

Cord Blood CD34⁺ Cells Differentiate into Dermal Dendritic Cells in Co-Culture with Cutaneous Fibroblasts or Stromal Cells

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The skin is a unique organ that contains two different subsets of dendritic cells, i.e., Langerhans cells and dermal dendritic cells. Our hypothesis is that cutaneous fibroblasts may affect the development of these dendritic cells. We cocultured cord blood CD34⁺ hematopoietic progenitor cells with several human cutaneous fibroblast cell lines without any exogenous cytokines for 3 wk. In this culture, hematopoietic progenitor cells increased in number from 20.1 ± 2.4 times, and produced aggregates of cells with dendritic processes. They were composed of $54.9 \pm 3.2\%$ HLA-DR⁺ CD14⁺ CD1a⁻ cells and $13.8 \pm 3.6\%$ HLA-DR⁺ CD1a⁺ cells, which also expressed CD11b and CD11c. There were significant numbers of factor XIIIa⁺ cells in the culture, whereas no Lag⁺ or E-cadherin⁺ cells were detected, and they were potent stimulators in allogeneic T cell activation. There was a significant difference in the ability to induce CD1a⁺ cells among different human cutaneous fibroblast cell lines. These CD1a⁺ cells lacked the expression of CD80, CD86, or CD83. In addition, half of them still expressed CD14. When these dendritic cells were cultured with tumor necrosis factor- α , however, they became mature dendritic

cells with augmented expression of CD86 and CD83 and with increased allogeneic T cell stimulation. The subsequent experiment using a dividing chamber, enzyme-linked immunosorbent assay for granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor, and the blocking studies with antibodies for these cytokines suggested that both the presence of direct contact between hematopoietic progenitor cells and human cutaneous fibroblast cell lines and macrophage colony-stimulating factor produced by human cutaneous fibroblast cell lines are required for their maximum growth and differentiation into CD1a⁺ dendritic cells, whereas macrophage colony-stimulating factor was solely responsible for their differentiation. These data suggest that cutaneous fibroblasts support the differentiation of dermal dendritic cells in addition to that of monocytes from hematopoietic progenitor cells by their direct contact with hematopoietic progenitor cells and by their macrophage colony-stimulating factor production. **Key words:** cadherin/factor XIIIa/granulocyte-macrophage colony-stimulating factor/Langerhans cells. *J Invest Dermatol* 118:450–460, 2002

Recent progress in the field of cytokine-derived dendritic cell (DC) growth and differentiation has elucidated that different combinations of hematopoietic growth factors, such as granulocyte-macrophage colony-stimulating factors (GM-CSF), interleukin (IL)-3, tumor necrosis factor (TNF)- α , stem cell factor, Flt3 ligand (Flt3L), and transforming growth factor (TGF)- β , can generate DC *in vitro* from bone marrow, cord blood, or adult blood CD34⁺ hematopoietic progenitor cells (HPC). (Caux *et al*, 1992, 1996a, b; Reid *et al*, 1992; Santiago-Schwarz *et al*, 1992; Romani *et al*, 1994; Bernhard *et al*, 1995; Siena *et al*, 1995; Szabolcs *et al*, 1995, 1996; Herbst *et al*, 1996; Rosenzweig *et al*, 1996; Strobl *et al*, 1996;

Strunk *et al*, 1996). In addition, these studies have increased our knowledge about the subsets of DC. DC comprise at least three distinct subsets, including two with a myeloid lineage and a third represented by lymphoid DC. Langerhans cells and interstitial DC constitute two types of immature myeloid DC *in vivo*. In *in vitro* cultures of CD34⁺ HPC in GM-CSF/TNF- α -supplemented medium, CD14⁻ CD1a⁺ Langerhans cell precursors and CD14⁺ CD1a⁻ precursors, which apparently differ from each other in their lineage commitment, emerge early during the culture. Langerhans cells precursors give rise to CD1a⁺ E-cadherin (E-cad)⁺ Birbeck granule (BG)⁺ but CD11b⁻ CD36⁻ factor XIIIa (FXIIIa)⁻ Langerhans cells, whereas CD14⁺ CD1a⁻ precursors differentiate into macrophage/monocyte and non-Langerhans cell interstitial DC with the help of M-CSF and GM-CSF/TNF- α , respectively (Caux *et al*, 1992; Strunk *et al*, 1996). These interstitial DC, which are characterized by the abundant expression of FXIIIa, CD1a, CD11b, and CD36 with the virtual absence of E-cad and BG, display striking similarities to dermal DC (Nestle *et al*, 1993; Caux *et al*, 1996a, 1997; de Saint-Vis *et al*, 1998).

In spite of these observations, however, we have not been able to identify which cytokines really function in these processes and what

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Abbreviations: BG, Birbeck granule; CFCL, cutaneous fibroblast cell lines; E-cad, E-cadherin; HPC, hematopoietic progenitor cells; Flt3L, Flt3 ligand; FXIIIa, factor XIIIa

