Expansion of dendritic cell precursors from human CD34+ progenitor cells isolated from healthy donor blood; growth factor combination determines proliferation rate and functional outcome

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Abstract: CD34+ haematopoietic progenitor cells, which circulate at extremely low frequencies in peripheral blood, are used to generate dendritic cells (DC) in vitro. Here, we describe a method to grow large numbers of DC precursors from these low frequent cells. Different combinations of early acting haematopoietic growth factors supported expansion of CD34+ cells. CD1a+ DC derived from precursors, expanded in fms-like tyrosine kinase-3 ligand (Flt3-L), stem-cell factor (SCF), interleukin (IL)-3, and IL-6, were less potent antigen-presenting cells (APC) compared to CD1a+ DC derived from precursors expanded in Flt3-L, trombopoietine (TPO), and SCF. Furthermore, the latter produced high levels of IL-12 and low levels of IL-10, a cytokine profile favorable for the priming cytotoxic T cells. In contrast, a mean increase of total cell number of 453-fold was obtained with Flt3-L, SCF, IL-3, and IL-6, and this increase was only 38-fold with Flt3-L, TPO, and SCF. Sequential cultures of both cocktails resulted in high numbers of potent APC, which can be useful DC-based cancer vaccines. J. Leukoc. Biol. 72: 321–329; 2002.

Key Words: tumor immunity · vaccination · cytokines

INTRODUCTION

The dendritic cell(s) (DC) is the most potent antigen-presenting cell (APC) and appears the sole cell type capable of inducing primary T cell responses. DC play a key role in delivering antigen from the periphery to the secondary lymphoid organs. Precursors, originating from the bone marrow, migrate to virtually every organ in the body, where immature DC take up surrounding antigens. After antigen uptake, the DC mature, costimulatory molecules (e.g., CD40, CD80, CD86) are up-regulated, and the DC migrate to the lymphoid tissues where they activate effector T cells [1–3]. Consequently, tumor antigen-loaded DC may be ideal cells for the generation and amplification of antitumor responses in a vaccination setting [4, 5]. Indeed, several clinical studies have shown distinct clinical responses after vaccination with tumor antigen-loaded, autologous DC [6–8]. Despite these successes, one of the problems remains the limited number of DC available for immunotherapy. As DC appear not only to be important for the initiation of specific cytotoxic T cell (CTL) responses but also for the maintenance of protective CTL [9], it may be crucial to repeatedly vaccinate cancer patients with tumor antigen-loaded DC. So far, most clinical tumor vaccination studies have been performed with monocyte-derived DC (MoDC), which have been characterized in detail with respect to antigen presentation and differentiation [10,11].

Here, we have explored another source of DC precursors, CD34+ haematopoietic stem cells (HPC), which, in contrast to monocytic precursors, proliferate when cultured in vitro. CD34+ HPC differentiate into CD1a+ DC after culture in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor α (TNF-α). [12]. In some studies, these cytokines were used in combination with interleukin-4 (IL-4) [13]. Several protocols have been described to expand CD34+ HPC. DC populations cultured from these expanded precursors are much more heterogeneous and less well defined than MoDC. Combinations of the early acting haematopoietic growth factors, fms-like tyrosine kinase-3 ligand (Flt3-L), trombopoietine (TPO), stem-cell factor (SCF), IL-3, and IL-6, resulted in the expansion of DC precursors [14, 15]. However, it is not clear which combination supports optimal proliferation of DC precursors, and comparative data on phenotype and APC function of the subsequently differentiated DC are limited. We have explored different combinations of these growth factors to induce optimal proliferation of CD34+ HPC isolated from G-CSF mobilized blood and non-mobilized blood obtained from healthy donors. The precursor cell populations subsequently obtained were differentiated into immature DC using GM-CSF and IL-4. Lipopolysaccharide (LPS) was used to demonstrate maturation. Here, we report that

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Received July 13, 2001; revised February 8, 2002; accepted April 8, 2002.
different cocktails of early acting cytokines propagate DC precursor proliferation, which subsequently differentiate into CD1a-positive DC expressing comparable levels of costimulatory molecules. However, the proportion and antigen-presenting capacity of these CD1a positive DC are noticeably different. Our results indicate that the potential to induce an allo- geneic mixed leukocyte reaction (MLR) and the capacity to restimulate memory cytotoxic T cells of the DC depend on the cytokines used for expansion of CD34+ HPC. Furthermore, the balance of IL-12 and IL-10 production by the DC after CD40 signaling is also dependent on the cytokine cocktail used to support expansion of DC precursors. As IL-10 is thought to be detrimental for antitumor immunity, caution is warranted, and APC function and cytokine production should be analyzed in vitro before these types of DC are used for immunotherapy. The protocol described here generates high yields of IL-12-producing DC from CD34+ HPC from nonmobilized, healthy donors.

