

# Unilineage monocytopenesis in hematopoietic progenitor culture: switching cytokine treatment at all Mo developmental stages induces differentiation into dendritic cells

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## Abstract

We have developed a new culture system whereby human hematopoietic progenitors purified from adult peripheral blood extensively proliferate and gradually differentiate into >95% pure monocytic (Mo) cells. At all developmental stages treatment with interleukin (IL)-4 + granulocyte-macrophage colony-stimulating factor or IL-4 + c-Kit-ligand + FLT-3 ligand switched the Mo precursors into dendritic cells (DCs). The switching capacity decreased only at the end of the culture, when most Mo cells matured to macrophages. Moreover, the Mo precursors were highly susceptible to transduction with lentiviral vectors: once switched to DCs, they maintained the transgene expression, as well as the phenotype and function of the DC lineage. Our results provide new insight into the potential role of the Mo lineage as a reservoir of DCs *in vivo*. Furthermore, the methodology for transduction of Mo precursors provides a tool to generate genetically modified, normally functioning DCs potentially useful for immunotherapy.

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**Keywords:** cytokines; dendritic cell differentiation

**Abbreviations:** APC, antigen-presenting-cell; BM, bone marrow; BSA, bovin serum albumin; DC, dendritic cell; FITC, fluorescein isothiocyanate; FL, FLT-3 ligand; GFP, green fluorescence protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; GM-CSFR, GM-CSF receptor; HLA, human leukocyte antigens; HPC, hematopoietic progenitor cell; IFN, interferon; IL, interleukin; KL, c-Kit-ligand; LPS, lipopolysaccharide; MACS, magnetic cell sorter; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; MLR, mixed leukocyte reaction;

MoAb, monoclonal antibody; Mo, monocytic; Mos, monocytes; NK, natural killer; PB, peripheral blood; PBMCs, PB mononuclear cells; PE, phycoerythrin; TGF, transforming growth factor; TNF, tumor necrosis factor

## Introduction

Dendritic cells (DCs) are sentinels for the immune system. Located in the periphery in an immature form, upon encounter of microbial antigens or proinflammatory cytokines, the DCs mature, process, and present the internalized antigens in combination with major histocompatibility complex (MHC) molecules. Mature DCs then prime naïve T cells, leading to both effector T-cell differentiation and memory T-cell expansion.<sup>1–4</sup>

In humans, DCs represent a heterogeneous population that may arise from different hematopoietic progenitors/precursors along distinct differentiation pathways. Two intermediate progenitors of DCs have been hypothesized: one expressing myeloid cells-related antigens, which gives rise to DC1 and intrathymic DC1-like cells, as well as monocytes (Mos) and granulocytic cells;<sup>5,6</sup> the other one with lymphoid but not myeloid potential, able to generate thymic and peripheral DC2 in addition to T, B and NK cells.<sup>7–9</sup> Even if some authors suggest that these intermediate progenitors belong to different lineages, they may represent different stages of maturation of a unique lineage.<sup>10–12</sup>

DCs derive in their ontogenic profile from stem cells committed to a hematopoietic developmental pathway.<sup>13–17</sup> The complex mechanisms at the basis of hematopoietic progenitor cells (HPCs) differentiation into specific lineages and the subsequent migration of the committed elements from bone marrow (BM) to peripheral districts, have been elucidated by *in vitro* and *in vivo* studies on the differentiative potential of intermediate progenitors, and by the phenotypical and functional characterization of the derived mature progeny.<sup>18,19</sup>

*In vitro* generation of DC populations has been described to occur in several culture systems. DCs can be obtained from HPCs isolated from cord blood, peripheral blood (PB) and BM,<sup>14,20–22</sup> or directly from PB mature Mos,<sup>23–25</sup> using different cytokines, in combination or alone.<sup>26–28</sup> Granulocyte-macrophage colony-stimulating factor (GM-CSF) + tumor necrosis factor (TNF)- $\alpha$  and GM-CSF + interleukin (IL)-4 were initially used to induce DC differentiation of HPCs and mature Mos, respectively.<sup>14,25</sup> Although GM-CSF is a relevant factor, at least in theory, its exclusive use *in vitro* could underestimate the DC differentiative potential of precursors, since some of them do not express the GM-CSF receptor (GM-CSFR). Recent reports<sup>29</sup> have demonstrated that an opportune mix of other cytokines can support DC differentiation. Among these cytokines, FLT-3 ligand (FL) was particularly interesting. Its ability to induce *in vitro* proliferation of

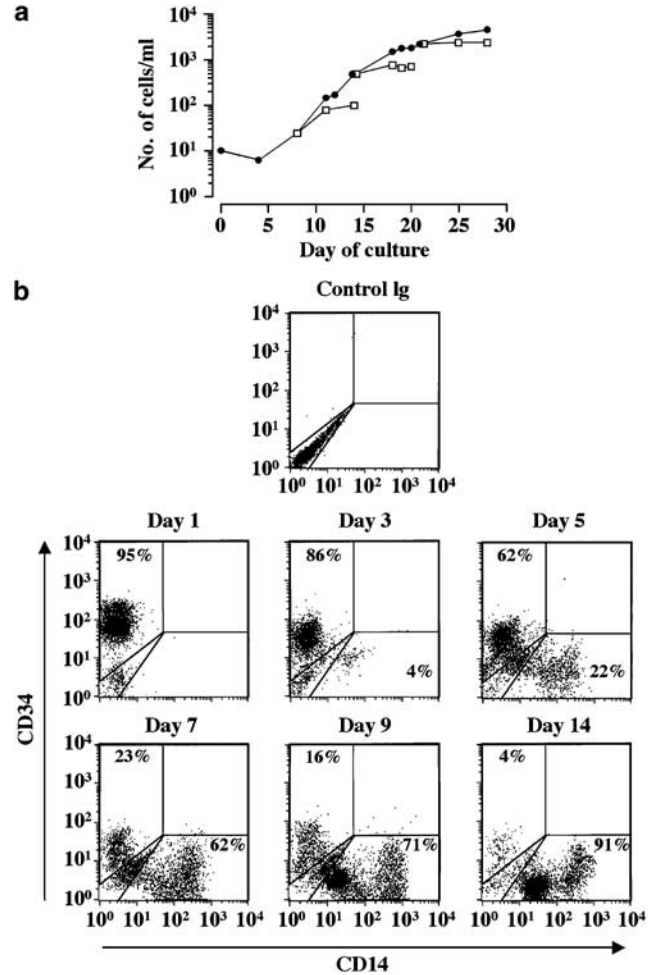
HPCs as well as to support, in conjunction with other specific cytokines, the differentiation of several hematopoietic lineages is well known.<sup>30</sup> *In vivo* studies have revealed its capability to enhance the number of circulating DCs both in mice<sup>31</sup> and in humans.<sup>32</sup> Furthermore, c-Kit-ligand (KL), known to induce cell proliferation in diverse hematopoietic lineages, has been used in several DC culture systems.<sup>33</sup>

