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Stem Cell Factor and FLT3-Ligand Are Strictly Required to Sustain the Long-Term Expansion of Primitive $CD34^+DR^-$ Dendritic Cell Precursors

Antonio Curti,[1] Miriam Fogli, Marina Ratta, Sante Tura, and Roberto M. Lemoli[2]

We studied cytokine-driven differentiation of primitive human $CD34^+HLA\text{-}DR^-$ cells to myeloid dendritic cells (DC). Hemopoietic cells were grown in long-term cultures in the presence of various combinations of early acting cytokines such as FLT3-ligand (FLT3-L) and stem cell factor (SCF) and the differentiating growth factors GM-CSF and TNF-α. Two weeks of incubation with GM-CSF and TNF-α generated fully functional DC. However, clonogenic assays demonstrated that CFU-DC did not survive beyond 1 wk in liquid culture regardless of whether FLT3-L and/or SCF were added. FLT3-L or SCF alone did not support DC maturation. However, the combination of the two early acting cytokines allowed a 100-fold expansion of CFU-DC for >1 month. Phenotypic analysis demonstrated the differentiation of $CD34^+DR^-$ cells into $CD34^-CD33^+DR^+CD14^+$ cells, which were intermediate progenitors capable of differentiating into functionally active DC upon further incubation with GM-CSF and TNF-α. As expected, GM-CSF and TNF-α generated DC from committed $CD34^+DR^+$ cells. However, only SCF, with or without FLT3-L, induced the expansion of DC precursors for >4 wk, as documented by secondary clonogenic assays. This demonstrates that although GM-CSF and TNF-α do not require additional cytokines to generate DC from primitive human $CD34^+DR^-$ progenitor cells, they do force terminal differentiation of DC precursors. Conversely, FLT3-L and SCF do not directly affect DC differentiation, but instead sustain the long-term expansion of CFU-DC, which can be induced to produce mature DC by GM-CSF and TNF-α. *The Journal of Immunology,* **2001, 166: 848–854.**

Dendritic cells (DC)[3] have been shown to be a distinct pathway of myeloid differentiation in human and animal models (reviewed in Ref. 1). DC precursors have been isolated within the $CD34^+$ cell fraction in bone marrow (BM), cord blood, and peripheral blood (PB) (2–8). Recent studies have also demonstrated that human hemopoietic $CD34^+$ progenitor cells expressing adhesion or costimulatory molecules such as CD18, CD86 (9), or CD40 (10) are strictly committed to the dendritic lineage. Further downstream, the mutually exclusive expression of CD1a and CD14 Ags identifies two types of intermediate precursors: $CD1a^+CD14^-$ and $CD1a^-CD14^+$. These cells mature into DC bearing phenotypic and functional characteristics typical of epidermal Langerhans cells or interstitial DC-like cells, respectively (4, 11). Only $CD14^+$ cells act as bipotent precursors capable of differentiating into macrophage-like cells in response to M-CSF (12).

Development of mature myeloid DC depends on stimulation with GM-CSF (1–3). In the murine model, mature granulocytes, monocytes, and DC have been obtained in semisolid culture medium of primitive MHC class II negative progenitors stimulated with GM-CSF (13). TNF-α is critical for the differentiation of DC from $CD34^+$ cells as it up-regulates GM-CSF receptor while down-regulating receptors for other lineage-restricted cytokines (14, 15). Moreover, TNF-α induces the expression of CD86 and CD40 on $CD34^+$ cells (10). In addition to GM-CSF and TNF-α, IL-3 has also been identified as a growth factor for DC development, specifically for the so-called "plasmacytoid T cells": these $CD4^+$ cells express HLA-DR, CD40, and costimulatory molecules upon incubation with IL-3; this effect is further increased by ligation of CD40 (16).

Stem cell factor (SCF; also known as c-*kit* ligand) and FLT3 ligand (FLT3-L) are two early acting cytokines sharing similar receptors that have tyrosine kinase activity and are expressed on primitive progenitor cells (reviewed in Ref. 17). Suspension cultures and clonogenic assays of human $CD34^+$ cells both suggest that addition of either FLT3-L or SCF leads to higher DC production than with GM-CSF and TNF-α (5, 6, 8, 18). However, it is still unclear whether FLT3-L and SCF are solely synergistic factors that enhance DC production by expanding already committed, lineage-restricted precursors or whether they act as permissive factors by inducing proliferation and perhaps self-renewal of very primitive hemopoietic cells that then become capable of responding to GM-CSF and TNF-α. It is also unknown whether GM-CSF and TNF-α are able to differentiate mature and functional myeloid DC from early DR^- human progenitors in the absence of early acting cytokines. In this regard, SCF cooperates with TNF-α and GM-CSF for inducing DC differentiation from primitive murine c-kit^+ $lineage^-$ BM cells (19).

In this study we attempted to address these questions by using long-term cultures of highly purified human $CD34^+DR^-$ and $CD34^+DR^+$ cells. Our results indicate that primitive, uncommitted, progenitor cells can be driven to generate DC by GM-CSF and TNF-α without FLT3-L or SCF. However, only FLT3-L and SCF

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[3] Abbreviations used in this paper: DC, dendritic cells; SCF, stem cell factor; FLT3-L, FLT3 ligand; BM, bone marrow; PB, peripheral blood; CFU-C, CFU-cell; rh, recombinant human; IMDM, Iscove's modified DMEM; SI, stimulation index; TT, tetanus toxoid; KLH, keyhole limpet hemocyanin.