MATERIALS AND METHODS

Cytokines

All cytokines were recombinant human material. Flt-3 L (R&D Systems, Abingdon, Oxon, UK) was used at 25 ng/ml; IL-3, IL-6 (R&D Systems), and SCF (PeproTech, Rocky Hill, NJ) were used at 10 ng/ml; and TPO (PeproTech) was used at 10 U/ml. IL-4 (CLJ, Amsterdam, The Netherlands) was used at 1000 U/ml, and GM-CSF (Schering-Plough, Kenilworth, NJ) was used at 100 ng/ml.

Purification and culture of CD34+ cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood of normal human volunteer donors by density centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway). CD34+ cells were purified from PBMC from healthy donors or from cryopreserved leucopheresis material from G-CSF-treated hematological cancer patients using the direct CD34+ progenitor cell magnetic cell sorter (MACS) isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's recommendations.

Isolated CD34+ cells were cultured at 1–2 × 10^5 cells per ml in 24-, 12-, or 6-well plates in Iscove's modified Dulbecco's medium (IMDM) containing 50 U/ml penicillin-streptomycin, 1.6 mM l-glutamine, 0.01 mM β-mercaptoethanol, and 10% fetal calf serum (FCS; complete medium), supplemented with various combinations of the cytokines as indicated in Results. Weekly, the precursor cells were harvested; adherent cells were released with 0.5 mM ethylenediaminetetraacetic acid and pooled with nonadherent cells. Global cell count and frequency of CD34+ cells were determined, and cellular density was adjusted to 1–2 × 10^5 cells/ml in complete medium with appropriate cytokines; cultures were maintained in this way for up to 6 weeks.

DC cultures

After 2–6 weeks culture, precursor cells were washed and subsequently set up at 2 × 10^5 cells per ml in complete medium containing GM-CSF and IL-4. After 1 week culture, the DC were analyzed. MoDC were used as control DC and as target cells in enzyme-linked immunospot (ELISPOT) assays. PBMC (2–5 × 10^6 per ml complete medium) were added to 2 × 10^5 cells/ml of MoDC. Additionally, we confirmed that all cell culture flasks (Nunc, Intermed, Denmark) at 37°C. Nonadherent cells were removed by washing with warm phosphate-buffered saline (PBS). The adherent cells were cultured for an additional 7 days in complete medium supplemented with GM-CSF and IL-4. Maturation was induced by an additional 48-h culture with 100 ng/ml LPS (Difco Laboratories, Detroit, MI).

Flow cytometry analyses and sorting CD1a+ DC

Expression of lineage and activation markers on CD1a+ DC was analyzed on a FACStar Plus using cellQuest fluorescent-activated cell sorter (FACS) analysis software (Becton Dickinson Erembodegem-Aalst, Belgium). Cells were double stained with the following monoclonal antibodies (mAb): fluorescein isothiocyanate-labeled anti-CD1a and phycoerythrin (PE)-labeled anti-CD14, anti-CD80, anti-CD86, and anti-human leukocyte antigen (HLA)-DR (all from BD Biosciences, Heidelberg, Germany) and anti-CD40 and anti-CD83 (Beckman Coulter, Nyon, Switzerland). DC (5·10^5) were labeled with anti-CD1a PE-conjugated antibodies (BD Biosciences). Cells were sorted on a FACStar Plus cell sorter (Becton Dickinson Erembodegem-Aalst) as CD1a+ and CD1a- cells. Cells were systematically reanalyzed after sorting, CD1a+ were 97.3± 3.0% pure, and CD1a- were 94.0± 1.9% pure (mean±SD of four experiments).

Induction of MLR

For allogeneic MLR, immature DC were irradiated (2500 rad) and added as stimulator cells to round-bottom, 96-well plates (Nuncelon Delta, Intermed, Denmark) at graded doses, reflecting the indicated responder-stimulator ratios (RS). Allogeneic PBMC were used as a source for responder cells, and 1 × 10^6 cells/well were added to the allogeneic DC. The cells were cultured for 3 days in complete medium, and during the last 18 h, [3H]thymidine (Amersham, Aylesbury, UK) was added (0.4 μCi/well) as previously described [16]. Subsequently, the cells were harvested onto fiberglass filters, and [3H]thymidine incorporation was determined using a flatbed liquid scintillation counter (Wallac, Turku, Finland).

