

***Ex vivo* Generation of Functional Dendritic Cells from Mobilized CD34⁺ Hematopoietic Stem Cells**

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The ability to generate dendritic cells (DCs) in sizeable numbers has enormous implications for the development of clinically-effective antigen presentation procedures for cancer immunotherapy. We evaluated the generation of immunostimulatory DCs from peripheral blood CD34⁺ cells collected from healthy donors. CD34⁺ cells purified from leukapheresis product were seeded at 1×10^4 cells/mL in complete medium supplemented with GM-CSF, TNF α , IL-4, c-kit ligand, and flt3 ligand (FL). By day 14 of culture in the presence of GM-CSF + TNF α , the total cell number increased by 23.4 ± 5.4 -fold compared to the starting number of CD34⁺ cells. When the c-kit and FL were added to GM-CSF and TNF α , the cell number increased by 109.8 ± 11.2 -fold without affecting the immunophenotype of recovered cells. Flow cytometric analysis indicated that cells with the markers of mature dendritic cells, ie, CD1a⁺CD14⁻HLA-DR⁺, and CD80⁺CD86⁺HLA-DR⁺, constituted $49.0\% \pm 7.5\%$, and $38.9\% \pm 6.5\%$, respectively. This pattern of expression of surface antigen was unchanged whether the c-kit ligand and/or FL was added. The irradiated CD1a⁺HLA-DR⁺ cells recovered from in vitro cultures elicit a vigorous proliferation of allogeneic peripheral blood T-cells, irrespective of cytokine combinations. These findings provide advantageous tools for the large-scale generation of DCs that are potentially usable for clinical protocols of immunotherapy or vaccination in patients undergoing cancer treatment.

Key Words: Dendritic cells, CD34, peripheral blood hematopoietic stem cells

Dendritic cells (DCs) represent potent antigen-presenting cells (APCs) for the induction of T-cell dependent immune response (Steinman, 1991; Caux *et al.* 1995). Only they can prime naïve T-helper lymphocytes, and they can also elicit a cytotoxic T-cell response to soluble antigens (Inaba *et al.* 1990; Liu and McPherson, 1993), whereas macro-

phage and activated B cells stimulate only primed cells. Characterization of DCs in humans has been confounded both by a lack of exclusive lineage-specific markers and by their low numbers and consequent difficult isolation as a pure population. Nonetheless, some common phenotypic patterns have appeared in recent studies (Lenz *et al.* 1993; Thomas *et al.* 1993): (1) the dendritic morphology by phase contrast, light, and transmission electron microscopy, (2) the expression of surface membrane CD1a antigen, high levels of major histocompatibility complex (MHC) class I and II antigens, and accessory molecules including CD40, CD80, CD86 as well as the lack of the monocyte-restricted CD14 antigen by immunofluorescence flow cyto-

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metry, (3) the efficient capacity to induce proliferation of allogeneic T-cells in primary mixed leukocyte reaction (MLR) and to process and present xenogenic antigens to autologous T-cells, and (4) the low phagocytic activity (Caux *et al.* 1995).

Several groups have succeeded in generating mature DCs from human (Caux *et al.* 1992; Santiago-Schwarz *et al.* 1992; Romani *et al.* 1994) and murine (Inaba *et al.* 1990) hematopoietic precursor cell populations by using exogenous GM-CSF and TNF α . GM-CSF and TNF α cooperate in the *ex vivo* generation of DCs from CD34⁺ hematopoietic progenitors retrieved from bone marrow (Reid *et al.* 1992), cord blood (Caux *et al.* 1992; Santiago-Schwartz *et al.* 1992), or peripheral blood (Romani *et al.* 1994; Strunk *et al.* 1996).

Experimental evidence in animals and initial clinical results support the working hypothesis that individuals vaccinated with *ex vivo*-generated DCs pulsed with tumor antigen can mount tumor-specific humoral and cellular responses and achieve tumor regression as well as protective immunity to tumor growth *in vitro* (Flamand *et al.* 1994; Hsu *et al.* 1995). The ability to generate DCs in sizeable numbers has enormous implications for the development of clinically-effective antigen presentation procedures for immunotherapy of hematologic malignancies and cancer. Therefore, in a similar clinical setting, one may envision manipulating mobilized CD34⁺ cells to differentiate into DCs *ex vivo* and using these cells as a tool for tumor vaccination. It is from this perspective that we evaluated the feasibility of effective *ex vivo* generation of DCs from mobilized CD34⁺ cells, and analyzed the immunophenotype and functional capacity of DCs generated by using various combinations of hemopoietic growth factors. Our results provided novel information on the characteristics of DCs which may be useful for their potential therapeutic purposes in hematologic malignancies and cancers.

MATERIALS AND METHODS

Cell collection and purification of CD34⁺ cells

Normal, healthy donors (n=7) for allogeneic peripheral blood stem cell (PBSC) transplantation were

injected with 10 μ g/kg of recombinant human granulocyte colony-stimulating factor (G-CSF) for 5 days. PBSCs were collected from leukapheresis products by centrifugation on Ficoll-Hypaque (Nyegaard, Oslo, Norway) to isolate mononuclear cells. CD34⁺ cells were positively purified by a high gradient magnetic cell separation system (MACS; Miltenyi Biotech GmbH, Gladbach, Germany) with the CD34 QBEND/10 antibody according to methods previously described (Min *et al.* 1995). Cells were analyzed by flow cytometry to assess purity. The mean percentage of purified CD34⁺ cells was 89% \pm 3%.