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are capable of maintaining the long-term expansion of DC precursors.

Materials and Methods

Purification of CD34$^+$ and CD3$^+$ cells and selection of CD34$^+$DR$^-$ progenitors

BM- or G-CSF-mobilized PBMC were obtained by gradient centrifugation (Lymphoprep; 1.077 g/ml; Nycomed Pharma, Oslo, Norway) from 12 healthy donors. Light-density cells were washed twice in PBS with 1% BSA (Sigma, St. Louis, MO), and CD34$^+$ or CD3$^+$ cells were highly purified from mononuclear cell fraction by a MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (20). Flow cytometric reanalysis of purified cell fractions was performed on a gated population set on scatter properties by using FACScan equipment (Becton Dickinson, Mountain View, CA) (20). A minimum of 10,000 events was collected in list mode on FACScan software. The CD34$^+$ cell fraction was further separated according to HLA-DR expression. Briefly, progenitor cells obtained by magnetic separation were incubated with anti-CD34 HPCA-2-FITC and IgG2a anti-DR$^-$PE mAbs (Becton Dickinson) for 30 min at 4°C, washed twice, and resuspended in culture medium containing 2% FCS (Sera-Lab, Crawley Down, Sussex, U.K.). Purified CD34$^+$DR$^-$ and CD34$^+$DR$^+$ cells were obtained by fluorescence-activated cell sorting by FACSVantage equipment (Becton Dickinson). Sort windows were established for the FITC (CD34) and PE (HLA-DR) fluorescence based on the fluorescence of control samples as previously reported (21). Aliquots of sorted cell fractions were reanalyzed by a FACScan or a FACSVantage to verify their purity, which was always >98%.

Cytokines

Five recombinant human (rh) cytokines were used in this study. SCF (Amgen, Thousand Oaks, CA) was used at 20 ng/ml. GM-CSF, IL-4 (both obtained from Genzyme, Cambridge, MA), and FLT-3L (Immunex, Seattle, WA) were used at 50 ng/ml. TNF-α (Innogenetics, Zwijndrecht, Belgium) was added at 25 ng/ml. Optimal concentrations of cytokines were chosen based on our previous experience (8). Stock solutions of the growth factors were stored at −80°C, and dilution vials were stored at −20°C until use. Growth factors were diluted in Iscove's modified DMEM (IMDM) with 2% FCS.

Long-term cultures of CD34$^+$DR$^-$ and CD34$^+$DR$^+$ cells

Stroma-free suspension cultures of BM and PB CD34$^+$DR$^-$ or DR$^+$ cells were initiated with IMDM supplemented with 10% FCS (Sera-Lab), L-glutamine, and antibiotics at an initial density of 1×10^4 cells/ml. All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere for at least 4 wk in the presence of optimized concentrations of the rh cytokines (see above). At weekly intervals, the culture medium was replaced by fresh medium and cytokines as indicated in *Results*, and hemopoietic cells were adjusted to the initial cell concentration. An aliquot of cell suspension was then plated in methylcellulose (see below) to evaluate the presence of secondary CFU-cell (CFU-C) and CFU-DC. Moreover, the generation of functionally active DC was assessed by phase-contrast microscopy, cytospin preparations, immunophenotyping, and MLR (see below).

Short-term colony assays

Hemopoietic cells were cultured in semisolid medium as previously described (8). Briefly, 2000–5000 CD34$^+$DR$^-$ or DR$^+$ cells were plated in duplicate in culture medium consisting of 1 ml of IMDM supplemented with 30% FCS, 10^{-5} mol/L 2-ME (Sigma), and 0.2 mmol/L bovine hemin (Sigma). The final concentration of methylcellulose was 1.1%. Cytokines were added as follows: TNF-α (10 ng/ml), GM-CSF (50 ng/ml), and SCF (20 ng/ml). CFU-DC were recorded as aggregates >50 cells after 12–14 days of incubation at 37°C in a fully humidified 5% CO_2 atmosphere. To confirm the dendritic origin of scored colonies, individual aggregates were plucked from methylcellulose under direct inspection by inverse-phase microscopy. Cells were then resuspended in IMDM-10% FCS, washed twice in the same medium, and cytocentrifuged onto glass slides. Morphology was assessed by May-Grunwald-Giemsa staining (8). To evaluate the clonogenic efficiency of CFU-granulocyte-macrophage (CFU-GM), burst-forming units erythroid (BFU-E), and multilineage colonies (CFU-Mix) (together referred to as CFU-C), hemopoietic cells were plated in duplicate in IMDM, FCS, BSA, and 2-ME as above with 0.2 mM bovine hemin (Sigma), 2 U/ml of rh erythropoietin (Dompè Biotec, Milan, Italy), and a selected batch of PHA-lymphocyte-conditioned medium (10% v/v). Methylcellulose final concentration was 1.32%. CFU-C were scored after 14 days of incubation at 37°C in a fully humidified 5% CO_2 atmosphere (20).

