日本財団補助金による
1999 年度日中医学協力事業報告書

－在留中国人研究者研究助成－

2000 年 3 月 14 日

財団法人 日中医学協会

理事長 中島 章 殿

研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ GM-CSF, IL-4, TNF-α 添加培養による臍帯血付着細胞からの樹状細胞の誘導

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有) ・ 無 (学会名・内容)

鄭 智茵、高橋益広、鳥羽 健、劉 愛春、オスマン・ヤーセル、高橋英伸、橋本誠雄、古川達雄、青木定夫、小池 正、相沢義房 末梢血および臍帯血由来樹状細胞の抗原取り込み能と提示能 第61回日本血液学会総会 平成11年4月19日－21日（東京）

鄭 智茵、高橋益広、劉 愛春、鳥羽 健、橋本誠雄、新国公司、古川達雄、青木定夫、小池 正、相沢義房 樹状細胞の抗原提示能に対する抑制因子の検討 第 41 回日本臨床血液学会総会 平成11年10月13-15日（秋田）

鄭 智茵、高橋益広、劉 愛春、新国公司、成田美和子、鳥羽 健、古川達雄、小池 正、相沢義房 IL-10 および UV-B で処理した樹状細胞を用いて T cell anergy の誘導 第62回日本血液学会総会 平成12年3月16日－18日（福岡）

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

ZHIYIN ZHENG, MASURIRO TAKAHASHI, KEN TOBA, AICHUN LIU, TATSUO FURUKAWA, TADASHI KOIKE, YOSHIFUSA AIZAWA: Generation of Dendritic Cells from Adherent cells of Cord Blood by culture with GM-CSF, IL-4 and TNF-α. J Hematotherapy (in press)

3．今後の研究計画

研究テーマ　白血病由来樹状細胞が分泌する白血病抗原/MHC 複合体の抗白血病免疫療法への応用

研究の背景　　樹状細胞は、抗原を提示するだけではなく、主要組織適合抗原（MHC）および抗原提示不可欠な co-stimulatory molecule と結合した抗原ペプチド複合体（エクソゾーム）を分泌しており、このエクソゾーム自体も抗原提示能を有することが明らかにされている。白血病由来樹状細胞から分泌される白血病抗原/MHC 複合体エクソゾームを抗白血病免疫療法における白血病抗原の提示に応用することができれば、従来のリンパ球や樹状細胞自体の輸注による免疫療法に比し、より実用的で広く普及できる一般的な治療法として白血病に対する免疫療法の確立が期待できる。

研究の目的と方法　1）白血病由来樹状細胞による細胞障害性 T りンパ球（CTL）　の誘導と樹状細胞が分泌するエクソゾームの精製。2）白血病由来樹状細胞が分泌するエクソゾームの抗原提示能と CTL の誘導。

4．研究指導者の意見

鄭　智茵さんは、中国浙江省中医学院附属浙江省中医院から新潟大学医学部内科学第一教室に留学し、医学部大学院博士課程を 2000 年 3 月 31 日に修了しす。博士論文のテーマは”Generation of Dendritic Cells from Adherent cells of Cord Blood by culture with GM-CSF, IL-4 and TNF-α.（GM-CSF, IL-4, TNF-αを加えた培養による臍帯血からの樹状細胞の誘導）”で、主に樹状細胞を応用した悪性腫瘍の免疫療法についての研究を精力的に行いました。その成果は、発表論文、学会発表等で国内外で明らかにされています。日本における最先端の研究を行い、この分野における進歩に十分な貢献をしましたし、中国に帰国後は中国における腫瘍免疫学の研究をリードするものと思います。鄭さんがこのような成果を得ることができましたのも、貴協会の補助金を頂いたことが、大きな要因になっていると思います。ここに謹んでお礼を述べさせていただきたいと思います。

研究指導者氏名　相澤義房　印

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、日中医学協会－日本財団補助金による旨を明記して下さい。

研究テーマ

GM-CSF, IL-４, TNF-α添加培養による臍帯血付着細胞からの樹状細胞の誘導

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要旨（日本語）

[目的] 臍帯血 CD34 陽性細胞からの樹状細胞の培養については報告されているが,臍帯血の単球からの樹状細胞の培養についての報告はない。簡便な方法で多数の樹状細胞を臍帯血から誘導することを目的として，臍帯血の付着細胞から樹状細胞の誘導を試みた。また，樹状細胞に腫瘍抗原等のパルスを行う際の好ましい培養条件である無血清培養での樹状細胞の誘導についても試みた。さらに，臍帯血付着細胞由来樹状細胞と末梢血付着細胞由来樹状細胞の抗原取り込み能と抗原提示能についても比較検討した。

[方法] 臍帯血と末梢血から付着細胞を分離し，10％FBS を添加した RPMI-1640 あるいは無血清メジウム（X VIVO-15）に GM-CSF, IL-4, TNF-α を添加して 7 日間培養により，樹状細胞の誘導を行った。また，TNF-α 添加（TNF-α を 5 日目に添加する）及び無添加培養で，血清の樹状細胞の誘導に及ぼす効果を検討した。誘導された細胞について細胞形態の観察，細胞表面形質の解析，FITC-dextran と Lucifer Yellow（LY）の抗原取り込み能及び MLC（mixed leukocyte culture）における抗原提示能の検討を行った。

[結果] 臍帯血付着細胞を GM-CSF, IL-4 を加えて 7 日間培養することにより，単球から樹状細胞への移行が見られた。それらの細胞は樹状細胞の形態を持ち，CD14 は陽性から陰性になった。また CD1a, CD80, CD86, HLA-DR 等の発現が増強した。GM-CSF, IL-4 で培養した臍帯血付着細胞に TNF-α を添加することにより，成熟樹状細胞のマーカーである CD83 と CMRF-44 の発現が明らかになると共に CD1a, CD80, CD86, HLA-DR の発現の増強も認められた。さらに，TNF-α を添加培養した細胞は TNF-α 無添加培養の細胞に比し，MLC における強い抗原提示能が認められた。臍帯血付着細胞に GM-CSF, IL-４, TNF-α を加えて培養することにより，1.2×10^7 個の臍帯血単核細胞から血清添加培養では $4.5\pm1.1\times10^5$, 無血清培養では $3.0\pm0.5\times10^5$ 個の樹状細胞が誘導できた。以上の結果より，無血清培養系でも臍帯血付着細胞から樹状細胞の培養は可能であった。

臍帯血と末梢血付着細胞から培養した樹状細胞の細胞表面形質，抗原取り込み能及び抗原提示能について比較検討では，臍帯血付着細胞由来樹状細胞は CD1a, CD80, CD83 などの発現が末梢血付着細胞由来樹状細胞に比し低下していたが，抗原取り込み能及び抗原提示能では，臍帯血付着細胞由来樹状細胞は末梢血付着細胞由来樹状細胞と同程度であった。

[結論] 臍帯血は末梢血と同様，GM-CSF, IL-４，TNF-α を加えた培養で単球から樹状細胞への移行が認められた。血清添加，無血清培養にかかわらず，臍帯血から十分量の樹状細胞の誘導が可能で，かつ誘導された樹状細胞は強い抗原取り込み能と抗原提示能が認められたことから，臍帯血由来樹状細胞の免疫療法への応用の可能性が想定された。

FEY WORDS

Dendritic cells, cord blood adherent cells, serum free culture, phagocytosis, antigen presentation, immunotherapy.

Generation of Dendritic Cells from Adherent Cells of Cord Blood by Culture with GM-CSF, IL-4 and TNF-α

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Running title: Dendritic cells from cord blood adherent cells

Key words: dendritic cells, cord blood adherent cells, serum free culture, phagocytosis, antigen presentation, immunotherapy.