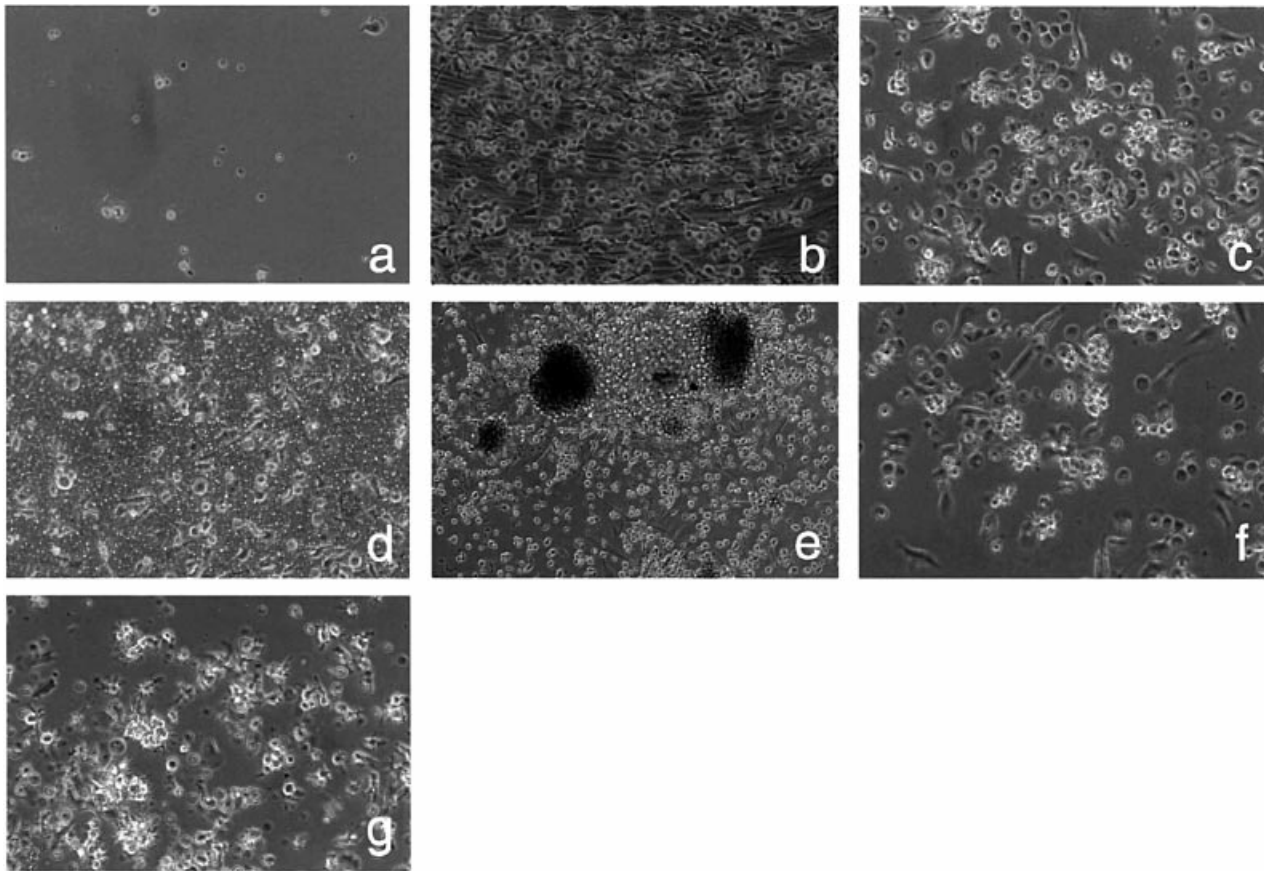


Figure 1. The coculture with cutaneous fibroblast cell lines supports the growth of CD34⁺ hematopoietic stem cells and their differentiation into DC. Three ml of NDF-1, NDF-2, CF-1, CF-2 or CF-3 (1×10^5 cells per ml) were seeded in 60×15 mm style Petri dishes. When these cells became confluent, they were irradiated with 2000 rad. Afterwards, 3×10^4 cells per ml of cord blood CD34⁺ HPC were added into these fibroblast cultures. Cultures were kept at 37°C in a 5% CO₂ humidified condition. Cells were recovered at 21–28 d of culture for analysis. As a control, CD34⁺ HPC were cultured with GM-CSF, TNF- α , and Flt3L. (a) The culture of CD34⁺ HPC without fibroblasts. (b) The coculture of CD34⁺ HPC with CF-2 (day 14). (c) The proliferating cells from the coculture of CD34⁺ HPC with CF-2 were harvested by gentle pipetting (day 20) and further cultured for 2 d without fibroblasts. (d) The proliferation of CD34⁺ HPC cultured in the upper chamber of a dividing chamber (day 20). (e) TNF- α stimulates DC derived from the coculture of CD34⁺ HPC with CF-2 to form cell aggregates. (f) TGF- β 1 does not significantly alter the morphology of DC derived from the coculture of CD34⁺ HPC with CF-2. (g) The culture of CD34⁺ HPC with GM-CSF, TNF- α , and Flt3L (day 13).

kinds of cells produce these relevant cytokines in the differentiation of DC from HPC *in vivo*. Recently, Miralles *et al* (1998) have reported that human thymic stromal cells induce both the proliferation of CD34⁺ HPC and their differentiation into DC *in vitro* without cytokines or serum supplementation.

Skin is a unique organ that contains two different subsets of DC, i.e., Langerhans cells in the epidermis and non-Langerhans cell dermal DC in the dermis. Therefore, our hypothesis is that cutaneous fibroblasts may affect the development of these DC. For this purpose, it is reasonable to examine the effects of dermal fibroblasts on the precursors of DC in the peripheral blood. Recently, Ito *et al* (1999) have identified the direct precursors of epidermal Langerhans cells in the peripheral blood as CD1a⁺ CD11c⁺ populations. In contrast, the precursors of dermal DC still remain unknown. Thus, in this study, we examined the effects of cutaneous fibroblasts on CD34⁺ HPC instead of examining those on the direct precursors in the peripheral blood. Here we report that several cutaneous fibroblast cell lines (CFCL) could definitely support the proliferation of CD34⁺ HPC and their differentiation into DC, especially CD1a⁺ E-cad⁺ BG⁺ FXIIIa⁺ CD11b⁺ CD11c⁺ non-Langerhans cell DC, without any exogenous cytokines. These DC had immature phenotypes, such as the lack of CD80, CD83, or CD86 expression, however, although they were potent antigen-presenting cells in the allogeneic T cell

stimulation. They were further matured with lipopolysaccharide (LPS) or TNF- α . The subsequent experiments to examine the mechanism for the effects of fibroblasts demonstrated that direct contact of CD34⁺ HPC with CFCL or M-CSF produced by CFCL was responsible for the optimal proliferation and differentiation of CD34⁺ HPC into CD1a⁺ E-cad⁺ BG⁺ FXIIIa⁺ CD11b⁺ CD11c⁺ non-Langerhans cell DC.

MATERIALS AND METHODS

Media and reagents The medium used in the study was RPMI-1640 including 25 mM Hepes buffer (Sigma, St Louis, MO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 1% penicillin, streptomycin, and fungizone antibiotic solution (Sigma), and 10% fetal calf serum (Bio West, Nuaille, France) (complete medium). The buffer used for the purification of CD34⁺ HPC from the cord blood was phosphate-buffered saline supplemented with 1% bovine serum albumin (less than 1 ng per mg of detectable endotoxin) (Sigma) and 5 mM ethylenediamine tetraacetic acid (MACS buffer).

We used the following monoclonal antibodies for immunostaining: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 antibody, FITC-conjugated anti-CD14 antibody, phycoerythrin (PE)-conjugated anti-CD11b antibody, PE-conjugated anti-CD19 antibody (Dakopatts, Glostrup, Denmark), FITC-conjugated anti-CD86 antibody, PE-conjugated anti-CD16 antibody, PE-conjugated anti-CD56 antibody

Table I. Effects of CFCL on the proliferation of CD34⁺ HPC and their differentiation into CD1a⁺ cells with or without direct contact^a

CFCL	Exp.	Coculture				Dividing chamber			
		Fold increases in cell no.	CD1a ⁺ cells (%)	CD1a ⁺ CD14 ⁺ cells (%)	CD14 ⁺ CD1a ⁺ cells (%)	Fold increases in cell no.	CD1a ⁺ cells (%)	CD1a ⁺ CD14 ⁺ cells (%)	CD14 ⁺ CD1a ⁺ cells (%)
NDF-1	1	n.c.	5.9	5.0	58.3	n.c.	0.6	n.c.	n.c.
NDF-1	2	9.0	3.4	1.9	40.8	n.c.	n.c.	n.c.	n.c.
NDF-1	3	9.0	6.2	1.8	52.8	1.2	n.c.	n.c.	n.c.
NDF-1	4	10.5	1.0	0.7	53.2	9.0	0.8	0.6	95.8
NDF-1	5	24.0	0.2	0.2	70.4	21.6	0.2	0.2	88.0
NDF-2	6	33.6	0.7	0.5	83.4	5.4	1.6	0.3	91.0
NDF-2	7	15.0	8.5	6.2	35.2	n.c.	0.2	n.c.	n.c.
NDF-2	8	18.0	2.1	1.5	47.9	6.0	n.c.	n.c.	n.c.
CF-2	1	27.0	28.0	7.5	57.6	n.c.	n.c.	n.c.	n.c.
CF-3	1	30.0	28.7	13.1	54.4	n.c.	n.c.	n.c.	n.c.
CF-3	2	16.5	32.7	16.8	51.9	4.8	4.9	1.6	82.3
CF-3	3	24.0	14.1	5.2	61.5	22.6	9.0	2.6	44.1
CF-3	4	21.0	23.6	9.5	48.5	3.0	17.7	1.6	63.8
CF-3	5	33.9	37.7	18.6	51.5	n.c.	n.c.	n.c.	n.c.
Mean ± SEM		20.1 ± 2.4	13.8 ± 3.6	6.3 ± 1.6	54.9 ± 3.2	9.2 ± 2.9	4.4 ± 2.2	1.2 ± 0.4	77.5 ± 8.1
GM-CSF, TNF- α , and Flt3L	1	21	5.8	4.9	84.9				
	2	64	3.88	3.0	77.2				

^an.c. not counted. CD34⁺ HPC were cocultured with CFCL for 21 d. Then the cells were recovered, counted and analyzed by flow cytometry. The increase in cell number was expressed as the fold increases of the numbers of recovered cells after culture against the numbers of plated CD34⁺ cells.

(PharMingen, San Diego, CA), FITC-conjugated anti-CD45 antibody, PE-conjugated anti-CD80 antibody, FITC-conjugated anti-HLA-DR antibody (Becton-Dickinson, San Jose, CA), FITC-conjugated or PE-conjugated anti-CD1a antibody, PE-conjugated anti-CD11c antibody, PE-conjugated CD83 antibody (Coulter, Hialeah, FL), FITC or PE-conjugated isotype-matched mouse control antibodies (IgG1 and IgG2a from PharMingen, and IgG2b from Ancell, Bayport, MN), monoclonal anti-human E-cad antibody (Takara Biomedicals, Tokyo, Japan), anti-BG-associated antigen antibody, Lag (gift of Dr F. Furukawa, Hamamatsu University, Sizuoka, Japan), and isotype control antibody (IgG1) (Sigma). We also used rabbit anti-human FXIIIa antibody (Berhingerwerke, Marburg, Germany; 1:800 dilution in final concentration) and rabbit anti-FVIII antibody (Dakopatts; 1:800 dilution) as a negative control. Recombinant human GM-CSF was a gift from Kirin Brewery (Tokyo, Japan) and recombinant human TNF- α , recombinant human M-CSF, recombinant human TGF- β 1, and recombinant human Flt3L were purchased from PepreoTech EC (London, U.K.).