In this study, we describe a liquid culture system that allows unilineage Mo differentiation/maturation from CD34<sup>+</sup> HPCs. Using two different cytokine combinations, Mo cells were induced, at different days of development, to differentiate into DCs. The results indicate that Mo precursors maintain the bipotential capability to generate not only Mos but also DCs throughout their differentiation/maturation, from early committed precursors to mature Mos. Furthermore, gene transfer experiments showed that Mo precursors and DCs obtained by this culture system are highly susceptible to lentiviral transduction. These studies provide a tool to analyze at cellular and molecular level the developmental relationship between the two lineages and to obtain large numbers of genetically modified DCs, which may be used in novel strategies of immunointervention.

## Results

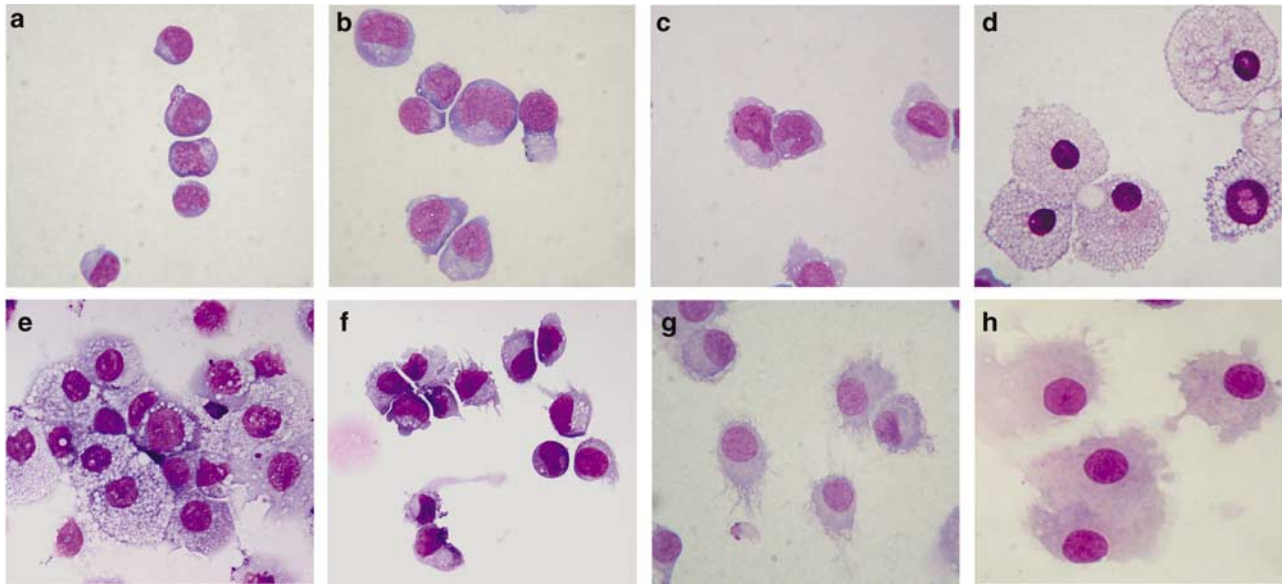
### Mo unilineage culture

Treatment of highly purified HPCs with optimal macrophage colony-stimulating factor (M-CSF), IL-6 and FL dosages in the presence of FCS (here referred to as 'primary culture') resulted in significant cell proliferation (i.e. 100-400-fold cell number amplification) (Figure 1a), as well as selective Mo differentiation/maturation (Figures 1b and 2a-d). In particular, in the first week of culture, HPCs proliferated and concomitantly differentiated towards the Mo lineage, as assessed by the progressive downmodulation of the HPC-associated CD34 antigen (from  $\geq 95\%$  CD34<sup>+</sup> cells at day 1 to  $\sim 20\%$  at day 7) and by the appearance of Mo-specific CD14 antigen, present on  $\sim 60\%$  of cells after 7 days of culture. In the very first days of culture (Figure 2a), morphological evaluation indicated that the cell population consisted of about 90% small blasts, while the remaining cells were mainly monoblasts, characterized by a deep blue cytoplasm and a large, eccentric and centrally indented nucleus with one or two large nucleoli, and rare promonocytes, that is, larger cells with a lobated nucleus and a clear blue cytoplasm containing few azzurrophilic granules. Noteworthy, the relative percentage of these cell types markedly changed from days 1 to 7 of culture, in correlation with the differentiative stage. In the second week, CD34 antigen expression was almost completely lost (being present on less than 4% of cells at day 14) whereas, at the same time, a gradual increase of CD14<sup>+</sup> cells could be observed. Early in the second week (Figure 2b), the culture approximately consisted of 15% blasts, 60% monoblasts, 15% promonocytes and 5% Mos. At days 13-15 (Figure 2c), cells were represented by about 5% monoblasts, 10% promonocytes, 65% Mos, recognizable by a reniform nucleus that occupies a half of the cell volume and by a finely granulated, gray blue cytoplasm, and about 15% macrophages, larger cells with an eccentric small nucleus and a



**Figure 1** Characterization of unilineage Mo culture. (a) Absolute numbers of total viable cells during Mo unilineage culture (●). Mo precursors were switched to DCs at different days of culture (8, 14 and 21 in this experiment) (□). Results are representative of seven independent experiments. (b) Kinetics of CD34 and CD14 expression on HPCs cultivated with FL, IL-6 and M-CSF. At the indicated time points, aliquots of cells were collected and double stained with anti-CD14-FITC and anti-CD34-PE MoAbs. Quadrants were set according to the fluorescence intensities of FITC- and PE-conjugated isotype-matched control IgGs. Results are representative of three experiments

cytoplasm highly vacuolated. In the third week, the developed progeny resulted in 95-100% CD14<sup>+</sup> cells, consisting mainly of mature Mos, and about 30% macrophages. Extending the time of culture up to 25-30 days (Figure 2d), the proliferation rate became consistently slower than before and the terminal differentiation of Mos to macrophages was almost complete. Indeed, at this time of culture, roughly a half of the cells expressed the CD86 antigen (data not shown). A more detailed phenotypical characterization was hampered by the absence of monoclonal antibodies (MoAbs) able to discriminate human Mo cells at the different maturative stages. In fact, CD31 was expressed on virtually all the cells during the entire culture period, while CD11b showed an upmodulation with kinetics that was very similar to that of CD14, but still not specific for the cells at the differentiative stages preceding that of mature Mos (data not shown).