ABSTRACT

Although dendritic cells (DC) can be cultured from cord blood (CB) $CD34^+$ progenitor cells, the generation of DC from CB monocytes has not been reported. In this paper, we explored to generate DC from CB monocytes for establishing the simple way to obtain substantial number of DC from CB. We isolated monocytes from CB mononuclear cells (CB-MNC) by plastic adherence method. These adherent cells (monocyte rich cells) were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBSM) or in serum free X-VIVO 15 medium (SFM) for 7 days, both of which contained 100 ng/ml GM-CSF and 10 ng/ml IL-4 with or without 10 ng/ml TNF-α (added at day 5). In the presence of GM-CSF and IL-4, CB adherent cells became nonadherent, acquired DC morphology, and showed the increased expression of CD1a, CD80, CD86 and HLA-DR; they lost membrane CD14 and some cells with the expression of CD83 and CMRF-44 were generated. With the addition of TNF-α to these cultures and culturing for further two days, the proportion of CD83 positive cells were elevated in both FBSM and SFM culture system, compared with the culture without TNF-α. In the culture with TNF-α, CD1a, CD80, CD86, HLA-DR and HLA-DQ expressing cells were markedly increased. TNF-α treated-cells were demonstrated to be stronger stimulators for proliferation of both allogeneic CB lymphocytes and PB lymphocytes than TNF-α nontreated-cells. The yield of $CD83^+$ DC at day 7 of cultures was $4.9 \pm 1.1 \times 10^5$ or $3.0 \pm 0.5 \times 10^5$ per 1.2×10^7 CB-MNC plated initially when cultured in FBSM or SFM, respectively. These results have shown that a substantial number of mature DC could be generated from CB adherent cells even by serum free culture.

We then compared these CB adherent cell-derived DC (CB-DC) with peripheral blood (PB) adherent cell-derived DC (PB-DC) in cell surface phenotype and function. We found day 7 CB-DC have lower expression of CD80, CD1a, CD83 and CMRF-44 than day 7 PB-DC, but CB-DC have similar capacity to stimulate the proliferation of both allo-CB lymphocytes and PB lymphocytes, compared with PB-DC. CB-DC cultured with GM-CSF and IL-4 have almost identical capacity of phagocytosis to take up FITC-dextran and Lucifer yellow (LY), compared with PB-DC.

In summary, Our findings suggested cord blood adherent cells, when cultured with GM-CSF, IL-4 and TNF-α, are a potent source of functional DC. Thus, CB-DC as well as PB-DC may represent valuable tools for immunotherapy.

INTRODUCTION

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the human immune system, for they are capable of initiating antigen-specific responses in naive T lymphocytes (1,2). This outstanding T cell-stimulatory capacity of DC makes them be used as nature adjuvant in therapeutic strategies against tumor and infectious agents (3,4,5).

The use of DC for clinical research and application has been hampered by their very low frequency in any organ or tissue. Recently, two approaches to effectively generate a substantial number of DC have been found. One of them is generating DC from $CD34^+$ hematopoietic precursors by using GM-CSF and TNF-α as key cytokines (6,7). Another approach is to start with $CD14^+$ precursors (monocytes) in peripheral blood and culture

them in a combination of GM-CSF and IL-4 (8,9). The latter method has been widely used and appears to be more suitable for clinical application due to a number of reasons. First, the $CD14^+$ precursors are abundant in peripheral blood, compared with the frequency of $CD34^+$ cells. Secondly, the preparation and culture of monocytes are easier to perform and are inexpensive (10). Thirdly, the DC generated by this approach appears homogeneous and fully differentiated (11). In addition, the APC function of them is similar to DC generated from $CD34^+$ precursors (10). Recent studies have demonstrated that these monocyte-derived DC as well as $CD34^+$ cell-derived DC pulsed with tumor antigens could induce specific cytotoxic T cells that posses antitumor effects in vitro and in vivo (12,13,14).

Although DC can be generated from $CD34^+$ progenitor cells from human CB, obtaining enough numbers of $CD34^+$ cells that can generate a number of DC for meeting the demands of clinical immunotherapy may be difficult. Because the volume of obtainable CB is limited to small volume in which the number of $CD34^+$ cells is low. Thus, looking for other DC source in CB may be necessary.

It has been known more percentage of monocytes exist in $CD34^-$cell population of CB, compared with PB. Caux et al. have proved the generation of DC from CB $CD34^+$ cells via $CD14^+$ cells (15), but the generation of DC from CB monocytes has not yet been reported. In this paper, we have explored to generated DC from monocytes in vitro not only for searching another DC source in CB, but also for providing simple and inexpensive approach to obtain a substantial number of DC from CB. We have investigated whether DC could be generated from adherent cells (monocyte rich fraction) in the presence of GM-CSF, IL-4 and TNF-α. The induction of DC from adherent cells under serum free culture

condition has been also investigated. Moreover, under the same cytokine condition, we have compared CB-DC with PB-DC in cell phenotypes, phagocytic activity and antigen presenting ability by using allo-MLR assay.

MATERIALS AND METHODS

Cell preparation and culture condition

Human CB was obtained from the placentas of normal full-term deliveries, following ethical committee approval. Healthy male and female laboratory personnel donated adult peripheral blood.

Mononuclear cells were obtained from all samples by centrifugation over Ficoll-Hypaque gradients (density=1.077 g/ml, Lymphoprep; Nycomed, Oslo, Norway). The cells were plated (1.2×10^6 cells/mL) in 100×20 mm tissue culture plates (Falcon, Becton Dickinson, NJ) in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/ml streptomycin (Biowhittaker, Walkersville, MD). After 2 h at 37℃, the nonadherent cells were gently removed. Adherent cells were cultured at 37 ℃, in humidified 5 % CO_2 in air, and in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) [=further on called as FBSM] , containing 100 ng/mL GM-CSF (Kirin Brewery, Maebashi, Japan) and 10 ng/mL IL-4 (Schering-Plough Research Institute, Kenilworth, NJ). 10 ng/mL TNF- α (Hayashibara Biochemical Labs, Okayama, Japan) was added to some of these cultures at day 5. After incubation for 7 days, cultured cells were harvested. In some experiments, cells were cultured for 7 days in serum free X-VIVO 15 medium (BioWhittaker,

Walkersville, MD) [=further on called as SFM] with the same concentrations of GM-CSF, IL-4 and TNF-α.

Morphological observation of cells

Cells before culture and cultured for 3 days and 7 days were centrifugated onto microscope slides using a Cytospin-2 centrifuge (Shandon Southern Products, Astmoor, U.K.), stained with May-Grunwald-Giemsa solution and observed on a microscope (Olympus, Tokyo, Japan).

Analysis of surface markers by flow cytometry

The expression of cell surface markers was analyzed by single-color immunofluorescence. Cells were harvested after culture for 1, 3, 5 and 7 days and washed with RPMI 1640 medium. 2×10^5 cells were pelleted and incubated with 0.5 % human immunoglobulin for 10 min at 4 ℃ to prevent non-specific binding of monoclonal antibody (mAb). For direct labeling, cells were incubated for 30 min at 4 ℃ in 2 % FBS containing PBS with the FITC- or PE-conjugated mAb (listed in Table 1), or with control irrelevant isotype-matched mAb. After washing, cells were resuspended in buffer, and analyzed with a FACScan (Becton Dikinson, Muntain View, CA). For CD83 (16) and CMRF-44 (17) labeling, cells were incubated with mAb HB15a or CMRF-44 for 30 min at 4 ℃, washed twice, blocked with 0.5 % human immunoglobulin for 10 min and then incubated with goat FITC-labeled anti-mouse IgG2b or IgM. MAbs of the respective isotype, but with irrelevant specificity, were included as control. After final wash, cells were suspended in

buffer and analyzed with a FACScan. For all labelling experiments, analysis was performed on the FACScan using LYSIS II software (Becton Dickinson). For each sample, not less than 5,000 events were acquired.