Immunophenotype studies

Dual color immunofluorescence was performed using the following panel of mAbs: PE- or FITC-conjugated anti-human CD1a (PharMingen, San Diego, CA); FITC-conjugated anti-human CD86 (PharMingen); FITC-BB1/B7 (anti-CD 80; Becton Dickinson); FITC-anti-human HLA-DR (Becton Dickinson); FITC- or PE-Leu-M3 (anti-CD14; Becton Dickinson); FITC-Leu-4 (anti-CD3; Becton Dickinson); PE-anti-human CD83 (Immunotech, Marseille, France); FITC-anti-human CD40 (PharMingen); and PE-anti-human Leu-M9 (anti-CD33; Becton Dickinson). Negative controls were isotype-matched, irrelevant mAbs (PharMingen and Becton Dickinson). Cells were incubated in the dark for 30 min at 4°C in PBS-1% BSA. After washing, cells were resuspended in PBS and 1% paraformaldehyde and analyzed as reported above.

FITC-dextran assay

To evaluate the capacity of uptake soluble Ags from the culture medium, DC were incubated with 1 mg/ml of FITC-dextran at 37°C or at 0°C for 1 h. Uptake was stopped by adding ice-cold PBS followed by four washes in a refrigerated centrifuge (22). Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson).

Activation of allogeneic and autologous T cell proliferation

To test their allogeneic stimulatory activity, DC were irradiated (3000 cGy) and tested as stimulators in primary MLR (8). Cells were resuspended in RPMI 1640, 25 mM HEPES, antibiotics, and 15% AB human serum that had been inactivated at 56°C for 30 min. Allogeneic PBMC (5×10^4) were mixed with decreasing numbers of stimulators in round-bottom 96-well plates for 6 days at 37°C in a 5% CO_2 humidified atmosphere. Cells were pulsed with 1 μCi/well [^{3}H]thymidine for 18 h before harvest on day 6. Where indicated, the stimulation index (SI) was calculated for each individual experiment as follows: SI = cpm (T cell responders + stimulators)/cpm (T cell responders).

Autologous MLRs were set up to demonstrate the capacity of cultured DC of processing and presenting nominal Ags to T cells (8). Briefly, 10^5 PB CD3$^+$ T cells were coincubated with decreasing numbers of autologous APC without Ags or with 50 μg/ml of keyhole limpet hemocyanin (KLH; Sigma) or 1 μg/ml of tetanus toxoid (TT; Calbiochem, La Jolla, CA). T cell proliferation was measured as follows: SI = cpm (T cell responders + Ag-pulsed stimulators)/cpm (T cell responders + stimulators).

Statistical analysis

The results are expressed as the mean ± SD of at least three different experiments. Results were analyzed with the paired nonparametric Wilcoxon rank sum test, and p values < 0.05 were considered significant.

Results

FLT3-L and SCF in combination expand CD34$^+$HLA-DR$^-$ CFU-DC in long-term cultures

We first established long-term cultures of primitive CD34$^+$HLA-DR$^-$ cells to determine the effects of FLT3-L, SCF, GM-CSF, and TNF-α on DC hemopoiesis, and specifically to assess whether the cytokines under study affect the balance between differentiation and self-renewal of DC precursors (Fig. 1). At weekly intervals, the suspension cultures were harvested, washed, and either replated in liquid suspension at the initial starting concentration with the same cytokine(s) (Fig. 1*A*) or plated in methylcellulose to quantify the expansion of hemopoietic CFU-C (Fig. 1*B*) or CFU-DC (Fig. 1*C*). This was evaluated by dividing the absolute number of CFU-C or CFU-DC generated during weekly expansion in suspension cultures by the baseline value of clonogenic progenitors.

In all of the cytokine combinations containing GM-CSF plus TNF-α, CFU-C production peaked at week 1 (Fig. 1*B*). Thereafter, erythroid and granulocytic progenitors rapidly declined and were no longer detectable in secondary clonogenic assays at week 3. The only cytokine that, by itself, was able to maintain CFU-C