**Figure 2** Morphology of cells from Mo culture and their derived DC progeny. Mo populations at day 2 (a), day 8 (b) and day 14 (c) of the primary culture. Typical macrophagic features appear after 25–30 days of culture in Mo medium (d). CD14<sup>bright</sup> cells (e) and CD14<sup>dim</sup> cells (f) sorted at day 14 of the Mo culture. DC populations, derived from day 14 Mo precursors after 5 day treatment with IL-4 + GM-CSF and IL-4 + FL + KL, respectively, and terminally activated by adding LPS (e and f) (original magnification  $\times 400$ )

In the majority of the experiments, we could detect CD14<sup>dim</sup> and CD14<sup>bright</sup> cells at different times of the Mo culture (see Figure 1b). Forward side scatter and morphological analysis indicated that they represent cell subsets at different maturative stages. In particular, almost all the CD14<sup>bright</sup> population consisted of more mature cells with premacrophagic features, that is abundant and highly vacuolated cytoplasm (Figure 2e), while the majority of the day 14-sorted CD14<sup>dim</sup> cells displayed a reniform nucleus and a pale and finely granulated cytoplasm, peculiar of Mo cells (Figure 2f).

Analysis of GM-CSFR expression revealed that this receptor was already detectable on Mo precursors at days 4–5 of culture and continued to be present during all the differentiative/maturative stages. Later on, about 70% of the total cells were GM-CSFR<sup>hi</sup> (see below).

Several DC-specific membrane antigens, such as CD40, CD80, CD83 were never detected on Mo precursors and mature Mos, while the expression of CD1a never exceeded 10% of the cell number. Finally, cell staining with CD15 MoAb during the entire culture period excluded the presence of basophils (data not shown).

Occasionally, at day 0 we observed  $\sim 1\%$  CD34<sup>+</sup>14<sup>+</sup> cells together with  $\geq 95\%$  CD34<sup>+</sup>14<sup>-</sup> cells (results not shown). To assess the relative contribution of CD34<sup>+</sup>14<sup>-</sup> and CD34<sup>+</sup>14<sup>+</sup> cells to generate the unilineage population described above, each cell type was sorted and utilized as a starting population for the Mo culture (and then switched to DC culture conditions, see below). The results obtained using CD34<sup>+</sup> or CD34<sup>+</sup>14<sup>-</sup> progenitors as starting population were overlapping, in terms of both proliferative and differentiative capabilities, whereas the CD34<sup>+</sup>14<sup>+</sup> subset did not show any growth potential in the presence of an Mo-specific cytokine cocktail and died within a few days (data not shown).

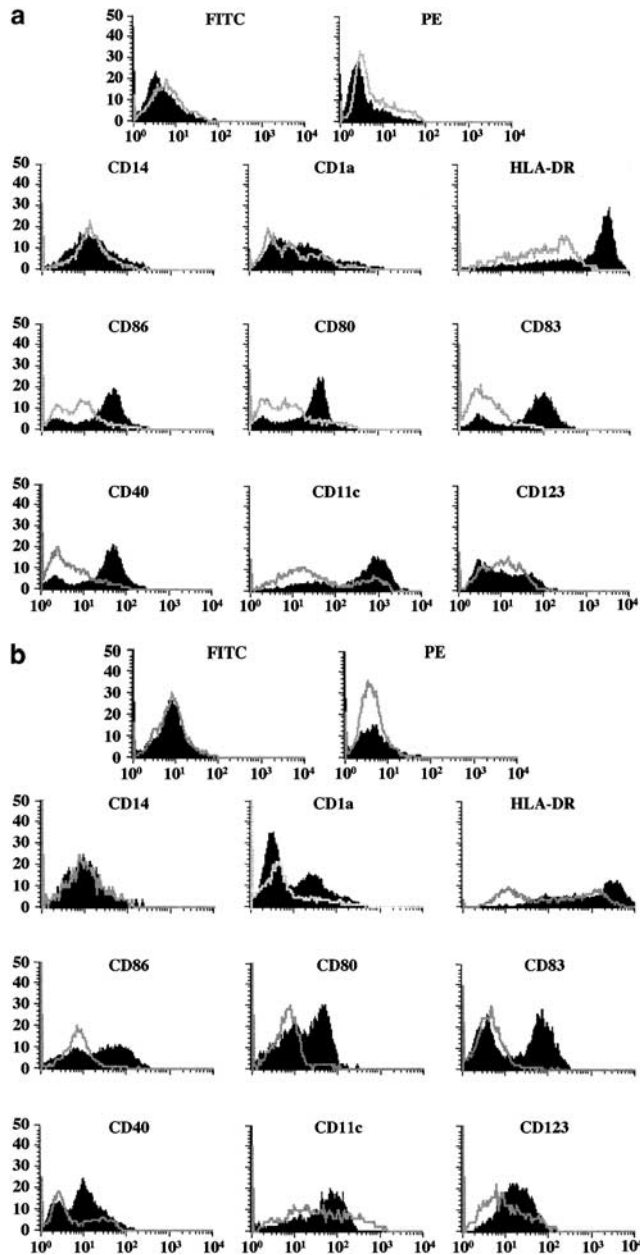
## Generation of DCs from Mo precursors

To identify at which differentiative stage(s) Mo cells could acquire the capacity to switch toward the DC lineage, Mo precursors between days 7 and 21 of culture were treated with either IL-4 + GM-CSF or IL-4 + KL + FL for 5 days. Samples of the cells were further treated overnight with lipopolysaccharide (LPS) to verify the capacity of acquiring the functional and phenotypical characteristics of mature DCs. The differentiation to DCs, irrespective of the culture medium used, was consistently coupled with a reduced proliferation rate with respect to Mo precursor growth (Figure 1a). This effect was particularly evident at the late phases of the primary culture. Treatment of Mo cells with both DC media gave rise to DCs, and the culture period required for the generation of typical DCs was dependent on the differentiative stage of the primary culture. In summary, days 7–10 Mo precursors required 10–13 days to differentiate into DCs, while days 13–21 Mo cells required a culture of 5–6 days. Results reported below refer mainly to Mo precursors induced at days 13–15.