Induction of IL-10 and IL-12p70 secretion

DC were harvested and washed, and 1–4 × 10^5 cells/well were stimulated in flat-bottom, 96-well culture plates (Nuncelon Delta) at a 1:1 ratio with irradiated (5000 rad) CD40L-transfected J558 cells (a generous gift of Prof. Dr. M. L. Kapsenberg, Amsterdam Medical Center, Amsterdam, The Netherlands) in complete medium in the presence or absence of 1000 U/ml interferon-γ (IFN-γ; Central Laboratory van de bloedtransfusie, Amsterdam, The Netherlands). Supernatants were harvested after 24 h, and the concentration of IL-10 (IL-10 enzyme-linked immunosorbent assay (ELISA) kit, CLB; according to the manufacturer’s recommendations) and IL-12p70 [17] was measured by ELISA.

Induction of influenza matrix protein-specific CD8+ T cells

CD4-depleted, autologous PBMC were in vitro restimulated with DC infected with 100 plaque-forming unit (pfu)/cell recombinant adenovirus containing the M1 matrix protein of the Haemophilus influenzae virus (RAd128, kindly provided by Drs. Rickards and Wilkinson, University of Wales, UK) at a R:S of 5:1 in complete IMDM supplemented with 10% human pooled serum (CLB) and 5 ng/ml IL-7 (R&D Systems). After 1 week culture, the T cells were analyzed for specificity in an IFN-γ ELISPOT assay, using in the case of HLA-A2.1-positive donors, autologous MoDC infected with wild-type (vv-wt) or recombinant vaccinia virus encoding the M1 matrix protein of the Haemophilus influenzae virus (vv-flu) [18]. In the case of HLA-A2.1-positive donors, the T2 cell line was used and loaded with the HLA-A2.1-binding M1-derived peptide (M1gp46) or as a negative control, the HLA-A2.1-binding HPV 16 E7-derived peptide (E711-26).

CD4 MACS depletion

PBMC were labeled with anti-CD4 antibody (BD Biosciences), washed, and subsequently labeled with goat anti-mouse IgG microbeads (Miltenyi Biotec), Cells were separated using MiniMACS separation columns (Miltenyi Biotec) according to the manufacturer’s recommendations.

RAd128 infection

DC were washed with serum-free medium and incubated at 37°C with 100 pfu/cell in serum-free medium containing lipofectamine (1.7 μg/1×10^6 pfu). After 2 h, the cells were washed with complete medium and incubated at 37°C in an incubator with 5% CO_2 humidified atmosphere.

vv Infection

MoDC were washed once in serum-free medium and incubated at 37°C with 5 pfu/cell of the vv-wt or the vv-flu virus. After 2 h, the cells were washed with complete medium and incubated at 37°C in an incubator with 5% CO_2 humidified atmosphere.
Multiscreen, 96-well filtration plates (Millipore, Molsheim, France) were coated for 3 h at room temperature or o/n at 4°C with the mAb 1-D1K (50 μl, 15 μg/ml in filtered PBS, Mabtech, Nacka, Sweden). Plates were washed six times with serum-free medium and subsequently blocked with filtered, complete medium with 10% FCS for 0.5 h at room temperature. Effector cells/well (7.5 × 10^5 l × 10^5) were incubated o/n with 1 × 10^7 target cells at 37°C in an incubator with 5% CO₂ humidified atmosphere. The cells were discarded, and the plates were washed six times with filtered 0.05% Tween 20 in PBS (PBS-T). The mAb (50 μl) 7-B6-1 (1 μg/ml in filtered PBS, Mabtech) was added to each well, and plates were left at room temperature for 2–4 h. After six washes with PBS-T, 50 μl streptavidin-alkaline phosphatase (1:1000 diluted in PBS, Mabtech) was added to each well, and plates were left at room temperature for 1–2 h. After six washes with PBS-T, 50 μl alkaline-phosphate reagent (AP conjugate substrate kit, Biorad, Hercules, CA) was added and left for 15 min–1 h, until spots had developed. The reaction was stopped by washing with tap water. Spots were counted by two independent observers.

Statistics
The number of DC derived from the different culture conditions, mean fluorescence intensity (MFI) of CD markers, MLR, and ELISPOT results was compared by Student’s two-tailed, paired or unpaired t-test as appropriate.

RESULTS
Expansion of DC precursors from G-CSF mobilized blood
Flt-3L (F), TPO (T), SCF (S), IL-3 (3), and IL-6 (6) are early acting cytokines that support the proliferation of DC precursors from CD34⁺ HPC. Two different cocktails, FTS (Flt3-L, TPO, SCF) and FS36 (Flt3-L, SCF, 3, 6), were compared for their capacity to induce proliferation of CD34⁺ HPC. CD34⁺ cells (1×10⁵) isolated from G-CSF mobilized donors cultured for 4 weeks with FTS resulted in a mean increase of total cell number of 38-fold, whereas cultures with FS36 resulted in a mean increase of total cell number of 43-fold (Fig. 1A and Table 1). Next, the precursor cell populations were washed and induced to differentiate into DC with GM-CSF and IL-4 (Fig. 2). CD1a expression was analyzed, as this is the only available marker solely expressed on immature DC and is a generally accepted marker for DC differentiation. The proportion of CD1a-positive DC that differentiated from FS36 precursors (FS36-DC) was lower (19%) than from the FTS precursor cell population (FTS-DC, 48.7%; Fig. 1B and Table 1).

