Transduction of CD34+ cells with lentiviral vectors enables the production of large quantities of transgene-expressing immature and mature dendritic cells

Patrick Salmon[1]
Jean-François Arrighi[2]
Vincent Piguet[3]
Bernard Chapuis[4]
Rudolf H. Zubler[4]
Didier Trono[1]
Vincent Kindler[4]*

[1]*Department of Genetics and Microbiology, Faculty of Medicine, Geneva University Hospital, Geneva, Switzerland*

[2]*Allergology Unit, Division of Immunology and Allergology, Faculty of Medicine, Geneva University Hospital, Geneva, Switzerland*

[3]*Dermatology Department, Faculty of Medicine, Geneva University Hospital, Geneva, Switzerland*

[4]*Division of Hematology, Faculty of Medicine, Geneva University Hospital, Geneva, Switzerland*

*Correspondence to: Vincent Kindler, Division of Hematology, Geneva University Hospital, 25 Micheli-du-Crest, 1211 Geneva 14, Switzerland.
E-mail: vincent.kindler@hcuge.ch

Abstract

Background   Genetically engineered dendritic cells (DC) presenting specific antigens to T cells may be of great interest for immunotherapy. For this reason, the production of transgene-expressing DC derived from CD34+ cells transduced either shortly after *ex vivo* purification or during their differentiation into DC were evaluated.

Methods   CD34+ cells were transduced with lentivectors encoding for GFP before or after 21 days of culture with FLT3-ligand, thrombopoietin and stem cell factor and induction into DC with GM-CSF+IL-4 (G4) or G4+TNF (GT4). GFP and DC-specific marker expression was assessed by flow cytometry, and allostimulatory capacity was evaluated on GFP+ and GFP− sorted cells.

Results   Immature (G4-induced) DC obtained from amplified CD34+ cells were transducible by lentiviral vectors while mature (GT4-induced) DC were rather refractory. Moreover, since differentiated DC did not proliferate, large quantities of vectors were required to generate transgene-expressing cells with this protocol. In contrast, greater numbers of both immature and mature GFP−expressing DC were obtained with CD34+ cells exposed to lentivector shortly after purification. By the time of DC induction, GFP+ cells had increased by approximately 170-fold. After DC induction with G4, 32% of CD1a+, HLA-DR+, or CD40+ cells expressed GFP. CD1a+E-cadherin+ GFP+ Langerhans-like DC were also obtained. Incubation with TNF induced mature CD83+GFP+ DC that displayed a higher allostimulatory capacity than cells induced with G4 alone.

Conclusion   The transduction of a small number of CD34+ cells with minimal doses of lentivector may allow for the production of a large number of DC expressing selected antigens useful for immunotherapy. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords   CD34+ cells; dendritic cells (DC); lentiviral transduction

Introduction

Dendritic cells (DC) are the only antigen (Ag) presenting cells capable of stimulating resting T cells [1]. For this reason, they represent a potent tool for the development of immunotherapeutic protocols against tumor cells [2,3].

DC originate from the bone marrow, and are found as immature, highly endocytosing cells throughout the organism [4]. Once heat shock, trauma, hypoxia or microorganism products are detected, DC mature, that is, their metabolism shifts toward a shutdown of endocytosis, and an increase in their antigen presenting capacity leading to T cell activation [5].

In vitro generated DC can be loaded with synthetic peptides whose sequence is derived from tumor antigens, but these are restricted to a given MHC phenotype [6,7]. To circumvent this restriction, DC can be incubated with tumor cell lysate [8,9] or apoptotic tumor cells [10] to uptake and process antigen, but the presence of determinants common to normal and malignant cells represent a potential risk of eliciting non-specific T cell responses. DC can also be induced to present a specific antigen via the transduction of a cDNA coding for a specific sequence. In that case, the protein processed by the host cells and derived peptides are automatically presented in the proper MHC context [11,12]. In addition, the risk of non-specific response is decreased since only the specific Ag is introduced into the DC. Vectors derived from adenoviruses and herpesviruses can transduce non-dividing cells including differentiated DC [12,13]. However, transduction with these vectors is transient and often associated with undesirable immunogenicity caused by remaining viral proteins [14,15]. In contrast, vectors derived from lentiviruses are able to provide stable transgene integration, devoid of unwanted immunogenicity in both dividing and non-dividing cells [16]. Recent developments of these vectors, i.e. multiply attenuated packaging constructs, self-inactivating vectors containing strong promoters, and stable packaging cell lines, have provided enhanced biosafety and efficiency [17–20]. The present study demonstrates that the transduction of a cord blood (CB) and granulocyte-colony stimulating-factor mobilized blood (MB) CD34+ cells with HIV-derived vectors allows for the generation of large numbers of transgene-expressing DC. Transduced CD34+ cells amplified in culture with FLT3-ligand, thrombopoietin and stem cell factor (FTS) produce large amounts of hematopoietic progenitors that give rise after induction with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (G4) or G4 plus tumor necrosis factor-α (GT4) to either immature or mature DC, expressing the transgene and displaying normal DC phenotype and function. Such results open new avenues for the use of autologous DCs in immunotherapy.

Materials and methods

Cytokines

All cytokines were recombinant human material: GM-CSF, Leucomax (10^7 U/mg) was from Essex Chimie & Sandoz (Basel, Switzerland) and used at a concentration of 20 ng/ml. Other cytokines were purchased from Peprotech EC (London, UK) and used at the following concentrations: FLT3-ligand (FLT3-L, 2×10^5 U/mg) 25 ng/ml, thrombopoietin (TPO, 10^6 U/mg) 10 U/ml, stem cell factor (SCF, 10^5 U/mg) 20 ng/ml (the combination of these three factors is abbreviated FTS thereafter) and interleukin (IL)-4 (2×10^6 U/mg) 20 ng/ml, tumor necrosis factor-α (TNF, 5×10^6 U/mg) 40 ng/ml (200 U/ml), and transforming growth factor β-1 (TGFβ, 2.5×10^7 U/mg) 10 ng/ml.