Ex vivo generation of DCs from CD34⁺ cells

Suspension cultures of purified CD34⁺ cells (1×10^4 cells/mL) were performed in 24-well tissue culture plates (Costar, NY, USA) in supplemented Iscove's modified Dulbecco's medium (IMDM; GIBCO, Gaithersburg, MD, USA)/10% fetal calf serum (FCS; GIBCO) in the presence of 100 U/mL penicillin, 100 μ g/mL streptomycin and different combinations of the following recombinant human cytokines: 100 ng/mL GM-CSF (Immunex, Seattle, WA, USA); 100 U/mL TNF α (Genzyme, Cambridge, MA, USA); 50 ng/mL IL-4 (R & D System, Minneapolis, MN, USA); 50 ng/mL *c-kit* ligand (Amgen, Thousand Oaks, CA, USA), and 100 ng/mL flt3 ligand (FL; Immunex) as described (Min *et al.* 1996). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was replaced every 4~5 days. When the cells became confluent, the nonadherent fraction was cultured in additional plates. The generation of DCs was monitored by phase-contrast microscopy of intact cultures daily and by three-color direct immunofluorescence of pooled adherent and nonadherent cells with the panel of monoclonal antibodies (MoAbs, Table 1). After 12~16 days of culture time, cells were harvested and used for phenotyping and functional assays.

Flow cytometry

Cell staining was performed using the panel of fluorescent (FITC, PE, and PerCP or CyChrome) conjugated mouse MoAbs listed in Table 1. Cells were incubated for 30 minutes at 4°C with con-

Table 1. List of monoclonal antibodies used in this study

Specificity	Monoclonal Antibody	Isotype and Subclass	Source
CD1a	HI149	IgG ₁	Pharmingen
CD3	Leu-4	IgG ₁	Becton Dickinson(BD)
CD4	Leu-3a	IgG ₁	BD
CD11b	Leu-15	IgG _{2a}	BD
CD11c	B-ly6	IgG ₁	Pharmingen
CD13	Leu-M7	IgG ₁	BD
CD14	Leu-M3	IgG _{2b}	BD
CD15	Leu-M1	IgM	BD
CD20	Leu-16	IgG ₁	BD
CD33	Leu-M9	IgG ₁	BD
CD34	HPCA-2	IgG ₁	BD
CD38	Leu-17	IgG ₁	BD
CD40	G44-26	IgG ₁	Pharmingen
CD44	HLe-1	IgG ₁	Pharmingen
CD45RO	Leu-54	IgG _{2a}	BD
CD54	Leu-19	IgG ₁	BD
CD56	IOL58	IgG ₁	BD
CD58	BB1	IgG ₁	Immunotech
CD80	IT2.2	IgM	Pharmingen
CD86	L243	IgG _{2b}	Pharmingen
HLA-DR	Leu-10	IgG _{2a}	BD
HLA-DQ		IgG ₁	BD

jugated MoAbs in phosphate buffered saline (PBS), 2% FCS. After washing, cells were resuspended in PBS and 1% paraformaldehyde and analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA). Negative controls were irrelevant MoAbs. Data acquisition was performed with Lysis 2.0 (BDIS). Forward light scatter, orthogonal light scatter, and three fluorescence signals were determined for each cell and the listmode data files were analyzed with the Paint-A-Gate^{Plus} software (BDIS). For sorting, day-14 cultured cells were washed, incubated at 4°C for 30 minutes with FITC-conjugated anti-CD1a, PE-conjugated anti-HLA-DR MoAb, or PE-conjugated anti-CD14 MoAb, and washed again. Cells were then sorted with the FACStar^{Plus} (BDIS).

Mixed lymphocyte culture (MLR)

The allogeneic MLR was used as a test of CD34⁺ PBSC-derived DC stimulatory function for resting T

cells. Naïve CD3⁺ T-cells ($1 \times 10^5/100 \mu\text{L}$) isolated through a high gradient magnetic cell separation system/anti-CD3 MoAb (Miltenyi Biotech GmbH) according to the method provided by the manufacturer from normal donor peripheral blood lymphocytes were used as responder cells. DC cultures were harvested after 12~16 days, sorted by using FACStar^{Plus} (BDIS) into CD1a⁺HLA-DR^{bright} cells, CD1a⁺CD14⁻ cells, CD1a⁺CD14⁺ cells, and CD1a⁻CD14⁺ cells, irradiated with 3,000 cGy, and added to the responder cells at different cell concentrations ($0.1 \times 10^3 - 30 \times 10^3/100 \mu\text{L}$). The assay was performed in 96-well round-bottomed plates (Corning, NY, USA) at 37°C. The medium used consisted of RPMI 1640 (GIBCO) with 10 mM L-glutamine, 200 U/mL penicillin, 200 U/mL streptomycin, 2.5×10^{-5} M 2-mercaptoethanol, and 10% human AB serum (GIBCO) without exogenous recombinant human interleukin-2 (IL-2).

T-cell proliferation was measured on day 5 of culture by an 18-hour pulse with ³H-thymidine (1 $\mu\text{Ci}/\text{well}$, specific activity 6.7 Ci/mmol; Amersham, UK) and counted. Data are shown as the mean of three replicates.