To investigate whether the FS36 precursors could still be directed into a precursor population containing more DC precursors, FS36 precursors were washed and subsequently cultured in FTS for an additional week (Fig. 2). This sequential culture resulted in an increased percentage of CD1a-positive DC (FS36/FTS-DC, 41.3%) compared with the FS36-DC cultures (19%) but equivalent to the FTS-DC cultures (48.7%, Fig. 1B and Table 1). As a consequence, the total calculated, absolute number of CD1a-positive DC obtained from 1 × 10⁶ CD34⁺ cells was higher in FS36/FTS-DC cultures (15.6×10⁶) compared with the FS36-DC (6.4×10⁶) and the FTS-DC (1.8×10⁶) cultures (Fig. 1C and Table 1). Thus, it was possible to profit from the better proliferation induction of the FS36 cocktail and the increased yield of CD1a-positive DC of the FTS cocktail.

CD34⁺ cells are present at a frequency of 0.05–0.2% in nonmobilized, healthy donor blood. To investigate whether the cytokine cocktails could induce nonmobilized CD34⁺ HPC to proliferate to a similar extent as the G-CSF mobilized CD34⁺ cells, CD34⁺ cells were isolated from ~90 ml blood from three healthy donors in five separate experiments and divided over three culture conditions, FTS, FS36, or FS36 followed by FTS (Fig. 2). The yield of precursors from 30 ml blood after 4 weeks of culture in FTS was 2.8 × 10⁶ cells, whereas after culture in FS36, the yield was 18.9 × 10⁶ cells (Fig. 1D and Table 1). As with the mobilized CD34⁺ cell cultures, the proportion of CD1a positive cells was higher in the FTS-DC cultures (32.6%) than in the FS36-DC cultures (12.6%). More CD1a positive DC differentiated from the FS36 precursors when they were cultured for an additional week in FTS (29.6%; Fig. 1E and Table 1), which is comparable to the percentages obtained in the FTS-DC cultures. Thus, the highest calculated absolute number of CD1a positive DC from nonmobilized CD34⁺ cells was obtained with a sequential culture of FS36 followed by FTS before differentiation was induced by GM-CSF and IL-4 (5.1×10⁶ CD1a⁺ DC). This yield of CD1a positive DC was significantly higher than the numbers obtained in the FTS-DC (0.9×10⁶; P=0.01) and FS36-DC (2.3×10⁶; P=0.01; Fig. 1F) cultures. Two healthy donors (HD1 and HD2) were analyzed in two separate experiments (Fig. 1, D–F). The same pattern emerges for each donor in both experiments and for all other donors tested (Fig. 1), although some interexperimental variations were observed.

Phenotypic analysis of precursor cells and DC
The precursor cells progressively lose most of the CD34 expression in all culture conditions and are over 95% positive for CD33, indicating that all precursors are from myeloid origin (Table 2). GM-CSF receptor expression is equally low in all three conditions (MFI<2; Table 2) and cannot easily explain the differences in CD1a-positive DC yield among the three culture conditions. The percentage of CD14 positive precursors was significantly lower in the FTS cultures compared with the FS36 and FS36/FTS cultures. CD11c expression was comparable on CD14 negative and CD14 positive precursors in all culture conditions. However, CD11b expression, a marker present on macrophages and suggested as being more highly expressed on APC progenitors arrested in their development, was significantly higher on the CD14-positive DC yield among the three culture conditions. The percentage of CD14 positive precursors was significantly lower in the FTS cultures compared with the FS36 and FS36/FTS cultures. CD11c expression was comparable on CD14 negative and CD14 positive precursors in all culture conditions. However, CD11b expression, a marker present on macrophages and suggested as being more highly expressed on APC progenitors arrested in their development, was significantly higher on the CD14-positive precursor cells in the FS36 cultures (Table 2). The higher content of CD14⁺/CD11b⁺ cells of the FS36 precursor cultures suggests that these cultures contain earlier DC precursors than the FTS and FS36/FTS cultures.