Isolation of CD34⁺ HPC Umbilical cord blood samples were obtained according to the institutional guidelines. Cells bearing CD34 antigen were isolated from mononuclear fractions through positive selection as described (Gatti *et al*, 2000). Briefly, mononuclear cells were prepared by discontinuous density gradient centrifugation using Lymphoprep (Nycomed Pharma As, Oslo, Norway). CD34⁺ HPC were isolated from these mononuclear cells, using a Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) and MiniMACS separation columns (Miltenyi Biotec) according to the manufacturer's protocol.

CFCL We used five human CFCL. Two different lots of human neonatal dermal fibroblasts (NDF-1 and NDF-2) were obtained from Kurabo Industries (Tokyo, Japan). We also isolated fibroblast cell lines (CF-1, CF-2, and CF-3) from long-term culture of single cell culture from the epidermal sheets of three different donors as described previously (Nakagawa *et al*, 1993; Iwagami *et al*, 1994).

Coculture of CD34⁺ HPC with cutaneous fibroblasts Three milliliters of NDF-1, NDF-2, CF-1, CF-2, or CF-3 (1×10^5 cells per ml) were seeded in either 60 \times 15 mm style Petri dishes or six-well flat bottom multiwell tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ). When these cells became confluent, they were irradiated with 2000 rad by a Softex (Tokyo, Japan). Afterwards, 3×10^4 cells per ml of cord blood CD34⁺ HPC were added to these

fibroblast cultures. Cultures were kept at 37°C in a 5% CO₂ humidified condition. Every 6–7 d after culture, 200–300 μ l of fresh complete medium were added to the cultures. Cells were recovered by gentle pipetting at 21–28 d of culture for analysis. In some experiments, neutralizing mouse anti-M-CSF antibody (10 μ g per ml) (R&D Systems, Minneapolis, MN), neutralizing rat anti-GM-CSF antibody (5 μ g per ml) (PharMingen), or isotype-matched control antibodies (PharMingen) was added to the culture.

Dividing chamber culture By using a six-well format cell culture insert with a 0.4 μ m pore-sized polyethylene terephthalate (PET) track-etched membrane (Becton Dickinson Labware) in six-well flat bottom multiwell tissue culture plates, CD34⁺ HPC were placed in the upper chambers that were separated from the confluent culture of irradiated CFCL at the bottom of the plates. The numbers of CD34⁺ HPC and CFCL, and the volume of culture medium in the dividing chamber culture were identical to those in the control coculture.

Culture with cytokines As control experiments, we cultured CD34⁺ HPC with a combination of GM-CSF (50 ng per ml), TNF- α (10 ng per ml), and Flt3L (50 ng per ml) in the complete culture medium for 6 d according to the procedure described by Jaksits *et al* (1999), which produced both CD1a⁺ E-cad⁺ BG⁺ Langerhans cells and CD1a⁺ CD11b⁺ CD36⁺ FXIIIa⁺ E-cad⁺ BG⁺ non-Langerhans cell DC. We also cultured CD34⁺ HPC with 50 ng per ml of GM-CSF or 100 ng per ml of M-CSF for 21 d. We changed one-half the volume of culture medium every 4 d with fresh medium containing the same concentration of GM-CSF or M-CSF. In some of the cultures with M-CSF, we added anti-M-CSF antibody or irrelevant control antibody (10 μ g per ml) once every 4 d.

Flow cytometry To determine the surface phenotypes of cultured CD34⁺ HPC, the cells harvested after culture were treated with a combination of FITC-conjugated antibody (25 μ g per ml) and PE-conjugated antibody (25 μ g per ml). For E-cad expression, the cells were first incubated with anti-E-cad antibody or isotype-matched control antibody, then blocked with mouse serum, and revealed by FITC-conjugated anti-mouse immunoglobulins (Tago Immunologicals, Camarillo, CA). After washing with phosphate-buffered saline supplemented with 1% bovine serum albumin and 0.02% NaN₃ (FACS buffer), the cells were analyzed by FACSscan using CellQuest software (Becton Dickinson). Dead cells were gated out after staining with 0.5 μ g per ml of propidium iodide solution.

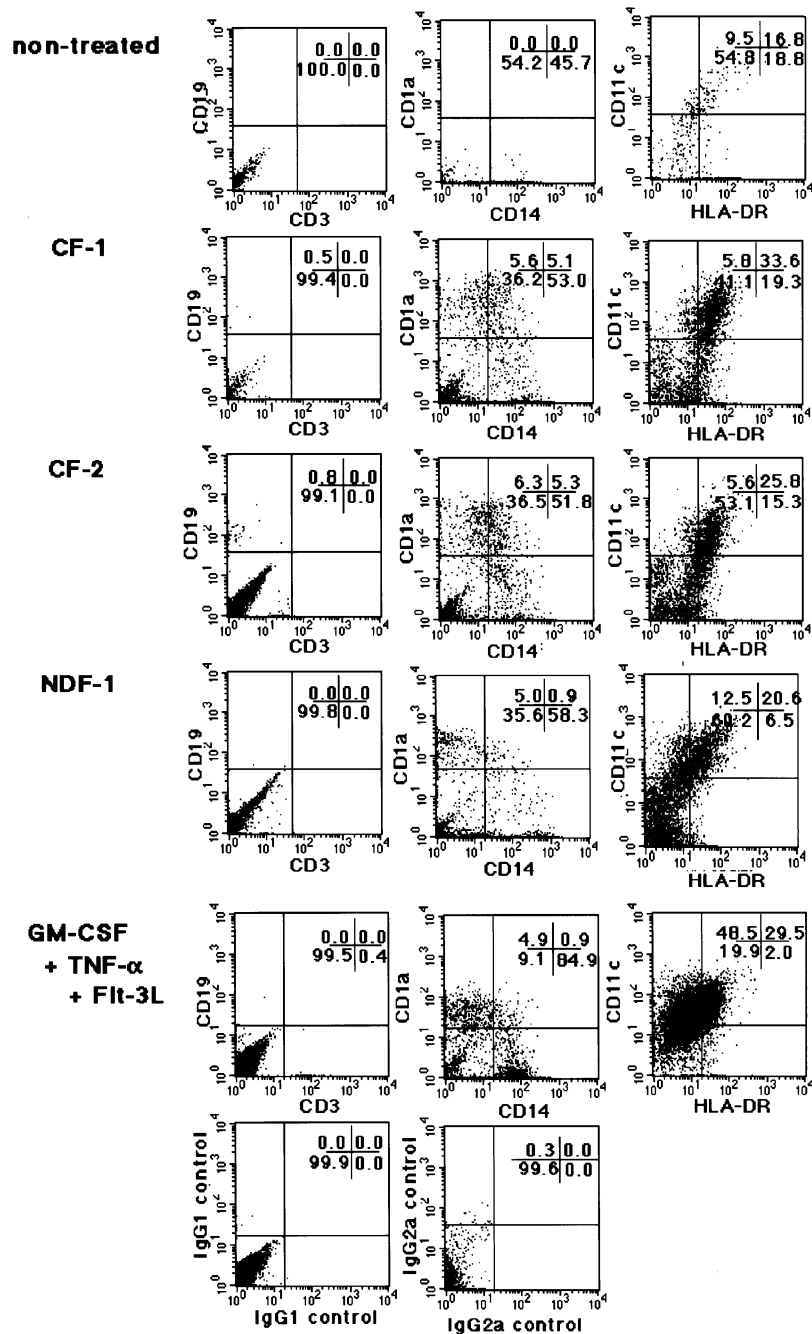


Figure 2. The coculture with CFCL induces the differentiation of CD34⁺ HPC into CD14⁺ monocytes and CD1a⁺ DC. CD34⁺ HPC were cultured alone in the complete medium for 7 d, with various CFCL (CF-1, CF-2, and NDF-1) without any exogenous cytokines for 21 d, or without CFCL in the presence of GM-CSF, TNF- α , and Flt3L for 10 d. After culture, the cells were recovered and analyzed by flow cytometry. These data are representative of three independent experiments.