RESULTS

Phase-contrast microscopy of CD34⁺ cells cultured in the conditions showed that, after 4~5 days, the cells differentiated into a heterogenous population comprising macrophage-like cells as well as elements with thin cytoplasmic processes peculiar to DCs. The latter cells developed in aggregates that were loosely adherent to the culture plates and increased in size until day 12~16 of culture. The typical morphologic appearance of day 14 culture is shown (Fig. 1A-1B). We noted weakly adherent cells with stellate, elongated cytoplasmic projections, as well as motile, veiled, nonadherent cells. The latter were characteristic of mature blood dendritic cells. The remaining cells were round, granulocytic-type cells and adherent monocytes/macrophages. When the CD34⁺ cells were cultured in the presence of GM-CSF and IL-4, irrespective of the addition of TNF α , *c-kit* ligand, or FL, the adherent macrophage-like cells looked markedly

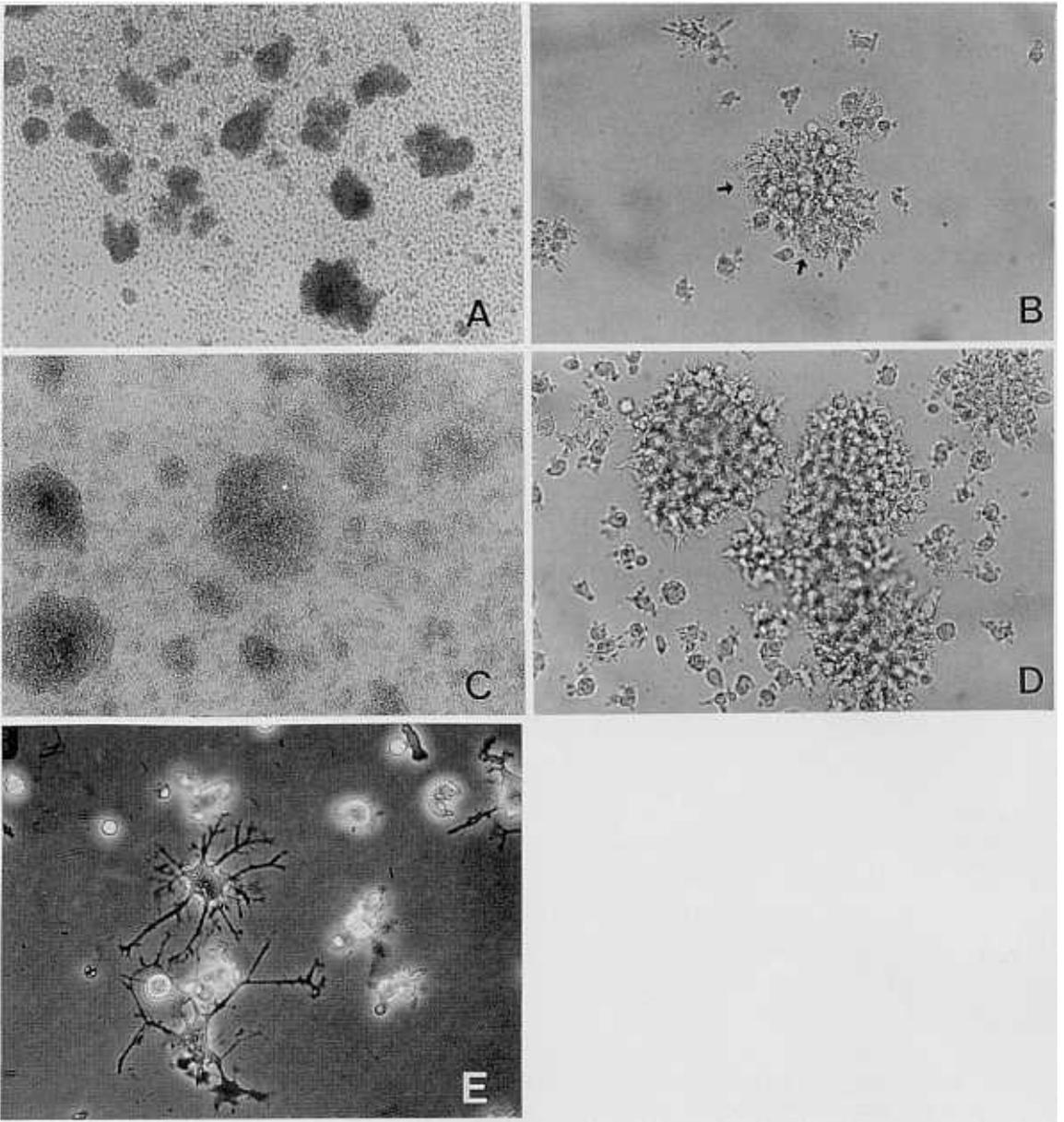


Fig. 1. Microscopic appearance of 14-day cultures. CD 34⁺ peripheral blood stem cells are cultured in the presence of GM-CSF and TNF α (A, B), or GM-CSF, TNF α , c-kit ligand, and flt3 ligand (C, D). Multiple nonadherent cell aggregates are visible under the inverted microscope (A). Higher magnification reveals that they display typical veils at their edge (arrow; B). When c-kit ligand and/or flt3 ligand are added to the basic cytokine combination GM-CSF and TNF α , the dendritic cell aggregates have become much larger (C), and they also display typical many processes (D). The phase contrast microscope shows that the cellular aggregates finally release typical single DC which display typical dendritic morphology with delicate membrane projections (E). (A and C) $\times 25$; (B and D) $\times 100$; (E) $\times 350$.

decreased in number compared to those cultures without IL-4. Upon the addition of *c-kit* ligand and/or FL to GM-CSF and TNF α , very large cell clusters were formed (Fig. 1C-1D). Those large cell clusters showed the typical DC morphology (Fig. 1E).