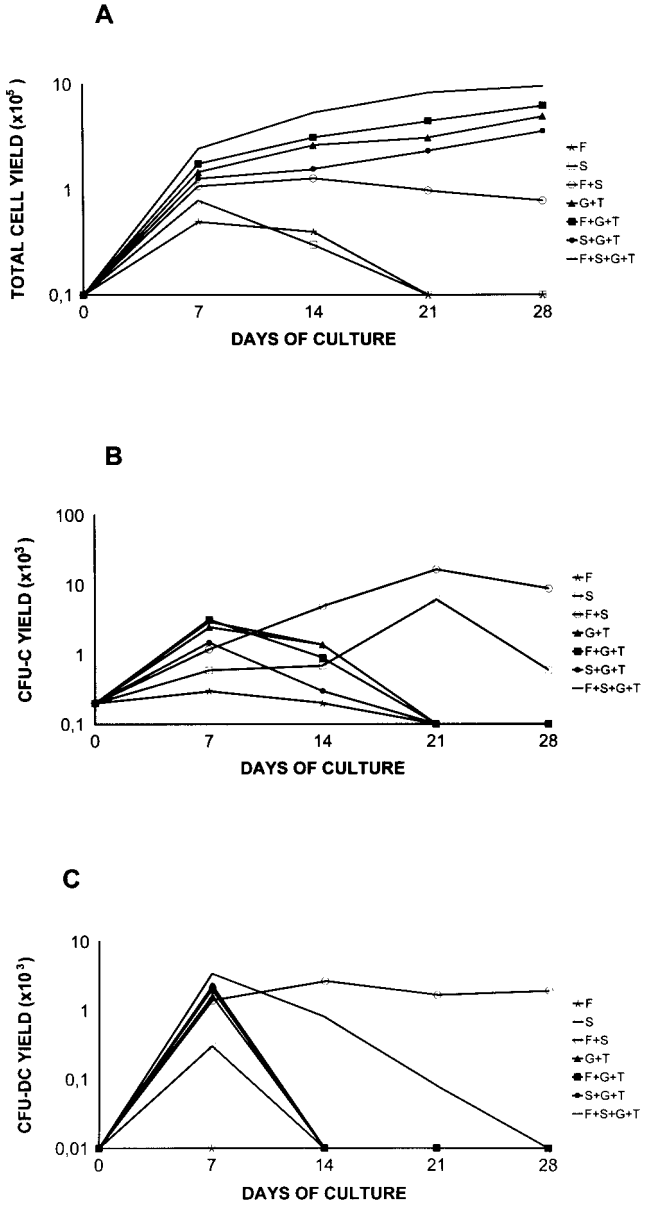

FIGURE 1. Generation of clonogenic progenitors from long-term liquid cultures of CD34$^+$HLA-DR$^-$ cells. DR$^-$ cells were cultured for 4 wk in the presence of cytokines FLT3-L (F), SCF (S), GM-CSF (G), and TNF-α (T). At weekly intervals, an aliquot of cell suspension was counted (*A*) and then plated in semisolid medium to evaluate the presence of secondary CFU-C (*B*) and CFU-DC (*C*). Only FLT3-L and SCF in combination (F+S) expanded and maintained the CFU-DC compartment up to 1 month of culture. The results reported the mean of eight different experiments.

output up to week 4 was SCF. However, its activity was significantly enhanced by the addition of FLT3-L.

A similar pattern was observed when the expansion of DC colonies was assessed (Fig. 1*C*). The differentiating factors GM-CSF and TNF-α did not maintain CFU-DC in culture for longer than 1 wk, and the addition of FLT3-L and SCF did not extend the survival of clonogenic precursors for >3 wk. Furthermore, neither FLT3-L nor SCF used alone supported the long-term production of CFU-DC. However, remarkably, in combination, FLT3-L and SCF expanded the pool of committed CFU-DC by 100-fold during the first and second week of suspension culture, and the output of DC colonies then remained stable for up to 1 month.

FLT3-L and SCF expand DC precursors that generate functional DC upon incubation with GM-CSF and TNF-α

We then determined whether CD34$^+$HLA-DR$^-$ cells that have been incubated with FLT3-L plus SCF and then with GM-CSF plus TNF-α are capable of turning into fully functional DC. Thus, suspension cultures were maintained in the presence of FLT3-L and SCF for 4 wk. At weekly intervals the cytokines were washed out and, in half of the wells, were replaced by GM-CSF and TNF-α for 7 days. Fig. 2 shows a representative example of the phenotype of hemopoietic cells after 14 days of culture with FLT3-L and SCF (similar data were observed up to week 4, data not shown). Flow cytometry (Fig. 2) demonstrated that CD34$^+$DR$^-$ cells differentiated into CD34$^-$CD33$^+$ DR$^+$CD14$^+$CD83$^-$CD86$^-$ cells. After one additional week of incubation with GM-CSF and TNF-α, these intermediate progenitors generated functionally active CD1a$^+$DR$^+$CD86$^+$ DC. The weak expression of CD83 Ag demonstrated that the DC did not fully mature in vitro, even though at the functional level they showed efficient stimulation of allogeneic and autologous T cells (see below). In this regard, it should be noted that the expression of CD83 on DC derived from CD34$^+$ cells has not consistently been reported in the literature.

The functional characterization of intermediate precursors and DC generated after the replacement of early acting cytokines with GM-CSF and TNF-α demonstrated that a significant proportion of cells were capable of uptaking soluble Ags as determined by the FITC-dextran assay (mean value of 40% of GM-CSF plus TNF-α-treated cells vs 2% of cells cultured in FLT3-L and SCF alone; $p < 0.01$). Furthermore, our data suggest that whereas myeloid cells cultured only with FLT3-L plus SCF provide only a weak stimulation for allogeneic T cells, those subsequently exposed to GM-CSF plus TNF-α become potent APC (Fig. 3). Interestingly, statistical analysis demonstrated that the latter were even more efficient stimulators of allogeneic T lymphocytes than the cells exposed to the differentiating factors from the beginning of the culture ($p < 0.05$; Fig. 3).

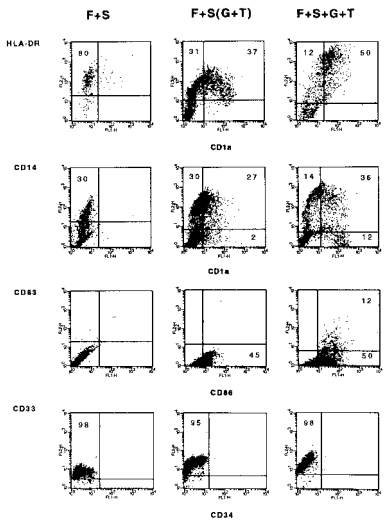

FIGURE 2. Phenotypic characterization of DC generated ex vivo in switch cultures. Highly purified CD34$^+$HLA-DR$^-$ cells were grown in the presence of FLT3-L (F) and SCF (S) for 2 wk. F+S were then replaced by GM-CSF (G) and TNF-α (T) for an additional week (F+S(G+T)). By comparison, the phenotype of DR$^-$ cells cultured in the presence of F+S+G+T from day 0 or maintained in F+S is also presented. The late addition of differentiating factors (G+T) to F+S resulted in the expression of DC-associated markers such as CD1a, HLA-DR, and the costimulatory molecule CD86. The percentages of positive cells in this representative experiment are also provided.