Confocal laser scanning microscopy Immunohistochemical analysis of FXIIIa and Lag expression was performed on cytospin slides for CD34⁺ HPC cocultured with CFCL and for those cultured with a combination of GM-CSF, TNF- α , and Flt3L. The slides were at first fixed and permeabilized by Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, An Der Grub, Austria). After blocking with goat serum, the slides were treated either with Lag or nonreactive mouse IgG1 antibody followed by FITC-conjugated anti-mouse immunoglobulins (Tago Immunologicals) or with anti-FXIIIa or anti-FVIII antibody followed by FITC-conjugated anti-rabbit immunoglobulins (Tago Immunologicals). The slides were mounted in permaFluor (Lipshaw Immunon, Pittsburgh, PA) containing 0.5 μ g propidium iodide per ml, and observed under a confocal microscope (Leica microscope and system GmbH, Wetzlar, Germany).

Stimulation with TGF- β 1, TNF- α , or LPS CD34⁺ HPC were harvested from the coculture and then stimulated in the complete culture medium with 10 ng TGF- β 1 per ml, 10 ng TNF- α per ml, or 10 ng LPS per ml for 48 h.

Allogeneic T cell stimulation Peripheral blood mononuclear cells were obtained by Ficoll Paque (Pharmacia) gradient centrifugation of heparinized blood. These peripheral blood mononuclear cells were treated with a pan T cell isolation kit (Miltenyi Biotec), according to the manufacturer's protocol. These enriched T cells consisted of more than 95% CD3⁺ cells. T cells (2×10^5 cells per well) were cocultured in 96-well flat-bottom microtiter plates with various numbers of cultured CD34⁺ HPC. After 4 d of culture at 37°C in a 5% CO₂ humidified atmosphere, the cells were pulsed with 5 μ Ci per ml of [³H]-thymidine (Radiochemical Center, Amersham, U.K.) for the last 16 h of culture. At the end of the culture period, the cells were transferred on to glass fiber and [³H]-thymidine incorporation was measured using a liquid scintillation counter. The results were expressed as the mean \pm SEM of triplicate cultures. To decrease the background proliferation, cultured CD34⁺ HPC were irradiated with 2000 rad with a Softex.

Enzyme-linked immunosorbent assay for GM-CSF and M-CSF production by fibroblasts NDF-1, NDF-2, CF-1, CF-2, and CF-3 were cultured in complete medium. After the cells became confluent,

CF-3

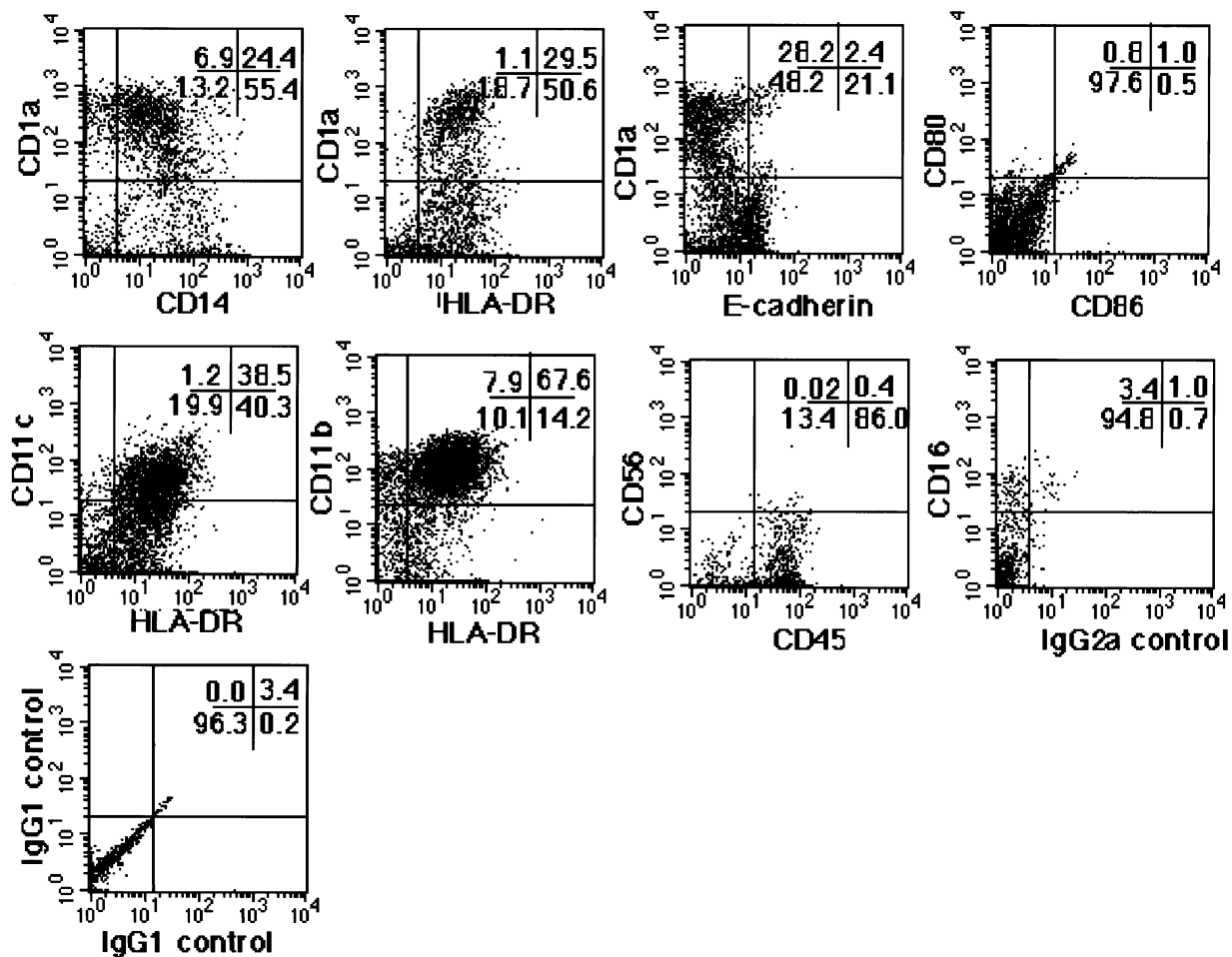


Figure 3. The coculture with CFCL induces the differentiation of CD34⁺ HPC into CD1a⁺ CD11b⁺ CD11c⁺ HLA-DR⁺ E-cad⁻ DC. CD34⁺ HPC were cultured with CF-3 without any exogenous cytokines for 21 d, and then the cells were analyzed by flow cytometry. These data are representative of four independent experiments.

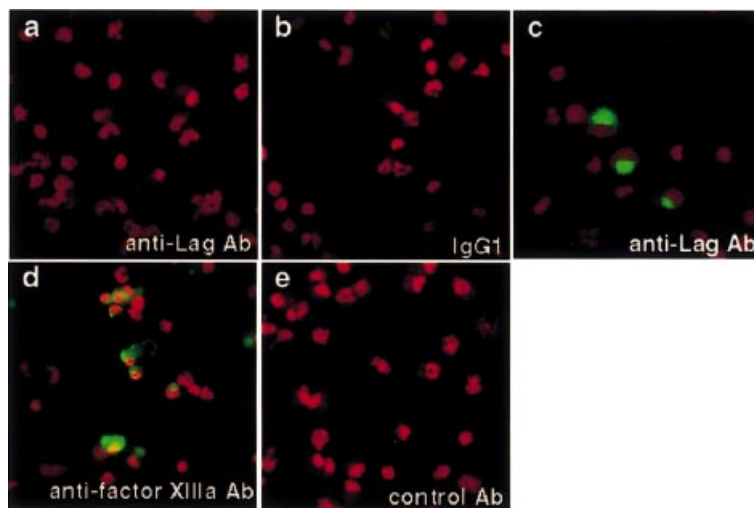


Figure 4. DC differentiated from CD34⁺ HPC by CFCL do not express BG-associated antigen, Lag, but express FXIIIa. The cytosin slides for CD34⁺ HPC cocultured with NDF-1 were examined for the expression of BG-associated antigen, Lag (a) and FXIIIa (d). (b, e) demonstrate DC stained with relevant control antibodies for anti-Lag antibody and anti-FXIIIa antibody, respectively. (c) shows DC differentiated from CD34⁺ HPC cultured with a combination of GM-CSF, TNF- α , and Flt3L stained with anti-Lag antibody. Similar data were also obtained by the coculture with other CFCL.

the cultures were replaced with new complete medium and further cultured for 1 wk. The recovered supernatants were measured for GM-CSF and M-CSF by enzyme-linked immunosorbent assay kits obtained

from R&D Systems, using 96-well microtiter plates, according to the manufacturer's instructions. The levels of GM-CSF and M-CSF were calculated by using a standard curve obtained with recombinant

GM-CSF (from 0 to 1000 pg per ml) and M-CSF (from 0 to 1000 pg per ml). The data were expressed the mean \pm SEM of triplicate determinations.