Flow cytometric measurement of endocytosis

The endocytic activity of the cells was measured as described (18). Briefly, cells were incubated in FBSM containing 0.5 mg/mL FITC-Dextran (Sigma, St.Louis, MO) and 0.5 mg/mL lucifer yellow CH, potassium salt (LY) (Molecular probes, Leiden, The Netherlands) for 30 min at 37 ℃. As a negative control, cells were incubated with FITC-Dextran or LY at 4 ℃. The cells were washed 4 times with cold PBS containing 2 % FBS and 2 mM sodium aside, and analyzed on a FACScan using LYSIS II software (Becton Dickinson).

Determination of the allostimulatory potential of DC rich cells

DC generated by the culture in FBSM or SFM in the presence or absence of TNF-α were used as stimulators in an allogeneic mixed leukocyte reaction (MLR). The nonadherent cells obtained from normal CBMNC and PBMNC by depleting adherent cells after culture for 2 hours were used as responder. Irradiated (30 Gy) DC rich cells of graded numbers ($10^3-3 \times 10^4$) were added to a constant number of nonadherent cells (1×10^5/well) in 96-well flat-bottom tissue culture plates containing RPMI 1640 medium containing 10 % FBS. After culture for 5 days, the cultured cells were pulsed with 1 μ Ci/well [methyl - ^{3}H] thymidine (Amersham, Buckinghamshire, England) for 16 hours and T cell proliferation was measured as ^{3}H-thymidine incorporation by using γ-scintillation

counter (Beckman, UK).

Statistical analysis

Student's t-test was used to compare CB-DC with PB-DC in percent positive cells and mean fluorescence intensity (MFI) of surface antigens. A two-way analysis of variance (ANOVA) was used to compare the antigen presentation between CB-DC and PB-DC, which were generated in either FBSM or SFM. The differences were considered as significant for $P < 0.05$.

RESULTS

Morphological changes and growth characteristics during culture

By the culture with GM-CSF and IL-4, most CB adherent cells developed into larger cells with widespread cytoplasmic projections and the cell nucleus became nonlobulated and round within 3 days. After culture for 7 days, the cells showed typical morphology of DC (Fig. 1). There was no difference in morphological characteristics between cultured cells in FBSM and SFM culture.

During culture, most cells cultured in either FBSM or SFM containing GM-CSF and IL-4 became apart from plastic dishes. Small cell aggregates consisting of dendritic like cells were observed after culture for 3 days. By addition of TNF-α, the cell aggregates enlarged in size (data not shown).

Phenotypes of cells cultured from CB adherent cells

Analysis by the flow cytometry showed that the frequency of large cells (gated cells in Fig. 2A) was about 20 % of the total viable population of CB adherent cells by forward and

side scatter analysis by the present procedure for obtaining adherent cells. The 75−90 % of cells was $CD14^+$, 4 %−11 % of cells was $CD3^+$ and 4 %−6 % was $CD19^+$ in large cell population. In contrasted with large cell population, the expression of $CD14^+$ cells was less than 5 % in small cell population (cells outside of gated area in Fig. 2A).

Compared with the starting cell population, cells which were cultured with GM-CSF and IL-4 for 3 days, showed a striking change in the forward and side scatter distribution. The cells in monocyte distribution (gated area in Fig. 2A) gradually increased their size and granularity (Fig. 2A−E). When analysis by FACScan demonstrated, on average, the large cells constituted about 30 % of the total cells at day 7 (Fig. 2E). Sequential analysis of cell surface phenotypes has been done in cells before and cultured for 3, 5 and 7 days. By culture with GM-CSF and IL-4, cord blood adherent cells formed aggregates, displayed dendritic morphology with cytoplasmic projections and became $CD14^-$ with increasing population of $CD1a^+$ cells. At early time of culture (day 3), the expression of CD14 was disappeared. The expression of CD83 of cultured cells remained to be low even on day 7 of culture (Fig. 3). In the presence of TNF-α (when added on day 5), the expression of CD80, CD86, HLA-DR and HLA-DQ of cultured cells was increased at day 7, compared with cells cultured without TNF-α. Especially, the expression of CD83 (specific mature DC marker) was up-regulated at day 7 of culture (Fig. 4). As shown in table 2, CD83 positive cells were 39.32 $\pm$ 5.12 % or 28.75 $\pm$ 7.01 % of total large cell population, in FBSM or SFM respectively, when cultured with GM-CSF, IL-4 and TNF-α for 7 days. When cultured in FBSM or SFM for 7 days, the yield of mature DC ($CD83^+$ cells) were 4.9 $\pm$ 1.1 $\times$ 10^5 or 3.0 $\pm$ 0.5 $\times$ 10^5 per 1.2 $\times$ 10^7 CB-MNC plated initially.

Mix leukocyte Reaction (MLR) studies

The response of CB nonadherent cells (data not shown) or PB nonadherent cells to allogeneic TNF-α treated CB adherent cells, which were generated in either FBSM or SFM condition, was significantly greater than that to fresh isolated allogeneic PBMNC or CBMNC at any stimulator: responder cell ratio. Moreover, CB-DC generated by culture in FBSM (data not shown) or SFM with TNF-α (added at day 5) for 7 days possessed enhanced capacity to stimulate an allogeneic MLR, compared with the cells cultured without TNF-α (Fig. 5). CB-DC cultured in SFM with TNF-α (added at day 5) for 7 days were demonstrated to have the equivalent activity of antigen presentation to CB-DC cultured in FBSM with TNF-α in allogeneic MLR (Fig. 5).

Comparative studies of phenotypes and functions in CB-DC and PB-DC

Based on these results obtained above, we then compared CB-DC derived from CB adherent cells with PB-DC derive from PB adherent cells in surface phenotypes, phogocytic activity and antigen presenting activity in MLR assay for detecting the differences of the levels of DC function. CB-DC and PB-DC were investigated at day 7 of culture containing GM-CSF and IL-4 with or without TNF-α. As shown in Table 3, CD86 and HLA-DR were almost equally expressed on CB-DC and PB-DC, when cultured in FBSM or in SFM. But the expression of CD80 in CB-DC was significantly lower than that of PB-DC in both the frequency and intensity under FBS culture condition. Moreover, the expression of CD1a and CD83 in CB-DC were significantly lower than those in PB-DC in the culture

with FBS. Although there was no significant difference between CB-DC and PB-DC in the expression CD80, CD1a and CD83 when cultured in SFM, but CB-DC also showed a tendency of lower expression of these surface markers than PB-DC. However, the expression of CMRF-44 in CB-DC prepared from SFM was significantly lower than that expression in PB-DC cultured in the same condition. These findings suggested CB-DC, prepared in FBSM and SFM seemed to have less mature phenotypes, compared with PB-DC. The expression of surface phenotypes such as CD1a, CD80 and CD83 were greater in both CB- and PB-DC cultured in FBSM condition compared with DC cultured in SFM condition.

There are two distinct mechanisms for DC to capture antigens. The first is a high level of fluid phase uptake via macropinnocytosis. The second mechanism of capture is mediated via mannose receptor (18). To determine whether CB-DC has phagocytic activity as well as PB-DC, we used two fluorescent molecules: LY, a nonspecific fluid phase marker, and FITC-dextran, which is mainly taken up via the mannose receptor. We have found that 60—95% or 40—95% of cells cultured from CB with GM-CSF and IL-4 for 7 days took up FITC-DX or LY, respectively. When cultured with GM-CSF and IL-4, 80—95% of total gated cells cultured from PB adherent cells took up both FITC-DX and LY. On the contrary, PB lymphocytes did not take up these two molecules, which demonstrated that FL-1 or FL-2 positivity of dendritic cells at 37 ℃ is not due to the attachment of these molecules to the surface of the cells but due to taking up of the molecules (Fig. 6).

