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Characterization of canine monocyte-derived dendritic cells with phenotypic and functional differentiation

Running title: An improve method to generate canine dendritic cells

Abstract

For therapeutic purposes, large numbers of dendritic cells (DC) are essential. However, the isolation efficiency of canine DC was low. In this study, we established a modified adherence step to reliably enrich CD14⁺ monocytes from peripheral blood mononuclear cells (PBMC). Canine DC were generated from the adherent monocytes. Adherent monocytes were cultured for 6 days in human GM-CSF, Flt3L, and canine IL-4. This improved procedure yielded 2–4 times more canine DC than other published methods. Canine DC characteristics, including surface phenotype and biological functions during maturation, were also investigated in this study. In canine immature DC (iDC), DLA class II, CD1a, CD11c, CD40, and CD86 expression was high, based on flow cytometry and RT-PCR assays. During maturation, which was stimulated by LPS, CD80 and CD83 were up-regulated in mDC. However, DLA class II, CD1a, CD11c, and CD40 did not increase in mature DC (mDC). Functional maturation was assessed by antigen uptake ability and the allogenic MLR. Our data showed that incubating canine iDC with LPS decreased antigen uptake and increased the immunostimulatory capacity, indicating that LPS accelerates DC maturation. The TNF- α gene expression was decreased during DC differentiation from monocytes confirm the functional difference between monocytes and DC. This new protocol markedly increased the cell number with *in vitro* culture of DC precursors and thus may facilitate the use of DC in clinical cellular immunotherapy.

Introduction

Dendritic cells (DC) plays a fundamental role both in innate and adaptive immune responses (1, 2) and also plays a critical role against tumor growth by presenting tumor-specific antigens and initiating an effective immune response (3). The effectiveness of DC as antigen-presenting cells (APC) provides the rationale as cellular adjuvants for inducing a durable, anti-tumor immune response. The precursors of DC circulate in blood with an intermediate differentiation/activation phenotype and represent a small (less than 1%) proportion of blood mononuclear cells (MNC). The precursors move from the circulation into the tissues in where they acquire a fully mature APC phenotype after interaction with pathogens or other stimuli. The key surface molecules such as CD40, CD80 and CD86, which are involved in costimulation and served as markers of mature DC are overexpressed on surface of differentiation/activation DC (4). A large quantity of DC cells is required for basic and clinical studies. The development of techniques to generate large numbers of DC in culture, from either proliferating CD34⁺ progenitors (5, 6) or non-proliferating CD14⁺ monocytic precursors is required for DC immunotherapy due to low DC numbers in cell tissues naturally (7, 8). Several studies have identified proliferating within the small CD34⁺ subfraction of cells in human blood (5, 9, 10). These can be stimulated with cytokines, particularly GM-CSF and TNF- α , to develop into potent dendritic cells over 1-2 weeks in culture (5). In addition, the removal of monocytes and lymphocytes from human blood has uncovered a small population of nonproliferating progenitors that require cytokines to develop into typical dendritic cells, about 10⁶ cells per 450-500 ml of peripheral blood (11-13). Human DC have been generated significantly larger numbers *in vitro* from adherent blood monocytes after culture with the cytokines GM-CSF and interleukin-4 (IL-4), about 3-8 $\times$ 10⁶ per 40 ml of blood (7, 8). Inaba et al.

(14, 15) isolated proliferating cells in mouse blood that differentiated into DC after 7 days of culture in presence of granulocyte macrophage-colony-stimulating factor (GM-CSF). In vitro-generated monocyte-derived DC have the ability to take up, process and present foreign antigens. Monocyte-derived DC are currently most widely used since they can be generated relatively simply without need for any cytokine (e.g. G-CSF) pretreatment of the donor. The resulting DC progeny are at present the most homogenous and best characterized DC population which have also been used in those trials (16-18). In contrast to monocyte-derived DC, the bone marrow-derived DC is too invasive for routine use.

Several studies have demonstrated that canine DC can be cultured from peripheral blood monocytes or bone marrow (19-24). However, isolation efficiency was low, and surface phenotype studies were incomplete (22, 23). Although Bonnefont-Rebeix et al. showed that anti-human CD86 antibody can cross-reactive to canine DC (24), other surface molecules of DC such as CD40, CD80 and CD83 that commonly used in humans for characterization of DC maturation state are still lack. In this study, we revealed a procedure for efficiently generating canine DC from peripheral blood mononuclear cells (PBMC) and showed the key surface markers that expression on canine DC such as CD40, CD80, CD86 and CD83. The method could generate DC 2–4 times more than other methods tested. We investigated two cross-reacting antibodies against canine CD40 and CD80. Using RT-PCR, CD83 was detected on mDC. The functions profiles of the DC obtained from PBMC were also determined. In addition we analysed the LPS response of DC to express TNF- α gene to verify the differentiation process from monocytes. This technology greatly facilitates the use of canine DC for immunotherapy study.

Materials and Methods

Source Animals and the Generation of Monocyte-Derived DC

Peripheral blood was obtained from seven healthy beagles. All dogs were dewormed regularly and vaccinated against distemper, leptospirosis, parvovirus, and canine hepatitis. PBMC were isolated from heparinized whole blood by standard gradient centrifugation with Ficoll-Hypaque (density: 1.077, Amersham Pharmacia Biotech, Piscataway, NJ) (25). PBMC were harvested from the interface, washed twice, and re-suspended in RPMI-1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and various concentrations of canine autologous serum or heat-inactivated fetal bovine serum (FBS, Life Technologies). For testing the isolation efficiency of adherence, 10 % FBS, 2 % FBS, 10 % autologous serum or 2 % autologous serum were used to prepare the adherent monocytes. The PBMC were allowed to adhere to a 25-cm² flask (1×10^7 cells/ml) for 24 h at 37 °C. To obtain immature DC (iDC), nonadherent cells were removed and adherent cells were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 800 U/ml human GM-CSF (Leucomax; Schering-Plough, Kenilworth, NJ), 500 U/ml canine IL-4 (R&D Systems, Minneapolis, MN), 200 ng/ml human Flt3 ligand (Flt3L, R&D Systems) and 10 % FBS for another 6 days (19, 26, 27). GM-CSF and Flt3L have been proven to be bioactive for canine cells (19). Fresh medium and cytokines were added every 3 days. To prepare mature, activated DC (mDC), iDC were incubated for 48 h with 10 µg/ml LPS (*Escherichia coli* serotype 0128:B12, Sigma, St. Louis, MO). At the end of the incubation period, the putative iDC and mDC were harvested for morphological, phenotypic or functional analyses.