By day 14 of culture in the presence of GM-CSF and TNF α , the total cell number increased by 23.4 \pm 5.4-fold (range, from 12- to 37-fold) compared to the starting number of CD34⁺ cells (Fig. 2). When IL-4 was added to GM-CSF alone or GM-CSF + TNF α , fewer DCs were developed. The increase in

the cell number was 4.9 \pm 2.1-fold and 11.9 \pm 3.8-fold, respectively. When the *c-kit* ligand or FL was added to the culture medium supplemented with GM-CSF, and TNF α from the start, the cells expanded 52.1 \pm 7.4-fold, and 32.4 \pm 9.3-fold, respectively. Cell cultures with *c-kit* ligand or FL in addition to GM-CSF + TNF α exhibited significantly higher DC growth than those with only the latter two growth factors ($p < 0.005$ for GM-CSF + TNF α vs. GM-CSF+TNF α +*c-kit* ligand or GM-CSF+TNF α +FL). The combination of FL and *c-kit* ligand simultaneously in addition to GM-CSF + TNF α resulted in a further enhancement of DC growth on day 14 of culture ($P < 0.005$ for GM-CSF + TNF α vs. GM-CSF+TNF α +*c-kit* ligand+FL). When the *c-kit* and FL were added to GM-CSF and TNF α , the total cell number increased by 109.8 \pm 11.2-fold compared to the starting CD34⁺ cell population (Fig. 2).

The initially purified CD34⁺ cells were CD38⁺ HLA-DR⁺ CD13^{lo} CD33^{lo} CD1a⁻ CD4^{-/lo} (data not shown). CD40, CD80, and CD86 cell surface antigens were not detectable by flow cytometry. Flow cytometric analysis of day 7 cultured cells indicated that CD1a⁺CD14⁻HLA-DR⁺ cells, CD80⁺CD1a⁺HLA-DR⁺ cells, and CD80⁺CD86⁺HLA-DR⁺ cells constituted 6.5% \pm 1.4%, 4.5% \pm 1.3%, and 3.4% \pm 0.5%, respectively, of the total cells in the presence of GM-CSF and TNF α (Table 2). By day 14, two populations of cells in suspension were individualized according to size and granularity. One displayed low light scatter signals characteristic of small agranular cells (R2; Fig. 3A) The other had high scatter and autofluorescence signals of large

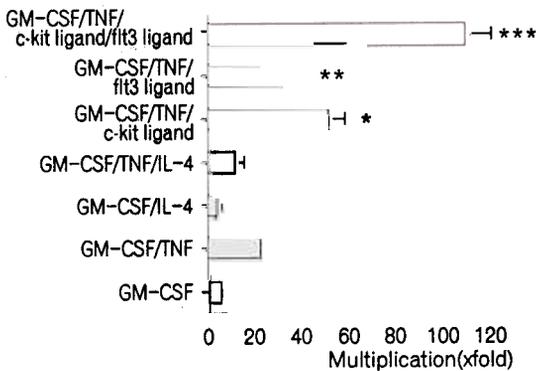


Fig. 2. Effects of cytokines on CD34⁺ peripheral blood hematopoietic cell growth. CD34⁺ cells purified from leukapheresis products were seeded at a density of 1 \times 10⁴ cells/ml. After 14 days suspension culture in cytokine-supplemented medium, the cells were counted. Data are expressed as the mean \pm SD of seven experiments. *, GM-CSF + TNF α + *c-kit* ligand vs GM-CSF + TNF α , $p < 0.005$; **, GM-CSF + TNF α + *flt3* ligand vs GM-CSF + TNF α , $p < 0.05$; ***, GM-CSF + TNF α + *c-kit* ligand + *flt3* ligand vs GM-CSF + TNF α , $p < 0.05$.

Table 2. Three-color flow cytometric analysis of CD34⁺ peripheral blood stem cells-derived dendritic cells according to the combination of cytokines

Phenotype	Days of culture	GM-CSF + TNF α	GM-CSF + TNF α + IL-4	GM-CSF + TNF α + <i>c-kit</i> ligand	GM-CSF + TNF α + F1t3 ligand	GM-CSF + TNF α + <i>c-kit</i> ligand + F1t3 ligand
CD1a ⁺ CD14 ⁻ HLA-DR ⁺	7	6.5% \pm 1.4%	13.2% \pm 4.5%	8.9% \pm 1.3%	6.1% \pm 2.2%	8.1% \pm 0.7%
	14	49.0% \pm 7.5%	60.2% \pm 11.2%	54.3% \pm 7.0%	48.2% \pm 13.2%	59.8% \pm 11.2%
CD80 ⁺ CD1a ⁺ HLA-DR ⁺	7	4.5% \pm 1.3%	5.4% \pm 2.1%	5.5% \pm 0.7%	4.7% \pm 0.6%	12.1% \pm 2.4%
	14	39.2% \pm 14.6%	44.3% \pm 9.1%	46.3% \pm 12.2%	38.8% \pm 7.7%	48.2% \pm 7.2%
CD80 ⁺ CD86 ⁺ HLA-DR ⁺	7	3.4% \pm 0.5%	2.4% \pm 0.5%	4.8% \pm 1.3%	3.6% \pm 0.7%	7.5% \pm 1.2%
	14	38.9% \pm 6.5%	46.7% \pm 8.6%	41.9% \pm 4.4%	44.4% \pm 6.5%	46.2% \pm 9.1%