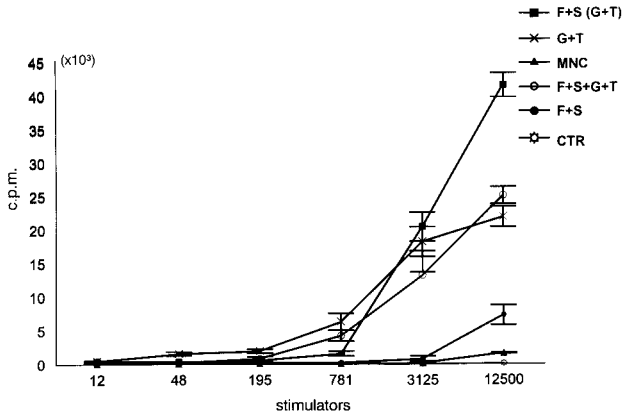

FIGURE 3. Alloreactivity of DC derived from intermediate precursors generated in presence of FLT3-L and SCF. $CD34^+HLA\text{-}DR^-$ cells were incubated for 14 days with early acting cytokines. Subsequently, in half of the wells FLT3-L (F) and SCF (S) were washed out and replaced by GM-CSF (G) and TNF-α (T) for an additional week. The cells were then tested for their capacity of stimulating T cells. Negative controls were represented by unmanipulated mononuclear cells and allogeneic PBMC alone (CTR), which always gave <2000 cpm. Positive controls were DC derived from $CD34^+HLA\text{-}DR^-$ cells incubated from the start with GM-CSF and TNF-α alone (G+T) or with FLT3-L + SCF + G + T (F+S+G+T). The results report the mean ± SD of eight different experiments. Cells grown in FLT3-L and SCF (F+S) were poor stimuli for T lymphocytes, whereas the addition of GM-CSF and TNF-α (F +S(G+T)) induced DC with a potent APC function. Statistical analysis demonstrated that DC primed with F+S and then switched to G+T were even more efficient stimulators of allogeneic T cells than cells always exposed to the differentiating factors from the beginning of the culture ($p < 0.05$).

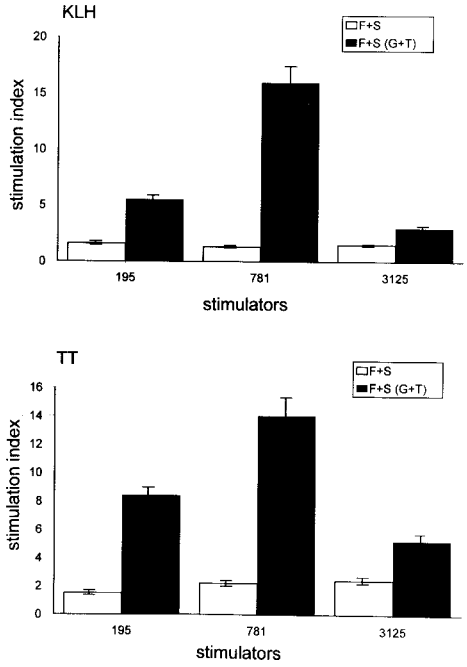

FIGURE 4. Capability of DC derived from intermediate precursors to present soluble Ags to autologous T cells. PBL (1×10^5) were incubated with increasing numbers of cells derived from $CD34^+HLA\text{-}DR^-$ cells cultured either with FLT3-L plus SCF (F+S) alone or with F+S followed by GM-CSF and TNF-α (F +S(G+T)). Coincubation was performed in the presence or absence of TT and KLH, and T cell proliferation was measured after 5 days. Autologous DC alone gave a mean of 3800 ± 1300 cpm when 3125 cells were used and lower values when decreasing numbers of DC were tested. The results report the mean ± SD of four different experiments. The SI using 3125 APC was significantly lower than that obtained using less DC ($p < 0.05$).

We also studied the capacity of the DC that had differentiated in the presence of GM-CSF plus TNF-α to process and present soluble Ags to autologous T cells (Fig. 4). Pulsing DC with either KLH or TT resulted in a significant proliferation of T lymphocytes for the induction of primary and secondary immune responses, respectively. Interestingly, a higher SI of T cells was observed when TT or KLH was presented by low numbers of DC ($p < 0.05$; Fig. 4). By contrast, myeloid cells maintained in culture with FLT3-L plus SCF did not show APC activity.

Taken together, these results indicate that FLT3-L and SCF do not induce DC differentiation from early DR^- progenitors. Rather, they expand a pool of myeloid cells (Fig. 2), with intrinsically poor APC activity (Figs. 3 and 4), which then become committed to erythroid, granulocytic, or dendritic lineage upon incubation with appropriate cytokines.

