

# Correlation between differentiation plasticity and mRNA expression profiling of CD34<sup>+</sup>-derived CD14<sup>-</sup> and CD14<sup>+</sup> human normal myeloid precursors

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Received 12.11.04; revised 14.4.05; accepted 19.4.05; published online 10.6.05  
Edited by G Cossu

## Abstract

**In spite of their apparently restricted differentiation potentiality, hematopoietic precursors are plastic cells able to trans-differentiate from a maturation lineage to another. To better characterize this differentiation plasticity, we purified CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors generated by 'in vitro' culture of human CD34<sup>+</sup> hematopoietic progenitors. Morphological analysis of the investigated cell populations indicated that, as expected, they consisted of granulocyte and monocyte precursors, respectively. Treatment with differentiation inducers revealed that CD14<sup>-</sup> cells were bipotent granulo-monocyte precursors, while CD14<sup>+</sup> cells appeared univocally committed to a terminal macrophage maturation. Flow cytometry analysis demonstrated that the conversion of granulocyte precursors to the mono-macrophage maturation lineage occurs through a differentiation transition in which the granulocyte-related myeloperoxidase enzyme and the monocyte-specific CD14 antigen are co-expressed. Expression profiling evidenced that the observed trans-differentiation process was accompanied by a remarkable upregulation of the monocyte-related MafB transcription factor.**

*Cell Death and Differentiation* (2005) 12, 1588–1600.

doi:10.1038/sj.cdd.4401679; published online 10 June 2005

**Keywords:** normal myeloid precursors; lineage switching; trans-differentiation

**Abbreviations:** A, absent; APCs, antigen-presenting cells; ATRA, all-*trans* retinoic acid; CB, cord blood; CFU-G, colony forming unit-granulocyte; CFU-GM, colony-forming unit-granulocyte/monocyte; CFU-M, colony forming unit-monocyte; CSF, colony-stimulating factor; CTP, cytidine 5'-triphosphate; D, decreased; DCs, dendritic cells; FcR, Fc receptor; FCS, fetal calf

serum; FITC, fluorescein isothiocyanate; Flt3-l, Flt3-ligand; G-CSF, granulocyte-colony-stimulating factor; I, increased; IL, interleukine; IMDM, Iscove's modified Dulbecco's medium; M, marginal; MACS, magnetic activated cell separation; Mas 5.0, Microarray Analysis Suite 5.0; M-CSF, macrophage-colony-stimulating factor; MFI, mean fluorescence intensity; MGG, May-Grunwald-Giemsa; MoAb, monoclonal antibody; MPO, myeloperoxidase; NC, not changed; OMIM, online Mendelian inheritance in man; P, present; PB, peripheral blood; PBS, phosphate-buffered saline; PE, phycoerythrin; SCF, stem cell factor; UTP, uridine 5'-triphosphate; VD, 1 $\alpha$ , 25 di-hydroxy-vitamin D3

## Introduction

Hematopoiesis is a hierarchically organized process by which pluripotent stem cells become gradually restricted in their differentiation potential, generating unipotent progenitors/precursors that in turn give rise to terminally differentiated blood cells.<sup>1,2</sup> A specific combination of transcription factors<sup>3–5</sup> and chromatin remodeling complexes<sup>6</sup> is responsible for the genetic program underlying the differentiation potentiality of each hematopoietic progenitor/precursor. Although this model would imply a univocal fate for a cell that has been already committed to a precise maturation lineage, several reports indicate that hematopoietic precursors are able to trans-differentiate from a lineage to another.<sup>7,8</sup> This biological property of hematopoietic cells, referred to as 'lineage switching' or 'intra-hematopoietic plasticity', is essentially based on the observation that a number of leukemic cell lines undergo phenotypic changes 'in vitro' as a result of specific manipulations, such as pharmacological treatments<sup>9,10</sup> and transcription factors overexpression.<sup>11,12</sup> By using this approach, lineage switches between erythroid and myeloid, megakaryocytic and erythroid, or even lymphoid and myeloid cell compartments have been reported.<sup>7</sup> The first demonstration of an intra-hematopoietic lineage switching is probably represented by the observation that HL60 leukemic myeloblasts (M2–M3 type granulocytic precursors), normally differentiating toward granulocytes upon treatment with all-*trans* retinoic acid (ATRA), undergo monocyte-macrophage maturation when stimulated with 1 $\alpha$ , 25 di-hydroxy-vitamin D3 (VD).<sup>10</sup> Since then, other granulocyte precursor cell lines such as the M2 type Kasumi-1 have been reported to undergo mono-macrophage differentiation upon VD stimulation.<sup>9</sup> Based on these observations, the late myeloblast to promyelocyte transition appeared to be the preferential context to induce the trans-differentiation of granulocyte precursors to monocytes, since more undifferentiated M0–M1-type (early myeloblastic) or more mature M3-type (promyelocytic) leukemic myeloid cell lines were unable to undergo the same destiny. More recently, data obtained by Nakamura *et al.*,<sup>13</sup>