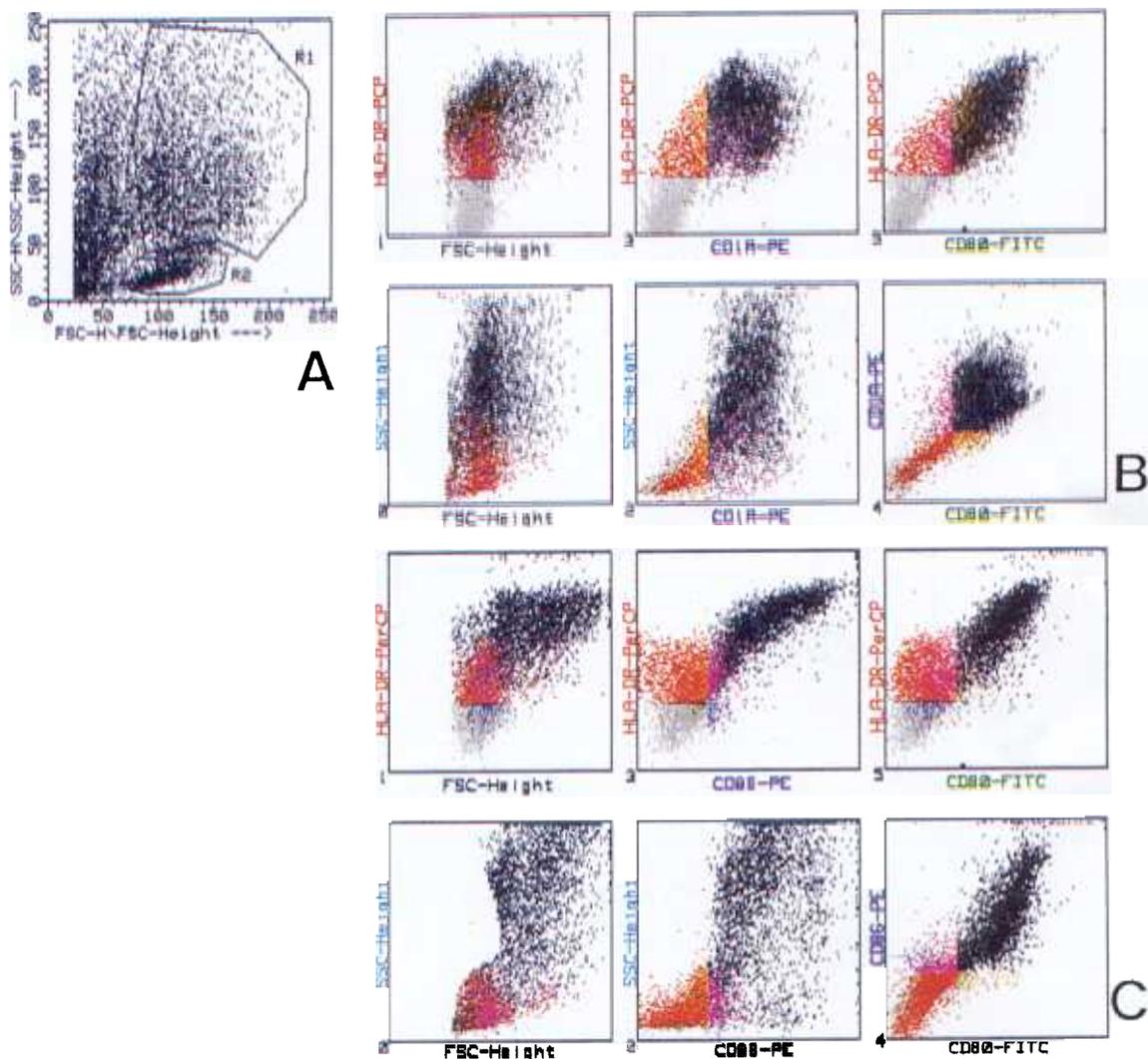


Fig. 3. Phenotypic characterization of day-14 progeny cultured from CD34⁺ peripheral blood stem cells in the presence of GM-CSF, and TNF α . Representative light scatter profile of the cells (A). Three-color cytogram of the cells labeled with CD80-FITC/CD1a-PE/HLA-DR-PerCP (B), and CD80-FITC/CD86-FITC/HLA-DR-PerCP (C). In this representative analysis, the predominant cell phenotypes are CD80⁺CD1a⁺HLA-DR⁺ (B; black dots, 54.3%), and CD80⁺CD86⁺HLA-DR⁺ (C; black dots, 48.9%). All the triple positive cells are in the R1 gate.