Morphological Examination

Each DC preparation was examined by phase contrast microscopy (Nikon, Tokyo, Japan). For cytological study, iDC were smeared on a glass slide, allowed to air-dry, and stained with May-Grunwald Giemsa stain (Diff-Quick; Gamidor, Abingdon, Oxfordshire, U.K.). For transmission electron microscopy (TEM), iDC pellets obtained after centrifugation (1000 x g) were fixed in 2.5 % glutaraldehyde in 0.1 M/pH 7.4 phosphate-buffered saline (PBS) at 4 °C overnight and post-fixed in 1 % osmium-tetroxide at room temperature for 1 h. The blocks of iDC were dehydrated in graded ethanol solutions, then they were embedded with Spurr's resin kit (EMS, Washington, PA). Ultrathin sections were cut with a diamond knife using Reichert-Jung Ultracut E (Reichert-Jung, Vienna, Austria). The sections were stained with a 5 % aqueous solution of uranylacetate for 20 min, and then they were stained with Reynold lead citrate for 4 min. After staining, the sections were examined with a JEOL 1200EXII transmission electron microscope (JEOL USA, Peabody, MA).

Flow Cytometry Analysis

Primary antibodies used for flow cytometry analyses are listed in Table 1. FITC-conjugated goat anti-mouse IgG antibody (Serotec, Oxford, U.K.) was used as the secondary antibody. DC were stained following procedures described previously (25). Briefly, for direct immuno-fluorescence analysis, cells (5×10^4) were washed twice with FACS buffer (PBS, 1 % BSA, 0.02 % sodium azide, pH 7.4). Cells were incubated for 30 min, on ice and in the dark, with isotype control or specific mouse monoclonal antibodies to detect CD1a, CD14, CD40, and CD80. For indirect immuno-fluorescence analysis, cells were incubated with isotype control or specific monoclonal antibodies against CD11c, and DLA II. Cells were washed and further stained with FITC-conjugated goat anti-mouse IgG for 30 min. Finally, all cells were

washed and suspended in FACS buffer containing 5 µg/ml propidium iodide. The surface immuno-fluorescence of 1×10^4 viable cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Fluorescence intensities were analyzed with Cell Quest Software (Becton Dickinson).

RT-PCR Analysis of CD80, CD83, and CD86 Expression in Canine DC

Because reagents for specifically staining canine CD86 and CD83 are not available commercially, the RT-PCR was used to identify the surface molecules CD80, CD83, and CD86. Total RNA was extracted from iDC and mDC with TRIzol (GIBCO-BRL, Grand Island, NY) and then reverse transcribed with SuperScript II reverse transcriptase (GIBCO-BRL) and oligo(dT) primers. The reverse-transcribed reaction products were subjected to PCR for 35 cycles, using Taq polymerase (TakaRa Shuzo, Kyoto, Japan) (28) and the primers for canine CD80, CD86, and CD83 genes. We designed RT-PCR primers between different exons to avoid any amplification of DNA. The PCR products were electro-phoresed in 1-% agarose gel and stained with ethidium bromide. The following were the sequences of the primers used: CD80 sense primer (5'-ATGGATTACA CAGCGAAGTG GAGAA-3') and anti-sense primer (5'-AGGCGCAGAG CCATAATCAC GAT-3'), CD83 sense primer (5'-CAGTCATATA AAAGCTATGG TGAGAT-3') and anti-sense primer (5'-AGATGAAAAG GCCCTGCTGG GG-3'), CD86 sense primer (5'-ATGTATCTCA GATGCACTAT GGAAC-3') and anti-sense primer (5'-TTCTCTTTGC CTCTGTATAG CTCGT-3').

Real-time PCR

Real-time PCR was performed as previously described (29). In brief, Primer Express software (Applied Biosystems) was used to design appropriate primers pairs. The primer sequences used were: β -actin forward- GACCCTGAAGTACCCATTGAG,

β -actin reverse- TTGTAGAAGGTGTGGTGCCAGAT; TNF- α forward-
GAGCCGACGTGCCAATG, TNF- α reverse- CAACCCATCTGACGGCACTA.

Real-time RT-PCR was performed on the ABI Prism 5700 (Applied Biosystems) using Sybr Green PCR Master Mix following the manufacturer's instructions. To normalize gene expression, a parallel amplification of endogenous and target genes was also performed. Amplifications without reverse transcription or template were included as negative controls. Relative quantitative evaluation of the amplification products was performed by comparing threshold cycle (ΔC_t), as previously described (Applied Biosystems user bulletin no. 2).

Measuring FITC-Dextran Uptake

Endocytic activity was assessed by incubating cells for 2 h with FITC-dextran (100 μ g/ml) (Sigma) at 4°C or 37°C. Cells were washed extensively with PBS. Non-specific binding of FITC-dextran to the cell surface was measured by incubating the cells 4°C (30).

Allogeneic MLR

Freshly prepared DC were washed and combined with untreated PBMC from an unrelated beagle. Mitomycin C-treated DC were added (in triplicates) in graded doses to 1×10^5 PBMC per well in 96-well flat-bottom plates and co-cultivated for 5 days in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 10 % FBS. Then, 1 μ Ci [3 H] thymidine (New England Nuclear, Boston, MA) was added to each well. After 16 h, cells were harvested, washed, transferred to glass fiber membranes (Canberra Packard, Schwadorf, Austria), and counted in scintillation mix (Canberra Packard, Schwadorf, Austria) in an imaging reader (Canberra Packard) (28, 31).

Statistical Analysis

All results were expressed as means $\pm$ SD and were analyzed with a two-tailed

Student's *t* tes. Differences were considered statistically significant at $P < 0.05$.

Results

A modified adherence step is sufficient to enrich DC precursors from PBMC

Monocytes for DC generation were isolated from canine PBMC. Following an adherence protocol, the results showed that the medium with 10% or 2% fetal bovine serum (FBS) caused low adherence efficiency. As described for human DC isolation (32, 33), medium with 2% autologous serum increased adherence efficiency of canine PBMC to 50 % more and generate more monocytes compared with 10% autologous serum (Table 1A). Although the medium without any serum increased the adherent cells, the percentage of monocytes were decreased (Table 1A). Therefore, 2% autologous serum was added into the medium for adherence step. Consequently, adherent PBMC were cultured with GM-CSF, IL-4, Flt3L, and 10% FCS for 6 days to obtain iDC. This procedure produced $0.5 - 1 \times 10^6$ cells per 20 ml of peripheral blood. To further increase the number of DC, Flt3L was used to test the ability during DC culture. In addition, human Flt3L (200 ng/ml) in the medium doubled the harvest of DC ($2.0 - 4.0 \times 10^6$) (Table 1B). Purity was assessed by staining slides with May-Grunwald Giemsa stain and 80–85 % of cells identified as the same cells by this method.

Morphology of Canine Monocyte-Derived DC

Phase contrast and light microscopy were used to examine the morphology of each DC preparation. Putative immature DC (iDC) usually exhibited typical dendritic cell morphology (Fig 1A, left) and adhered to the bottom of the flask in clusters (Fig1A, right). Although the cells varied in size and shape, each cell had numerous dendrites radiating from the surface and a large lobulated nucleus (Fig 1B). Ultra-structurally, iDC cytoplasmic dendrites had surface irregularity consisting of long, narrow

cytoplasmic protrusions. Randomly selected specimens were examined by TEM (Fig 1C). These iDC had a plasmacytoid appearance and contained extensive amounts of endoplasmic reticulum (Fig 1C).