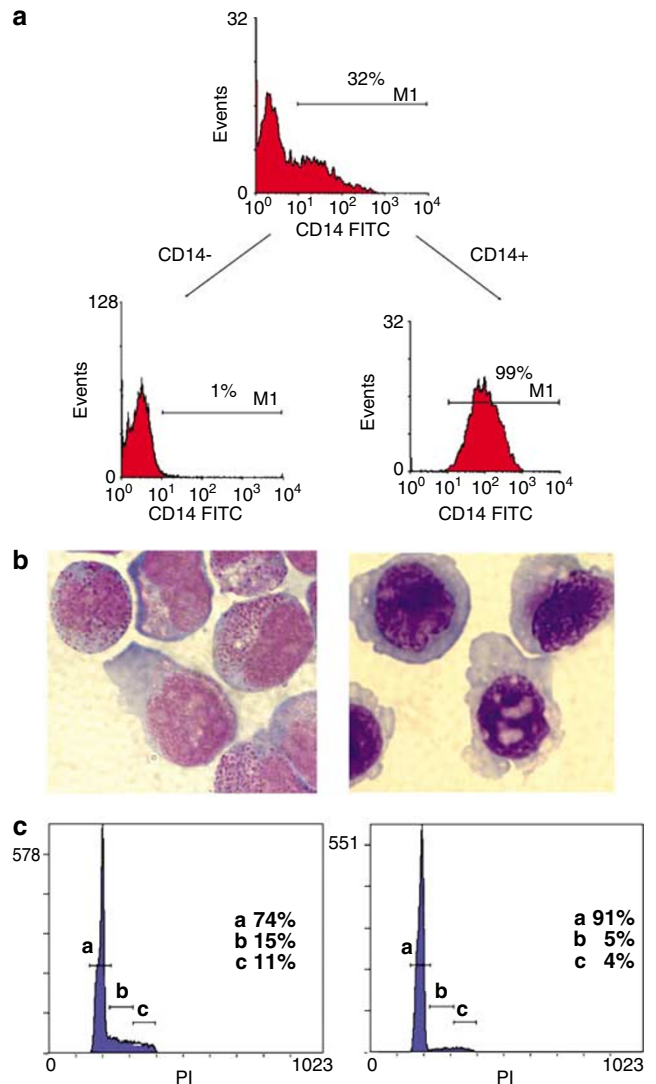
subsequently confirmed in our laboratory,<sup>14</sup> have evidenced that the conversion of granulocyte precursors to the mono-macrophage maturation lineage in response to VD treatment can also be reproduced using primary myeloblasts/promyelocytes, raising the possibility that such trans-differentiation events might occur 'in vivo' where they would assume a physiological relevance. Myelopoiesis, in fact, is believed to begin from a bi-potent progenitor (colony-forming unit-granulocyte/monocyte (CFU-GM)) giving rise to unipotent progenitors that are committed to the granulocyte (colony forming unit-granulocyte (CFU-G)) and mono-macrophage (colony forming unit-monocyte (CFU-M)) lineages. Further differentiation of these elements to the more mature myeloid precursors is normally accompanied by the loss of CD34 expression, the restriction of differentiation potentiality and the acquisition of a peculiar morphology.<sup>15,16</sup> The demonstration of a myeloblast-promyelocyte to mono-macrophage differentiation conversion directly implies the existence of a 'bi-potent precursor' that is distinct and hierarchically downstream with respect to the CFU-GM progenitor. In spite of its importance this process has been, to date, only observed as a consequence of treatment with the VD nuclear hormone<sup>14</sup> and the possible role played to this regard by more conventional hematopoietic growth factors, as lineage-specific colony-stimulating factors (CSFs), remains to be established. In addition, the molecular mechanisms underlying this differentiation transition are substantially unknown. To address these issues, we used a liquid culture model of cord blood (CB) CD34+ hematopoietic stem/progenitor cells due to its capacity to recapitulate myelopoiesis 'in vitro'. In fact, under precise culture conditions,<sup>14,17</sup> these cells are able to generate CD34- myeloid precursors that are committed along both the granulocyte and mono-macrophage differentiation pathways, that is, the proper contexts to analyze the lineage-switching processes. Based on CD14 expression pattern, generally considered as an early and specific marker of monocyte commitment,<sup>18</sup> we purified CD14- and CD14+ myeloid precursors generated by 'in vitro' culture of CD34+ cells, characterized their phenotypes, analyzed their differentiation capacity upon treatment with several inducing agents and studied their expression profiles by means of the Affymetrix microarray methodology. The results obtained indicated that, in spite of their granulocyte precursor phenotype, CD14- cells are plastic cells able to undergo either granulocyte or monocyte differentiation, whereas CD14+ cells appear as monocyte-like precursors univocally committed toward the macrophage maturation lineage. Microarray analysis indicated Maf family proteins, and the MafB member in particular, as key regulator transcription factors for mono-macrophage differentiation.