granular cells (R1; Fig. 3A). All day 14 cells were CD33⁺, confirming the myeloid lineage of these cells, but B-cell, T-cell, or NK cell markers (CD20, CD3, and CD56) were not detected (data not shown). The CD1a⁺CD14⁻HLA-DR⁺ fraction expressed CD45RO, CD11b, CD11c, CD40, CD54, and CD58 (data not shown). This phenotype is consistent with the published phenotype of mature

blood dendritic cells. Cells with the markers of mature dendritic cells, ie, CD1a⁺CD14⁻HLA-DR⁺ cells, CD80⁺CD1a⁺HLA-DR⁺ cells, and CD80⁺CD86⁺HLA-DR⁺ cells constituted 49.0% \pm 7.5%, 39.2% \pm 14.6%, and 38.9% \pm 6.5% of the bulk day 14 progeny, respectively (Table 2). The generation of mature DCs was dependent on the duration of *in vitro* culture. For example, the proportion of CD80⁺

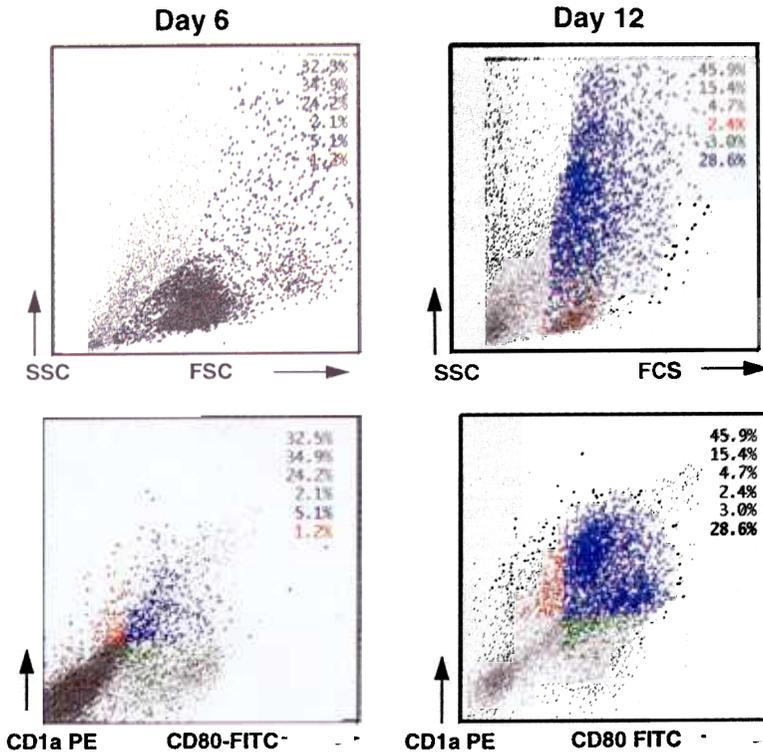


Fig. 4. A representative three-color cytogram showing the changes of CD80, CD1a, and HLA-DR expression on CD34⁺ peripheral blood stem cells-derived cells after culture as indicated (day 6 and 12). The cytokines used were GM-CSF, TNF α , IL-4, *c-kit* ligand, and *flt3* ligand. The proportion of triple positive (CD80⁺CD1a⁺HLA-DR⁺) cells of day 12 (Blue dots) are increased compared to that of day 6 (28.6% vs 5.1%). Green dots are CD80⁺CD1a⁻HLA-DR⁺ cells, and red dots are CD80⁻CD1a⁺HLA-DR⁺ cells.

CD1a⁺HLA-DR⁺ cells on day 12 was increased compared to that of day 6 (Fig. 4). Upon the addition of IL-4 to cultures containing GM-CSF and TNF α , the CD1a⁺CD14⁻HLA-DR⁺ cells constituted 13.2% \pm 4.5% of total cells, expressing lower levels of CD14. The addition of *c-kit* ligand, *flt3* ligand, or *c-kit* ligand + *flt3* ligand to the GM-CSF + TNF α did not show any difference compared to those cultures to which only GM-CSF + TNF α were added with respect to the proportion of DCs, such as CD1a⁺CD14⁻HLA-DR⁺, CD80⁺CD1a⁺HLA-DR⁺, and CD80⁺CD86⁺HLA-DR⁺ (Table 2). The majority of CD1a⁺ cells (82% \pm 11%) were positive for CD4, which is known to be expressed by cultured DC (data not shown). The vast majority of CD1a⁺

HLA-DR⁺ cells were CD80⁺ (Fig. 3B). Most of the CD80⁺ cells were CD86⁺ and HLA-DR⁺ (Fig. 3C).

Allogeneic primary MLR was performed to determine whether the *in vitro* generated CD1a⁺HLA-DR⁺ cells were capable of inducing a primary T-cell response. Experiments showed that cells recovered from *in vitro* cultures of CD34⁺ PBSC in GM-CSF/TNF α \pm IL-4 elicited a vigorous proliferation of allogeneic T-cells (Fig. 5). Dendritic cells generated in the presence of *c-kit* ligand and FL showed equivocal primary T-cell stimulatory activity (Fig. 5). The MLR-stimulatory capacity of GM-CSF/TNF α -induced PBSC-derived cells (CD1a⁺CD14⁻, and CD1a⁺CD14⁺) was compared with that of CD14⁺CD1a⁻ monocytes and CD20⁺ B-lympho-