DC Phenotype

Eight commercial monoclonal antibodies were determined whether have the activities to cross-react with canine DC surface antigens (Table 2). The antibody clones for CD1a, CD14, CD40 and CD80 could cross-reacted with canine cells. Based on forward and side scattering characteristics, fresh dog PBMC included mainly lymphocytes and monocytes with few granulocytes and RBC (Fig 2A, left). After 6 days of culture, all cells were distinctly larger, non-lymphoid appearance with characteristic of DC (Fig 2A, right). The expression of CD14, a monocyte marker, on PBMC and the putative iDC were measured. CD14 expression of non-adherent and adherent cells during the first day of DC culture were also measured. CD14 was expressed in 10 to 15 % of PBMC, while non-adherent PBMC was negative for CD14 with high CD3 and CD21, and adherent cells expressed very high levels of CD14 with negative for CD3 and CD21 (Figs 2B and 2C). Those characterizations were strongly suggesting that adherent cells were monocytes origin on the first day of culture and the non-adherent cells were lymphocytes. The CD14 became low expression in the cells 6 days later, those are iDC (Fig 2B). iDC homogeneity, as determined by flow cytometry, quick stain of iDC suspension smear, and CD14 negativity, confirmed the absence of other cell types in iDC and mDC. The iDC expressed high levels of CD1a ($72.9 \pm 1.06\%$), CD11c ($29.48 \pm 1.42\%$), CD40 ($65.3 \pm 1.48\%$), and DLA class II molecules ($68.72 \pm 1.33\%$) (Fig 3). Two days after LPS treated, the levels of CD1a ($81.77 \pm 1.32\%$), CD40 ($75.92 \pm 1.48\%$), DLA class II molecules ($73.68 \pm 2.16\%$) and CD80 ($16.7 \pm 1.58\%$) were significantly more increased ($P < 0.05$), which indicated DC maturation. The level

of CD11c was expression constantly during DC maturation ($29.99 \pm 5.49\%$) (Fig 3A). In human, CD83 expression is strongly up-regulated during DC maturation process (34). Because of the restrictions associated with using monoclonal antibody to determine expression of CD83 in canine DC, RT-PCR were used to determine the state of DC maturation. As a control for the quality of the RNA preparation and PCR analyses, β -actin was amplified in all samples from reverse transcribed RNA. Polymerase chain reaction analysis was also performed using samples containing RNA that was not reverse transcribed to exclude amplification of contaminating DNA. Through RT-PCR, we found that LPS-treated mature DC (mDC) expressed high levels of CD80, CD83, and CD86, but iDC expressed CD80 and CD86 only (Fig 3B). CD86 were expression constantly between iDC and mDC.

Endocytotic Activity

Immature DC display potent endocytic activity, which decreases upon maturation. To study the endocytic capacity of iDC and mDC, we evaluated iDC and LPS-treated mDC endocytotic activity by measuring the phagocytosis of mannose receptor-mediated of FITC-dextran (Fig 4A). LPS-treated mDC showed low levels of FITC-dextran uptake ($38.1\% \pm 0.38\%$; $n=5$) compare with iDC ($55.9\% \pm 2.87\%$; $P=0.01$ vs mDC; Student's *t* test).

Determination of stimuli for maturation of dendritic cells

To test whether maturation could be increased the immunostimulatory capacity, an allogeneic MLR was used to evaluate the stimulatory capacity of DC with allogenic PBMC as responder cells. Mitomycin C-treated autologous fresh PBMC from the same donors were used as controls. Compared to mitomycin C-treated autologous fresh PBMC or mitomycin C-treated iDC, mitomycin C-treated mDC were able to stimulate allogenic PBMC very efficiently ($P<0.05$) (Fig 4B).

Confirmation of dendritic cells differentiation and functional difference

Flow cytometry revealed that monocytes expressed high levels of CD14. Monocytes that differentiated into DC undergo upregulation of CD1a and CD40, as well as a loss of CD14. As illustrated in Fig 5A, the monocytes differentiated into DC that decreased the expression of CD14 and did not increase during DC maturation. We compared the LPS response of monocytes, iDC and mDC. Cells were stimulated with LPS and the expression of TNF- α mRNA was analysed by real-time RT-PCR. Monocytes and iDC were stimulated 48h with LPS, iDC became mDC. LPS-treated monocytes expressed high levels of TNF- α after LPS stimulation (Fig 5B). iDC and mDC were less sensitive to LPS stimulation (Fig 5B).

Discussion

This paper described a modification method of generating canine DC by enriching adherent cells for large scale isolation and culture of monocytes from PBMC.

Phenotypic evidence indicates that the great majority of adherent cells from canine PBMC were non-lymphoid cells and positive for monocytes that were CD3 and CD21 negative and CD14 positive. The morphologic studies by light and electron microscopy revealed a great similarity of these canine cells to iDC from humans and mice.

Immunologically, the iDC from canine PBMC were positive for CD1a, CD11c, CD40 and DLA Class II molecules. Through RT-PCR, DC also expressed CD80, CD83, and CD86, which are used to define the maturity of DC. Thus, the adherent canine monocytes that cultured for 6 days in DC medium were iDC. Two days after LPS treated, a significant decrease of dextran intake and increase of allogenic reaction, support the view that the LPC-treated iDC were becoming mature. The high levels expression of CD83 provide further evidence to prove that LPS-treated iDC were matured.

DC play a major role in the generation of immunity against tumor cells. DC mainly resides in tissues, and they represent only a small portion (less than 1 %) of peripheral blood leukocytes. For both experimental and therapeutic purposes, a procedure for efficiently generating large numbers of DC is crucial. In the literature, there have been several papers that described the methods of *in vitro* culture of canine DC(19, 21-23), three of them were related to the generation of monocyte-derived DC. Canine monocyte-derived DC have been generated using only phyto-hemagglutinin (PHA) (21) or canine GM-CSF, IL-4, and human Flt-3L (22). The lack of data to clarify the percentage of T cells and the purity of DC as cultured with PHA makes it difficult to evaluate the efficiency of DC generation. Catchpole et al. (2002) used 10% culture

supernatants of recombinant Chinese hamster ovary cells expressing canine IL-4 and GM-CSF as their sources of the cytokines in generating canine DC. The precise concentrations or IU of these cytokines in the supernatants were not determined in the report. Ibisich et al. described a procedure to generate canine DC, but their DC expressed high level of CD14 that made us hard to define the different stages of canine DC(23). The purification of monocyte is the most important step for generation of DC. We found in our experiments that 2% autologous serum achieved twice the amount of attachment efficiency ($10.8 \pm 2.4 \%$) compared with 10 % FBS ($5.1 \pm 2.5 \%$) with the highest monocyte content ($88.35 \pm 4.19 \%$) among all the tested groups. Canine autologous serum helps monocytes adherent, and this simple procedure doubled the numbers of adherent cells from PBMC. Thus, it was one of the key ingredients for increasing the total number of DC. Flt3L is an important regulator of DC growth and a critical cytokine for *ex vivo* expansion of DC in both mice and humans(26, 35). We found that adding of Flt3L to the medium generated 2–4 times more canine iDC than the medium without Flt3L.