GM-CSF and TNF-α do not require FLT3-L or SCF to differentiate $CD34^+DR^-$ cells into fully functional DC

In preliminary experiments, highly purified $CD34^+DR^-$ cells were grown in liquid culture in the presence of GM-CSF with and without TNF-α. The addition of TNF-α was found to be essential to generate putative DC (data not shown). The phenotypic profile of myeloid cells cultured with GM-CSF plus TNF-α is shown in Fig. 5. After 7 days of culture (Fig. 5*A*), the cells had lost the CD34 Ag and acquired HLA-DR. They now also expressed myeloid Ags such as CD33 and CD14, whereas expression of CD83, CD40, and CD1a Ags was weak or absent. At this stage the cells did not show any APC ability. After 12–14 days of culture, flow cytometry revealed up-regulation of all the surface markers generally expressed by $CD34^+$ cell-derived DC ($CD1a^+$, $CD83^{+/-}$, $CD40^+$, $CD86^+$, $HLA\text{-}DR^{2+}$) and down-regulation of CD14 (Fig. 5*B*). The alloreactivity of DC generated from $CD34^+HLA\text{-}DR^-$ progenitor cells in the presence of GM-CSF plus TNF-α was well documented by one-way MLR assay and was not altered by the addition of FLT3-L or SCF into the culture (Fig. 3, and data not shown). Furthermore, the DC efficiently presented TT and KLH to autologous T cells (Fig. 6), especially when low numbers of APC were used ($p < 0.04$).

Thus, our results demonstrate that the combination of GM-CSF and TNF-α induces differentiation of early DR^- hemopoietic progenitor cells into functional DC, and that this process does not require FLT3-L or SCF. However, long-term culture and secondary clonogenic assays (Fig. 1) indicate that GM-CSF and TNF-α do force terminal differentiation of $CD34^+HLA\text{-}DR^-$ DC precursors, whereas FLT3-L and SCF seem to maintain their self-renewal.

SCF maintains CFU-DC in long-term cultures of $CD34^+DR^+$ cells, and its activity is enhanced by FLT3-L

In the last set of experiments, we assessed the cytokine requirement for the expansion of CFU-DC and the production of mature and functional DC from progenitors more mature than $CD34^+HLA\text{-}DR^-$ cells. To this end, long-term cultures of $CD34^+HLA\text{-}DR^+$ cells were established as shown in Fig. 7. Whereas expansion of more primitive DR^- cells required the presence of both FLT3-L and SCF, when SCF was used alone on DR^+ cells, a 10-fold increase of CFU-DC was observed at week 4. However, the activity of SCF was significantly augmented by FLT3-L (Fig. 7). Weekly determination of the phenotypic (data not shown) and functional characteristics of cultured cells indicated that the primary differentiation of DC required the presence of both

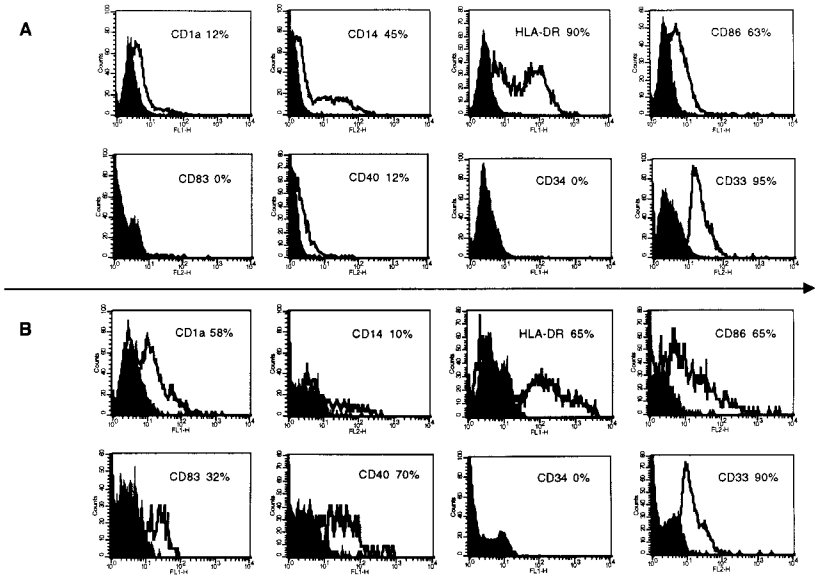

FIGURE 5. Phenotypic characterization of DC generated ex vivo from highly purified CD34$^+$HLA-DR$^-$ cells in the presence of GM-CSF and TNF-α for 7 (*A*) and 14 (*B*) days. Overlay diagrams show the expression of the relevant Ag vs negative controls. The percentages of positive cells in this representative experiment are also provided. As for HLA-DR Ag, the percentage of positive cells did not change significantly between days +7 and +14. However, the mean fluorescence intensity (MFI) value increased from 250 to 500 at day +14.

GM-CSF and TNF-α. SCF (with or without FLT3-L) did not induce DC differentiation directly, but instead expanded DC precursors into DC, which on exposure to GM-CSF plus TNF-α developed APC activity (Fig. 8). Cells incubated with SCF alone again turned out to be weak stimulators for T lymphocytes. Finally, as reported elsewhere (8), when TNF-α was replaced by IL-4 (two experiments) we observed a high yield of phenotypically characterized, CD14$^-$, fully functional DC (data not shown).

Discussion

Whereas GM-CSF appears to be the key factor for promoting DC generation, the effects of early acting cytokines like SCF and FLT3-L on DC hemopoiesis are still partially unclear. Both of these cytokines enhance production of DC from CD34$^+$ cells mediated by GM-CSF plus TNF-α and in combination they produce an additive response (5, 6, 8, 17, 18). Moreover, in combination with GM-CSF plus TNF-α, SCF induces DC formation from uncommitted CD34$^+$CD38^{dim} human thymic precursors (23). In vivo, treatment of mice with FLT3-L results in a dramatic increase in the number of functionally mature myeloid and lymphoid DC (24). Conversely, FLT3-L-deficient mice show a reduced number of DC (25). In humans, recent data indicate that FLT3-L expands the number of circulating, functionally competent DC in vivo (26). However, it remains to be determined whether FLT3-L and SCF directly affect DC differentiation and whether they stimulate early hemopoietic cells to generate committed CFU-DC.