The allogeneic T lymphocyte-stimulating capacity of CB-DC and PB-DC was evaluated comparatively. As illustrated in Fig. 7, both PB-DC and CB-DC induced a significant ^{3}H

thymidine uptake. In contrast, CB-MNC and PB-MNC showed much less stimulating activity in MLR on a per cell number basis, compared with PB-DC and CB-DC. No significant difference in the antigen presentation was found between PB-DC and CB-DC, which were generated in either FBSM or SFM condition.

DISCUSSION

In this paper, we have shown CB monocytes can differentiate to $CD83^+$ mature DC in the presence of GM-CSF, IL-4 and TNF-α. This differentiation of CB monocytes to DC has been proved by continuous phenotype analysis with FACScan described in this paper. The disappearance of CD14 and the increase of the expression of CD80, HLA-DR and CD1a in cultured cells were also demonstrated in our study. TNF-α could induce immature DC to mature DC. When TNF-α was added to the culture supplemented with GM-CSF and IL-4, the expression of CD80, CD86, HLA-DR, HLA-DQ and CD1a in cultured cells were further up regulated as well as mature DC markers, such as CD83 and CMRF-44. Together with forward and side scatter analyses, these changes of surface phenotypes of cultured cells suggested that the whole population of CB monocytes gradually differentiated to mature DC. PB monocytes were demonstrated to differentiate to DC using the precise analysis of surface phenotypes of cultured cells by Woodhead et al (19). Our findings revealed that the process of CB monocyte differentiation to DC is similar to that of DC differentiation from PB monocytes.

Although methods for growing DC have usually included FBS in the culture medium, it is desirable to avoid FBS, because FBS has antigenicity (20), when cultured DC would be

applied to immunotherapy. SFM supplemented with GM-CSF, IL-4 and TNF-α was used as DC culture medium in the present study. $3.0 \pm 0.5 \times 10^5$ $CD83^+$ DC per 1.2×10^7 MNC were generated in culture of CB adherent cells for 7 days. The antigen presentation of CB-DC, which were prepared in FBSM and SFM, were tested in MLR, which demonstrated no significant difference. These results suggested that a substantial number of functional CB-DC could be obtained even by serum free culture condition.

The potency of DC as antigen-presenting cells (APC) is correlated with their constitutive expression of high levels of cell surface MHC class Ⅰ and Ⅱ antigens and costimulatory molecules such as CD80 (21) and CD86 (1). These molecules serve to initiate and stabilize DC interaction with T cell through the corresponding ligands in T cells. Prior results (22) have shown that DC isolated from CB expressed significantly lower levels of HLA-DR, compared with adult blood DC. In our study, CB-DC that were prepared by either FBSM or SFM has exhibited the almost equal frequency and intensity in the expression of HLA-DR and CD86 to PB-DC prepared by the same culture condition, but CB-DC showed lower expression of CD80, CD1a, CD83 and CMRF-44 than PB-DC. Although CD1a, CD83 and CMRF-44 are believed to be associated with antigen presentation of DC, the precise function is not fully understood. However, no significant difference could be observed between CB-DC and PB-DC in trigging allogeneic lymphocyte proliferation, despite reduced expression of CD80, CD1a, CD83 and CMRF-44 in CB-DC. These findings suggested that CD86 might compensate the costimulatory function of CD80 in CB-DC as reported by Lanier et al. (23) who have demonstrated that CD80 and CD86 provide similar costimulatory signals for T cell proliferation.

DC which were cultured from adherent cells with GM-CSF and IL-4 are thought to be immature DC (24). Immature DC have stronger ability for antigen uptake and processing than mature DC. We found that CB-DC generated from adherent cells in the presence of GM-CSF and IL-4, could efficiently capture both FITC-dextran and LY, and this activity of endocytosis by CB-DC was almost equal to that of PB-DC.

In conclusion, we have demonstrated that a substantial number of mature CB-DC can be generated from CB adherent cells in the presence of GM-CSF, IL-4 and TNF-α. Moreover, a large numbers of CB-DC have been generated from adherent cells even by serum free culture. Although the expression of CD80, CD1a, CD83 and CMRF-44 was lower in CB-DC than in PB-DC, CB-DC have similar capacity to stimulate the proliferation of allogeneic lymphocytes and have almost identical activity of endocytosis to take up FITC-dextran and LY, compared with PB-DC. These results indicated CB adherent cells were thought to be a source of functional DC for immunotherapy. Further studies need to be undergone for detecting the possibility of applying DC derived from CB monocytes for immunotherapy.

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Figures and Tables

Fig. 1. DC generated from CB adherent cells by culture in FBSM containing GM-CSF and IL-4 for 7 days. DC like cells showing veils at their edges were observed.

Fig. 2. The transition process of CB adherent cells to CB-DC has been examined. The results are expressed as forward scatter versus side scatter dot-plots of adherent cells before culture (A) and cultured in FBSM supplemented with GM-CSF and IL-4 for 1 day (B), 3 days (C), 5 days (D) and for 7 days (E). The distribution of gated cells at each duration of culture demonstrated the shift of monocytes before culture to DC after culture for 3 days and thereafter. The results shown are from one experiment and are representative of five independent experiments.

Fig. 3. The changes of surface phenotypes in CB adherent cells cultured in FBSM supplemented with GM-CSF and IL-4. The expressions of CD1a, CD14, CD80, CD83, CD86, HLA-DR and HLA-DQ in cells before culture (day 0) and cultured for 3, 5 and 7 days were analysed using the FACScan. The large cells gated as in Fig. 2E were analysed for surface phenotypes in the present study. Open histograms: labeling with control irrelevant mAb; solid histograms: staining with FITC-or PE-labeled relevant mAb as indicated. The results shown are from one experiment and are representative of five independent experiments.

Fig. 4. The surface phenotype of CB adherent cells cultured in FBSM containing GM-CSF

and IL-4 with or without TNF-α (added at day 5) for 7 days. The large cells gated as in Fig. 2E were analysed. Open and solid histograms are defined as in Fig. 3. The results shown are from one experiment and are representative of five independent experiments.

Fig. 5. The antigen presenting capacity of CB-DC cultured with or without TNF-α in FBSM or SFM. CB adherent cells were cultured for 7 days in SFM or FBSM in the presence of GM-CSF and IL-4 with or without TNF-α (see the Materials and Methods). DC obtained from these cultures were used to stimulate 1×10^5 allogeneic PB nonadherent cells (see the Materials and Methods) and were compared with adult PBMNC. The results are shown as mean cpm of triplicate ^{3}H-thymidine incorporation. Data are from one experiment representative of five independent experiments.

Fig. 6 Phagocytosis of FITC-dextran (A) and LY (B) by PB lymphocytes, CB-DC and PB-DC. Cells were incubated for 30 min at 4 ℃ or 37 ℃ with 0.5 mg/ml FITC-dextran or 0.5 mg/ml LY. Cells were washed 4 times with cold PBS and were then analyzed by FACScan, as described in the materials and methods. The x-axis is a logarithmic scale of fluorescence intensity and the y-axis represents cell counts. The figure depicts representative data from seven independent expriments.

Fig. 7. The antigen presenting capacity of TNF-α treated CB-DC, TNF-α treated PB-DC, PBMNC or CBMNC, which were tested in MLR. 1×10^5 allogeneic CB or PB nonadherent cells were used as responder. DC prepared in FBSM (A) or SFM (B) were used as stimulators. The mitotic responses were measured by the uptake of

^{3}H-thymidine on day 5 of culture. The results are representative for more than four independent experiments (the number of experiments is shown in the Figure) and are expressed as mean cpm ± SEM of ^{3}H-thymidine incorporation.