Previous studies of canine DC surface phenotypes have focused on CD1a, CD11c, DLA Class II and CD86(22-24). We demonstrated that, in addition to CD1a, CD11c, CD86 and DLA Class II, canine iDC expressed high levels of CD40. We found the levels of CD1a, CD40, CD80, and CD83 on mDC were significantly increased over iDC, but levels of CD11c, CD86 and DLA Class II were similar in both iDC and mDC. These observations are unusual because in humans and mice the levels of surface antigens mentioned above usually increased significantly during DC maturation (36). These differences among species merit further study. The mRNA data from the RT-PCR confirmed that CD83 expression increased during canine DC maturation. Canine mDC expressed CD80 and CD83 mRNA, but iDC did not express CD83 mRNA. Similar

results were observed for human and mouse DC (14, 33). Functional maturation was assessed by antigen uptake ability and the allogenic MLR. Our data proved that incubating canine iDC with LPS decreased antigen uptake and increased the immunostimulatory capacity, indicating that LPS accelerates iDC maturation. Dextran-FITC uptake and immunostimulatory assay were often used to assay DC function in other species. Our data suggests that these methods are also good for the canine DC functional assay.

DC have multiple functions, with different subtypes performing different functions. Specialized DC subtypes represent different activation states of a single lineage. The functional differences among subtypes depend entirely upon local environmental signals. We investigated the LPS response of monocytes versus DC in terms of TNF- α gene expression. DC were less responsive to LPS, which can be explained by the low CD14 expression on DC compared for monocytes. Those low expression of CD14 and TNF- α gene expression confirmed that DC were differentiation from monocytes successfully.

In summary, we developed a method for efficiently generating canine DC from PBMC. Autologous serum and Flt-3L were important in increasing the number of canine iDC generated from PBMC. The development of method of improving ex vivo amplification of canine DC is important for immunotherapy of canine diseases. Canine iDC surface antigen expression patterns were well defined in our study. This new protocol may facilitate the use of DC in clinical cellular immunotherapy.

References

1. Hart, D.N. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 1997;90:3245-3287.
2. Clark, G.J., Angel, N., Kato, M., Lopez, J.A., MacDonald, K., Vuckovic, S., Hart, D.N. The role of dendritic cells in the innate immune system. *Microbes Infect* 2000;2:257-272.
3. Steinman, R.M. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991;9:271-296.
4. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., Palucka, K. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767-811.
5. Caux, C., Dezutter-Dambuyant, C., Schmitt, D., Banchereau, J. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 1992;360:258-261.
6. Siena, S., Di Nicola, M., Bregni, M., Mortarini, R., Anichini, A., Lombardi, L., Ravagnani, F., Parmiani, G., Gianni, A.M. Massive ex vivo generation of functional dendritic cells from mobilized CD34+ blood progenitors for anticancer therapy. *Exp Hematol* 1995;23:1463-1471.
7. Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M., Schuler, G. Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994;180:83-93.
8. Sallusto, F., Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 1994;179:1109-1118.
9. Caux, C., Massacrier, C., Dezutter-Dambuyant, C., Vanbervliet, B., Jacquet, C., Schmitt, D., Banchereau, J. Human dendritic Langerhans cells generated in vitro from CD34+ progenitors can prime naive CD4+ T cells and process soluble antigen. *J Immunol* 1995;155:5427-5435.
10. Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., Banchereau, J. Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* 1994;180:1263-1272.
11. O'Doherty, U., Peng, M., Gezelter, S., Swiggard, W.J., Betjes, M., Bhardwaj, N., Steinman, R.M. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* 1994;82:487-493.
12. O'Doherty, U., Steinman, R.M., Peng, M., Cameron, P.U., Gezelter, S., Kopeloff, I., Swiggard, W.J., Pope, M., Bhardwaj, N. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J Exp Med* 1993;178:1067-1076.
13. Thomas, R., Davis, L.S., Lipsky, P.E. Isolation and characterization of human peripheral blood dendritic cells. *J Immunol* 1993;150:821-834.
14. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., Steinman, R.M. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176:1693-1702.
15. Inaba, K., Steinman, R.M., Pack, M.W., Aya, H., Inaba, M., Sudo, T., Wolpe, S.,

- Schuler, G. Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med* 1992;175:1157-1167.
16. Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E.B., Steinman, R.M., Enk, A., Kampgen, E., Schuler, G. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999;190:1669-1678.
17. Dhodapkar, M.V., Krasovsky, J., Steinman, R.M., Bhardwaj, N. Mature dendritic cells boost functionally superior CD8(+) T-cell in humans without foreign helper epitopes. *J Clin Invest* 2000;105:R9-R14.
18. Dhodapkar, M.V., Steinman, R.M., Sapp, M., Desai, H., Fossella, C., Krasovsky, J., Donahoe, S.M., Dunbar, P.R., Cerundolo, V., Nixon, D.F., Bhardwaj, N. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest* 1999;104:173-180.
19. Hagglund, H.G., McSweeney, P.A., Mathioudakis, G., Bruno, B., Georges, G.E., Gass, M.J., Moore, P., Sale, G.E., Storb, R., Nash, R.A. Ex vivo expansion of canine dendritic cells from CD34+ bone marrow progenitor cells. *Transplantation* 2000;70:1437-1442.
20. Weber, M., Lange, C., Gunther, W., Franz, M., Kremmer, E., Kolb, H.J. Minor histocompatibility antigens on canine hemopoietic progenitor cells. *J Immunol* 2003;170:5861-5868.
21. Yoshida, H., Momoi, Y., Taga, N., Ide, K., Yamazoe, K., Iwasaki, T., Kudo, T. Generation of canine dendritic cells from peripheral blood mononuclear cells. *J Vet Med Sci* 2003;65:663-669.
22. Catchpole, B., Stell, A.J., Dobson, J.M. Generation of blood-derived dendritic cells in dogs with oral malignant melanoma. *J Comp Pathol* 2002;126:238-241.
23. Ibisch, C., Pradal, G., Bach, J.M., Lieubeau, B. Functional canine dendritic cells can be generated in vitro from peripheral blood mononuclear cells and contain a cytoplasmic ultrastructural marker. *J Immunol Methods* 2005;298:175-182.
24. Bonnefont-Rebeix, C., de Carvalho, C.M., Bernaud, J., Chabanne, L., Marchal, T., Rigal, D. CD86 molecule is a specific marker for canine monocyte-derived dendritic cells. *Veterinary Immunology and Immunopathology*;In Press, Corrected Proof.
25. Liao, K.W., Hung, S.W., Hsiao, Y.W., Bennett, M., Chu, R.M. Canine transmissible venereal tumor cell depletion of B lymphocytes: molecule(s) specifically toxic for B cells. *Vet Immunol Immunopathol* 2003;92:149-162.
26. Peretz, Y., Zhou, Z.F., Halwani, F., Prud'homme, G.J. In Vivo Generation of Dendritic Cells by Intramuscular Codelivery of FLT3 Ligand and GM-CSF Plasmids. *Molecular Therapy* 2002;6:407-414.
27. Sombroek, C.C., Stam, A.G., Masterson, A.J., Loughheed, S.M., Schakel, M.J., Meijer, C.J., Pinedo, H.M., van den Eertwegh, A.J., Scheper, R.J., de Gruijl, T.D. Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. *J Immunol* 2002;168:4333-4343.
28. Chi, C.H., Wang, Y.S., Lai, Y.S., Chi, K.H. Anti-tumor effect of in vivo IL-2 and GM-CSF electrogene therapy in murine hepatoma model. *Anticancer Res* 2003;23:315-321.
29. Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*