Antibodies/immunoreactants

The following materials were used in the present study. Specific PE-labeled mAbs: anti-CD34 (mIgG1, clone 8G12), and anti-CD14 (mIgG2b, MoP9), from Becton-Dickinson, (Mountain View, CA, USA); anti-CD1a (mIgG1, clone BL6) and anti-CD83 (mIgG2b, clone HB15a) from Immunotech (Marseilles, France); and anti-CD86 (mIgG2b, clone IT2.2) from Pharmingen (San Diego, CA, USA). Specific biotin-labeled mAbs: anti-CD14 (mIgG2a, clone UCHM1) from Ancell (Bayport, MN, USA); anti-HLA-DR (mIgG2b, clone B8.12.2) from Immunotech; anti CD40 (mIgG1, clone RVS-M) from Serotec (Oxford, UK); and anti E-cadherin (mIgG1, clone 67A4) from BMA Biomedicals AG (Augst, Switzerland). Monoclonal isotype controls: PE and biotin-labeled mIgG1, and biotin-labeled mIgG2b from Dako A/S (Glostrup, Denmark); biotin-labeled mIgG2a from Ancell; PE-labeled mIgG2b from Immunotech. Other reactants: allophycocyanin (APC)-labeled streptavidin from Pharmingen; polyclonal mouse IgG reagent grade from Sigma Chemical Co. (St Louis, MO, USA); anti-CD34 mIgG coated M450 Dynabeads from Dynal A/S (Oslo, Norway); uncoupled anti-HLA-DR (mIgG2a, clone L-243) given by Dr J. Pieters of the Basel Institute for Immunology, Basel, Switzerland; and PE-labeled polyclonal goat anti-mouse Ab from Jackson Immunoresearch (West Grove, PN, USA). Both anti-CD40 and anti E-cadherin monoclonal antibodies were labeled in house using a kit from Molecular Probes (Eugene, OR, USA).

Purification of CD34+ cells

Cord blood (CB), and mobilized blood (MB) samples were obtained according to institutional guidelines of the ethics commission and purified as described elsewhere [21]. Briefly, mononuclear cells (MNC) were recovered after Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation, and CD34+ cells were purified using anti-CD34 M450 Dynabeads with a ratio of one bead : four cells for 30 min at 4°C under gentle agitation. Further steps were undertaken as recommended by the manufacturer. At the end of the procedure, cells were immediately washed, and analyzed by flow cytometry. CD34+ cells represented $80 \pm 5\%$ and $96 \pm 3\%$ of the total for CB and MB, respectively (mean $\pm$ SD of seven and five experiments, respectively).

Cell transduction and cultures

Vectors

Production of HIV-derived vectors pseudotyped with the VSV G envelope protein was achieved by transient co-transfection of three plasmids into 293T epithelial cell line as described previously [16]. The HIV vector plasmids were derivatives of the original pHR' backbone [16] with the following modifications. The HIV-derived vector referred to as HIV-EF1 in the present work is a self-inactivating (SIN) [17] HIV-vector containing the EF1α promoter derived from the pEF-BOS plasmid [22] (nucleotides 373–1561 of the human elongation factor EF1α gene, Gene Bank Accession No. J04617) and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) inserted between the reporter gene and the 3' LTR as described previously [23]. The HIV-derived vector referred to as HIV-PGK in the present work is a is a HIV-vector derived from the original pHR' backbone [16] but containing the phosphoglycerate kinase (PGK) promoter (nucleotides 424–930 of the murine PGK gene, Gene Bank Accession No. M18735). After transient transfection of the three plasmids by calcium phosphate in 293T cells, the supernatant was harvested, concentrated by ultracentrifugation, and resuspended in serum-free medium (CellGro®SCGM; CellGenix, Freiburg, Germany). Viral stocks were stored at $-70°C$ and titers determined by transduction and flow cytometry analysis of GFP expression in HeLa cells as described previously [20].

Transduction of early hematopoietic progenitors

CD34+ cells (10^5) were seeded in 96-well plates (flat bottom) in 100 μl CellGro medium supplemented with antibiotics (Gibco-BRL, Life Technologies Ltd, Paisley, Scotland, UK), with 10^{-4} mole/l [M] of dithiothreitol (DTT; Fluka Biochemika, Buchs, Switzerland) and TPO as described elsewhere [20]. After overnight incubation, 10^6 HeLa-transducing units of vector were added per well, and the volume was adjusted to 200 μl with CellGro medium containing TPO. This corresponds to a multiplicity of infection (MOI) of 10. After 24 h, cells were washed, diluted to 400 μl in IMDM supplemented with 10% fetal calf serum (FCS) and antibiotics (all from Gibco-BRL), and 1×10^{-4} M DTT with FTS. A global cell count was done every 5–7 days, and the cellular density was adjusted to 2×10^5 cells/ml. Bead-purified cells were also directly cultured in FTS medium containing FCS, and transduced on Day 3. After 14–21 days of culture, cells were either maintained in culture in the original conditions, or induced to differentiate into DC. Alternatively, cells were cryopreserved in IMDM 20% FCS, 10% DMSO (Merck, Darmstadt, Germany) for later induction into DC.

Induction of DC

Fresh or frozen cells recovered from primary culture were washed, counted and seeded at 2×10^5 cells/ml in 24-well plates containing 1 ml of either IMDM supplemented with 10% FCS or Yssel serum-free medium (Diaclone SA, Besançon, France) supplemented with DTT and anti-biotics. Cells were induced with GM-CSF plus IL-4 (G4), or G4 plus TNF (GT4), and analyzed after 6 days of induction.

Transduction of differentiated DC

Bead-purified CD34+ cells were cultured for 2–3 weeks in FTS as described above. Cultured cells were washed, adjusted to 2×10^5 cells/ml in IMDM 10% FCS and incubated for 72 h with G4 or GT4 to generate immature and mature DC, respectively. Vector was added at this time. Cultures were supplemented with fresh cytokines 24 h later, and cells were further cultured for 3–4 days.