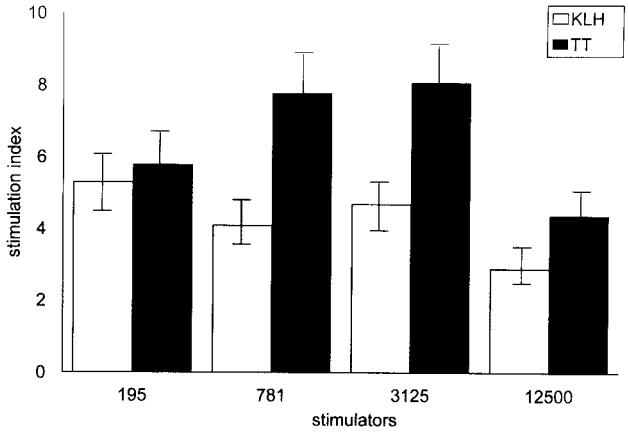

FIGURE 6. Capability of DC derived from CD34$^+$HLA-DR$^-$ cells incubated with GM-CSF and TNF-α to present soluble Ags to autologous T cells. PBL (1×10^5) were incubated with increasing numbers of DC in the presence or absence of TT and KLH, and T cell proliferation was measured after 5 days. Autologous DC alone gave a mean of 5700 $\pm$ 1800 cpm when 12,500 cells were used, and lower values when decreasing numbers of DC were tested. The results report the mean $\pm$ SD of six different experiments. Similar results were obtained when FLT3-L and SCF were added to GM-CSF and TNF-α (data not shown). The SI using 12,500 APC was significantly lower than that obtained using less DC ($p < 0.04$).

In this study, we used long-term liquid cultures of primitive human CD34$^+$HLA-DR$^-$ progenitors and more mature CD34$^+$HLA-DR$^+$ cells to investigate: 1) the precise role of FLT3-L and SCF on DC maturation pathway, and 2) the cytokine requirements of early hemopoietic cells for their differentiation into mature and functional myeloid DC. Notably, CD34$^+$HLA-DR$^-$ cells are practically the most immature population of hemopoietic progenitors (with the possible exception of a small subset of CD34$^-$ cells), containing as they do a cellular subset with the properties of putative stem cells (27).

Our results provide evidence that highly purified CD34$^+$HLA-DR$^-$ cells can differentiate into DC bearing typical morphology and surface markers after 14 days of culture with GM-CSF plus TNF-α. Moreover, allogeneic and autologous MLR both demonstrated that the resulting DC were fully functional APC. Remarkably, when higher numbers of DC were used to stimulate autologous T cells the SI fell off. This paradoxical finding might be explained by the potent immunostimulatory activity of the DC produced following treatment with GM-CSF plus TNF-α. Indeed, we found that when the APC/T cell ratio was 1:30 or greater, the DC induced significant proliferation of T lymphocytes even in the absence of soluble Ags (KLH and TT). However, it is also possible that the DC might have picked up xenogenic proteins (e.g., FCS-derived ones) during their development in vitro, and that this might have enhanced their nonspecific immunogenicity.

Interestingly, the cytokine-driven differentiation of human primitive hemopoietic cells into DC did not require the addition of FLT3-L or SCF. However, the results of secondary clonogenic assays indicated that the combination of GM-CSF and TNF-α does induce terminal differentiation of DC precursors, which were no