In addition, the receptor for SCF (c-kit), IL-3 (CD123), and IL-6 (CD126) was expressed on precursors, which were in culture for up to 4 weeks, regardless of the cytokine combination used (data not shown). FLT3 (receptor for Flt3-L) expression was low or undetectable at any time (on CD34⁺ HPC directly after isolation and on the precursors cultured for up to 4 weeks; data not shown). However, it was previously shown that despite low levels of FLT3 expression, Flt3-L exerted

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synergistic effects in combination with other growth factors on proliferation of CD34+ HPC [19]. After culture in GM-CSF and IL-4, a subpopulation of the cells acquired the typical DC morphology characterized by high side-scatter (SSC) values on the FACS. Phenotypic analyses showed that these were CD14-negative in all culture conditions, and CD1a expression was higher in the FTS and FS36/FTS conditions compared with the FS36 condition. The expression of the costimulatory molecules (CD40, CD80, CD86) and HLA-DR was, in general, comparable in all conditions analyzed (Fig. 3A). In some experiments, a slight increase in expression was observed on DC differentiated from precursors that were expanded in FS36/FTS. The immature DC derived from all precursor culture conditions were negative for CD83 (Fig. 3A); 48-h LPS maturation induced CD83 expression and increased expression of CD40, CD80, CD86, and HLA-DR on gated DC (Fig. 3B), indicating that these cells have all the phenotypic characteristics of classic DC. An example of FTS-derived immature and mature DC is shown, but the same effect was observed on mature DC derived from FTS and FS36 cultures.

**FTS-DC and FS36/FTS-DC induce strong, allogeneic MLR**

Blood from healthy donors was used for further functional assays. Immature DC cultures derived from the three precursor culture conditions were used as stimulator cells in allogeneic MLR. When the total DC population was used, the FTS-DC \((P<0.05)\) and FS36/FTS-DC \((P<0.10)\) induced a slightly but reproducibly stronger MLR than the FS36-DC (Fig. 4A). To investigate whether this difference was simply a result of the quantitative difference (lower percentage of CD1a+ DC) between the different culture conditions or whether there was also a qualitative difference, DC cultures were sorted in a CD1a positive and a CD1a negative fraction and were used separately.
that the CD1a (Fig. 4C; open bars). These results indicate that FTS and FS36/FTS-CD1a DC have a cytokine profile that is in favor of type 1 cellular immunity, and FS36-CD1a+ DC have a cytokine profile that favors type 2 cellular immunity.

**FTS-DC and FS36/FTS-DC activate memory CTL in vitro**

CTL are believed to be the most important effector cell in tumor immunity. To compare CTL stimulatory capacity of the different DC populations, CD1a+ DC were infected with a recombinant adenovirus encoding the matrix protein of the Haem influenza virus (M1) and were used to stimulate CD8 positive T cells from healthy donors. After 1 week culture, T cells were analyzed for IL-10 and GM-CSFR expression. From the MLR results, it is clear that the CD1a negative cells are not potent APC. Thus, in all probability, the CD1a+ cells truly represent the APC in these heterogeneous populations. Therefore, sorted CD1a+ DC were analyzed for IL-10 and IL-12 production. Immature CD1a+ DC were stimulated with the CD40L transfected cell line J558 for 24 h in the presence or absence of IFN-γ, and the production of IL-10 and IL-12 was measured in the supernatants. In the absence of IFN-γ, the production of IL-12 was below detection level in all culture conditions (Fig. 5A, open bars). In the presence of IFN-γ, FS36-CD1a+ DC produced considerably less IL-12 compared to the CD1a+ DC derived from the FTS and FS36/FTS cultures (Fig. 5A, solid bars). In contrast, FS36-CD1a+ DC produced higher levels of IL-10 (Fig. 5B). As expected, the difference was most striking in the absence of IFN-γ, a type-1, T cell-mediated immunity (CMI) response-skewing cytokine (open bars). These results indicate that FTS and FS36/FTS-CD1a+ DC have a cytokine profile that is in favor of type 1 cellular immunity, and FS36-CD1a+ DC have a cytokine profile that favors type 2 cellular immunity.

**TABLE 1. Recovery of Number of Precursors, Percentage, and Calculated Number of CD1a-Positive DC after Culture in GM-CSF and IL-4**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th># Precursors ($\times 10^6$)a mean (range)</th>
<th>% CD1a$^b$ mean (range)</th>
<th># CD1a+ ($\times 10^5$)c mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobilized donors$^d$:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTS</td>
<td>3.8 (3.5–4.2)</td>
<td>48.7 (21–65)</td>
<td>1.8 (0.9–2.3)</td>
</tr>
<tr>
<td>FS36</td>
<td>45.3 (18.2–90)</td>
<td>19 (10–30)</td>
<td>6.4 (4.7–9)</td>
</tr>
<tr>
<td>FS36/FTS</td>
<td>45.3 (18.2–90)</td>
<td>41.3 (27–67)</td>
<td>15.6 (7.5–27)</td>
</tr>
<tr>
<td>Healthy donors$^e$:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTS</td>
<td>2.8 (0.5–5.8)</td>
<td>32.6 (11–58)</td>
<td>0.9 (0.2–1.5)</td>
</tr>
<tr>
<td>FS36</td>
<td>18.9 (2.9–31.1)</td>
<td>12.6 (7–15)</td>
<td>2.3 (0.3–7.2)</td>
</tr>
<tr>
<td>FS36/FTS</td>
<td>18.9 (2.9–31.1)</td>
<td>29.6 (20–41)</td>
<td>5.1 (0.9–7.2)</td>
</tr>
</tbody>
</table>