Flow cytometric analysis/sorting

Cultured cells were washed, re-suspended at 3×10^4–1×10^5 in 50 μl cold isotonic saline containing 3% FCS and 200 μg/ml mouse IgG, and incubated for 10 min on ice. Specific labeled monoclonal antibodies or appropriate isotypic controls were added, and cells were further incubated on ice for 25 min. Cells were washed twice, and incubated with streptavidin-APC diluted 1/300 for 20 min at 4°C. Cells were washed once and incubated in 300 μl saline-3% FCS containing 10 μg/ml 7-amino-actinomycin D (7AAD) (Sigma). Cells were fixed with 0.5% para-formaldehyde and analyzed within 20 min on a FACScalibur. Cell debris and dead cells were eliminated from the analysis using gates on 7AAD low cells [24] and on forward and side scatters, respectively. Data were analyzed using WINMDI software by J. Trotter at Scripps Institute (La Jolla, CA, USA). Cell sorting of GFP+ and GFP− cells was done on a FACStar+ machine using FL-1 channel to record GFP fluorescence.

Immunostaining

Cells were cytospun on coverslips and air dried. The cells were fixed with 2% paraformaldehyde for 20 min at room temperature and then processed as described previously [25]. Briefly, the cells were permeabilized with 0.1% NP-40 and washed with PBS containing 3% bovine serum albumin (BSA). The anti-HLA-DR mAb (L-243) was used at the dilution 1:20. Secondary antibodies were used at a dilution of 1:200. Samples were analyzed on a Zeiss Axiovert microscope equipped with a cooled charge-coupled-device (CCD) camera.

Statistical analysis

Means and standard deviations are shown as a measure of data dispersion. One-way analysis of variance was done to evaluate differences of allostimulatory activity between GFP+ and GFP− cultures in the MLR. Mann-Whitney and Wilcoxon non-parametric tests were used for other data.

Results

GFP+ and GFP− cells proliferate with similar kinetics in FTS culture

Purified CD34+ cells were transduced with lentiviral vectors and cultured for 4 days with FTS. By then, the total cell number had increased by a factor of 6.2 ± 2, and $59 \pm 20\%$ of the cells were CD34+. GFP+ cells represented $24 \pm 7\%$ of the total cell number [mean $\pm$ SD of five experiments, using a multiplicity of infection (MOI) of 10 with HIV-EF1]. No differences were seen in the percentage of GFP+ cells between HIV-EF1 or HIV-PGK vectors, but the median fluorescence intensity of GFP, as previously reported [20], was higher with HIV-EF1 (data not shown). Cells maintained their proliferation in FTS cultures for a few weeks (Figure 1), but the proportion of GFP+ cells decreased with time. After 12 days of culture, $17 \pm 4\%$ of the cells were GFP+. Typically, this resulted on Day 30 in a 415-fold increase in the GFP+ cell number for a 980-fold increase in total cell number (Figure 1). These values are in agreement with long-term cultures involving the differentiation of transduced CD34+ cells into the erythroid, megakaryocytic, granulocytic, and monocytic pathways [20].

Transduction of differentiated DCs by HIV vectors is poorly efficient

After a few weeks of culture with FTS, hematopoietic progenitors amplified from non-transduced CD34+ cells were induced into immature DC with GM-CSF and IL-4 for 6 days (G4), or into mature DC with GM-CSF, IL-4, and TNF for 6 days (GT4). These culture conditions are known to generate immature and mature DC, respectively
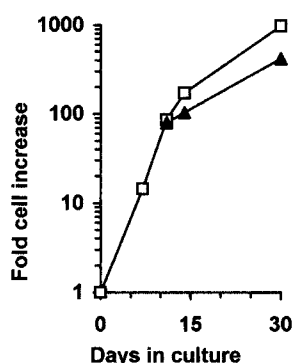
Figure 1. Maintenance of GFP expression in FTS cultures. Bead-purified CD34+ cells from CB were transduced with HIV-EF1 after 24 h of culture in serum-free medium containing TPO, as described in Materials and methods. GFP expression and cell growth were assessed at regular intervals during culture with FTS by flow cytometry and Trypan blue-negative cell count, respectively. Total cell growth (open squares) and GFP+ cell growth (solid triangles) are represented. GFP+ cell growth was extrapolated from the GFP+ cell frequencies directly obtained from flow cytometry analysis and from total cellular counts. These data are representative of five experiments

[26]. Cultures that were induced into DC with G4 exhibited a high proportion of CD1a+ HLA-DR+ and CD83− cells (Figure 2A, first row of histograms). When exposed to GT4, the CD1a+ cell fraction slightly decreased, while HLA-DR+, and in particular CD83+ cells, were increased (Figure 2A, second row of histograms). To evaluate the effect of transduction on differentiating DC, HIV-EF1 vector was added on Day 3 of DC induction, and cells were analyzed on Day 6. In this setting, 14% of the cells induced with G4 were GFP+ after 6 days of DC induction, and 11% were GFP+CD1a+. Globally, CD1a+ and HLA-DR+ cell numbers were close to that of untransduced cells induced in similar conditions while CD83+ cells were increased. CD1a+GFP+ and HLA-DR+ GFP+ cells were detected, but CD83+ cells were mostly GFP-negative (Figure 2B, first row of histograms). Only 6.5% of the cells induced with GT4 and transduced on Day 3 were GFP+, and 4.4% were GFP+CD1a+. CD1a+ and HLA-DR+ cell numbers were slightly lower and CD83+ cells were more frequent than the corresponding non-transduced cultures (Figure 2B, second row of histograms). Therefore the exposure of DC to lentiviral vectors lead to the expression of the transgene in both G4 and GT4 cultures, but the efficiency of transduction was rather low in these conditions. In addition, incubation with the vectors increased the number of CD83+GFP− cells in G4 cultures.

Generation of GFP-expressing DC after transduction and amplification of precursors

Alternatively, CD34+ precursors were transduced, expanded with FTS, and then differentiated into DCs (see Materials and methods). The percentage of GFP+ cells was maintained during the differentiation process (Table 1). Few cultures showed an increase of GFP+ cell frequency after DC induction, but on the whole this was statistically not significant. Immature DCs were obtained after incubation of FTS-amplified cells with GM-CSF plus IL-4 for 6 days (Figure 3, upper row). Mature DCs were obtained after incubation of FTS-amplified cells with GM-CSF plus IL-4 for 3 days, and with GM-CSF plus IL-4 and TNF for another 3 days of culture (Figure 3, lower row, and Table 2). Total GFP expression was similar in both conditions (Figure 3, left column, monoparametric histograms). G4 cultures contained a significant fraction of CD1a+, HLA-DR+ and CD40+ cells that expressed GFP (Figure 2, 2D plots, and Table 1). G4+TNF cultures also contained CD1a+, HLA-DR+ and CD40+ cells expressing GFP. In addition, CD83+GFP+ and CD86+GFP+ cells that were low or absent in G4 cultures were detected, indicating that mature GFP+ cells were generated in these conditions. CD14+ cell frequency was low in both types of culture.