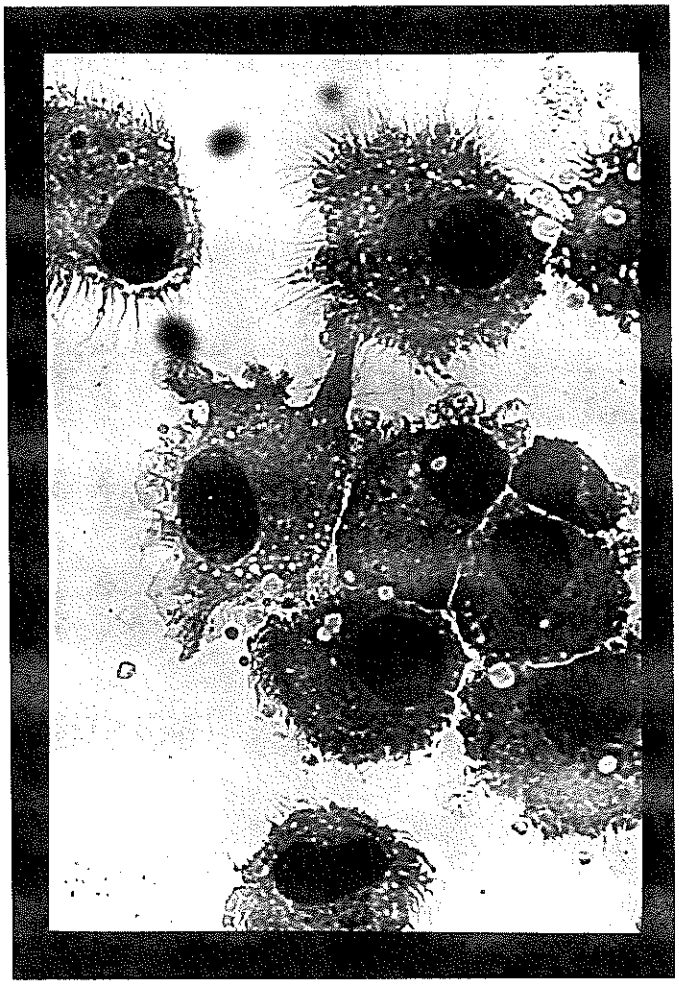

Figure 1.

Figure 2

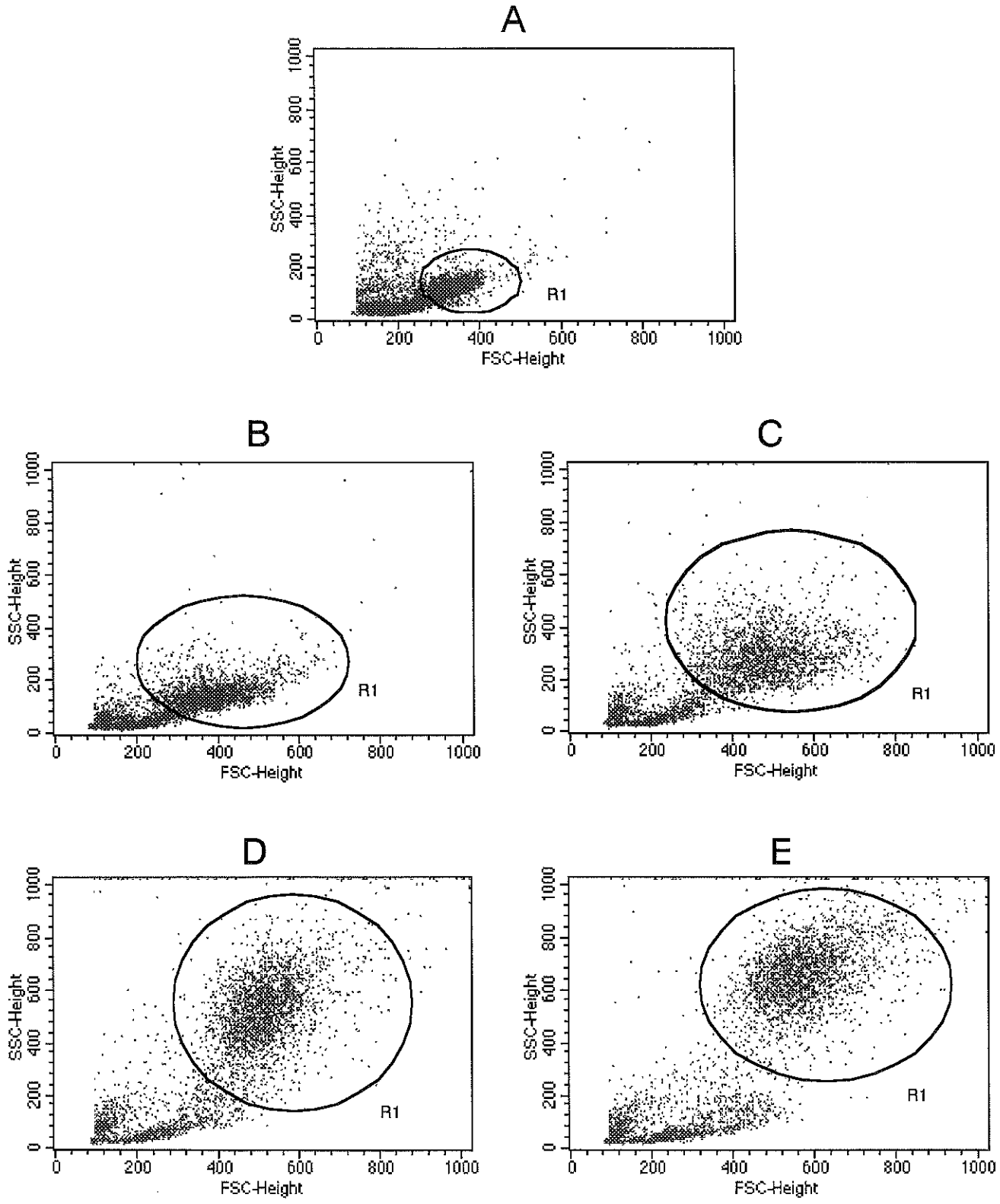

Figure 3

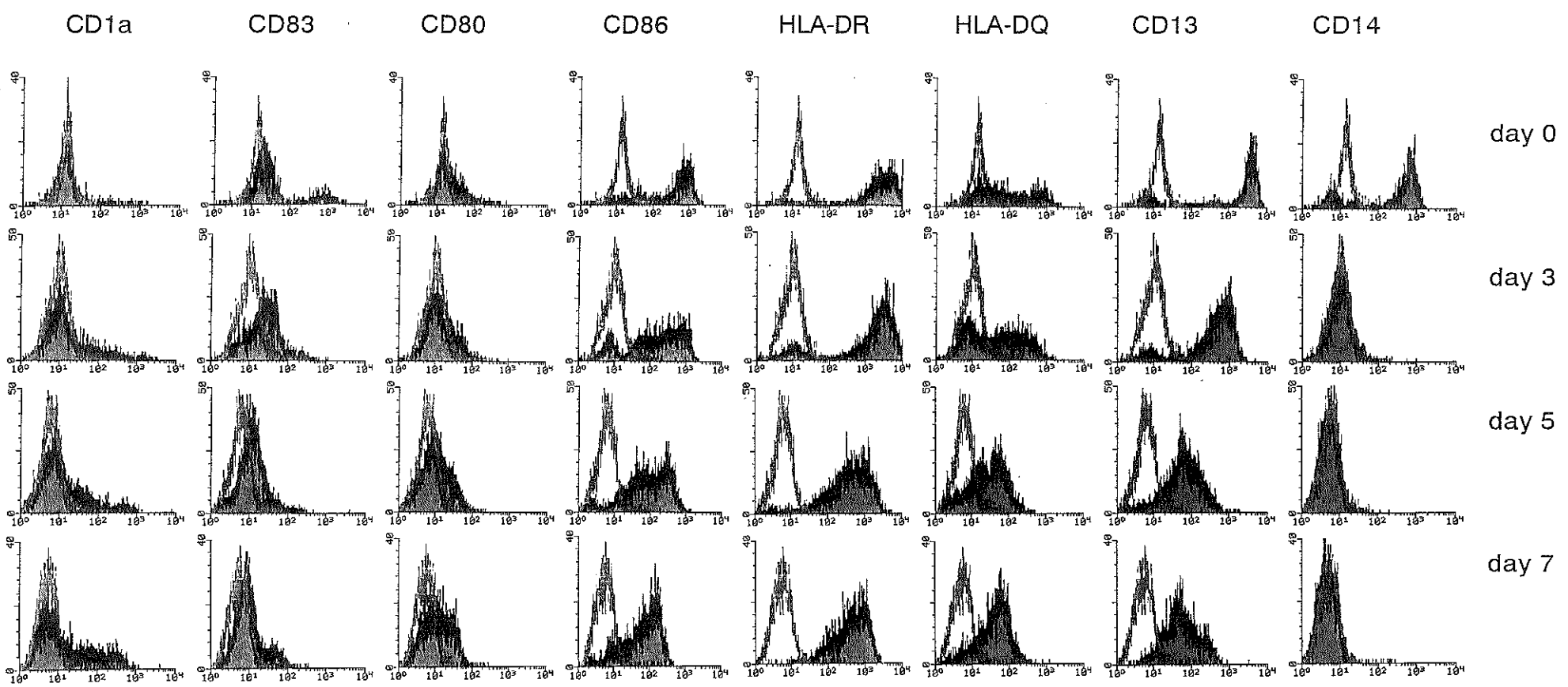
CD1a
CD83
CD80
CD86
HLA-DR
HLA-DQ
CD13
CD14
day 0
day 3
day 5
day 7