## Results

### Culture of CB CD34+ hematopoietic progenitors and purification of CD14+ and CD14- cell precursors

To obtain homogeneous populations of CD14+ and CD14- myeloid precursors, CB CD34+ hematopoietic stem/progenitor cells were preliminarily expanded/differentiated in

liquid culture in the presence of cytokines, as described in the Materials and Methods section. A 7-days culture in these experimental conditions led to the virtually complete loss of CD34 expression (not shown) and to the induction of the monocyte-specific CD14 antigen in 30±5% of cells (Figure 1a), as expected on the basis of previously reported observations.<sup>14,17</sup> These results imply the differentiation of hematopoietic CD34+ progenitors to the more mature precursor stage, that is to say the proper context needed to



**Figure 1** CD14+ and CD14- myeloid precursor purification, morphological analysis and cell cycle distribution. Cord blood CD34+ hematopoietic progenitors were differentiated to the myeloid precursor stage of hematopoiesis by a 7-day liquid culture in the presence of the proper cytokines mixture and then separated into the CD14+ and CD14- cell fractions (see Materials and Methods and Results for details). (a) Results of flow cytometry analysis, performed to estimate CD14 positivity, on unseparated (upper histogram) and separated (lower histograms) day 7 myeloid precursors. (b) Morphological analysis of purified CD14- (left) and CD14+ (right) cells, respectively, appearing as granulocyte precursors at the late myeloblast/promyelocyte of differentiation and monocyte-like precursors. (c) Flow cytometry evaluation of cell cycle distribution performed on CD14- and CD14+ cells upon propidium iodide staining (left and right, respectively); (a), (b) and (c) line markers, respectively, measure G0/G1, S and G2/M cell cycle phases