Morphological modifications started very early, that is 2–3 days after resuspending the cells in the DC specific media; after additional 3 days of culture cells acquired the distinct DC morphology, which differed in the two culture conditions. Namely, the DCs obtained after IL-4 + GM-CSF treatment were characterized by a monocytoid nucleus and an abundant, pale basophilic cytoplasm, endowed with a minor azzurophilic granulation, irregular margins and fine cell processes, often polarized (Figure 2g); whereas the DCs obtained after incubation with IL-4 + KL + FL were larger, with a more abundant and pale cytoplasm and margins shaped as lamellipodia or veils (Figure 2h).

For a better characterization of the two DC populations, extensive phenotype analyses were conducted at the end of

culture, before and after LPS stimulation. Figure 3 demonstrates a representative experiment, where Mo precursors at day 14 of culture were induced to differentiate into DCs by IL-4 + GM-CSF (Figure 3a) or IL-4 + KL + FL (Figure 3b) treatment. The data indicate that CD14 antigen was significantly, if not completely, downmodulated and that the mean fluorescence intensity (MFI) of CD14<sup>+</sup> DCs was always lower than that of CD14<sup>+</sup> Mo cells, being about 30 and 70, respectively



**Figure 3** Phenotypic profiles of DCs derived from Mo precursors. DCs were obtained from Mo precursors treated with GM-CSF and IL-4 (a) or KL + FL + IL-4 (b). Cells were analyzed before (gray line) and after (filled plot) 16 h LPS stimulation, by flow cytometry, for surface antigens expression. The following FITC-conjugated MoAbs: CD14, CD1a, HLA-DR, CD86, CD80, and PE-conjugated MoAbs: CD83, CD40, CD11c, CD123, were used. Appropriate conjugated isotype-matched IgGs were used as control. Data from one representative experiment out of five are reported

(mean from four experiments). Moreover, the retention of CD14 was more evident when the switching of Mos to DCs was performed later during the culture period (days 17–21). DCs obtained upon IL-4 + GM-CSF culture showed an increase up to 30–65% of the CD1a marker. After LPS stimulation, an upregulation of DC markers (i.e. CD80, CD40, CD83, CD86 and human leukocyte antigens (HLA)-DR) was observed. The phenotypic profile of the DCs derived from Mo precursors treated with IL-4 + KL + FL was similar, with the exception of CD83 and CD40 that were expressed by a lower percentage of cells (i.e. 26% ± 8 and 20% ± 6 *versus* 60% ± 15 and 55% ± 14, respectively).

The CD11c antigen, variably expressed on the starting Mo population, was virtually present on all the derived DCs, whereas CD123 marker, namely the  $\alpha$  chain of IL-3 receptor, highly expressed on plasmacytoid DCs, was present even if at low intensity of fluorescence on 30–40% of cells. The apparent discrepancy between this latter result and the myeloid origin of the DCs was further investigated. We found that, at odds with plasmacytoid control DCs isolated from PB mononuclear cells (PBMCs), our DC cultures were BDCA-2 and BDCA-4 negative and unable to undergo phenotypical maturation and interferon (IFN)- $\alpha$  secretion upon CpG stimulation (data not shown). The negative results in these assays were consistent with the myeloid origin of DCs and excluded a plasmacytoid DC differentiation from CD34-derived Mo precursors.

Interestingly, the immunofluorescence analyses indicated that the capability of Mos to differentiate into DCs was completely lost at the end of the culture (days 25–30) when the majority of cells were adherent macrophage-like cells. To confirm this observation, the adherent and nonadherent cells of the primary culture were separated at day 20 and induced into DCs by cultivating them with IL-4 + GM-CSF for 6 days. The cell population derived from the adherent fraction was about 85% CD14<sup>+</sup> and less than 1% CD1a<sup>+</sup>. On the contrary, DCs derived from the nonadherent fraction were 25 and 55% CD14<sup>+</sup> and CD1a<sup>+</sup>, respectively (Figure 4). The same results were obtained by cultivating the two populations in the presence of IL-4 + KL + FL (data not shown).

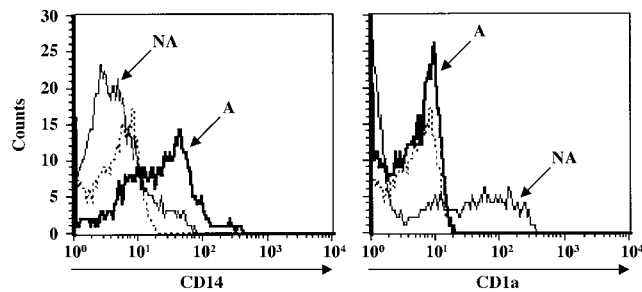
### Correlation between the expression of GM-CSFR and DC induction by IL4 plus GM-CSF treatment

Since we observed that early Mo precursors (days 7–10) needed a longer culture period to switch to DCs, as compared to late Mo cells (days 13–21), we tested a hypothetical correlation of this finding with the GM-CSFR expression on the cell surface. As described above, the GM-CSFR appeared very early in Mo cultures and both GM-CSFR<sup>hi</sup> and GM-CSFR<sup>lo</sup> cell populations were under constant observation from days 7 to 14. Double staining of day 14 cells with anti-CD86 (highly expressed on macrophages) and anti-GM-CSFR MoAbs detected the presence of CD86<sup>-</sup> GM-CSFR<sup>-</sup>, CD86<sup>-</sup> GM-CSFR<sup>+</sup> and CD86<sup>+</sup> GM-CSFR<sup>+</sup> cell populations, which represented 26, 47 and 27%, respectively, of the total cell number (Figure 5, left panel). These fractions were sorted, analyzed by cell morphology and then incubated with IL-4 + GM-CSF. Giemsa staining revealed the following morphological composition: 15% monoblasts, 60% promonocytes