Figure 4

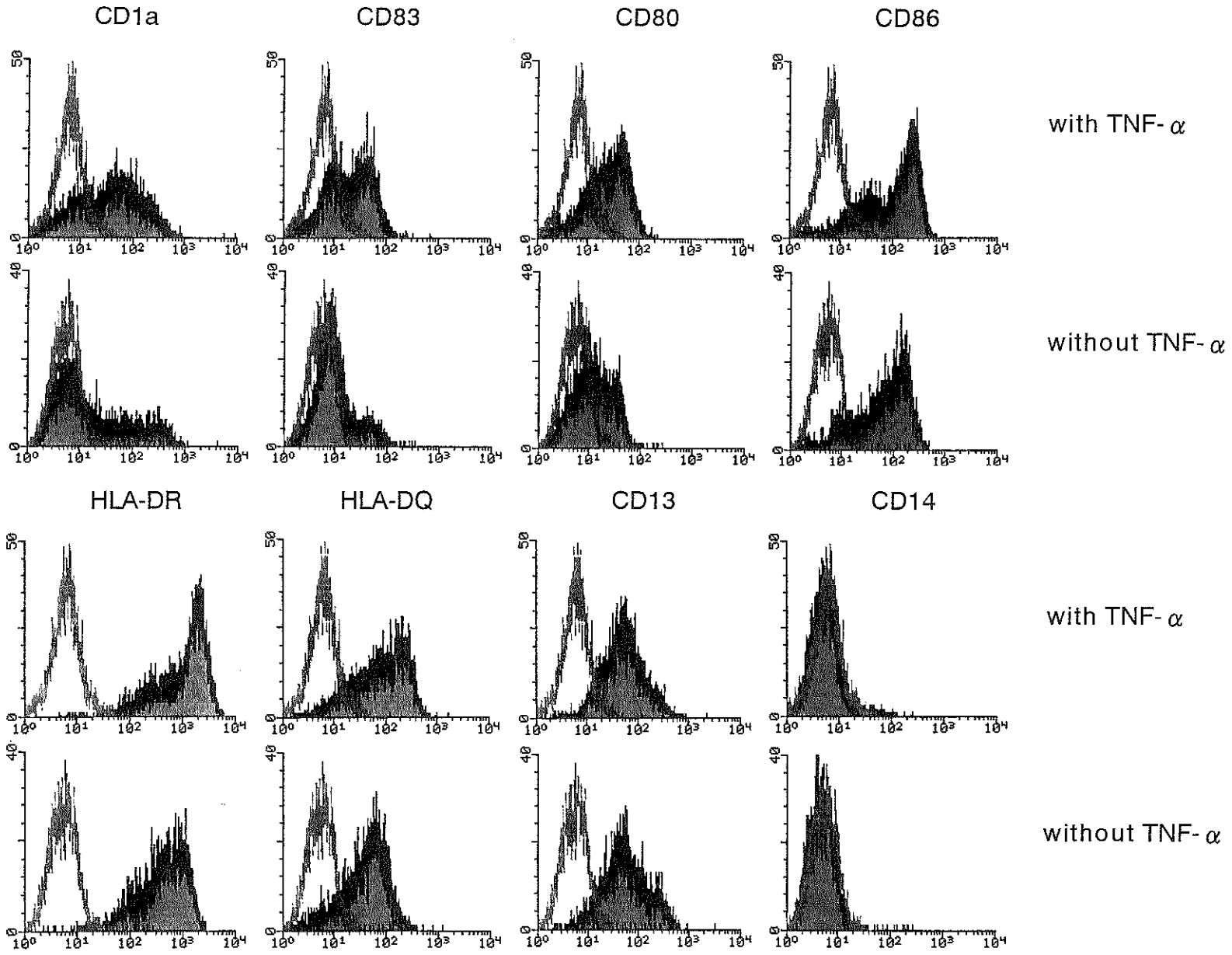
CD1a
CD83
CD80
CD86
with TNF-α
without TNF-α
HLA-DR
HLA-DQ
CD13
CD14
with TNF-α
without TNF-α

Figure 5

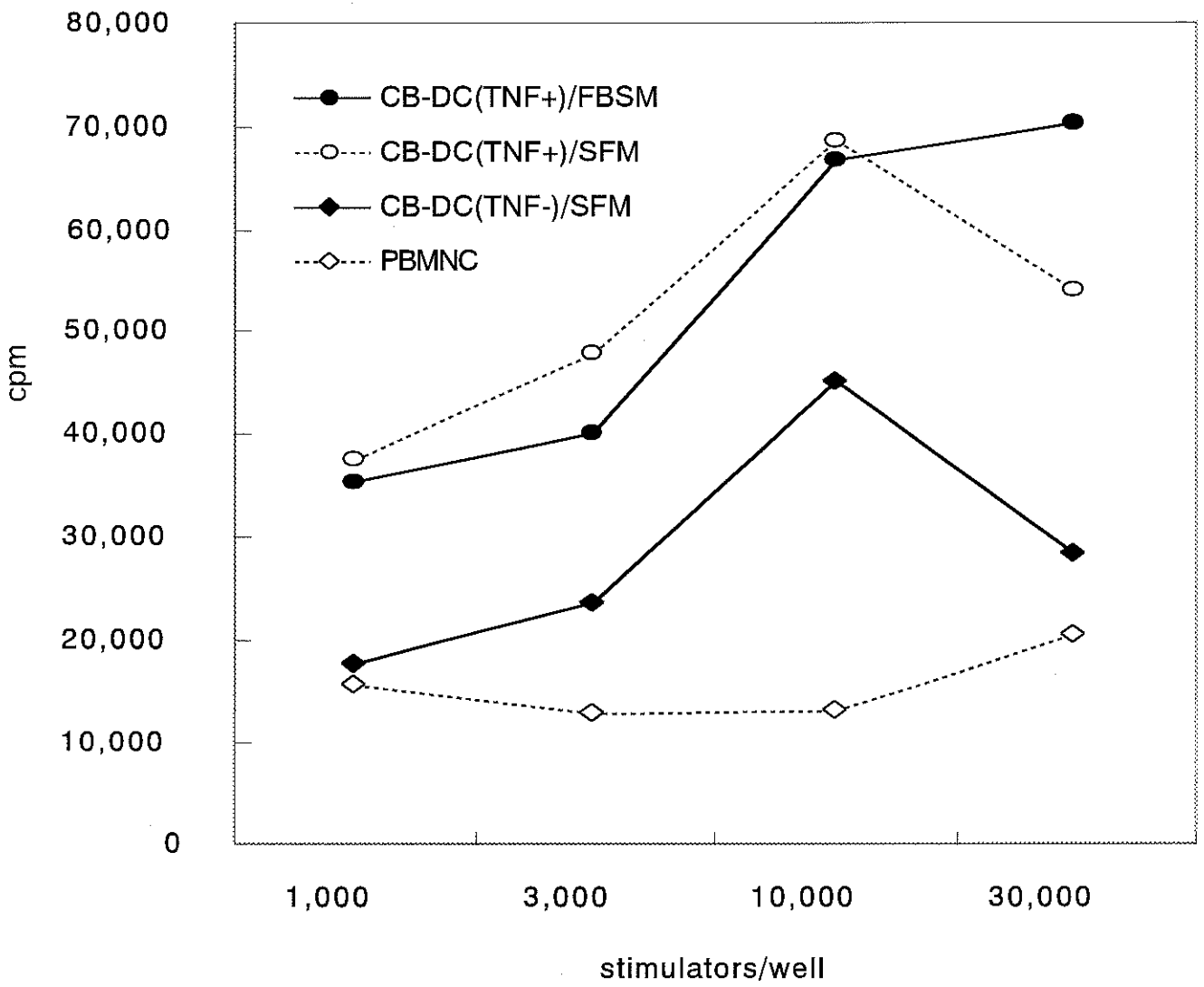
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
cpm
CB-DC(TNF+)/FBSM
CB-DC(TNF+)/SFM
CB-DC(TNF-)/SFM
PBMNC
1,000
3,000
10,000
30,000
stimulators/well