RESULTS

CD34⁺ HPC differentiate into CD1a⁺ non-Langerhans cell DC and CD14⁺ monocytes in the coculture with CFCL To determine whether CFCL support the development of DC from CD34⁺ HPC, CD34⁺ HPC were cocultured with irradiated human CFCL. When CD34⁺ HPC were cultured alone in the complete medium, most of them died within 7 d (**Fig 1a**). In contrast, when they were cultured with CFCL without any exogenous cytokines, the growth of both adherent and nonadherent cells became recognizable within 7 d and they continued growing for at least 1 mo (**Fig 1b**). When these growing cells were recovered from the coculture with gentle pipetting and further cultured without fibroblasts, they made small aggregates of DC (**Fig 1c**). CD34⁺ HPC expanded 20.1 ± 2.4 -fold in the coculture ($n = 13$) (**Table I**), whereas CD34⁺ HPC cultured without CFCL in the presence of a combination of GM-CSF (50 ng per ml), TNF- α (10 ng per ml), and Flt3L (50 ng per ml) in the complete culture medium for 6 d expanded 21- and 64-fold in two different experiments and produced aggregates of DC (**Fig 1g**).

Immunophenotypic analysis of the cocultured cells (**Fig 2**) revealed that they were composed of $54.9 \pm 3.2\%$ CD14⁺ cells and $13.8 \pm 3.6\%$ CD1a⁺ cells, in which $6.3 \pm 1.6\%$ were CD1a⁺ CD14⁻ cells (**Table I**). In addition, 20–34% were HLA-DR⁺ CD11c⁺ cells. There were no CD3⁺ or CD19⁺ cells. These phenotypic characteristics were similar to those of CD34⁺ HPC cultured with a combination of GM-CSF, TNF- α , and Flt3L,

which was reported to induce the differentiation of CD34⁺ HPC into DC by Jaksits *et al* (1999), although the cocultured cells contained more CD1a⁺ CD14⁺ cells than CD34⁺ HPC cultured with a combination of cytokines. There was a significant difference in the ability to induce the differentiation of CD34⁺ HPC into CD1a⁺ cells among the five different CFCL. Namely, CF-3 induced the differentiation into CD1a more efficiently than NDF-1 ($p = 0.01$).

The coculture of CD34⁺ HPC with CFCL induced aggregates of CD1a⁺ cells with DC morphology. Therefore, we clarified further their phenotypic characteristics. In **Fig 3**, showing representative data of four different cocultures with CF-3, it is demonstrated that CD1a⁺ cells in the coculture, which were 50–60% of CD14⁺ or 40–50% CD14⁻, expressed HLA-DR antigen, but did not express E-cad, CD80, or CD86. Although we did not perform double staining of anti-CD1a antibody with anti-CD11b antibody or anti-CD11c antibody in this experiment, the data showing that CD1a⁺ cells expressed HLA-DR, whereas most of HLA-DR⁺ cells expressed CD11b or CD11c, suggested the expression of CD11b and CD11c on CD1a⁺ cells. We observed similar phenotypic characteristics in the cells cocultured with other CFCL.

Next, we examined immunohistochemically the expression of BG-associated antigen, Lag, and FXIIIa on CD34⁺ HPC cocultured with CFCL. In **Fig 4**, it is shown that they contained 40% of FXIIIa⁺ cells, but no Lag⁺ cells. In contrast, CD34⁺ HPC cultured with a combination of GM-CSF, TNF- α , and Flt3L contained Lag⁺ cells. These phenotypic analyses suggested that CFCL supported the differentiation of CD34⁺ HPC into CD1a⁺ E-cad⁻ Lag⁻ FXIIIa⁺ CD11b⁺ CD11c⁺ HLA-DR⁺ cells, which were reported as the phenotype of non-Langerhans cell

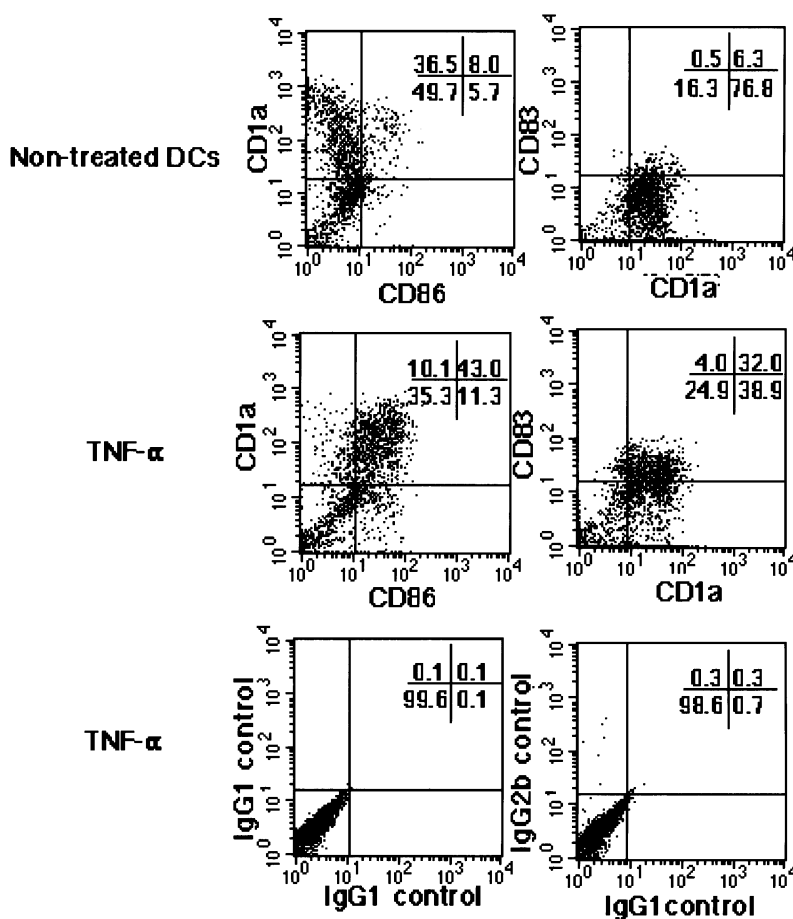


Figure 5. DC differentiated from CD34⁺ HPC by CFCL increase their CD86 expression by TNF- α . CD34⁺ HPC were harvested from the coculture after 21 d and then stimulated in the complete culture medium with 10 ng per ml TNF- α for 48 h. Their surface expression of CD83 and CD86 on CD1a⁺ cells was examined by flow cytometry. These data are representative of three independent experiments.

DC by Caux *et al* (1996a), although the coculture with CFCL produced significant numbers of CD14⁺ CD1a⁺ cells.

The effects of cytokines on DC obtained from the coculture system The coculture of CD34⁺ HPC with CFCL induced both CD1a⁺ CD14[−] cells and CD1a⁺ CD14⁺ cells. Caux *et al* (1996a), have reported that CD1a⁺ CD14⁺ cells are in the middle of differentiation of CD14⁺ DC precursor subsets to CD1a⁺ CD14[−] DC. In addition, none of the CD1a⁺ cells derived from CD34⁺ HPC cocultured with CFCL expressed CD80 or CD86. These data suggested that DC obtained from the coculture were still immature. Therefore, to examine whether these immature DC can be matured, the cells recovered from the cocultures were treated with TNF- α . The treatment of the cells with TNF- α for 2 d

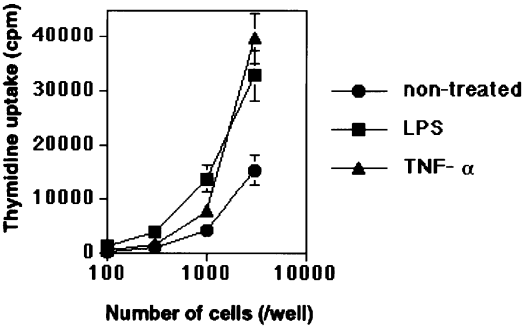


Figure 6. DC differentiated from CD34⁺ HPC by CFCL are potent antigen-presenting cells in allogeneic mixed lymphocyte culture. Purified CD3⁺ T cells (2 × 10⁵ cells per well) were cocultured in 96-well flat bottom microtiter plates with various numbers of CD34⁺ HPC cocultured with CF-3. After 4 d of culture at 37°C in a 5% CO₂ humidified atmosphere, the cells were pulsed with 5 μ Ci per ml of [³H]thymidine during the last 16 h of culture. The mean \pm SEM from three replicates were taken from each sample culture. To decrease the background proliferation, cultured CD34⁺ HPC were irradiated with 2000 rad. In some experiments, cultured CD34⁺ HPC were prestimulated with 10 ng per ml of LPS or TNF- α for 48 h before T cell stimulation. These data are representative of four independent experiments.

produced large aggregates of DC (**Fig 1e**) that expressed CD86 and CD83 (**Fig 5**), suggesting the induction of DC maturation. Moreover, to examine whether these immature DC acquire the Langerhans cells phenotype, we cultured them with TGF- β 1. We found that the treatment of the cells with TGF- β 1 did not induce any significant morphologic changes (**Fig 1f**), nor the expression of E-cad or Lag (data not shown).