$^a$ Total number of precursors after 4 weeks culture in FTS: Flt3-L, TPO, and SCF; FS36: Flt3-L, SCF, IL-3, and IL-6; or FS36/FTS: 3 weeks in FS36 and 1 week in FTS.

$^b$ Percentage of CD1a-positive cells in the different culture systems after DC differentiation with GM-CSF and IL-4.

$^c$ Calculated total number of CD1a-positive DC in GM-CSF/IL-4-treated cultures.

$^d$ The mean of three experiments of G-CSF-mobilized donors is shown; cultures were started with 1 cell isolated from 30 mL blood.

$^e$ Compared with FTS precursor-derived DC, $P = 0.01$, paired Student’s $t$-test; compared with FS36 precursor-derived DC, $P = 0.01$, paired Student’s $t$-test.

**TABLE 2. CD Marker Expression on DC Precursors after 4 Weeks Culture in FTS, FS36, or FS36/FTS**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>FTS</th>
<th>FS36</th>
<th>FS36/FTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>4.6 (3.4)</td>
<td>6.5 (3.4)</td>
<td>6.5 (3.4)</td>
</tr>
<tr>
<td>CD33</td>
<td>95.0 (3.2)</td>
<td>97.1 (1.5)</td>
<td>95.8 (2.5)</td>
</tr>
<tr>
<td>CD14</td>
<td>60.3 (3.7)</td>
<td>81.1 (2.2)</td>
<td>77.7 (6.0)</td>
</tr>
<tr>
<td>Mean MFI (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSFR</td>
<td>1.5 (0.1)</td>
<td>1.9 (0.5)</td>
<td>1.7 (0.2)</td>
</tr>
<tr>
<td>CD14+</td>
<td>12.4 (3.0)</td>
<td>32.7 (4.6)</td>
<td>15.9 (6.0)</td>
</tr>
<tr>
<td>CD11b</td>
<td>24.3 (1.5)</td>
<td>32.3 (5.6)</td>
<td>28.7 (4.7)</td>
</tr>
<tr>
<td>CD11c</td>
<td>10.7 (2.4)</td>
<td>15.3 (2.2)</td>
<td>13.9 (2.1)</td>
</tr>
<tr>
<td>CD11c</td>
<td>8.6 (0.6)</td>
<td>10 (3.1)</td>
<td>10.6 (0.6)</td>
</tr>
</tbody>
</table>

FTS: Precursors cultured for 4 weeks in Flt3-L, TPO, and SCF; FS36: precursors cultured for 4 weeks in Flt3-L, SCF, IL-3, and IL-6; FS36/FTS: precursors cultured for 3 weeks in FS36 and 1 week in FTS.

$^a$ Compared with FS36-derived precursors, $P = 0.001$, unpaired Student’s $t$-test; compared with FS36/FTS-derived precursors, $P = 0.013$, unpaired Student’s $t$-test.

$^b$ Compared with FTS-derived precursors, $P = 0.03$, unpaired Student’s $t$-test; compared with FS36/FTS-derived precursors, $P = 0.09$, unpaired Student’s $t$-test. These data are the mean ± SD of three experiments.
cells were analyzed for antigen specificity against peptide-loaded T2 cells for HLA-A2.1-positive donors or MoDC infected with a recombinant vv encoding the M1 protein (in the case of HLA-A2.1-negative donors) in IFN-γ ELISPOT assays. Significantly more antigen-specific spots were seen after re-stimulation with FTS-CD1a+ DC (P < 0.01) and FS36/FTS-CD1a+ DC (P < 0.05) compared with FS36-CD1a+ DC (Fig. 6). These results indicate that the FTS- and FS36/FTS-derived DC are potent APC, and FS36-derived DC are not.