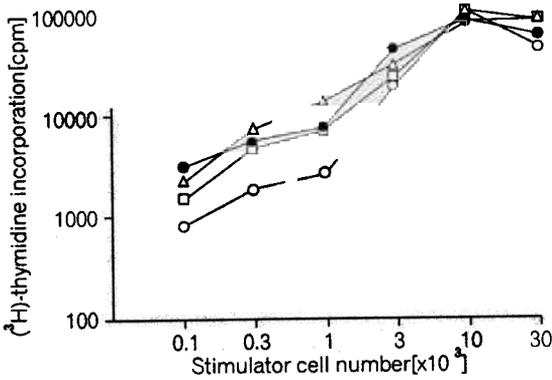


Fig. 5. The MLR-stimulatory capacity of cytokine-activated CD34⁺ peripheral blood stem cells. Graded numbers of CD1a⁺HLA-DR⁺ sorted cells were used after 2 weeks of culture with GM-CSF/TNF α (squares), GM-CSF/TNF α /IL-4 (circle), GM-CSF/TNF α /c-kit ligand (triangle), or GM-CSF/TNF α /c-kit ligand/flt3 ligand (solid circle) to stimulate 10⁵ allogeneic T-cells. Cells were pulsed with [³H]-thymidine for 18 hours after 5 days of culture.

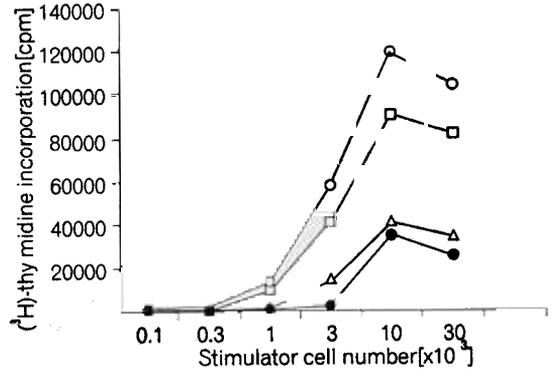


Fig. 6. The MLR-stimulatory capacity of GM-CSF + TNF α -activated CD34⁺ peripheral blood stem cells is critically dependent of the presence of CD1a⁺ cells. Graded numbers of CD1a⁺CD14⁻ cells (circle), and CD1a⁺CD14⁺ cells (square) as well as freshly isolated, autologous blood CD1a⁻/CD14⁺ monocytes (triangle) and CD20⁺ B-cells (solid circle) were analyzed for their capacity to stimulate 10⁵ allogeneic T-cells. Cells were pulsed with [³H]-thymidine for 18 hours after 5 days of culture.

cytes from peripheral blood of the same donor. Over the entire range of stimulator cell concentrations, the *in vitro* generated CD1a⁺ (CD14⁻ or CD14⁺) cells were greatly superior to the other populations in stimulating naïve allogeneic CD3⁺ T-cells (Fig. 6).

DISCUSSION

DCs represent a broad class of ubiquitous cells, which results in a phenotypic diversity, albeit with common morphologic and functional characteristics such as a superior antigen-presenting capacity (Caux *et al.* 1995). Therefore, the different methods and culture conditions that have been used to obtain DCs, especially from peripheral blood, do not usually result in fully homogenous populations, which may account for some of the disparate findings as to their characteristics. Nonetheless, the dendritic cells generated *in vitro* can be described by the following findings: a) typical dendritic morphology undistinguishable from cutaneous Langerhan cells as evidenced by phase contrast-, or light microscopy; b) high levels of MHC class II HLA-DR, -DP, and -DQ molecules, costimulatory mo-

lecules CD40, CD80, and CD86. Expression of surface membrane CD1a antigen, which is considered a hallmark DC marker, is expressed; c) capacity to induce proliferation of allogeneic T-cells in primary MLR at significantly higher levels than nondendritic myeloid cells; and, most importantly, d) capacity to process and present antigens to T-cells as evidenced by the elicitation of class II HLA-DR-restricted proliferation of CD4⁺ autologous T-cells (Steinman, 1991; Caux *et al.* 1995). This study was designed to determine the optimal modalities for the large-scale procurement of functional DCs *ex vivo* from mobilized peripheral blood CD34⁺ cells in a sufficiently practical manner to envision their exploitation for potential therapeutic purposes. Among the recently prospected sources of DCs, namely bone marrow, cord blood, and mobilized peripheral blood, the latter is certainly the richest and most accessible in all patients with cancer. The functional defect of DCs, which was inherent in patients with cancer, was recovered when the DCs were generated *in vitro* from CD34⁺ mobilized hematopoietic stem cells of the same patient (Siena *et al.* 1995).