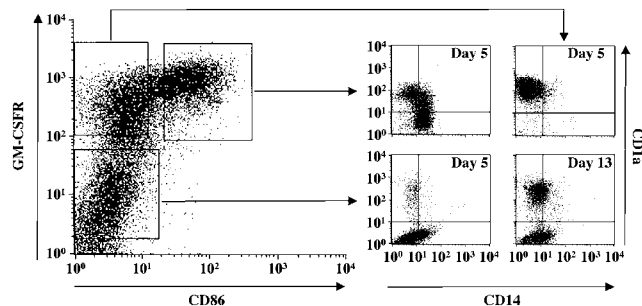
and 25% Mos in CD86<sup>-</sup> GM-CSFR<sup>-</sup> fraction; 15% promonocytes, 75% Mos and 10% macrophages in CD86<sup>-</sup> GM-CSFR<sup>+</sup> population; 10% Mos and 90% macrophages in CD86<sup>+</sup> GM-CSFR<sup>+</sup> subset. After 5 days of IL-4 + GM-CSF treatment, virtually all the cells derived from the CD86<sup>-</sup> GM-CSFR<sup>+</sup> fraction were CD1a<sup>+</sup> CD14<sup>-</sup>, whereas the majority of those derived from the CD86<sup>+</sup> GM-CSFR<sup>+</sup> fraction were CD14<sup>+</sup>, with a low percentage of CD1a<sup>+</sup> cells. The CD86<sup>-</sup> GM-CSFR<sup>-</sup> population consisted of more immature cells, in fact at least 13–15 days of IL-4 + GM-CSF treatment were needed for the appearance of DCs, as measured by the expression of CD1a marker, which reached 50% of positive cells (Figure 5, right panel). Unfortunately, it was not possible to further examine the CD1a marker expression of this cell population because after 25–30 days of culture (i.e. 14 days for *in vitro* differentiation in Mo conditions plus 2 weeks as DC culture) cells began to die.

### Functional activities of DCs derived from Mo precursors

Antigen uptake is a typical immature DC activity. DCs derived from Mo precursors, induced to differentiate either with



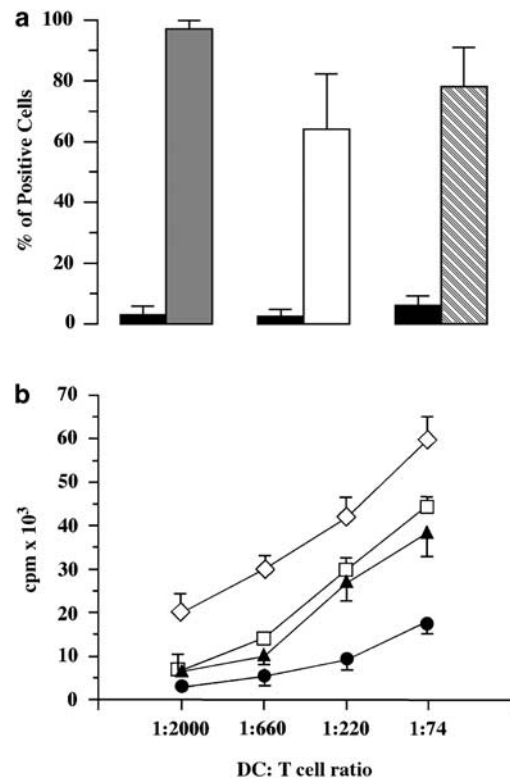
**Figure 4** Phenotypic analysis of DCs derived from adherent (a) and not adherent (NA) cell fractions of Mo precursors. At day 20 of primary culture, the nonadherent cell fraction was separated from the adherent one by removing the supernatant. After two washings with PBS, the remaining adherent cells were detached by gentle scraping. Then, the two cell fractions were induced to DC differentiation with GM-CSF + IL-4 and, after a 5-day culture, examined for expression of CD14 (left panel) and CD1a (right panel) antigens. Hatched line indicates the isotypic control. A representative experiment is shown



**Figure 5** DC differentiative potential of Mo cells. Identification of three distinct subsets of Mo precursors at day 14 of culture, based on GM-CSFR and CD86 expression (left panel). Sorted cells were seeded in the presence of GM-CSF + IL-4 and after 5 days analyzed for CD1a and CD14 expression by double color fluorescence (right panel). The phenotypic profile of sorted GM-CSFR<sup>-</sup> CD86<sup>-</sup> cells was repeated at day 13 of culture

IL-4 + GM-CSF or with IL-4 + KL + FL, were tested for their macropinocytotic capability by measuring the uptake of fluorescein isothiocyanate (FITC)-conjugated dextran. Flow cytometry analysis showed that 45–90% of immature DCs efficiently captured FITC-dextran. DCs derived from PB Mos were also tested as positive control (Figure 6a).

The most distinctive feature of mature DCs is their ability to strongly stimulate the activation of allogenic T cells. This functional activity was assessed by the mixed leukocyte reaction (MLR) assay, which revealed a comparable stimulatory capability of DCs derived from Mo precursors treated with both GF combinations. Interestingly their activity was consistently higher than that of PB mature Mos, but slightly weaker with respect to DCs derived from PB mature Mos, included as a reference population (Figure 6b). The latter results are explained by the asynchronism of the Mo precursor cultures giving rise to the DC populations. In fact, as shown in Figures 3a and b, not all the Mo precursors undergo differentiation into the dendritic lineage at the same time, therefore the real number of professional antigen-presenting-cells (APCs) is lower than that of the reference population.



**Figure 6** DCs derived from Mo precursors are functionally active. (a) FITC-dextran uptake by DCs derived either from PB Mos (■) or from Mo precursors (□), both cultivated with GM-CSF and IL-4, and by DCs derived from Mo precursors and cultivated with IL-4 + KL + FL (▨). As control, identical cell samples were incubated with FITC-dextran at 4°C (■). (b) Induction of allogenic T-cell activation by Mo precursors (●), DCs derived from Mo precursors and incubated 6 days either with GM-CSF plus IL-4 (□), or with the GF cocktail (▨) and PB-derived DCs (◇). <sup>3</sup>H-thymidine incorporation is expressed as the mean of quadruplicates ± S.D. The amount of <sup>3</sup>H-thymidine incorporated by T cells and DCs cultured in the absence of stimulator and responder cells, respectively, were assumed as background values