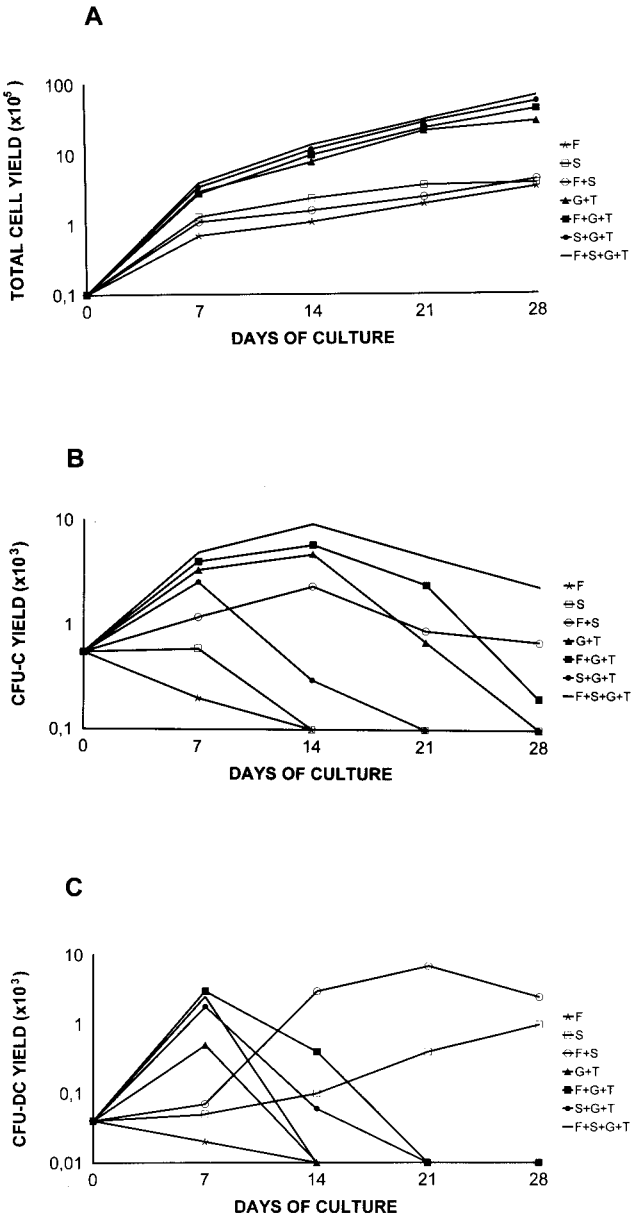

FIGURE 7. Generation of clonogenic progenitors from long-term liquid cultures of $CD34^+HLA\text{-}DR^+$ cells. DR^+ cells were cultured for 4 wk in the presence of cytokines FLT3-L (F), SCF (S), GM-CSF (G), and TNF-α (T). At weekly intervals, an aliquot of cell suspension was counted (*A*) and then plated in semisolid medium to evaluate the presence of secondary CFU-C (*B*) and CFU-DC (*C*). The CFU-DC compartment was expanded and maintained up to 1 month of culture only in presence of SCF, and the results were improved by the addition of FLT3-L to SCF (F+S). The results report the mean of eight different experiments.

longer detectable in the liquid cultures beyond 1 wk. Taken separately, neither FLT3-L nor SCF had any direct effect on DC production. In contrast, our results clearly demonstrate that in combination, they cooperate in expanding DC precursors from $CD34^+HLA\text{-}DR^-$ progenitor cells and in maintaining their long-term production. The cultured cells retained their growth potential and were not committed to a specific lineage as long as they remained in the presence of FLT3-L plus SCF. Subsequent addition of differentiating cytokines seems to be responsible for the irreversible commitment of $CD33^+CD14^+HLA\text{-}DR^+$ intermediate precursors into the DC lineage. Thus, FLT3-L and SCF do not merely act as "synergistic" factors, amplifying the activity of GM-

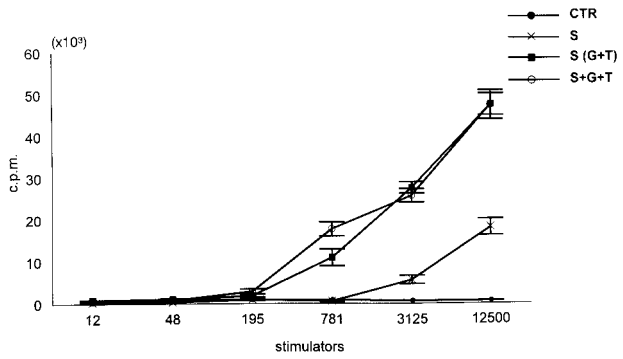

FIGURE 8. Alloreactivity of DC derived from intermediate precursors generated in presence of SCF. $CD34^+HLA\text{-}DR^+$ cells were incubated for 14 days with SCF (S). Subsequently, in half of the wells the cytokine was washed out and replaced by GM-CSF (G) and TNF-α (T) for an additional week. The cells were then harvested and tested for their capacity to stimulate T cells. Negative controls (CTR) were represented by PBMC alone, which gave <1000 cpm. Positive controls were DC derived from $CD34^+HLA\text{-}DR^+$ cells incubated from the beginning with S+G+T. The results report the mean $\pm$ SD of 10 different experiments. Cells cultured with S alone were poor stimuli for T lymphocytes, whereas the addition of differentiating factors (i.e., G+T) induced DC with a potent APC function.

CSF plus TNF-α on committed DC. Rather, they appear to be critical for the recruitment of early cells (and perhaps for the maintenance of their self-renewal). This seems to allow the expansion of more mature precursors, which then become responsive to lineage-restricted cytokines. This pattern resembles results obtained when the early acting cytokines were tested on differentiation lineages other than DC (17), as also described in this paper (see Fig. 1).

When long-term cultures were established with more mature $CD34^+HLA\text{-}DR^+$ cells, we found that SCF alone was capable of expanding CFU-DC (although its activity was further enhanced by FLT3-L). This finding may indicate that whereas the recruitment of earlier $HLA\text{-}DR^-$ progenitors requires multiple signals, stimulation of already committed cells can be achieved with a single cytokine like SCF, which in this context seems to provide a more potent stimulus than FLT3-L for myeloid DC differentiation.

In conclusion, we have described a long-term liquid culture system suitable for studying in detail the clonal development of DC precursors from primitive $CD34^+HLA\text{-}DR^-$ cells in vitro. Based on this assay, we have also established the role of the early acting factors FLT3-L and SCF in the DC differentiation pathway. In particular, although GM-CSF and TNF-α do not require additional cytokines to generate DC from primitive human $CD34^+DR^-$ progenitor cells, they do force terminal differentiation of DC precursors. Conversely, FLT3-L and SCF do not directly affect DC differentiation, but instead sustain the long-term expansion of CFU-DC, which can be induced to produce mature DC by GM-CSF and TNF-α. This study was not designed to address the issue of how committed lymphoid CFU-DC derived from primitive hemopoietic cells respond to FLT3-L and SCF. We are currently investigating this question in the human system on the basis of current knowledge regarding the role of FLT3-L on the expansion of lymphoid DC in the murine model (24),

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