DISCUSSION

Tumor antigen-loaded DC vaccination has been shown to be effective in experimental mouse tumor models in prophylactic [20] as well as in therapeutic [21, 22] settings. Clinical trials have shown promising results [6–8] but leave ample room for improvement. Although comparative studies are limited, recent data suggest that repeated vaccination with antigen-loaded CD1a+ DC may improve the outcome of DC-based tumor vaccination [9]. In most studies, five to nine doses of antigen-loaded DC are administered, resulting in partial and complete responses in some patients [6–8]. However, results of a study vaccinating melanoma patients suggest that it may be necessary to vaccinate up to 12 times to induce a complete response and to induce relatively high frequencies of tumor antigen-specific CTL, which can be measured ex vivo with tetramers [23]. Repeated vaccination requires many DC, hence ex vivo generation of large numbers of DC is essential. In addition, high levels of costimulatory molecule expression and potent T cell stimulatory capacity are essential properties of DC used for induction of tumor-specific immunity [4, 5]. Furthermore, it has recently been proposed that analogous to Th1-helper cell type 1 (Th1) and Th2, type 1- and type 2-inducing DC can be identified, although it is not yet clear whether the two types reside from a separate [24] or common precursor [25]. IL-12, which is important for CTL priming, is considered to be crucial in tumor immunity, and type 2 responses, mediated by IL-10 in general, are regarded as inhibitory. It is therefore important

Fig. 3. FACS analysis of DC. (A) Minor differences in expression of DC markers on GM-CSF/IL-4-differentiated, immature DC cultures obtained from the three precursor culture conditions. Expression of CD14, CD1a, CD40, CD80, CD83, CD86, and HLA-DR (thick lines) is shown on cells in the DC gate (high SSC) derived from FS36/FTS, FS36, or FTS precursor culture conditions. Thin lines represent the cells stained with isotype-control antibodies. A representative result of six experiments is shown. (B) Expression of costimulatory molecules is increased on mature FTS-derived DC. FTS-derived DC cultures were cultured for an additional 48 h with LPS. CD40, CD80, CD83, and HLA-DR expression is up-regulated, and CD83 expression is neo-expressed after LPS-induced maturation (thick lines). Thin lines represent the cells stained with isotype control antibodies. A representative result of four experiments is shown.

Fig. 4. Immature DC derived from the different cultures after differentiation with GM-CSF and IL-4 were used as stimulator cells in an alloreactive MLR. (A) Total DC population; FTS-DC versus FS36-DC: P < 0.05; FS36-DC versus FS36/FTS-DC: P < 0.10 at five out of six PBMC:DC ratios; and FTS-DC versus FS36/FTS-DC: P > 0.10. (B) CD1a- DC population; all groups: P > 0.10, and (C) CD1a+ DC population; FTS-DC versus FS36-DC and FS36/FTS-DC versus FS36-DC: P < 0.001 at five out of six PBMC:DC ratios; FTS-DC versus FS36/FTS-DC: P > 0.10. Mean cpm ± SD of triplicates is shown. This is a representative result of three experiments.
that the DC generated for the use in cancer immunotherapy produce high levels of IL-12 and low levels of IL-10.

Previous studies have analyzed various combinations of growth factors to expand CD34\(^+\) progenitor cells isolated from cord blood or from G-CSF mobilized blood for the generation of DC [14, 15, 26]. However, comparative data on expansion rates, the yield, phenotype, and function of the DC derived from these various expansion protocols are limited. Furthermore, recent studies have shown that G-CSF treatment mobilizes Th2-inducing DC, which do not produce IL-12. In addition, high numbers of IL-4- and IL-10-producing CD4\(^+\) cells, which are negative for the IL-12 receptor, are found after G-CSF mobilization [27, 28]. The aim of this study was therefore to test whether it is possible to generate large numbers of potent, type 1, CMI-inducing DC from CD34\(^+\) cells isolated from peripheral blood without G-CSF mobilization to reduce the risk of Th2 immune deviation as well as the burden on cancer patients. In these initial, in vitro studies, we have made use of FCS-containing media to determine the cytokine cocktail for optimal proliferation of DC precursors. LPS was used to show that it was possible to induce maturation in the obtained DC populations. For further clinical studies, we will culture the cells in clinically approved, serum-free media and subsequently vaccinate with DC matured with TNF-\(\alpha\) or CD40L.

We reached lower expansion of CD34\(^+\) cells isolated from PBMC from G-CSF mobilized donors using FTS than have been obtained by others with CD34\(^+\) HPC isolated from cord blood [14]. Cord blood-derived CD34\(^+\) HPC have been suggested to have significantly higher replicative potential than adult haematopoietic cells because of age- and proliferation-related loss of telomeric DNA [29], which may well explain the observed differences.