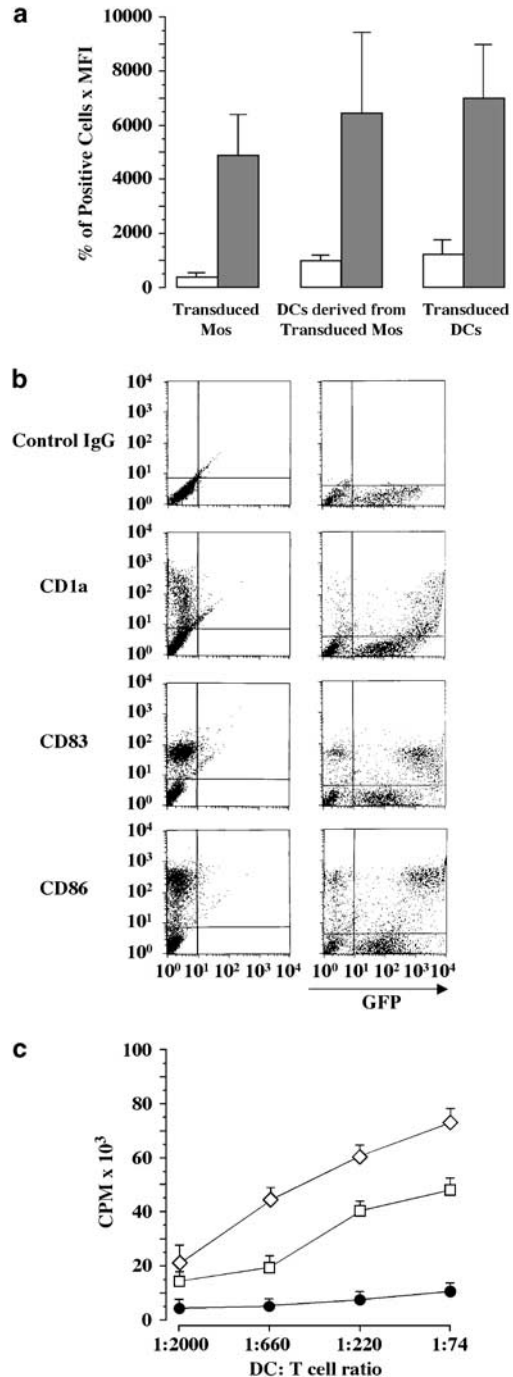
## Transgene expression in Mo precursors, PB Mos and DCs

To evaluate the susceptibility to lentiviral transduction of Mo cells and DCs and to establish optimal conditions for generation of gene-modified cells, Mo precursors were infected at days 10–14 of culture and successively grown either in Mo medium or in the presence of IL-4 + GM-CSF. Alternatively, they were first induced towards the DC lineage, by a 5-day treatment with IL-4 plus GM-CSF, and then infected. A similar schedule of infection was followed for mature Mos, as they were either infected soon after their isolation from PB, and then cultured in Mo or in DC condition, or were first induced to differentiate into DCs and then infected. At 3–10 days after two rounds of transduction, cells were analyzed for green fluorescence protein (GFP) and lineage markers expression. The overall transduction efficiency of the cell cultures derived from PB Mos was very low (GFP-positive cells ranged from 17 to 26%), particularly in terms of MFI (ranging from 23 to 33). On the contrary, cell populations derived from Mo precursors were all transduced at significant levels, being GFP positive:  $35 \pm 18\%$  of Mo precursors,  $40 \pm 23\%$  of DCs derived from transduced Mo precursors and  $53 \pm 20\%$  of transduced DCs. It was also constantly observed that the GFP-positive cells derived from Mo precursors showed a MFI considerably higher than that of the analogue cell populations derived from PB Mos, and that directly transduced DCs displayed a MFI slightly lower than that of DCs derived from transduced Mo precursors. In Figure 7a the product between the percentage of GFP-positive cells and the corresponding MFI value is reported. The lower transduction efficiency observed in some experiments was found to be correlated with the lower titer of the lentiviral vector preparations used. Phenotypical analysis demonstrated that gene transduction did not impair the expression of typical DC markers, since CD1a, CD83 and CD86 were present on about 35, 50 and 60%, respectively, of both transduced and untransduced cells (Figure 7b). In addition, antigen uptake and APC activities were both comparable to those displayed by the untransduced DCs (data not shown and Figure 7c).

## Discussion

The present study provides evidence that DCs are generated from Mo cells at any stage of their differentiation/maturation pathway. Our results provide novel insight into the development of DCs, showing that both Mo progenitors/precursors and circulating Mos can directly differentiate into DCs.

First, we characterized the optimal culture conditions to generate a progeny of virtually pure Mos from highly purified CD34<sup>+</sup> cells. In a preliminary report<sup>34</sup> we described the synergistic effect of FL and M-CSF on HPCs to stimulate their Mo differentiation, coupled with a moderate proliferation, resulting in 28-fold amplification of the initial cell number. In this study, we show that addition of IL-6, known to induce proliferation of myeloma cells and differentiation of macrophages in several myeloma cell lines,<sup>35–37</sup> strongly improved Mo cell proliferation up to 400-fold amplification. Moreover, this culture system allowed the maturation of Mos into



**Figure 7** Transduction of Mo and DC cultures. (a) Flow cytometry analysis of transduced Mos, DCs derived from transduced Mos and transduced DCs, all obtained from PB (□) or Mo precursors (■). Values of the histogram represent the product between the percentage of GFP-positive cells and the corresponding MFI. The mean of six experiments ± S.D. is shown. (b) Phenotypic profiles of DCs derived from untransduced (left panels) and transduced (right panels) Mo precursors seeded in GM-CSF and IL-4. After 16 h LPS stimulation, DCs were analyzed by flow cytometry for the surface antigen expression of CD83, CD86 and CD1a. Appropriate conjugated isotype-matched IgGs were used as control. Data from one representative experiment out of two are reported. (c) Induction of allogenic T-cell activation by transduced Mo precursors (●) and DCs derived from either transduced Mo precursors (□) or PB Mos (◇). <sup>3</sup>H-thymidine incorporation is expressed as the mean of quadruplicates ± S.D. Results are representative of one experiment out of two

macrophages, as assessed by morphological and phenotypical analyses. Thus, the methodology described here provides a homogeneous and large cell population pertaining to the Mo-macrophage lineage, which allows dissection of the molecular and cellular mechanisms underlying monocytopenesis. The differentiative stages of Mo lineage were characterized by a gradual decrease of CD34 and a progressive increase of CD14 antigen expression. Noteworthy, the membrane expression of these two molecules was mutually exclusive, as we never observed a CD34<sup>+</sup>14<sup>+</sup> cell population in the differentiating cultures. A CD34<sup>+</sup>14<sup>+</sup> circulating cell population, able to differentiate into DCs when cultured in the presence of GM-CSF, has been described before:<sup>38</sup> it was hypothesized to represent an intermediate stage of differentiation between HPCs and mature Mos or DCs. Apparently, our Mo culture conditions hamper the growth of double-positive CD34<sup>+</sup>14<sup>+</sup> cells; further studies may allow the identification of the GF/cytokine cocktail permissive for the *in vitro* production of this cell population, thus allowing the study of its role in both DC and Mo differentiative pathways.