- 2001;25:402-408.
30. Kiertscher, S.M., Luo, J., Dubinett, S.M., Roth, M.D. Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. *J Immunol* 2000;164:1269-1276.
31. Shen, W., Ladisch, S. Ganglioside GD1a impedes lipopolysaccharide-induced maturation of human dendritic cells. *Cell Immunol* 2002;220:125-133.
32. Bender, A., Sapp, M., Schuler, G., Steinman, R.M., Bhardwaj, N. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J Immunol Methods* 1996;196:121-135.
33. Romani, N., Reider, D., Heuer, M., Ebner, S., Kampgen, E., Eibl, B., Niederwieser, D., Schuler, G. Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J Immunol Methods* 1996;196:137-151.
34. Zhou, L.J., Tedder, T.F. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J Immunol* 1995;154:3821-3835.
35. Chakravarty, P.K., Alfieri, A., Thomas, E.K., Beri, V., Tanaka, K.E., Vikram, B., Guha, C. Flt3-ligand administration after radiation therapy prolongs survival in a murine model of metastatic lung cancer. *Cancer Res* 1999;59:6028-6032.
36. Yao, V., Platell, C., Hall, J.C. Dendritic cells. *ANZ J Surg* 2002;72:501-506.

TABLE 1.**A. Adherence of PBMC in different amount of fetal calf serum or autologous serum**

		% of PBMC	Lymphocytes ^d	Monocytes ^e
10% FBS ^a	Nonadherent cells ^b	93.4 ± 2.5	67.74 ± 12.38	17.43 ± 3.8
	Adherent cells ^c	6.6 ± 2.5	2.54 ± 0.92	95.01 ± 0.65
2% FBS	Nonadherent cells	95.4 ± 3.3	69.67 ± 12	16.07 ± 3.43
	Adherent cells	4.6 ± 3.3	7.20 ± 3.68	89.88 ± 3.02
10% autologous serum	Nonadherent cells	92.3 ± 3.2	70.55 ± 11.04	14.38 ± 4.8
	Adherent cells	7.6 ± 3.2	8.48 ± 1.21	86.34 ± 2.17
2% autologous serum	Nonadherent cells	84.6 ± 2.4	73.48 ± 10.34	13.9 ± 3.15
	Adherent cells	15.3 ± 2.4	8.49 ± 5.33	88.35 ± 4.19
no serum	Nonadherent cells	71.4 ± 8.4	71.05 ± 4.65	11.72 ± 3.67
	Adherent cells	28.6 ± 8.4	60.72 ± 21.43	33.39 ± 20.12

^a Medium (RPMI-1640) supplemented with 10% FBS, 2% FBS, 10% autologous serum, 2% autologous serum, or no serum

^b Fraction of cells harvested after incubation in plastic culture flasks. Flasks were washed extensively with PBS.

^c Fraction of cells that were still adherent after extensive washing. Cells were harvested after incubation with EDTA for 30 min (n = 3).

^d Fraction of lymphocytes in adherent or nonadherent cells cultured with different concentrations of FBS or autologous serum.

^e Fraction of monocytes in adherent or nonadherent cells cultured with different concentrations of FBS or autologous serum.

B. Yield of DC (%) cultured in different cytokine-containing media (n = 3)

	DC yield / 20 ml blood	% enrichment
IL-4 + GM-CSF ^a	0.5~1 × 10 ⁶ cells	80~85% ^b
IL-4 + GM-CSF + Flt3L	2~4 × 10 ⁶ cells	80~85%

^a DC cultured from adherent PBMC in different kinds of media supplemented with 2% autologous serum, IL-4 (500 U/ml), and GM-CSF (800 U/ml) with or without Flt3L (200ng/ml) and harvested at day 6.

^b Percentage of cells.

Table 2 List of monoclonal antibodies used for surface phenotype assays

Specificity	Clone	Ig class	Source
Human CD1a-FITC	NA1/34	Mouse IgG2a	Serotec ^a
Canine CD4	YKIX 302.9	Rat IgG2a	Serotec
Canine CD8	YCATE 55.9	Rat IgG1	Serotec
Canine CD11c	CA11.6A1	Mouse IgG1	Serotec
Human CD14	TUK4	Mouse IgG2a	DAKO ^b
Human CD40-FITC	LOB7/6	Mouse IgG1	Serotec
Human CD80-FITC	16-10A1	Hamster IgG2a	Pharmingen ^c
Human CD83-FITC	HB15e	Mouse IgG1	Pharmingen
Human CD83-FITC	HB15a	Mouse IgG2b	Immunotech ^d
Human CD86-RPE	BU63	Mouse IgG1	Serotec
Canine DLA class II	CA2.1C12	Mouse IgG1	Serotec
Mouse DEC205	NLDC-145	Rat IgG2a	Serotec

^a Kidlington, UK; ^b Glostrup, Denmark; ^c San Diego, CA; ^d Marseilles, France

Figure 1.

