DC on days 4, 6 and 9. Values are means ± SEM of DC from seven healthy individuals. Different letters with their symbols (i.e., #, ≠ and ¥, are viability, purity and CD11c, respectively) indicate significance ($p < 0.001$).

Other CD11c$^+$ expressing cells, including neutrophils, were excluded during PBMC isolation, therefore the CD11c$^+$ we detected was solely on MoDC. Technically, the long processes of DC usually are problematic for flow cytometry, which makes analysis of DC more difficult compared to other blood cells (Mehrzad and Zhao 2008, 2011, 2014, 2015, Mohammadi et al. 2014). Therefore, the FSC-SSC gate for flow cytometry assays of DC must be very large.

The time of harvest (incubation duration) of isolated monocytes to produce iMoDC also is critical

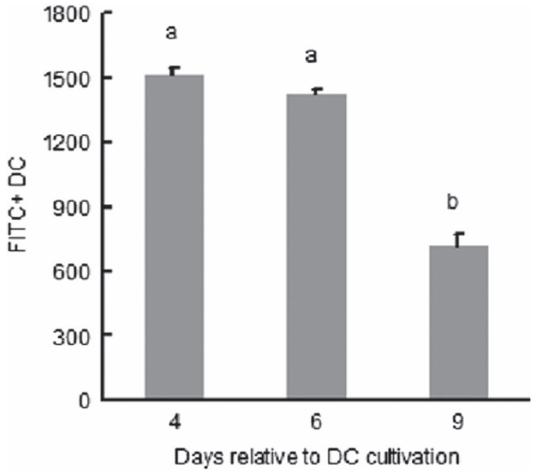

Fig. 5. Cultivation period, i.e., days 4, 6 and 9, affects the phagocytic activity of MoDC. Bars represent the number of FITC-positive MoDC. The data are presented as the mean percent ± SEM (n = 7). Different letters on the bars indicate significant differences ($p < 0.05$).

for DC culture. We investigated factors including viability and necrosis, CD11c expression, purity and phagocytic capacity of iMoDC on days 4, 6 and 9 of monocyte culture, and confirmed these factors using macroscopic and flow cytometric assays. On day 4, despite the presence of distinct membrane protrusions or dendrites, a structural hallmark of DC, and high viability and high purity (≥ 90%), the expression of the surface CD11c molecule did not reach the maximum level by day 4 (approximately 60%); therefore, the generated cells were not fully functional DC.

When we harvested MoDC at 9 days, our functional assays showed that the DC had substantially lost their viability (approximately 30%, with 70% necrosis), surface marker CD11c (approximately 50%) and phagocytic capacity (Figs 4, 5), which indicated low functional capacity of MoDC by day 9 of cultivation. On days 4 and 6, MoDC exhibited great phagocytic capacity in our functional test.

We found that approximately 6 days in culture are required to produce functional iMoDC with the appropriate expression of surface marker, $CD11c^+$ (approximately 95%) and > 90% purity and viability, which suggests that iMoDC require at least 6 days of cultivation to reach their fully functional state.

The greatest percentage of viability, purity and $CD11c^+$ expression on day 6 rather than day 9 might be due, in part, to the long dendrites, space constraint and poor provision of nutrients or secretion of some harmful metabolites in the media. Although we did not evaluate DC function on days 7 and 8, it is possible that those incubation periods may be the peak harvest time; this requires further evaluation.

To produce immunologically functional MoDC with maximal viability, purity and phagocytic capacity it is necessary to culture highly purified $CD14^+$ peripheral blood monocytes for 5 − 6 days. Prolonged cultivation without refreshing DC medium causes substantially decreased functional characteristics, as we observed on day 9 of culture; these functional changes require further molecular analyses to determine the underlying causes.

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