Second, we induced Mo cells at various stages of differentiation to switch into DCs by using IL-4 + GM-CSF or IL-4 + KL + FL. After 5 days, cells acquired all morphological, phenotypical and functional features of DCs. They expressed all typical DC markers and, as expected, CD11c was present on virtually all DCs. On the other hand, 30–40% of cells expressed low levels of CD123, a marker that defines plasmacytoid DCs,<sup>39,40</sup> but were BDCA-2 and BDCA-4 negative and unable to undergo phenotypical maturation and IFN- $\alpha$  secretion upon CpG stimulation. These results are consistent with the myeloid origin of DCs and exclude the plasmacytoid DC differentiation from HPC-derived Mo precursors. Mo precursors-derived immature DCs were able to internalize soluble antigens and, once completely mature, displayed a strong APC activity in allogeneic MLR assay. Noteworthy, we constantly detected <10% of CD1a<sup>+</sup> cells in Mo cultures. It seems unlikely that these few CD1a<sup>+</sup> cells represent a major source of DCs in the cultures: indeed, after a 5-day treatment with IL-4 + GM-CSF or IL-4 + KL + FL approximately 60 or 30% of the cells, respectively, expressed this marker, in spite of a very low cell proliferation rate. The bipotential capability of Mo precursors to generate both Mos/macrophages and DCs was maintained until approximately 20–25 days; thereafter, Mos undergo maturation into macrophages and their capability to differentiate into DCs is almost completely lost.

Although a wide variety of cytokine combinations have been described to induce mature Mos or HPCs to switch into DCs, the majority of reports indicate GM-CSF and IL-4 as essential factors for DC differentiation. Other cytokines shown to play a role in generating DCs from several precursors, under various culture conditions, include IL-2, IL-3, IL-6, IL-7, IL-12, IL-13, CD40 ligand, FL and transforming growth factor (TGF) $\beta$ , alone or in combination.<sup>29</sup> In particular, FL has a significant effect on DC development both *in vivo* and *in vitro*. Maraskovsky *et al.*<sup>32</sup> demonstrated that administration of FL to healthy volunteers was followed by a significant increase of circulating DCs, still very efficient as APCs. Furthermore, Strobl *et al.*<sup>41</sup> reported the cooperation between FL and TGF $\beta$ 1 in potentiating the development and the cluster

formation of DCs. Here we used FL together with KL, which enhances cell proliferation in several hematopoietic cell lineages<sup>42</sup> and also promotes DC replication *in vivo*.

Our findings further show that the capability of Mo precursors to generate DCs after treatment with IL-4 + GM-CSF is correlated with the elevated GM-CSFR expression on the cell surface. From days 7 to 14 of Mo culture, we observed the presence of GM-CSFR<sup>lo</sup> and GM-CSFR<sup>hi</sup> cells. Sorting of these cell populations, and their subsequent treatment with IL-4 + GM-CSF, demonstrated a higher susceptibility of GM-CSFR<sup>hi</sup> cells to differentiate into DCs, as compared to GM-CSFR<sup>lo</sup> cells, which require a longer culture time. It should be pointed out that the differentiation/maturation of Mo cells was not totally synchronous, as observed by the gradual modulation of all the analyzed membrane markers. This feature may explain why not all Mo precursors simultaneously differentiate into DCs. At late culture stages, cells acquired the morphological and phenotypical properties of macrophages and lost their differentiative potential towards the DC lineage. This observation was confirmed by sorting the cells expressing the CD86 macrophage marker, or by isolating the adherent cell fraction appearing late in the culture. Upon DC induction with IL-4 + GM-CSF, both the sorted GM-CSF<sup>+</sup>CD86<sup>+</sup> and the adherent cells did not differentiate into DCs: conversely, they maintained a high CD14 antigen expression and showed the typical morphology of macrophages.

Caux *et al.*<sup>14</sup> demonstrated that CD14<sup>+</sup> precursors deriving from CD34<sup>+</sup> cells possess a bipotential Mo/macrophagic and DC differentiative capability, when selectively induced. Our studies, while in line with these observations, provide novel methodology and insight into DC development. The culture conditions described by Caux *et al.*<sup>14</sup> and our group are basically different, in terms of cytokine treatment and, mainly differ in the pathway to DC differentiation. We induced HPCs to differentiate first along the Mo lineage and then into DCs by using two different cytokine cocktails, whereas they committed the CD34<sup>+</sup> cells directly towards the DC pathway by adding KL + GM-CSF + TNF $\alpha$ . More important, the CD14<sup>+</sup> precursors described by Caux *et al.* differ from those described in this study, in that they lack proliferative capacity, whereas in our culture conditions acquisition of the CD14 antigen was not associated with a decrease in the proliferation rate.

On the basis of our results, we suggest that immature Mos may represent a reservoir of DC precursors that, upon specific stimulation, generate functional DCs. In addition to the *in vitro* data mentioned above, studies in mice demonstrated that circulating Mos may differentiate into DCs.<sup>43</sup> On the other hand, surgical stress induces a rapid increase of the number of circulating DCs but not of mature Mo cells, thus indicating that the number of circulating DCs and Mos may be differentially regulated.<sup>44</sup> This rise of DCs was hypothetically explained by the existence of preformed DCs, margined in 'storage' sites, that could be rapidly mobilized to prepare the host's immune defense against trauma.<sup>44</sup> Our results additionally, or alternatively, suggest that Mo precursors, stored in the BM, may represent a remarkable DC source, more rapidly available than HPCs.

We have also established a new methodology to efficiently generate transduced DCs by a two-step culture method: first,

expansion of Mo precursors from HPCs; second, efficient gene transduction of Mo precursors using lentiviral vector, followed by their differentiation into DCs. This methodology differs from those previously described for transgene expression in DCs,<sup>45–47</sup> and provides the basis for gene transfer studies on Mo precursors to investigate the functional role of specific genes involved in the regulation of Mo and/or DC differentiation.