We also tested whether our protocol could generate CD1a+E-cadherin+ cells expressing GFP, i.e. DC related to Langerhans cells (LC) [27,28]. After amplification of
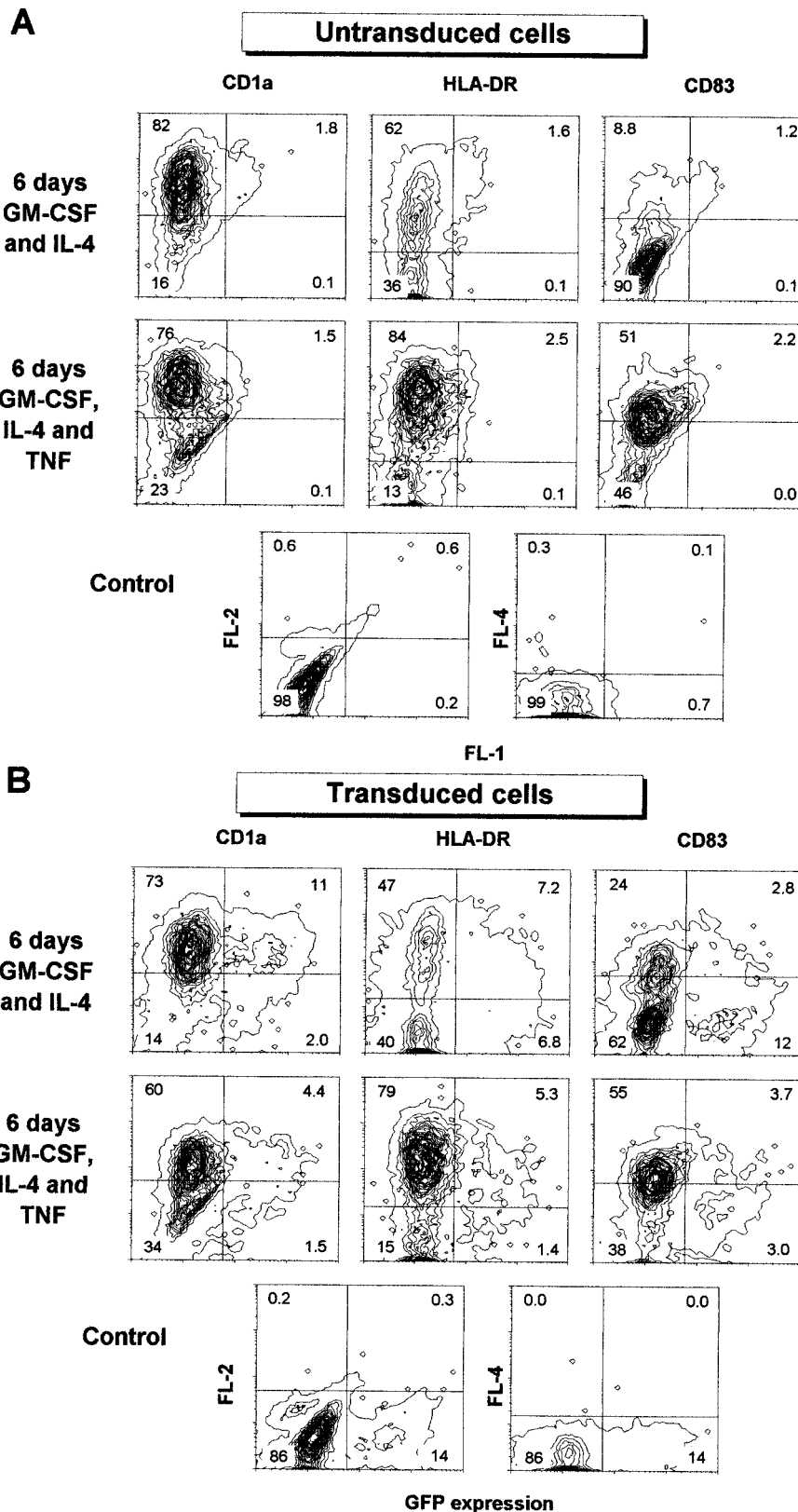
Figure 2. Transduction of DC after expansion and differentiation. CD34+ cells from MB were cultured for 21 days in FTS. Cells were induced into DC for 6 days with either G4 or GT4 and analyzed by flow cytometry. (A) Untransduced (control) cells; 2D contour plots in a linear setting show background values for FL-1 on the horizontal axis, and either CD1a, HLA-DR, or CD83 values on the vertical axis. Isotypic controls for both PE- and biotin-labeled antibodies are shown underneath. (B) Cells were induced into DC as in (A), but HIV-EF1 vector was added on Day 3. 2D Contour plots show GFP expression on the horizontal axis, and either CD1a, HLA-DR, or CD83 values on the vertical axis. Isotypic controls are as in (A). These data are from one experiment of three. Identical results were obtained with CB cells

Table 1. Frequency of GFP + cells during cultures with FTS, and after DC induction[a]

| | GFP + cells (%) | | | | | |
| | At first analysis | | Before DC induction | | After DC induction | |
Experiment	GFP + total	Day	GFP + total	Day	GFP + total	GFP + CD1a +
1	23	4	20	12	31	15
2	32	4	17	12	21	9.1
3	24	4	22	28	33	21
4	26	5	16	22	15	9.5
5	13	4	12	12	5	1.3

[a]Bead-purified CD34 + CB cells were transduced and cultured in FTS. The percentage of GFP + cells was determined either shortly after transduction (left column), after 12–28 days of FTS culture before DC induction (centre column), or after 6 days of DC induction (right column).
GFP + total, Total percentage of GFP-expressing cells; GFP + CD1a +, percentage of GFP + cells expressing CD1a; Day, days of culture in FTS at the time of GFP expression assessment.

either CB or mobilized CD34 + cells with FTS and differentiation with G4 in the presence (data not shown) or absence of serum, a significant fraction of CD1a + E-cadherin + cells expressing GFP was obtained (Figure 4, upper panels). The percentage of these cells could be further increased with the adjunction transforming growth factor β-1 (TGFβ) to G4 (Figure 4, lower panels).