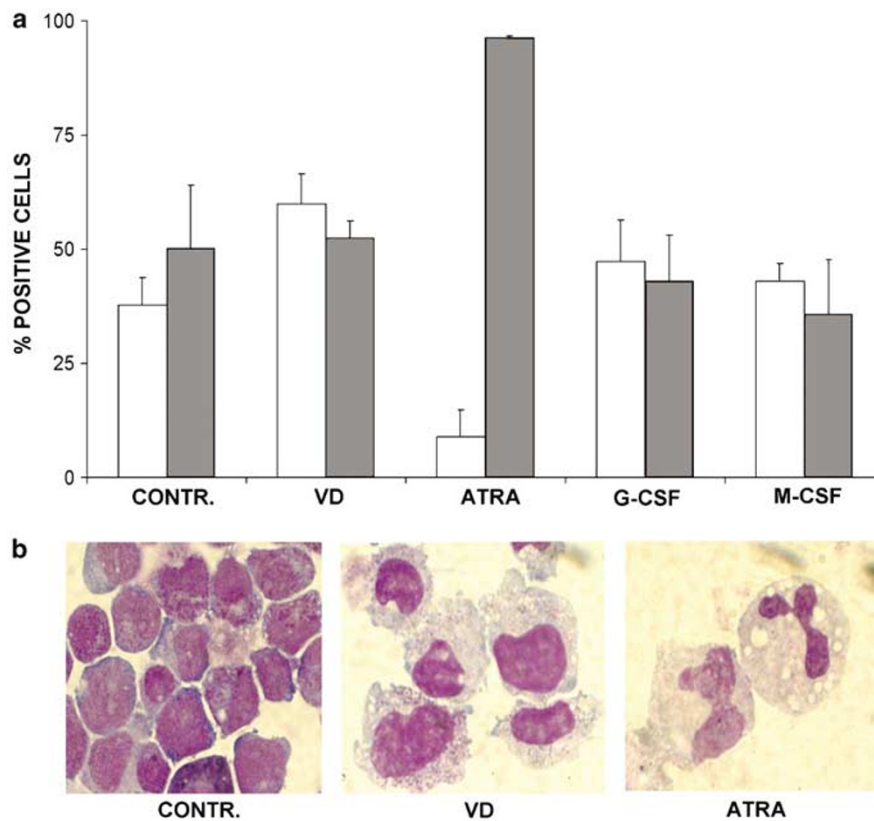
analyze mechanisms underlying the trans-differentiation of myeloblasts to monocytes-macrophages. Immunomagnetic separation of CD14<sup>+</sup> and CD14<sup>-</sup> fractions allowed to obtain highly purified cell populations, as assessed by flow cytometry analysis (Figure 1a). May–Grunwald–Giemsa (MGG) staining of these cells showed that the CD14<sup>-</sup> population was highly enriched in late myeloblast-promyelocytic elements, whereas CD14<sup>+</sup> cells essentially exhibited a monoblast-promonocytic morphology (Figure 1b). Interestingly, a small cell subset (<10%) of this last population displayed a late myeloblast morphology characterized by the presence of azurophilic granules in their cytoplasm (not shown). Cell cycle analysis performed by propidium iodide staining evidenced that CD14<sup>+</sup> cells were virtually arrested in the G0/G1 phase, whereas the CD14<sup>-</sup> population retained a normal proliferation capacity (Figure 1c). These results were also confirmed by cell growth experiments showing that CD14<sup>-</sup> precursors underwent  $10 \pm 2$  cell number expansions over a 2-week period in liquid culture, while CD14<sup>+</sup> precursors were totally unable to proliferate (data not shown).

### Differentiation capacity of CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors

To analyze the trans-differentiation capacity of CD34<sup>+</sup>-derived CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors, they were cultured in the presence of 'early acting' hematopoietic cytokines (see Materials and Methods) and exposed to

differentiation inducers as VD<sup>19,20</sup> and ATRA<sup>21</sup> nuclear hormones and macrophage-colony-stimulating factor (M-CSF)<sup>22</sup> and granulocyte-colony-stimulating factor (G-CSF)<sup>23</sup> lineage-specific CSFs. The extent and nature of differentiation were then assessed by immune phenotype and morphology examination.

Flow cytometry analysis performed at day 7 post-purification indicated that CD14<sup>-</sup> myeloid precursors under basal conditions, that is, cultured in the presence of 'early-acting' hematopoietic cytokines but without specific differentiation inducers, underwent a clear induction of CD14 antigen expression ( $37 \pm 6\%$ ) (Figure 2, panel a, CONTR.). This percentage was remarkably increased by VD treatment ( $61 \pm 7\%$ ), while it was strongly inhibited by stimulation with ATRA ( $9 \pm 6\%$ ) (Figure 2, panel a, VD and ATRA). Treatment with M-CSF and G-CSF affected only marginally CD14 expression ( $43 \pm 4$  and  $47 \pm 9\%$ , respectively) in comparison to control cells (Figure 2, panel a, M-CSF, G-CSF). As expected, stimulation of CD14<sup>-</sup> myeloid precursors with ATRA significantly enhanced CD38 antigen expression ( $96 \pm 0.5\%$  versus  $50 \pm 14\%$  of control cells). Consistently with these data, cytological examination, performed 14 days post-purification, evidenced that VD-treated CD14<sup>-</sup> cells assumed a clear monocyte/macrophage morphology (Figure 2, panel b, VD) whereas control cells were still represented by a heterogeneous mixture of myeloid precursors (Figure 2, panel b, CONTR.) and ATRA stimulation resulted in a typical neutrophil morphology (Figure 2, panel b, ATRA).