## Materials and Methods

### HPC, PB monocyte and plasmacytoid DC isolation

HPCs were purified from PB of healthy donors as previously described.<sup>48</sup> After a density gradient centrifugation on Lympholyte-H (Cedarlane, Hornby, Ont., Canada), PBMCs were harvested and CD34<sup>+</sup> cells were purified using the direct CD34<sup>+</sup> magnetic cell sorter (MACS) isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell population obtained consisted mainly of blasts (>95%) as evaluated by clonogenic assay<sup>49</sup> and morphological analysis. In some experiments, the CD34<sup>+</sup> population was further sorted by FACSVantageSE or FACSDIVA (Becton-Dickinson) yielding >99% pure CD34<sup>+</sup> cells, utilizing anti-CD34 (clone 563) MoAb. Mature Mos were isolated from PB of healthy adult donors. Mononuclear cells were first fractionated on Lympholyte-H gradient and then isolated with CD14<sup>+</sup> Microbeads kit (MACS of human leukocytes, Miltenyi Biotec). The resulting population consisted of more than 96% Mos, as confirmed by May-Grünwald Giemsa (Sigma-Aldrich, Steinheim, Germany) staining, forward and side scatter in flow cytometry and immunophenotype. Plasmacytoid DCs were isolated from PB mononuclear cells with the BDCA-4 Cell Isolation Kit, Human (Miltenyi Biotec).

### Mo unilineage culture

Mo unilineage cultures were obtained by cultivating  $2 \times 10^5$  HPCs/ml in the medium previously described<sup>49</sup> supplemented with 20% heat-inactivated FCS, 100 ng/ml FL, 250 U/ml M-CSF<sup>34</sup> and 1 ng/ml IL-6 (Peprotech, Rocky Hill, NJ, USA). The cells were cultivated in a fully humidified atmosphere containing 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>, and, three times a week, counted and adjusted to  $2 \times 10^5$  cells/ml. At different culture times after day 7, cells were either maintained in Mo culture condition or induced to differentiate into DCs.

### DC generation and maturation

Cells recovered at different times of Mo culture or mature Mos isolated from PB were extensively washed, counted, and seeded at  $2\text{--}3 \times 10^5$ /ml in RPMI 1640 supplemented with 10% FCS and containing either IL-4 (50 ng/ml) and GM-CSF (50 ng/ml) or IL-4 (10 ng/ml), KL (1 ng/ml) and FL (1 ng/ml) (Peprotech). After additional 6 days of culture, DCs were induced to terminal maturation by an overnight incubation with 1  $\mu$ g/ml of LPS (Sigma-Aldrich). In some experiments, cells were stimulated with 3 or 5  $\mu$ g/ml of CpG oligodeoxynucleotide. The CpG 2006 oligodeoxynucleotide, TCGTCGTTTTGTCGTTTTGTCGT<sup>50</sup> was purchased from MWG biotech AG (Ebersberg, Germany). IFN- $\alpha$  secretion was measured by Hu-IFN- $\alpha$  ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ USA).

### Immunofluorescence and morphological analyses

Cells ( $5 \times 10^4$ ) were stained with the following FITC- or PE-conjugated MoAbs: CD1a, CD11c, CD11b, CD31, CD14, CD34, CD40, CD80, CD83,

CD86, CD123, MHC class I, MHC class II (Pharmingen, San Diego, CA, USA), anti-GM-CSFR (Serotec Ltd, Oxford, UK) BDCA-2 and BDCA-4 (Miltenyi Biotec). The appropriate conjugated isotype-matched IgGs were used as control. After incubation at 4°C for 30 min with saturating concentrations of MoAbs or isotypic controls, cells were washed twice with PBS/0.1% bovin serum albumin (BSA) and analyzed on a FACScan (Becton-Dickinson). GFP-transduced cells ( $5 \times 10^4$ ) were washed with PBS/BSA, resuspended and fixed in 0.2 ml of 1% paraformaldehyde in PBS (Sigma Diagnostics, St Louis, MO, USA) and analyzed by FACScan.

Cells ( $2 \times 10^4$ ) collected at different days of culture were cytocentrifuged onto glass slides, stained with May-Grünwald Giemsa, and identified by morphological analysis.

### Cell sorting

The CD34<sup>+</sup>14<sup>+</sup> and CD34<sup>+</sup>14<sup>-</sup> cell fractions were sorted 2 days after CD34<sup>+</sup> HPC purification. Mo cells at day 12 of culture were labeled with the appropriate MoAbs and then separated into CD86<sup>-</sup>GM-CSFR<sup>-</sup>, CD86<sup>-</sup>GM-CSFR<sup>+</sup>, CD86<sup>+</sup>GM-CSFR<sup>+</sup> fractions. Reanalysis of the sorted populations showed that purity was higher than 98%.

### FITC-dextran uptake

Cells ( $5 \times 10^4$ ) were incubated with 1 mg/ml of FITC-dextran (Sigma-Aldrich) for 30 min at either 37 or 4°C. After several washings with cold PBS/0.1% BSA the intensity of cell fluorescence was measured by flow cytometry. The level of antigen uptake was indicated by fluorescence difference between the test at 37°C and the control tubes at 4°C for each sample.

### Allogeneic MLR assay

T lymphocytes ( $1 \times 10^5$ ) isolated from PB of healthy donors, as previously described,<sup>51</sup> were seeded in each well of round-bottomed 96-well microtiter plates and incubated 6 days at 37°C in the presence of graded numbers of 16 h LPS-stimulated DCs or Mos. During the last 18 h of incubation, cells were pulsed with methyl-<sup>3</sup>H thymidine (1  $\mu$ Ci/well, ICN Biomedicals, Irvine, CA, USA), harvested using a 96-well cell harvester (Tomtec, Hamden, CT, USA) and the thymidine incorporation was measured in a Microbeta counter (Wallac, Turku, Finland). Tests were performed in quadruplicate and the results are expressed as mean cpm  $\pm$  S.D.

### Production of the lentivirus containing GFP

Lentiviral vectors were prepared by cotransfecting 3.5  $\mu$ g of vesicular stomatitis virus G protein envelope-encoding plasmid, 6.5  $\mu$ g of the packaging construct and 10  $\mu$ g of the cytomegalovirus-GFP transfer vector into human kidney 293T cells, through the LipofectAMINE 2000 kit (Invitrogen, Frederick, MD, USA) following manufacturer's recommendations. Supernatants were collected 36–40 h post-transfection, clarified and stored at -80°C until use. Viral titers of supernatants were usually in the range of  $8 \times 10^5\text{--}8 \times 10^6$  transducing units (TUs)/ml.

### Gene transduction

Transduction of cells by lentiviral vectors was performed by adding 1–2 ml of viral supernatant and 4  $\mu$ g/ml of polybrene to  $3 \times 10^5$  cells (multiplicity of infection from 5 to 50) and centrifuging at 600  $\times$  g for 90 min at 32°C. A second cycle of infection was performed after overnight incubation in the

appropriate culture medium. The efficiency of the infection was evaluated by flow cytometry as percentage of GFP positive cells.

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