Collectively, these results show that large quantities of transgene-expressing DCs can be produced using this transduction-expansion-induction protocol. Moreover, they show that integration of the lentiviral transgene into DC precursor genome does not interfere with cellular differentiation into phenotypically immature and mature DC, and that the transgene expression is not affected by the whole process.

GFP-expressing DC obtained from transduced CD34 + cells are functional *in vitro*

To test whether the expression of the transgene did not alter the function of *in vitro* generated DC, progenitors amplified in FTS cultures from transduced CD34 + cells were sorted into GFP + and GFP − cells and induced into immature and mature DC as described above. More than 95% of GFP + sorted cells expressed the transgene after DC induction with G4, and the proportion of CD1a + cells was similar in GFP + and GFP − cells (Figure 5A). The allostimulatory activity of DC was evaluated in a mixed leukocyte reaction (MLR). Graded numbers of either immature or mature irradiated DC generated from sorted GFP + or GFP − precursors were incubated with allogeneic T lymphocytes, and DNA synthesis was analyzed after 5 days. Transgene-expressing, G4-induced cultures

produced, as their GFP − counterpart a lower allostimulatory activity than G4 + GT4 induced cultures (Figure 5B), indicating that TNF induced both a phenotypical and a functional maturation of transgene-expressing DC. The allostimulatory activity of GFP + cultures in the presence of TNF was significantly higher at several effector/target ratios (i.e. 1/40, 1/80, 1/320, and 1/640; $p < 0.05$) compared to GFP − cultures. This may be related to the immunogenicity of the GFP [29]. The redistribution of MHC-II molecules toward the cell surface, known as a specific feature of mature DC [26], was also evaluated by immunofluorescence. In agreement with flow cytometric analysis, HLA-DR positive cells were detected in both G4 and G4 + GT4 induced DC, but only in the latter conditions HLA-DR molecules were located on the cell membrane and on protruding dendrites (Figure 5C). Thus, according to these data, GFP + DC generated from CD34 + transduced progenitors display similar *in vitro* functions to untransduced DC.

Discussion

Recently, lentiviral vectors have been used with success to transduce DC differentiated *in vitro* from normal peripheral blood CD14 + precursor [30,31]. These results were extended to show that DC generated from CD34 + cells were also transducible by lentiviral vectors. A proportion of immature, G4-induced DC could be transduced if the viral vector was added 3 days before the end of the induction. However, in these culture conditions, the frequency of CD83 + mature DC was much higher compared to the cultures induced with G4 but in absence of lentiviral vector. This suggests that the virus may be

Table 2. Frequency of cell surface markers induced after FTS expansion and DC induction of CD34 + CB cells transduced with a lentiviral vector[a]

	CD1a	CD14	HLA-DR	CD83	CD86	CD40
G4 6 days	40 (5.7)	14 (5.8)	77 (10)	6.7 (2.7)	14 (4.0)	45 (5)
G4 3 days, GT4 3 days	58 (21)	7.4 (7.5)	84 (11)	50 (26)	47 (18)	74 (23)