Substantial progress in the generation and

characterization of cells of the DC system has only recently been gained by the demonstration that the cytokine combination GM-CSF plus TNF α can induce development of DCs from purified CD34⁺ progenitor cells *in vitro* (Caux *et al.* 1992; Reid *et al.* 1992; Santiago-Schwarz *et al.* 1992; Szabolcs *et al.* 1995). In the presence of GM-CSF plus TNF, the overall expansion varies between 20- and 50-fold of the starting number of CD34⁺ cells. Our study showed that the total cell number of day 14 increased by 23.4-fold in the presence of GM-CSF and TNF α , compared to the starting number of CD34⁺ cells. We observed that the addition of the early acting cytokine *c-kit* ligand and/or FL to the culture medium further boosts GM-CSF- plus TNF α -dependent DC development. When the *c-kit* ligand or FL was added to the culture medium supplemented with GM-CSF and TNF α from the start, the cells expanded 52.1-fold, and 32.4-fold, respectively. The combination of FL and *c-kit* ligand simultaneously in addition to GM-CSF + TNF α resulted in the further enhancement of DC growth on day 14 of culture. When the *c-kit* and FL were added to GM-CSF and TNF α , the total cells number increased by 109.8-fold compared to the starting CD34⁺ cell population without affecting the immunophenotype of recovered cells. Recent results show that *c-kit* ligand enhances proliferation of the mixed colony-forming unit (CFU)-DC/monocyte (MO) or the pure CFU-DC (Santiago-Schwartz *et al.* 1995; Young *et al.* 1995), and that DC maturation may continue until DC-T-cell interactions occur (Caux *et al.* 1994). This is due to crosslinking of CD40 which upregulates expression of the costimulatory molecules CD80 and CD86. The recently-cloned FL shows structural homology with *c-kit* ligand and signals via Flt3 A member of the class III tyrosine kinase receptor family that also includes *c-kit* and *c-fms* (Rosnet *et al.* 1991). *c-kit* ligand and FL similarly induce *in vitro* proliferation of highly purified immature and committed myeloid colony-forming cells in synergism with a number of other growth factors (Shah *et al.* 1996; Shapiro *et al.* 1996) and increase yields of CD1a⁺ DCs in culture of CD34⁺ cells (Siena *et al.* 1995; Maraskovsky *et al.* 1996). In our study, we evaluated the effect of FL in the absence or presence of *c-kit* ligand on the generation of cells with the markers

of mature dendritic cells, ie, CD1a⁺CD14⁻HLA-DR⁺ cells, CD80⁺CD1a⁺HLA-DR⁺ cells, and CD80⁺CD86⁺HLA-DR⁺ cells instead of evaluating just CD1a⁺ cells. We found that the proportion of those DCs candidates was unchanged whether the *c-kit* ligand and/or FL was added to the GM-CSF and TNF α . In this study, CD1a⁺CD14⁻HLA-DR⁺ cells, CD80⁺CD1a⁺HLA-DR⁺ cells, and CD80⁺CD86⁺HLA-DR⁺ cells constituted 49.0%, 39.2%, and 38.9% of the bulk day 14 progeny, respectively. These findings translate into a substantial expansion of DCs from CD34⁺ PBSC *in vitro*. It is not clear at this time why we could obtain higher yields of DCs compared to other trials. Because the purity of isolated CD34⁺ cells were high in our study, it seems that the amount of DC growth strongly correlated with the degree of purity of the starting CD34⁺ population. And the frequent change of media supplemented with cytokines could be associated with the higher yield of DCs.

It has been shown that IL-4 induced the differentiation of DCs from peripheral blood monocytes *in vitro*. But the effect of IL-4 on the generation of DCs from CD34⁺ PBSCs should be evaluated. We observed that when IL-4 was added to GM-CSF alone or GM-CSF + TNF α , fewer DCs were developed. The fold increase in the cell number was just 4.9% and 11.9%, respectively. Because IL-4-supplemented cultures were essentially devoid of CD14⁺ cells, IL-4 was thought to inhibit growth of the monocyte-, macrophage cell lineage as others have suggested (Jansen *et al.* 1989; Lauener *et al.* 1990). Although IL-4 reduced the percentage of CD14⁺ cells in the progeny cells, the significance of the reduction in CD1a⁺CD14⁺ cells is not clear at this time. According to this study, CD1a⁺CD14⁺ cells were not significantly different from CD1a⁺CD14⁻ cells in their capacity to stimulate allogeneic T-cells. The additive or synergistic effect of *c-kit* ligand and/or FL on the generation of DCs *in vitro* was also shown in the settings of cultures containing IL-4.

At a functional level, we have shown that DCs generated in the presence of GM-CSF/ TNF α \pm IL-4 are potent stimulators of primary MLR. Over the entire range of stimulator-cell concentrations, the *in vitro* generated CD1a⁺ (CD14⁻ or CD14⁺) cells were greatly superior to the other populations in

stimulating naive allogeneic CD3⁺ T-cells. The addition of c-kit ligand and/or FL does not have any effect on the GM-CSF/ TNF α \pm IL-4-induced DCs' T-cell stimulation activity on the cell basis. Recent data suggested that DCs derived from CD34⁺ circulating stem cells could potentially present certain antigens to autologous T-cells *in vivo*, which may enhance weak ongoing secondary T-cell responses against the autologous tumors (Huang *et al.* 1994; Mayordomo *et al.* 1995; Henderson *et al.* 1996). Since the expansion *ex vivo* of CD34⁺ PBSCs results in more than a 100-fold total cell expansion, our approach to generating DCs from mobilized CD34⁺ PBSCs promises to produce high numbers of autologous DCs for clinical use in immunotherapy. To induce immune responses in patients suffering from weakly immunogenic tumors, these DCs which were expanded *ex vivo* could be mixed either with irradiated autologous tumor cells (Reis *et al.* 1993), soluble tumor proteins or tumor-associated peptides (Mayordomo *et al.* 1995; Porgador and Gilboa, 1995; Celluzzi *et al.* 1996). The study on the generation of autologous cytotoxic T-cells against specific tumors using DCs is ongoing in our laboratory. This novel approach to immunotherapy of cancer may prove to be particularly useful in patients with minimal residual disease in hematologic malignancies.

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