Antigen presentation by DC differentiated from the coculture of CD34⁺ HPC with CFCL Next, we studied the antigen-presenting function of these DC differentiated from CD34⁺ HPC cocultured with CFCL. When they were cultured with allogeneic T cells, they stimulated the proliferation of allogeneic T cells in a concentration-dependent fashion (**Fig 6**). Even at 1000 cells per well, these cultured CD34⁺ HPC containing 10% CD1a⁺ cells significantly stimulated allogeneic T cells. Preculture of these cells with LPS or TNF- α significantly augmented the proliferation.

Both M-CSF produced by CFCL and direct interaction between CD34⁺ HPC and CFCL are required for the maximum proliferation of CD34⁺ HPC As the coculture of CD34⁺ HPC with CFCL supported the growth and differentiation of CD34⁺ HPC into CD1a⁺ DC and CD14⁺ monocytes, we next investigated whether direct contact of CD34⁺ HPC with CFCL was required for the proliferation and differentiation of CD34⁺ HPC. Using dividing chambers separated with semipermeable membranes, we cultured CD34⁺ HPC in the upper chamber and CFCL in the lower chamber. This culture condition still supported the growth of CD34⁺ HPC, but the magnitude of cell proliferation was less than one-half of that of the coculture (**Table I**). Statistical analysis using the Mann–Whitney U test indicated a significant difference in the growth of CD34⁺ cells between the coculture and the culture using the dividing chamber ($p < 0.01$). When examined under an inverted microscope (**Fig 1d**), most of the cells in the upper chamber were adherent and macrophage-like in morphology. Furthermore, by flow cytometry, the percentage of CD1a⁺ cells in the upper chamber was 4.4 ± 2.2 , which was significantly less than the 13.8 ± 3.6 that was noted in the coculture ($p < 0.05$) (**Table I** and **Fig 7**). These data suggested that the direct contact of CD34⁺ HPC with CFCL was required for

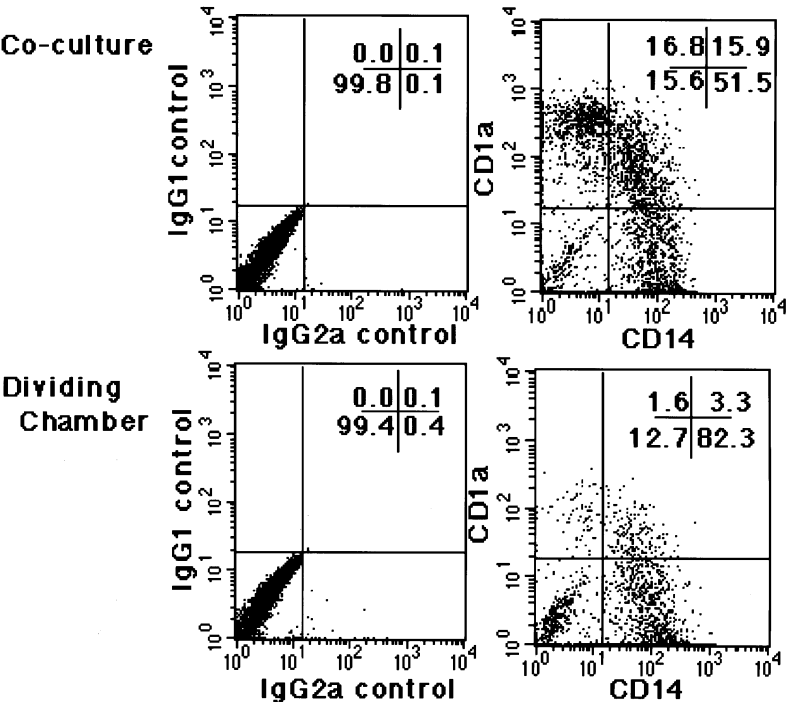


Figure 7. Direct contact between CD34⁺ HPC and CFCL are required for their optimal differentiation into CD1a⁺ DC. Using dividing chambers separated with semipermeable membranes, we cultured CD34⁺ HPC in the upper chamber and CF-3 in the lower chamber. After 21 d of culture, the cells were recovered and analyzed by flow cytometry for the expression of CD1a and CD14. Similar data were also obtained by the coculture with other CFCL. These data are representative of four independent experiments.

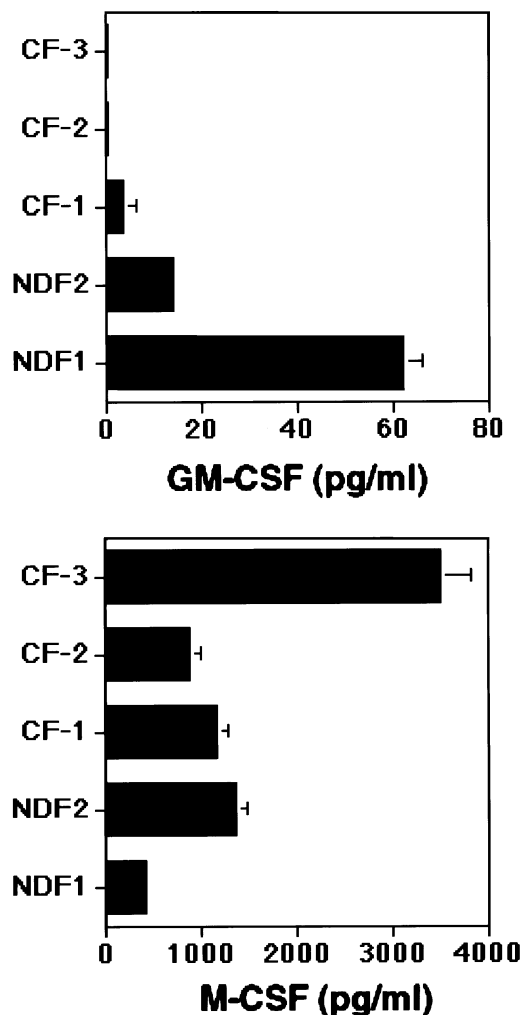


Figure 8. The production of GM-CSF by CFCL does not correlate with their ability to induce DC from CD34⁺ HPC. NDF-1, NDF-2, CF-1, CF-2, and CF-3 were cultured in complete medium. After the cells became confluent, the cultures were replaced with new complete medium and further cultured for 1 wk. The recovered supernatants were measured for GM-CSF and M-CSF by enzyme-linked immunosorbent assay. The levels of GM-CSF and M-CSF were calculated by using a standard curve obtained with recombinant GM-CSF (from 0 to 1000 pg per ml) and M-CSF (from 0 to 1000 pg per ml). Data are representative of two independent experiments, and reported as the mean \pm SEM of triplicate determinations.

their maximum proliferation and differentiation into CD1a⁺ cells, although soluble factors also played a significant part in these processes. As there are several papers that reported the role of GM-CSF and M-CSF for the growth or differentiation of DC (Caux *et al*, 1992; Reid *et al*, 1992; Santiago-Schwarz *et al*, 1992; Takashima *et al*, 1995; Kamps *et al*, 1999), we measured the production of GM-CSF and M-CSF from CFCL (Fig 8). NDF-1, NDF-2, and CF-1 produced low but detectable levels of GM-CSF, whereas CF-2 or CF-3 did not produce it at all. On the other hand, all the CFCL secreted a significant amount of M-CSF. Among CFCL, CF-3 produced more than two times more M-CSF than did the other CFCL, which agreed well with the potent ability of CF-3 to support CD34⁺ cell growth and differentiation. These data suggested that M-CSF, but not GM-CSF, might be responsible for the proliferation or the differentiation of CD34⁺ HPC. It was further confirmed by the experiment using anti-M-CSF antibody (Fig 9). Namely, when we added anti-M-CSF antibody to the

coculture of CD34⁺ HPC with CF-3, anti-M-CSF antibody partially but still significantly suppressed the proliferation of CD34⁺ HPC. When we added 100 ng M-CSF per ml in the culture of CD34⁺ HPC as a control, there occurred their significant proliferation, but the number of recovered cells was almost half of that of the coculture with CF-3. In addition, in this culture, anti-M-CSF antibody completely abrogated the proliferation of CD34⁺ cells.