[a]Purified CD34 + CB cells were transduced and amplified in FTS cultures. After 2–4 weeks in FTS, cells were induced into immature DC with a 6-day incubation in G4, or into mature DC with a 3-day incubation in G4, followed by a 3-day incubation in GT4. Flow cytometry analyses were performed by gating on GFP + cells (FL-1), and recording specific immunostaining in FL-2 and FL-4. FL-3 was used to exclude dead cells using 7AAD. These data are the mean of Experiments 1, 2, and 5 in Table 1, with 31%, 21%, and 5% of the cells expressing GFP after DC induction, respectively. SD are given in parentheses.
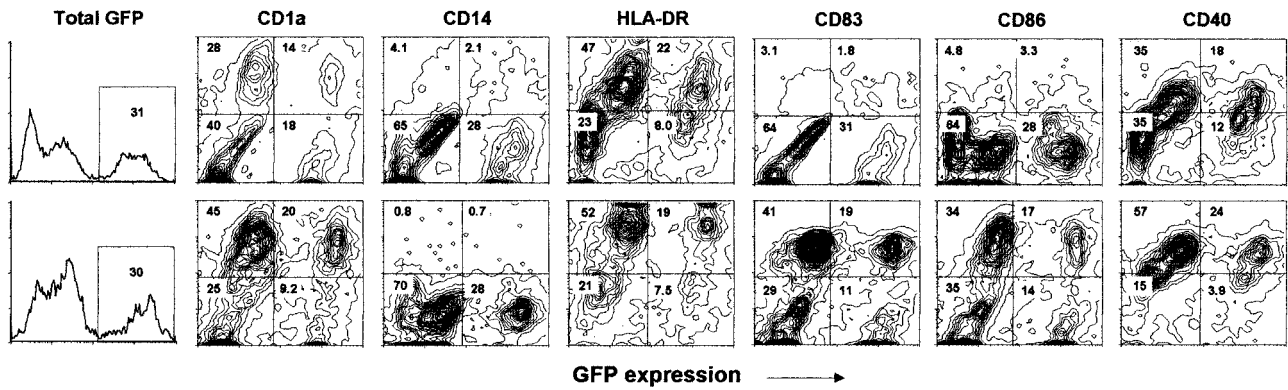
Figure 3. Generation of GFP-expressing DC from transduced, amplified precursor. CD34+ CB cells were infected with HIV-EF1 as described in Materials and methods, and cells were amplified for 13 days in culture with FTS. At that time, cell number had increased by a factor of 160-fold. Cells were then induced, either into immature DC with G4 for 6 days (upper panel), or in mature DC with G4 for 6 days and the adjunction of TNF for the last 3 days of culture (lower panel). Surface markers relevant to DC phenotype were then identified by flow cytometry using anti-CD1a, anti-CD83, and anti-CD86 mouse monoclonal antibodies labeled with phycoerythrin, and anti-CD14, anti-HLA-DR, and anti-CD40 mouse monoclonal antibodies coupled to biotin, and revealed by subsequent incubation with allophycocyanin-labeled streptavidin (see Materials and methods). The monoparametric histograms on the left show the overall expression of GFP in the cultures. 2D Contour plots are as in Figure 2, with GFP expression on the horizontal axis, and specific markers on the vertical axis. These data are from a single experiment of three
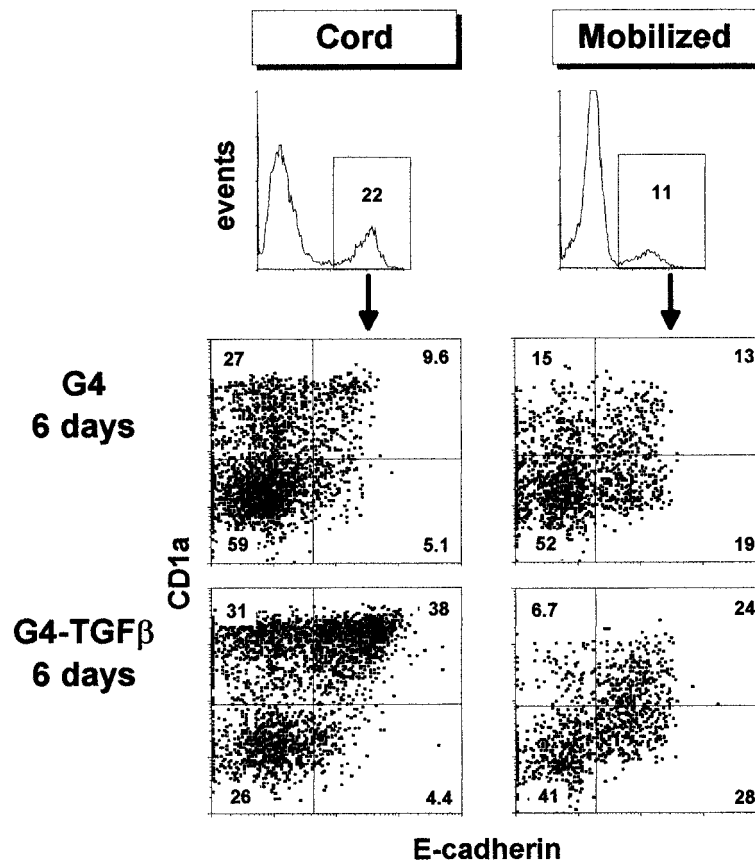

Figure 4. Production of E-cadherin+CD1a+ DC expressing GFP in serum-free conditions. Bead-purified CD34+ cells from either CB (cord, left panels) or MB (mobilized, right panels) were transduced with either HIV-EF1 or HIV-PGK vectors, respectively, and amplified in FTS in the presence of 10% FCS. After 14 days of culture, cells were induced into DC in serum-free conditions. The percentage of GFP+ cells in each type of cellular suspension is shown at the top of the figure (monoparametric plots). The 2D dot plots show the expression of CD1a and E-cadherin in the gated GFP+ cells cultured for 6 days with either GM-CSF plus IL-4, or GM-CSF plus IL-4 and TGFα. These experiments are representative of three experiments for CB and two experiments for MB
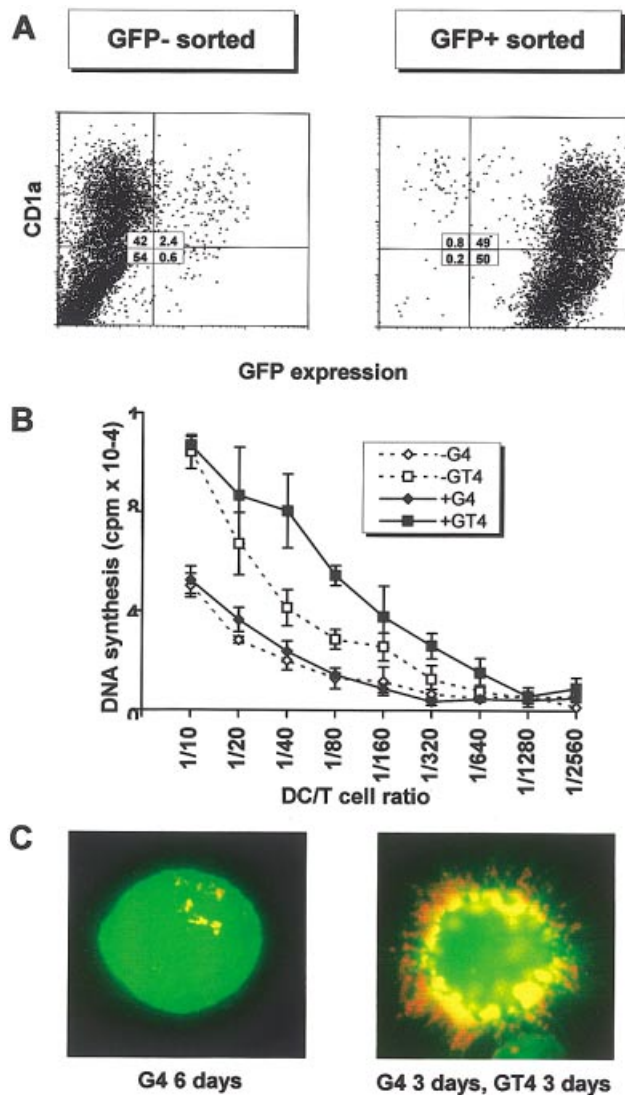
Figure 5. Functional properties of GFP-expressing DC. CD34+ cells from CB were transduced as described above, and cultured with FTS. After 9 days, growing DC progenitors were sorted into GFP+ or GFP− cells and further cultured for 7 days. Both subsets were induced into DC with G4 for 6 days, or G4 for 6 days and the adjunction of TNF for the last 3 days of culture. (A) CD1a expression in GFP+ and GFP− sorted cells after a 6-day incubation with G4. (B) GFP+ and GFP− sorted cells induced with G4 or G4 plus TNF were submitted to allogenic MLR. Dotted lines and open symbols represent GFP− sorted cells, while solid lines and closed symbols represent GFP+ sorted cells. DNA synthesis by T cells alone, or DCs alone, represented 298 ± 60 cpm, or 93 ± 30 cpm (mean $\pm$ SD of four wells), respectively. (C) GFP+ sorted DC induced with G4 or G4+GT4 as above were cytospun and stained with a HLA-DR-specific antibody. G4-induced DCs show a modest yellow fluorescence corresponding to the addition of the cytosolic GFP (green) staining and HLA-DR (red) staining, whereas G4+GT4-induced DC show a strong yellow staining radially distributed and a red staining in the dendrites, showing that HLA-DR molecules are present at a high density on the cell membrane. Magnification $540 \times$. All of these data are from one experiment, representative of two experiments. Error bars represent SD in (B)