Figure 6.

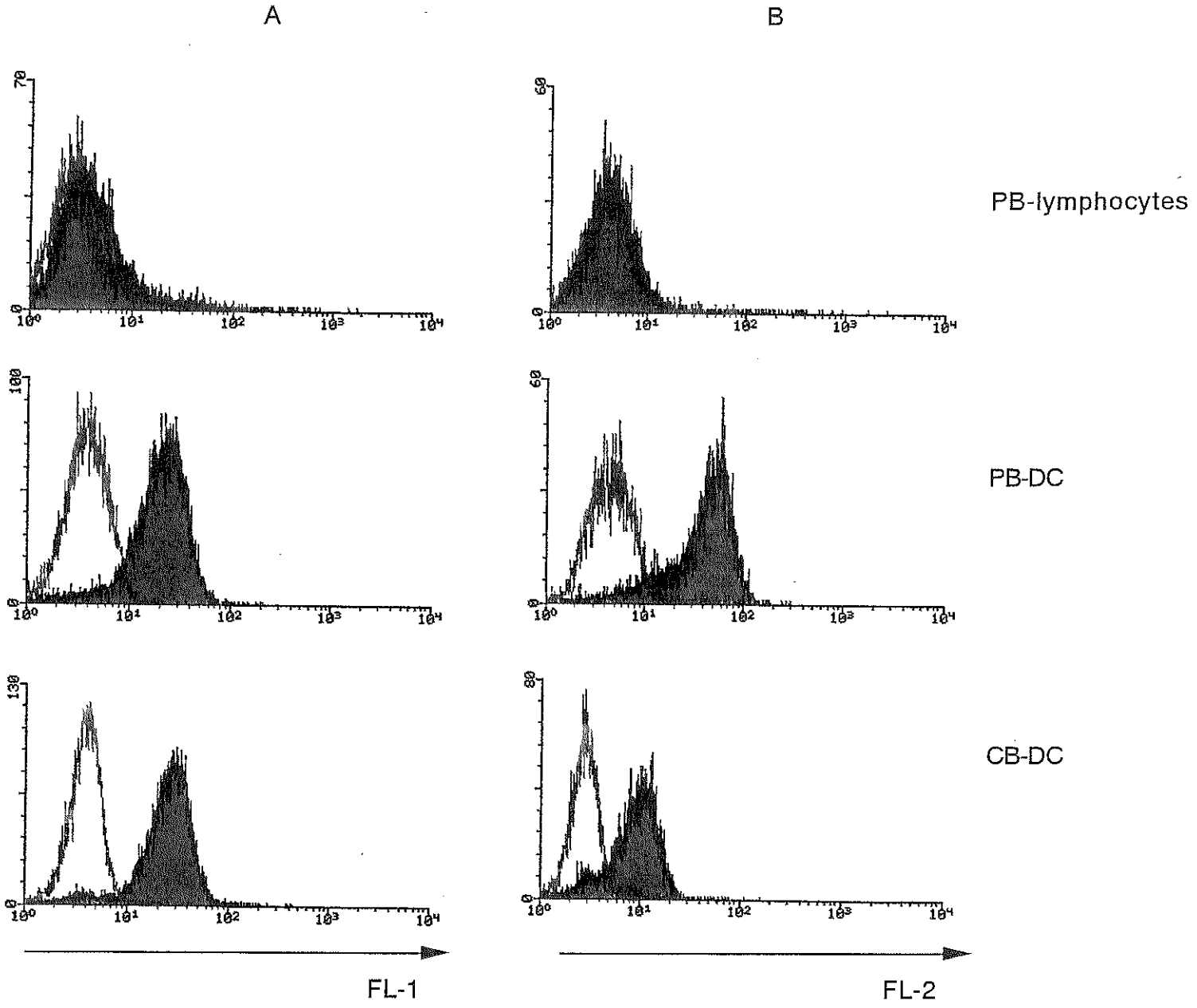

Figure 7.