M-CSF produced by CFCL induces CD1a⁺ DC from the culture of CD34⁺ HPC As M-CSF produced by CFCL was found to be at least partially responsible for the induction of CD34⁺ HPC proliferation by the coculture of CFCL, we examined the role of M-CSF on the differentiation of CD34⁺ HPC into CD1a⁺ DC. When we added anti-M-CSF antibody, anti-GM-CSF antibody, or irrelevant isotype control antibody to the culture of CD34⁺ HPC with CF-3, only anti-M-CSF antibody completely suppressed the differentiation of CD1a⁺ cells (Fig 10). Therefore, we checked the effect of M-CSF on the culture of CD34⁺ HPC. M-CSF could induce the cells showing phenotypes almost identical with those of the cocultured cells from CD34⁺ HPC (Fig 10). Namely, they induced CD1a⁺CD14⁻, CD1a⁺CD14⁺, or CD1a⁻CD14⁺ cells. In contrast, GM-CSF only induced CD1a⁺CD14⁺ cells. In addition, the cells induced by M-CSF were Lag⁻, but FXIIIa⁺.

DISCUSSION

So far, most of the studies on DC growth and differentiation have been performed with *in vitro* culture systems using a various combinations of cytokines. Recently, however, Regnier *et al* (1997) have demonstrated that CD34⁺ HPC cultured on epidermis that was reconstructed on dead de-epidermized cellular human dermis produced CD1a⁺ HLA-DR⁺ BG⁺ Langerhans cells without any exogenous cytokines. This study suggests that only a microenvironment that is composed of keratinocytes can induce the differentiation of HPC into Langerhans cells. In spite of this observation, however, at least two questions remain unanswered. The first one is that, as their culture system did not contain any dermal cell components, it is not clear whether a reconstituted skin microenvironment with keratinocytes plus fibroblasts might induce the differentiation of HPC into dermal DC in addition to Langerhans cells. The second question is how this culture system induced the differentiation of HPC into Langerhans cells. Namely, this study did not address whether soluble factors secreted by the reconstructed epidermis or the direct contact of CD34⁺ HPC to some components of this three-dimensional culture mediated the differentiation of HPC into Langerhans cells. In this study, we could demonstrate that the simple coculture of CD34⁺ HPC with cutaneous fibroblasts induced their differentiation into CD1a⁺ E-cad⁻ BG⁻ but CD11b⁺ CD11c⁺ FXIIIa⁺ non-Langerhans cell interstitial DC without any exogenous cytokines. Nestle *et al* (1993) reported that dermal DC were composed of three different subsets, FXIIIa⁺ CD1a⁺ non-Langerhans cell interstitial DC, and two other unique populations that were FXIIIa⁺ CD1a⁻ CD14⁻ cells and FXIIIa⁺ CD1a⁻ CD14⁺ cells. Interestingly, at least phenotypically, our culture system using cutaneous fibroblasts could induce all of these three subsets of dermal DC. Moreover, these three different components were recognizable even after 3 wk of culture. These data suggest that the dermis containing numerous fibroblasts supplies the environment for the differentiation of dermal DC. Our subsequent studies further elucidated that the maximum growth for CD34⁺ HPC and their differentiation into CD1a⁺ cells require both their direct contact with CFCL and M-CSF produced by CFCL, whereas their differentiation into CD1a⁺ E-cad⁻ BG⁻ FXIIIa⁺ CD11b⁺ CD11c⁺ non-Langerhans cell DC was solely mediated by M-CSF.

It is well known that M-CSF is produced by a variety of cell types, including fibroblasts, bone marrow stromal cells, T cells, endothelial cells, hepatocytes, and that of monocyte/macrophage lineage (Rambaldi *et al*, 1987; Praloran *et al*, 1990; Clinton *et al*,

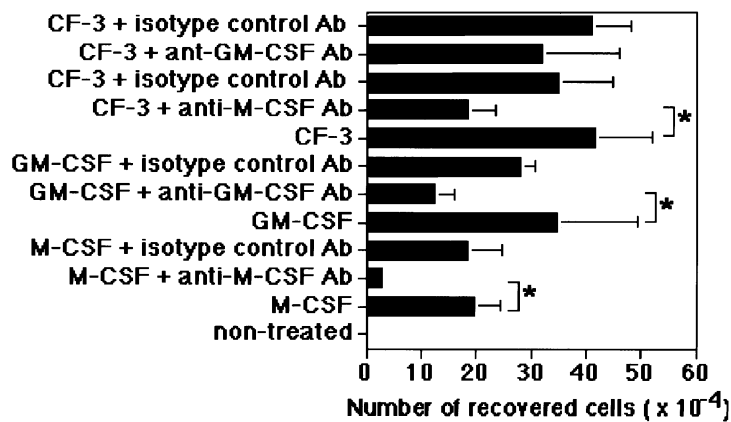


Figure 9. Anti-M-CSF antibody partially suppresses the proliferation of CD34⁺ cells cocultured with CF-3. We cultured CD34⁺ HPC with CF-3 or M-CSF (100 ng per ml) in the presence or absence of anti-M-CSF antibody or irrelevant antibody for 21 d. For the coculture with CF-3, half the volume of the culture medium was replaced with the same volume of fresh medium every 7 d. For the culture with GM-CSF or M-CSF, we changed one-half the volume of culture medium every 4 d with fresh medium containing the same concentration of GM-CSF or M-CSF. In some of the cultures with M-CSF, we added anti-M-CSF antibody or irrelevant control antibody (10 µg per ml) every 4 d. After culture, we counted the number of recovered cells. Data are reported as mean ± SD of three independent experiments. *p < 0.05 by paired Student's t test.

1992; Tobler *et al*, 1992; Tsukui *et al*, 1992; Nakajima *et al*, 1994). Therefore, it is reasonable to speculate that M-CSF affects the differentiation of DC; however, there is a controversy about the effects of M-CSF on DC growth and differentiation. Xu *et al* (1995) succeeded in generating long-term DC lines (XS series) from murine epidermis. To establish and maintain these cell lines, M-CSF produced by their feeder cell lines, called NS lines, is indispensable (Takashima *et al*, 1995). Furthermore, they demonstrated that XS lines as well as epidermal Langerhans cells express M-CSF receptor, and that M-CSF stimulated the proliferation of XS lines. In addition, Kamps *et al* (1999) have demonstrated that instead of GM-CSF, M-CSF also could induce DC in conjunction with TNF-α and IL-4. Moreover, Dalloul *et al* (1999) demonstrated that M-CSF augmented the production of DC from the cultures of thymic CD34⁺ CD1a[−] cells with stem cell factor⁺ Flt3[−] ligand⁺ IL-7. In contrast, Menetrier-Caux *et al* (1998) reported the inhibitory effects of M-CSF or IL-6 produced by tumor cells on DC differentiation from CD34⁺ HPC. In this experiment, however, they always examined the effects of M-CSF on DC differentiation in cultures containing GM-CSF and TNF-α; they did not examine the effects of M-CSF alone on CD34⁺ HPC. This study demonstrated that both the coculture with CFCL and the culture with M-CSF induced CD1a⁺ CD14[−] DC. Interestingly, although we cultured CD34⁺ HPC for more than 3 wk in our experiments, these DC remained immature without the expression of CD80, CD83, or CD86. Moreover, nearly half of the CD1a⁺ cells were still CD14[−]. It suggested that M-CSF induced DC, especially dermal DC, and kept them in an immature state, although these immature DC could exhibit a potent antigen-presenting function and be matured by TNF-α stimulation.