maturating on its own. The origin of this spontaneous activation is not firmly identified but is likely to come from the large number of defective viral particles present in these preparations. Indeed, using a short exposition to the virus Gruber *et al.* recently showed that spontaneous DC maturation could be reduced [30]. Conversely, the incubation of mature GT4-induced DC with lentiviral vectors lead to a low frequency of transgene-expressing cells compared to that of G4-induced DC. This could be related, as already reported for CD14+ peripheral blood-derived mature DC, to the inefficient integration of the HIV genome in such cells [30–32]. This suggests that the maintenance of DC into an immature state is crucial to preserve their transducibility by HIV vectors. Therefore, the efficient production of mature, genetically modified DC has to be realized via the transduction of immature DC followed by their maturation. This approach requires a very precise schedule and, in addition, depends on large quantities of transducing material since differentiated DC no longer proliferate [33].

In view of these observations, a protocol involving the transduction of CD34+ precursors rather than that of *in vitro* differentiated DC was set up, taking into account the fact that CD34+ cells proliferate extensively in FTS and generate large numbers of various types of hematopoietic progenitors, including DC progenitors [21,34]. Contrary to the first protocol based on transduction of already differentiated DC, progenitors amplified from already transduced CD34+ cells were efficiently differentiated into immature GFP-positive DCs using GM-CSF and IL-4 (only 5% of CD83+ and 8% of CD86+ cells were generated under these conditions; Figure 3). Moreover, these cells exhibited a moderate allostimulatory capacity in the MLR assay. The addition of TNF to GM-CSF and IL-4 induced the generation of a large number of mature DCs, as indicated by the increased expression of HLA-DR, CD83, and CD86 (Figure 3), as well as by the enhancement of allostimulatory capacity (Figure 5) in these cultures. Taken together, these data indicate that this approach allows for the production of both immature and mature transgene-expressing DCs and may be of particular importance if *in vitro* engineered DC with a specific state of maturation are required for immunotherapy. Previous studies using either classical retroviruses or lentiviruses have showed that transgene-expressing DC could be obtained from transduced CD34+ progenitors after incubation with GM-CSF and TNF [35,36]. However, it is known that cultures of CD34+ cells with GM-CSF and TNF produce only a small amplification of DC numbers relative to the input in CD34+ cells and lead to a heterogeneous population of immature and mature DC, due to the spontaneous DC maturation associated with this system [37,36]. The procurement of well-defined immature and mature DC may require further cell sorting in this system.

Our HIV-derived vectors are designed to achieve both high transgene expression and maximal biosafety. This is possible by using strong promoters such as EF-1α and the self-inactivating cassette (SIN) which minimizes the risk that replication-competent recombinants emerge from the vector manufacturing system. Such improved vectors have been shown to be very efficient in driving transgene

expression in *in vitro* generated erythroid cells, megakaryocytes, granulocytes, and macrophages derived from CD34+ transduced cells [20]. In the present study, progenitor amplification and DC induction did not significantly alter the proportion of GFP-expressing cells, suggesting that the transgene was stably inserted in the eukaryotic genome, and that the promoters (EF-1α and PGK) were not silenced by the differentiation process. Also, the active transcription of the transgene did not alter the phenotype nor the function of the DC.

It was also possible to generate with GM-CSF, IL-4, and TGFα-1 [39,40], in serum-free conditions, significant amounts of CD1a+ GFP+ cells expressing E-cadherin, a molecule specifically expressed on immature CD1a+ Langerhans epidermal dendritic cells (LC) [27,28]. To our knowledge, this observation has not been reported elsewhere, and may be of importance since LC are the effector cells of the DNA vaccination process. LC resident in the skin are transfected by the gun shooting of DNA-coated gold particles, and subsequently migrate to the draining lymph node where they present the antigen corresponding to the transfected DNA to T cells [41,42]. However such transfection is not very efficient [43], and it would be interesting to evaluate if the subcutaneous injection of cellular suspensions enriched in engineered LC could favorably replace *in situ* DNA shooting. It may be possible that *in vitro* generated LC may be more efficient to present antigens and induce a tumor rejection within the epidermal environment than dermal-related DC, and would thus represent the best choice for the immunotherapy of melanoma or other skin malignancies. Also, it has been possible to achieve efficient expression of GFP in DC obtained from CB and MB that have been differentiated into DC in serum-free conditions. Though still not corresponding to GMP requirements, these data show that it is possible to generate human DC expressing a specific transgene in a fully autologous setting, using completely defined culture conditions.

Altogether, the present report provides the evidence that gene transfer into HPC using HIV-based vectors, followed by extensive proliferation, represents a realistic approach to the production of mature DCs for immunotherapy.

Acknowledgements

References

1. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; **9**: 271–296.

2. Schuler G, Steinman RM. Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J Exp Med* 1997; **186**: 1183–1187.

3. Reid CD. The biology and clinical applications of dendritic cells. *Tranfus Med* 1998; **8**: 77–86.

4. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245–252.

5. Cella M, Sallusto F, Lanzavecchia A. Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol* 1997; **9**: 10–16.

6. Mayordomo JI, Zorina T, Storkus WJ, *et al*. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour activity. *Nat Med* 1995; **1**: 1297–1302.