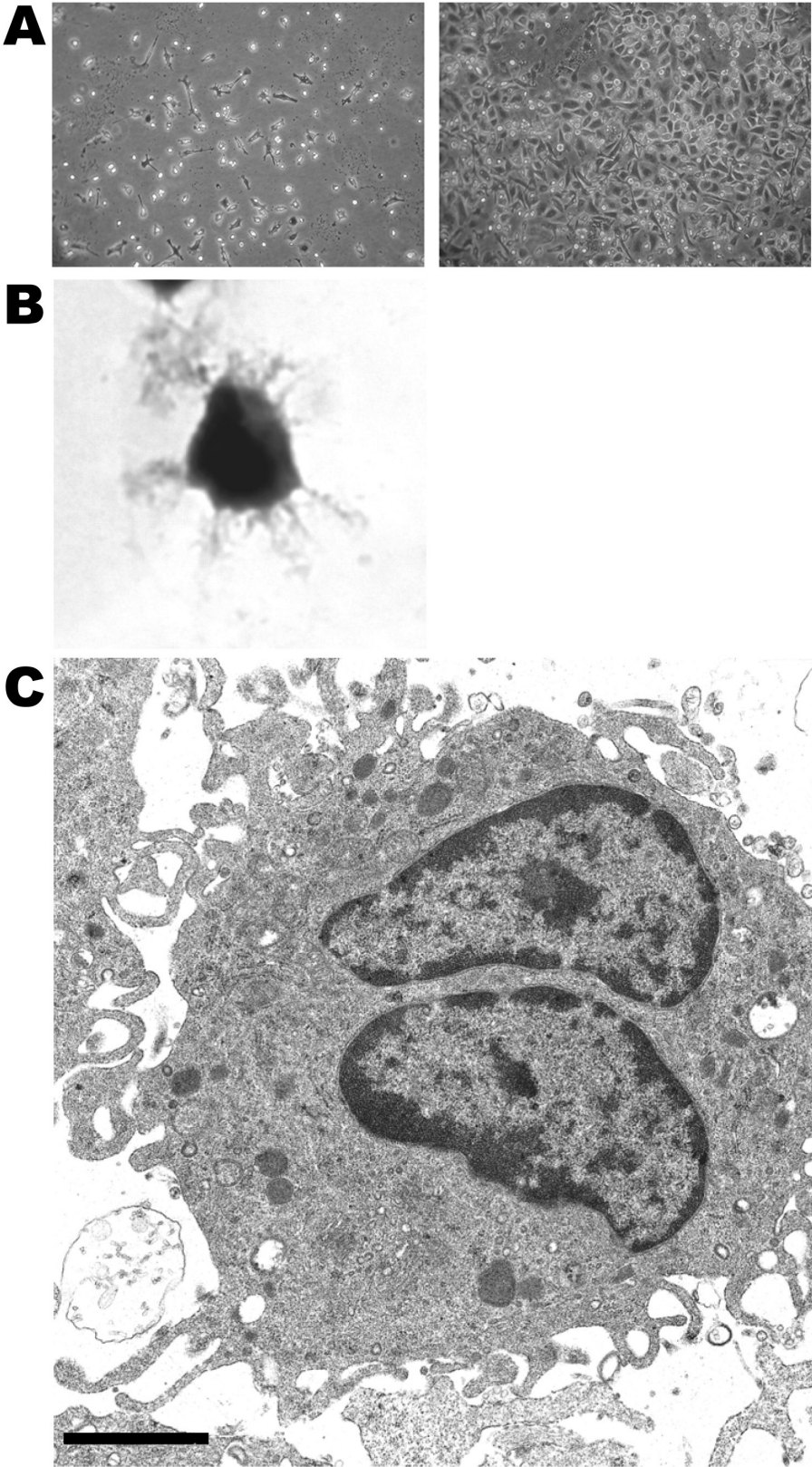


Figure 1. The morphology of canine monocyte-derived DC. PBMC were cultured with GM-CSF, IL-4, and Flt3-ligand. (A) After 6 days, adherent clusters of cells and dendrite morphology were observed (phase contrast, x25). (B) May-Grunwald Giemsa stained DC (x1000). (C) Electron micrographs of DC. The bar represents 1 μ m. Each image represents more than six different fields of DC from two separate PBMC preparations.

Figure 2.

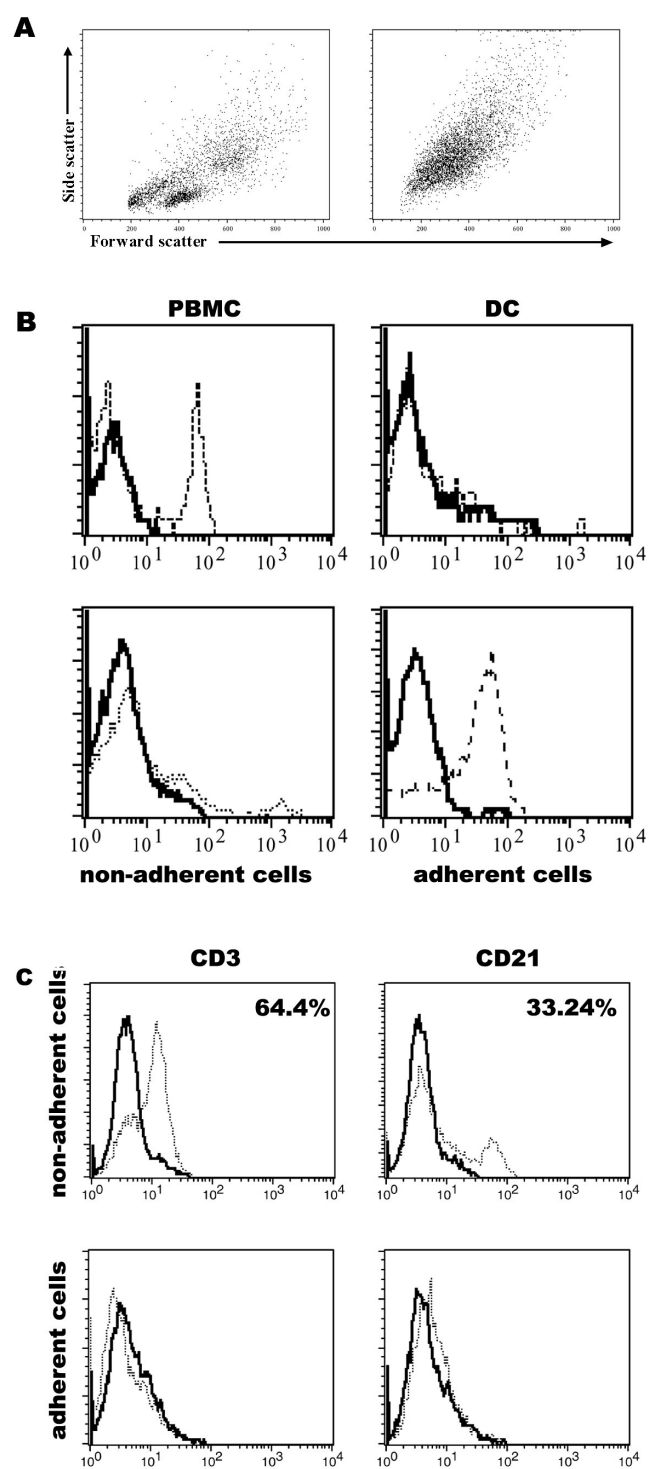


Figure 2. Analysis of CD14 expression on PBMC and DC by flow cytometry.

(A) Flow cytometry analysis of DC generated from PBMC cultured for 6 days with GM-CSF, IL-4, and FltL3. (B) Expression of CD14 by PBMC, nonadherent cells, adherent cells, and DC were determined by flow cytometry (dashed line). (C) Expressions of CD3 and CD21 by non-adherent cells and adherent cells were determined by flow cytometry (dashed line). Non-adherent and adherent cells were obtained from PBMC after incubation for 24 h at 37 °C in 25-cm² flask (2.5 x 10⁶ cells/ml). In these representative histograms, the control isotype is shown with a solid black line.