Although higher numbers of precursors were obtained with FS36, the percentage of CD1a\(^+\) cells obtained after subsequent differentiation with GM-CSF and IL-4 was considerably lower compared with FTS-DC cultures. Furthermore, sorted FS36-CD1a\(^+\) DC are poor inducers of alloreactive MLR and also showed a low capacity to restimulate memory CTL. In contrast,
FTS-CD1a+ DC were shown to be very potent stimulators of allogeneic and antigen-specific T cells. Thus, the APC function of these phenotypically similar DC was distinctly different. This distinct difference in function may be mediated by the differential cytokine production profile of both DC types. The predominant production of IL-10, which is a type 2 cytokine [30], by FS36-DC may cause the apparent, impaired function of these DC. On the other hand, the predominant production of IL-12, a type 1 cytokine [31] may contribute to the potent APC function of FS36/FTS-DC and FTS-DC. As expected, the type 1 skewing cytokine IFN-γ enhanced IL-12 and reduced IL-10 production [31, 32]. As the expression of costimulatory molecules and HLA-DR on the CD1a+/DC derived from the different precursor culture conditions was quite similar, we investigated whether there were any differences in phenotype of the precursor cells. CD11b is a marker highly expressed on macrophages [33] and on a subset of inhibitory APC progenitors, thought to be arrested in their development [34] and, as such, can be considered as a marker of more immature DC precursors. CD11b was expressed significantly higher on the CD14 positive cells in the FS36 cultures, indicating that the FTS cocktail induces differentiation into more mature DC precursors expressing lower levels of CD11b. Thus, immature FS36 precursors differentiate into DC with a weak antigen-presenting capacity, producing relatively high levels of the type 2 cytokine IL-10, and the more mature FTS precursors differentiate into potent APC, producing relatively high levels of the type 1 cytokine IL-12.

IL-6 has been identified as a tumor-derived factor that suppresses the development of DC [35], and our results point toward a role for IL-6 in inhibiting the development of DC precursors as well. Addition of IL-6 to FTS almost triples the recovery of precursors. However, after subsequent differentiation with GM-CSF and IL-4, the percentage of CD1a positive DC is reduced by half. Conversely, if IL-6 is left out of the FS36 cocktail, the recovery of precursors is reduced, and CD1a expression is slightly increased upon maturation. CD11b expression is increased on precursor cells when IL-6 is added to the FTS cocktail. Precursors cultured in FS3 have an even higher CD11b expression, which is still further increased when IL-6 is added to the cocktail (not shown). Thus, it appears that IL-3 and IL-6 inhibit maturation of the precursors and consequently support proliferation.

Recent work showing that IL-6 switches the differentiation of monocytes toward macrophages instead of DC [36] and the higher CD11b (a macrophage marker) expression on CD14+ precursor cultures in the presence of IL-6 also strongly suggest that IL-6 plays a role in the inhibition of DC development from precursors cultured in the presence of IL-6. Others have used IL-6 in combination with SCF and IL-3 (S36) [15, 23] or hyper-IL-6 in combination with SCF and Flt3-L (FSf6) [26] for 1 week to expand G-CSF mobilized CD34+ HPC followed by a 3-week DC differentiation in the presence of GM-CSF and IL-4. Expansion with S36 resulted in comparable percentages of CD1a+ DC as our protocol. After expansion with the FSf6 combination, no CD1a+ DC were obtained. Our results show that IL-6 has an inhibitory effect on the development of DC precursors. However, the prolonged culture in GM-CSF and IL-4 (3 weeks) may reverse the negative effects of IL-6, as did an additional week of culture in FTS in our protocol. Indeed, an additional week of culture in FTS induced further maturation of the FS36 precursors, and the subsequently differentiated DC cultures contained proportionally the same numbers of CD1a+ DC, which were functionally the same as FTS-DC.

The prolonged, in vitro culture and the possibility to freeze aliquots of precursors facilitate repetitive vaccination with DC grown from the same batch of precursors. The advantage of avoiding G-CSF mobilization is twofold: G-CSF mobilizes apart from CD34+ cells, also Th2-inducing DC [27, 28]. As Th1 cells are important for the control of tumor growth, and Th2 responses are considered to be detrimental, this is a side effect to be avoided in cancer patients. Second, G-CSF treatment puts a considerable burden on patients.

In conclusion, expansion of CD34+ HPC with different cytokine cocktails generates different DC precursor populations, which, although they have similar phenotypes, exhibit striking differences in function. FTS-DC and FS36/FTS-DC are potent APC and are of use in cancer immunotherapy, and FS36-DC are weak APC, which produce high levels of IL-10 and may be more favorable for the treatment of autoimmune disease. The sequential culture method is particularly appropriate for immunotherapeutical approaches, because relatively large numbers of DC can be generated, which are needed for repetitive vaccination. Furthermore, this is, to our knowledge, the first study showing that it is possible to obtain sufficient numbers of DC precursors and DC from CD34+ HPC isolated from a limited amount of peripheral blood without G-CSF mobilization. Clinical protocols, making use of the DC generated with the described protocol using clinically approved, serum-free media, are being developed for the adjuvant treatment of solid tumors.

ACKNOWLEDGMENTS

We thank Monique Momé-van Muijnen for excellent technical assistance.

REFERENCES