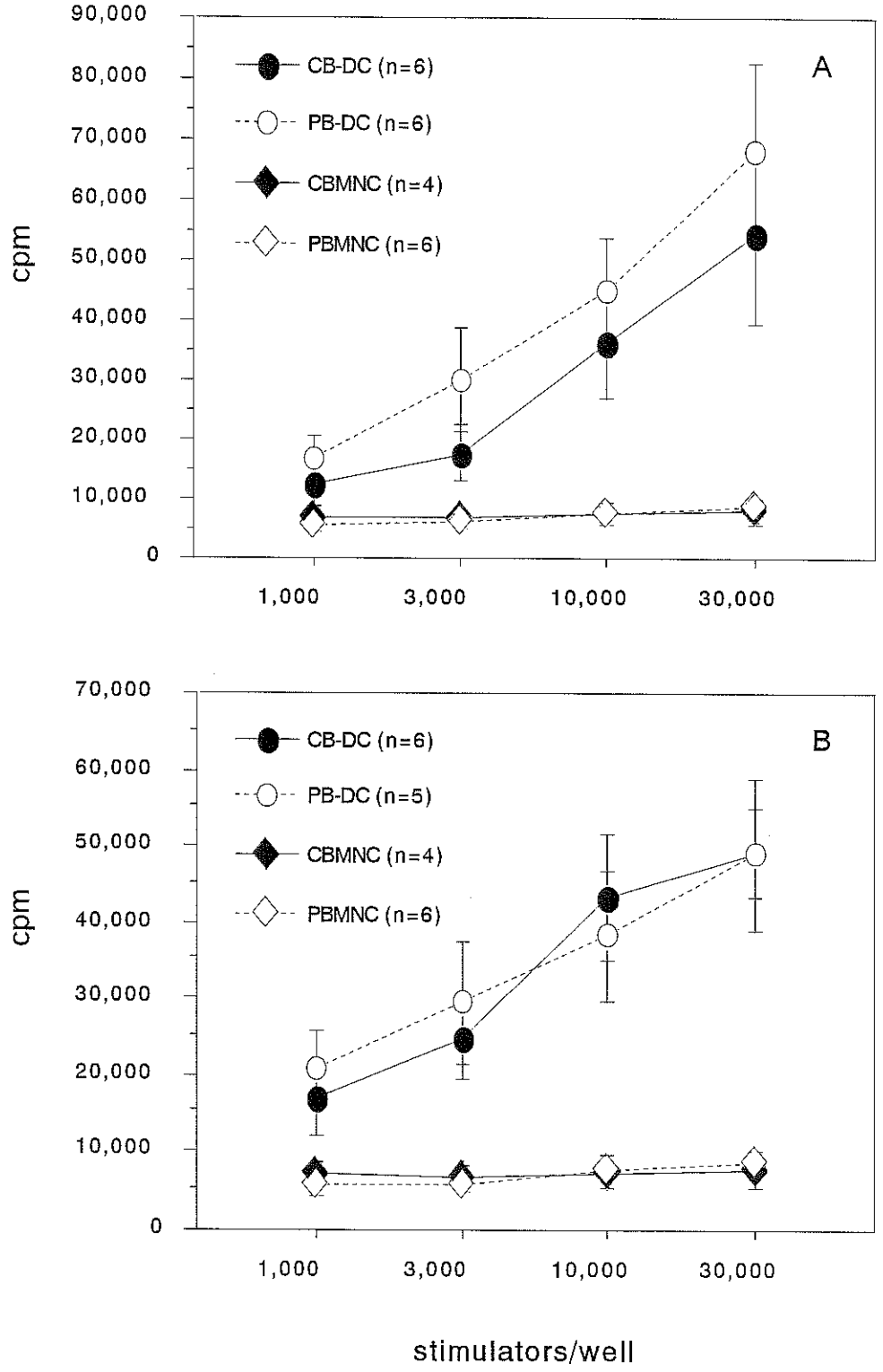
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
cpm
CB-DC (n=6)
PB-DC (n=6)
CBMNC (n=4)
PBMNC (n=6)
A
1,000
3,000
10,000
30,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
cpm
CB-DC (n=6)
PB-DC (n=5)
CBMNC (n=4)
PBMNC (n=6)
B
1,000
3,000
10,000
30,000
stimulators/well

TABLE 1. MONOCLONAL ANTIBODIES USED IN THE PRESENT STUDY

Surface antigens	Monoclonal antibodies	Suppliers
Antigens for myeloid cells		
CD13	Leu-M7	Becton Dickinson
CD33	Leu-M9	Becton Dickinson
Antigens for monocytes cells		
CD14	Leu-M3	Becton Dickinson
dendritic cell marker		
CD1a	T6	Coulter
CD83	HB15A	Immunotech
CMRF-44	CMRF-44	Dr. Derek Hart (Christchurch, NZ)
Co-stimulatory molecules		
CD80	L307.4/MAB104	Becton Dickinson
CD86	IT2.2	PharMingen
MHC class II		
HLA-DR	HLA-DR	Becton Dickinson
HLA-DQ	Leu-10	Becton Dickinson
Antigens for T and B cells		
CD3	Leu-4	Becton Dickinson
CD19	Leu-12	Becton Dickinson
CD20	Leu-6	Becton Dickinson

TABLE 2. GENERATION OF CD83 POSITIVE CELLS IN THE CULTURE OF CORD BLOOD ADHERENT CELLS, WHICH WERE SEPARATED FROM 12×10^6 MONONUCLEAR CELLS, USING SERUM FREE X VIVO-15 MEDIUM OR RPMI-1640 WITH 10% FBS CONTAINING GM-CSF, IL-4 AND TNF-A (ADDED AT DAY 5) FOR 7 DAYS

Exp. No.	Culture method	Total cell No.($\times 10^6$)	% gated big cells[a]	% $CD83^+$cells	Total No. of $CD83^+$ cells ($\times 10^6$)
1	10%FBS[b]	5.3	22.2	33	0.39
2	10%FBS	3	16.4	57	0.28
3	10%FBS	4.6	41.4	38	0.73
4	10%FBS	2.9	56.9	22.9	0.38
5	10%FBS	2.7	29.1	40	0.31
6	10%FBS	8.2	23.8	45	0.87
Mean±SEM		4.45±0.95	31.63±6.70	39.32±5.12	0.49±0.11
7	X VIVO-15[c]	5	36.2	18	0.33
8	X VIVO-15	2.5	25.7	33	0.21
9	X VIVO-15	4.1	22.4	44	0.4
10	X VIVO-15	3.4	38	20	0.25
Mean±SEM		3.75±0.61	30.6±4.44	28.8±7.01	0.30±0.05

[a]% gated large cells, larged cells, which were gated in dot plot display figured for foreward scatter and side scatter of FACS analysis and used for expression of monoclonal antibodies such as anti-CD83 Ab.

[b]X VIVO-15, serum free X VIVO-15 medium

[c]10 % FBS, RPMI-1640 with 10 % FBS

TABLE 3. COMPARISON OF PECENT POSITIVE CELLS AND MFI OF EACH SURFACE ANTIGEN BETWEEN CB-DC AND PB-DC CULTURED IN FBSM OR SFM WITH GM-CSF, IL-4 AND TNF-A (ADDED AT DAY 5) FOR 7 DAYS[a]

Surface antigens	Culture method	% Positive cell (n[b])			MFI (n)		
		CB-DC[c]	PB-DC[d]	*P*	CB-DC	PB-DC	*P*
CD1a	10 % FBS[e]	47.0±7.6 (6)	87.5±5.0 (6)	<0.005	92.2±40.4 (6)	291.0±54.8 (6)	<0.05
CD80	10 % FBS[f]	46.8±3.3 (4)	94.8±1.7 (6)	<0.0001	41.5±6.0 (4)	72.5±9.2 (6)	<0.05
CD83	10 % FBS	49.3±5.2 (8)	79.5±5.0 (8)	<0.001	60.0±17.6 (8)	55.1±7.3 (8)	NS
CD86	10 % FBS	79.8±11.7 (4)	88.0±4.7 (4)	NS[g]	138.5±15.9 (4)	129.8±19.6 (4)	NS
CMRF-44	10 % FBS	45.2±10.3 (5)	73.3±5.4 (4)	NS	41.4±15.6 (5)	56.8±10.5 (4)	NS
HLA-DR	10 % FBS	95.8±3.9 (4)	99.2±0.5 (6)	NS	794.5±242.6(4)	835.0±172.5(6)	NS
CD1a	X VIVO-15	17.2±5.3 (5)	28.8±8.4 (5)	NS	43.2±15.6 (5)	43.8±16.3 (5)	NS
CD80	X VIVO-15	28.3±18.1 (4)	34.0±7.8 (4)	NS	30.8±5.8 (4)	43.5±12.9 (4)	NS
CD83	X VIVO-15	22.8±7.1 (4)	36.3±11.4 (4)	NS	26.5±11.6 (4)	61.0±23.5 (4)	NS
CD86	X VIVO-15	85.7±6.3 (3)	67.0±9.5 (3)	NS	88.0±30.6 (3)	76.0±28.5 (3)	NS
CMRF-44	X VIVO-15	23.3±7.5 (4)	77.7±8.4 (3)	<0.005	31.8±12.2 (4)	53.7±11.2 (3)	NS
HLA-DR	X VIVO-15	98.7±0.3 (3)	99.0±0.6 (3)	NS	482.3±50.7 (3)	418.0±97.8 (3)	NS

[a]All values are given as the mean ± SEM.

[b]n, number of experiments

[c]CB-DC, dendritic cells derived from cord blood adherent cells

[d]PB-DC, denritic cells derived from peripheral blood adherent cells

[e]10 % FBS, RPMI-1640 with 10 % FBS

[f]X VIVO-15, serum free X VIVO-15 medium

[g]NS, no significent difference