Figure 3.

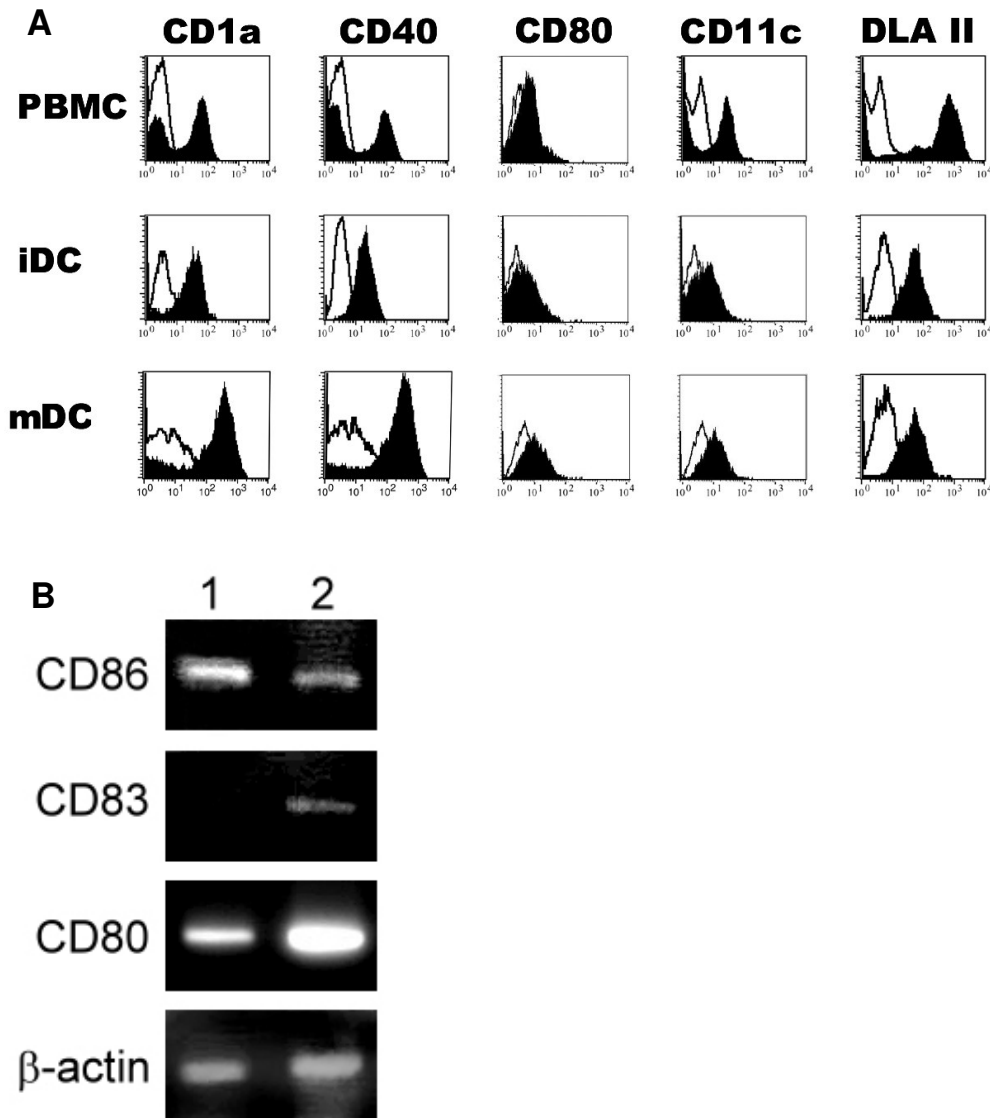


Figure 3. Surface marker expression on DC. (A) Phenotype profiles of PBMC, immature DC, and mature DC. Flow cytometry analysis of LPS-induced surface marker expression in DC. After 6 days of culture, iDC were incubated for 48 h in medium (iDC), with 10 μ g/ml LPS (mDC). After treatment, cells were harvested and analyzed for surface markers by FACSCalibur. (open curve, isotype control mAbs; solid curve, specific mAbs). (B) CD80, CD83, and CD86 mRNA expression in canine DC.

Immature DC were incubated for 48 h in medium (lane 1) or with 10 µg/ml LPS (lane 2). The total RNA of DC was extracted and subjected to RT-PCR. The primers used in this experiment were designed for CD80, CD83, CD86, and β -actin.

Figure 4.