Studies (Takahashi *et al*, 1993; Witmer-Pack *et al*, 1993; Usuda *et al*, 1994) using op/op mice, which cannot produce functional M-CSF, have demonstrated normal differentiation of DC without M-CSF, although reduced density (Witmer-Pack *et al*, 1993) and abnormal morphology (Takahashi *et al*, 1993) of epidermal Langerhans cells were also reported. These data mainly focused on epidermal Langerhans cells, however, but not on dermal DC. Therefore, these studies still cannot answer the question of whether M-CSF influence the differentiation of dermal DC *in vivo*.

On the other hand, the experiments using a dividing chamber in this study also suggested the influence of direct contact of CD34⁺ HPC with fibroblasts on their proliferation. As the integrins, especially β1 integrin(s), were reported to be involved in the regulation of HPC proliferation and differentiation (reviewed in Coulombel *et al*, 1997), we speculated that the growth of HPC supported by their direct contact with fibroblasts was mediated through the interaction of β1 integrin(s) on CD34⁺ HPC with the extracellular matrix produced by fibroblasts. Indeed, Staquet *et al*

(1997) have demonstrated that fibronectin upregulated the *in vitro* generation of DC from CD34⁺ HPC. When we added anti-β1 integrin antibody in this coculture system, however, we could not detect any inhibitory effects on the proliferation of CD34⁺ cells or their differentiation into CD1a⁺ cells (data not shown). So far, we have been unable to find out any candidates that cause the proliferation and differentiation of CD34⁺ cells supported by their direct contact with cutaneous fibroblasts.

We found a significant difference in the ability of different CFCL to induce CD1a⁺ DC. CF-3, which was obtained from the long-term culture of single cells from epidermal sheets, was much more efficient than the neonatal fibroblast cell lines. Previous studies (Nakagawa *et al*, 1993; Aiba *et al*, 1994, 1995; Iwagami *et al*, 1994) have reported that cutaneous fibroblast cell lines obtained by this procedure have immunologic functions distinct from those of the fibroblast cell lines obtained by standard enzymatic treatment of the dermis. Namely, they promote the growth of γ/δ T cell receptor-positive cells in mice and produce various cytokines in addition to autologous T cell activation after interferon-γ treatment in humans. In this study, although a considerable amount of M-CSF production was demonstrated in the culture supernatants of all CFCL, CF-3 produced more than two times M-CSF than the two other NDF lines, and induced significantly more vigorous proliferation of CD34⁺ HPC than the other NDF lines. These data again suggest the presence of heterogeneity in dermal fibroblasts in terms of their immunologic functions. In this study, we examined the effects of fibroblasts obtained from the skin. Dermal DC belong to interstitial DC, which distribute in various nonlymphoid tissues. Therefore, it was reasonable to speculate that fibroblasts from other organs can induce DC from CD34⁺ HPC in a coculture system. We cocultured CD34⁺ HPC with fibroblasts from the colon and lung. Expectedly, these cultures could induce the proliferation of CD34⁺ HPC and their differentiation into CD1a⁺ DC, although the percentage of CD1a⁺ cells in them was about one-third of that in the coculture with NDF-2 (data not shown).

Finally, despite the recent evidence presented for direct precursors of Langerhans cells in the peripheral blood (Ito *et al*, 1999), we did not obtain any information about the direct precursors of dermal DC. Sallusto and Lanzavecchia (1994) and Romani *et al* (1994) reported that even CD14 monocytes could be differentiated into DC in a manner similar to dermal DC in a culture with GM-CSF and IL-4. Our present data suggest that the environment that allows the direct contact of HPC with cutaneous fibroblasts can induce their differentiation into CD1a⁺ dermal DC. The next step of our research is to determine or find out the precursors of dermal DC in the peripheral blood.

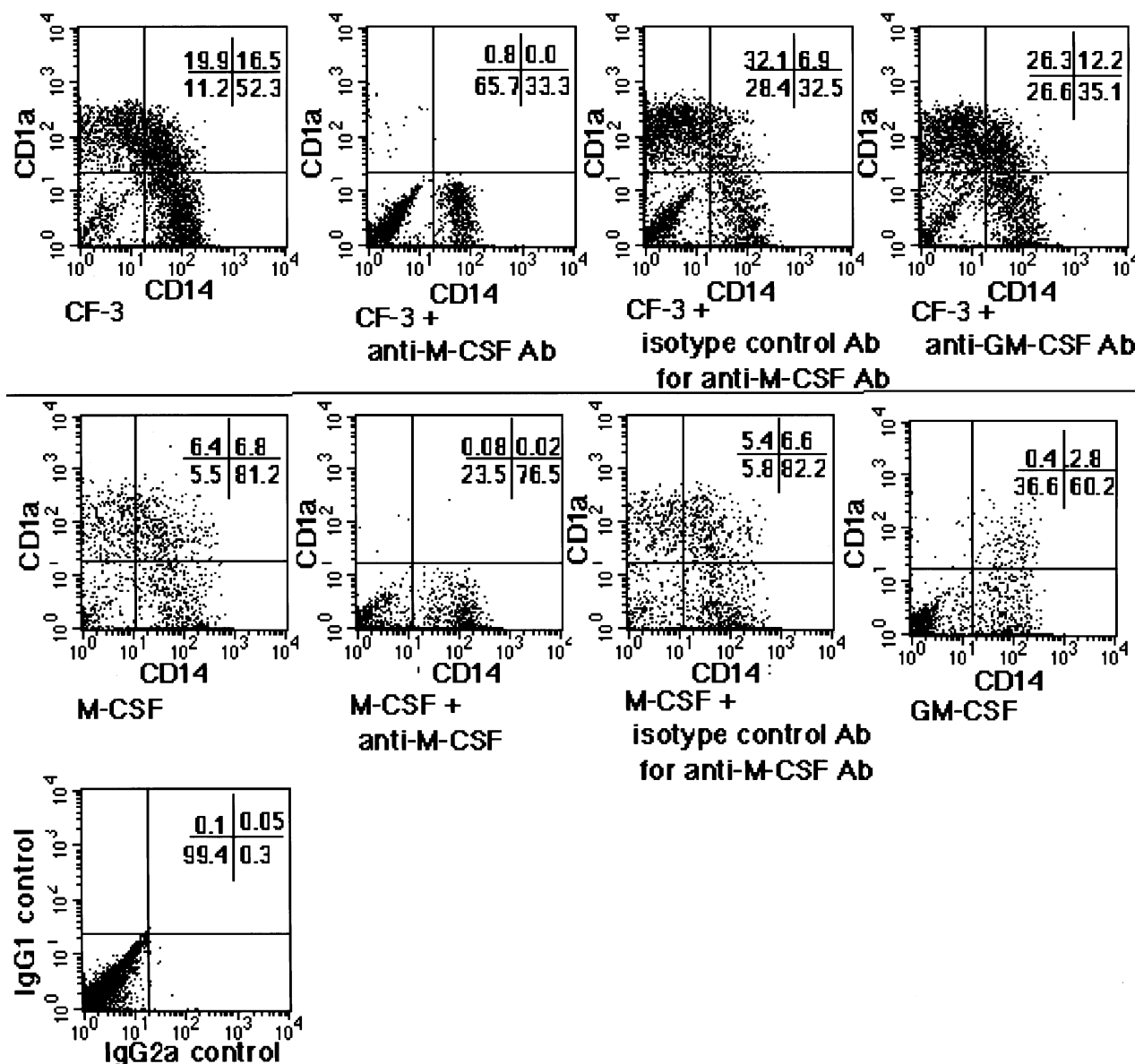


Figure 10. M-CSF is mainly responsible for the differentiation of CD34⁺ HPC into CD1a⁺ cells. We cultured CD34⁺ HPC with CF-3, M-CSF (100 ng per ml), GM-CSF (50 ng per ml) in the presence or absence of anti-M-CSF antibody or irrelevant antibody for 21 d. For the coculture with CF-3, 200–300 μ l of fresh complete medium were added to the cultures every 6–7 d after culture. For the culture with M-CSF or GM-CSF, we changed one-half the volume of culture medium every 4 d with fresh medium containing the same concentration of M-CSF or GM-CSF, respectively. In some of the cultures with M-CSF, or GM-CSF, we added anti-M-CSF antibody, anti-GM-CSF antibody or irrelevant control antibody once every 4 d. After culture, we analyzed their surface phenotype by flow cytometry.

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