7. Pogador A, Gilboa E. Bone marrow-generated dendritic cells pulsed with class-I restricted peptides are potent inducers of cytotoxic T-lymphocytes. *J Exp Med* 1995; **182**: 225–260.

8. Fields RC, Shimizu K, Mule JJ. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor responses *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 1998; **95**: 9482–9487.

9. Nestle FO, Alijagic S, Gilliet M, *et al*. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998; **4**: 328–332.

10. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998; **392**: 86–89.

11. Reeves ME, Royal E, Lam JS, *et al*. Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. *Cancer Res* 1996; **56**: 5672–5677.

12. Song W, Kong HL, Carpenter H, *et al*. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induces protective and therapeutic antitumor immunity. *J Exp Med* 1997; **186**: 1247–1256.

13. Coffin RS, Thomas S, Thomas NSB, *et al*. Pure populations of transduced primary human cells can be produced using GFP expressing herpes virus vectors and flow cytometry. *Gene Ther* 1998; **5**: 718–722.

14. Yang Y, Nunes FA, Berencsi K, *et al*. Cellular immunity to viral antigens limits E1-deleted adenovirus for gene therapy. *Proc Natl Acad Sci U S A* 1994; **91**: 4407–4411.

15. Gahery-Segard H, Juillard V, Gaston G, *et al*. Humoral immune response to the capsid components of recombinant adenoviruses: routes of immunization modulate virus-induced Ig subclass shifts. *Eur J Immunol* 1997; **27**: 653–659.

16. Naldini L, Blomer U, Gallay P, *et al*. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.

17. Zufferey R, Nagy D, Mandel RJ, *et al*. Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nat Biotechnol* 1997; **15**: 871–875.

18. Zufferey R, Dull T, Mandel RJ, *et al*. Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J Virol* 1998; **72**: 9873–9880.

19. Klages N, Zuffrey R, Trono D. A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Mol Ther* 2000; **2**: 170–176.

20. Salmon P, Kindler V, Ducrey O, *et al*. High level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood* 2000; **96**: 3392–3398.

21. Arrighi JF, Hauser C, Chapuis B, *et al*. Long-term culture of human CD34+ progenitors with FLT3-ligand, thrombopoietin, and stem cell factor induces extensive amplification of a CD34-CD14– and a CD34-CD14+ dendritic cell precursor. *Blood* 1999; **93**: 2244–2252.

22. Mizushima S, Nagata S. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 1990; **18**: 5322.

23. Zufferey R, Donello E, Trono D, *et al*. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 1999; **73**: 2886–2892.

24. Schmid I, Uittenbogaart CH, Keld B, *et al*. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods* 1994; **170**: 145–157.

25. Piguet V, Wan L, Borel C, *et al*. HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. *Nat Cell Biol* 2000; **2**: 163–167.

26. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus inter-leukin 4 and downregulated by tumor necrosis factor α. *J Exp Med* 1994; **179**: 1109–1118.

27. Blauvet A, Katz SI, Udey MC. Human Langerhans cells express E-Cadherin. *J Invest Dermatol* 1995; **104**: 293–296.

28. Udey MC. Cadherins and Langerhans cell immunobiology. *Clin Exp Immunol* 1997; **107** (Suppl. 1): 6–8.

29. Stripecke R, Carmen-Villacres M, Skelton D, *et al*. Immune response to green fluorescence protein: implications for gene therapy. *Gene Ther* 1999; **6**: 1305–1312.

30. Gruber A, Kan-Mitchell J, Kuhen KL, *et al*. Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response *in vitro*. *Blood* 2000; **96**: 1327–1333.

31. Schroers R, Sinha I, Segall H, *et al*. Transduction of human PBMC-derived dendritic cells and macrophages by an HIV-1-based lentiviral vector system. *Mol Ther* 2000; **1**: 171–179.

32. Granelli-Piperno A, Chen D, Moser B, *et al*. The HIV-1 life cycle is blocked at 2 different points in mature dendritic cells. *Adv Exp Med Biol* 1997; **417**: 415–419.

33. Ardeshna KM, Pizzey AR, Thomas NSB, *et al*. Monocyte derived dendritic cells do not proliferate and are not susceptible to retroviral transduction. *Br J Haematol* 2000; **108**: 817–824.

34. Piacibello W, Sanavio F, Garetto L, *et al*. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. *Blood* 1997; **89**: 2644–2653.

35. Szabolcs P, Gallardo HF, Ciocon DH, *et al*. Retrovirally transduced human dendritic cells express normal phenotype and potent T-cell stimulatory capacity. *Blood* 1997; **90**: 2160–2167.

36. Evans J, Cravens P, Lipsky P, *et al*. Differentiation of lentivirus vector-marked dendritic cells derived from human CD34+ cells. *Hum Gene Ther* 2000; **11**: 2483–2492.

37. Santiago-Schwarz F, Belilos E, Diamond B, *et al*. TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J Leukoc Biol* 1992; **52**: 274–281.

38. Caux C, Vanbervliet B, Massacrier C, *et al*. CD34+ hemato-poietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNFα. *J Exp Med* 1999; **184**: 695–706.

39. Borowski TA, Letterio JJ, Farr AG, *et al*. A role for endogenous transforming growth factor β1 in Langerhans cell biology: the skin of transforming growth factor β1 null mice is devoid of epidermal Langerhans cells. *J Exp Med* 1996; **184**: 2417–2422.

40. Geissmann F, Prost C, Monnet JP, *et al*. Transforming growth factor β1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 1998; **187**: 961–966.

41. Condon C, Watkins SV, Celluzi CM, *et al*. DNA-based immunization by *in vitro* transfection of dendritic cells. *Nat Med* 1996; **2**: 1122–1128.

42. Takashima A, Morita A. Dendritic cells in genetic immunization. *J Leukoc Biol* 1999; **66**: 350–356.

43. Akbari O, Panjwani N, Garcia S, *et al*. DNA vaccination: Transfection and activation of dendritic cells as a key event for immunity. *J Exp Med* 1999; **189**: 169–177.