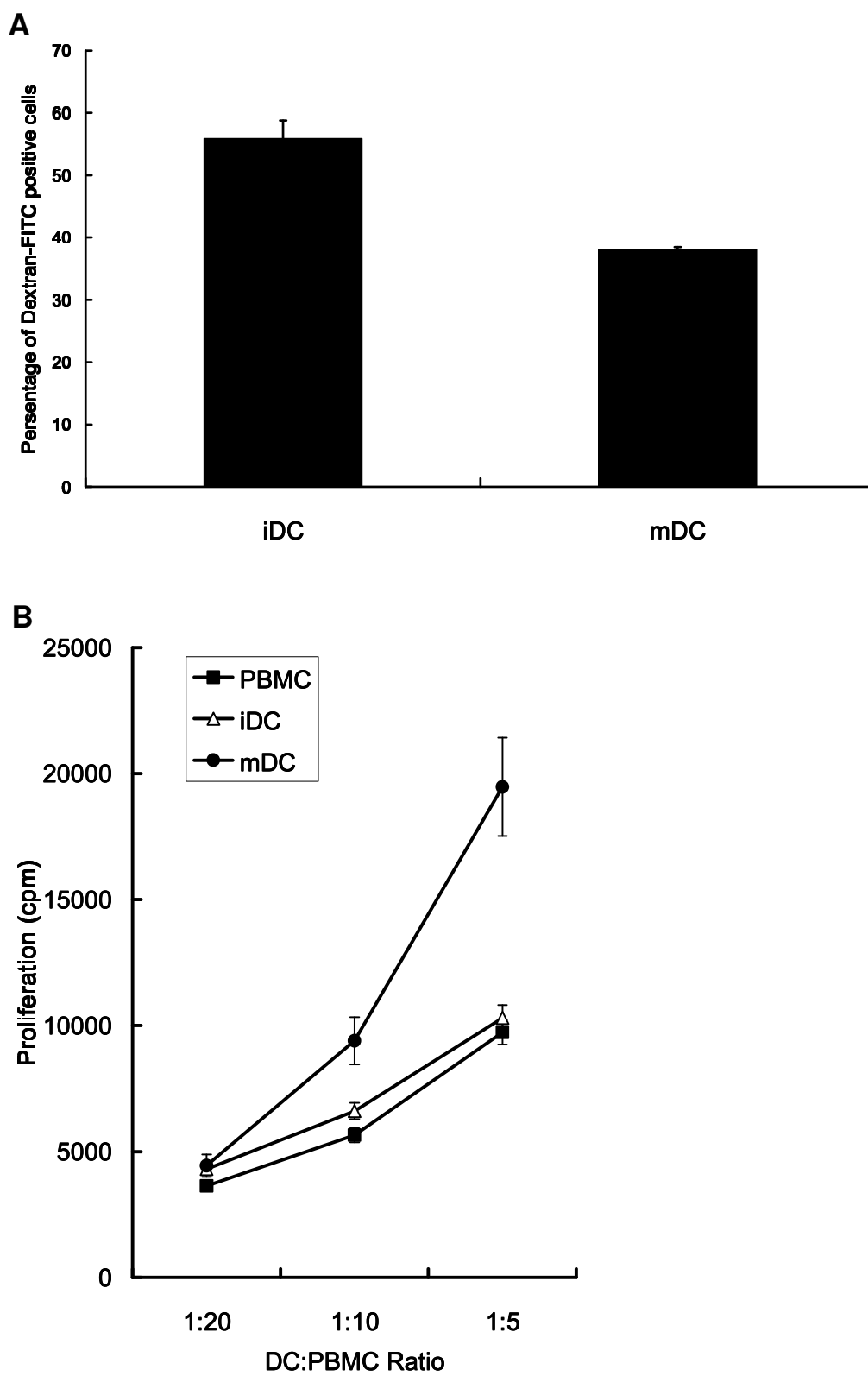


Figure 4. Functional activity assay of DC. (A) Endocytic activity of DC stimulated by LPS. DC were generated from PBMC cultured for 6 days with GM-CSF, IL-4, and FltL3. mDC obtained from iDC were incubated with 10 μ g/ml LPS (DC + LPS) for 48 h and reacted with 100 μ g/ml FITC-Dextran at 4°C or 37°C for 2 h and then analyzed by flow cytometry. (B) Allogeneic MLR induced by DC with various treatments. Immature DC generated with GM-CSF, IL-4, and Flt3L were activated with LPS and cultured with allogeneic PBMC. All treatment were incubated 104 hours before 3 H-thymidine was added at 1 μ Ci/ml for 16 hours before harvested and counted. Data are the means $\pm$ SD cpm of triplicate wells of three independent experiments.

Figure 5.

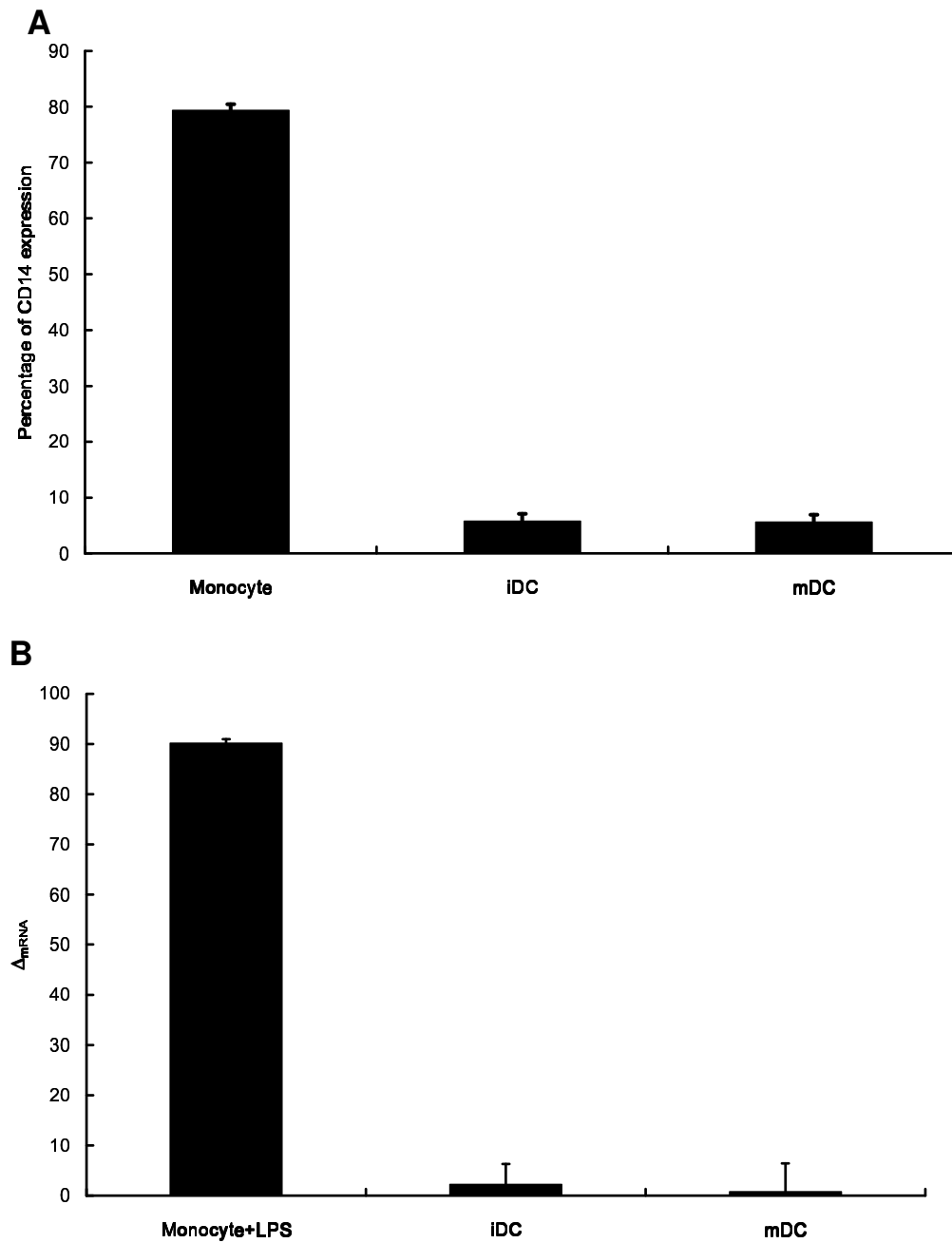


Figure 5. (A) Expression of CD14 by monocyte, iDC and mDC were determined by flow cytometry. Flow cytometry analysis was performed as described in the Materials and Methods. The percentage of fluorescence positive cells $\pm$ SD out of at least three experiments is shown. The percentage of fluorescence positive cells of the isotype

control antibody was subtracted. (B) LPS-induced TNF- α mRNA expression on monocytes, iDC and mDC. Canine PBMC were incubated for 48h with 10 μ g/ml LPS (PBMC+LPS). Immature DC were incubated for 48 h in medium (iDC) or with 10 μ g/ml LPS (mDC). Total RNA was extracted for real-time RT-PCR quantification. The relative quantity of TNF- α mRNA was normalized to that of β -actin mRNA. Results are expressed as relative change in mRNA expression (Δ_{mRNA}) and shown as means $\pm$ SD; $n = 5$.