**Figure 2** Differentiation capacity of CD14<sup>-</sup> myeloid precursors in response to treatment with nuclear hormones and CSFs. (a) Flow cytometry analysis of CD14 (white bars) and CD38 (grey bars) positivity, performed after a 7-day stimulation of CD14<sup>-</sup> cells with VD, ATRA, G-CSF and M-CSF. Results were reported as mean  $\pm$  S.E.M. and compared with control untreated cells (CONTR.). (b) Morphological examination of control (CONTR.), VD-treated (VD) and ATRA-stimulated (ATRA) CD14<sup>-</sup> cells

Interestingly, the trans-differentiation of CD14<sup>-</sup> cells toward the mono-macrophage lineage was not observed when VD stimulation was performed on cells purified at days 14 and 21 of culture, that is, on more mature cell contexts displaying a myelocyte/granulocyte morphology and a virtually abrogated proliferation activity (data not shown). Similarly, neutrophilic polymorphonuclear leukocytes, purified from adult peripheral blood (PB), did not convert to the mono-macrophage phenotype upon treatment with VD (data not shown).

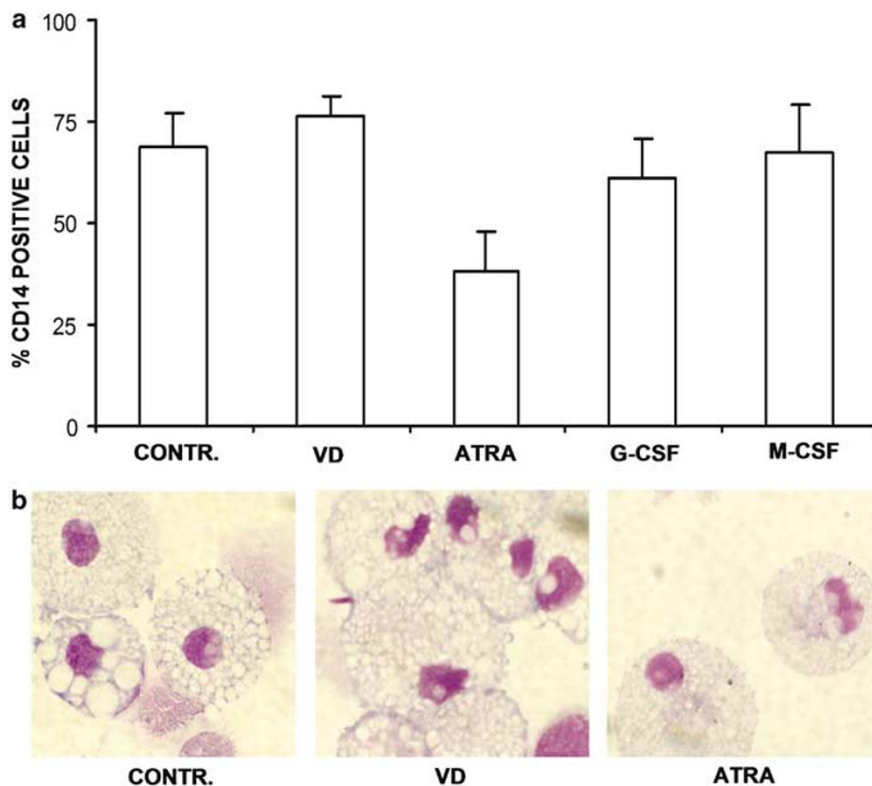
Regardless of the analyzed culture conditions (CONTR., ATRA, VD, CSFs), CD14<sup>+</sup> myeloid precursors always appeared as 'foamy' macrophages at day 7 post-purification (Figure 3, panel b) showing, at the same time, comparable values of CD14 positivity ( $61 \pm 9$  to  $76 \pm 5\%$ ), although ATRA determined a clear downregulation of this antigen ( $38 \pm 10\%$ ) (Figure 3, Panel a).

Treatment with CSFs substantially did not modify cell morphology as compared to control cells in both CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors (data not shown).

### Flow cytometry analysis of myeloperoxidase (MPO) expression in myeloblasts trans-differentiating to monocytes

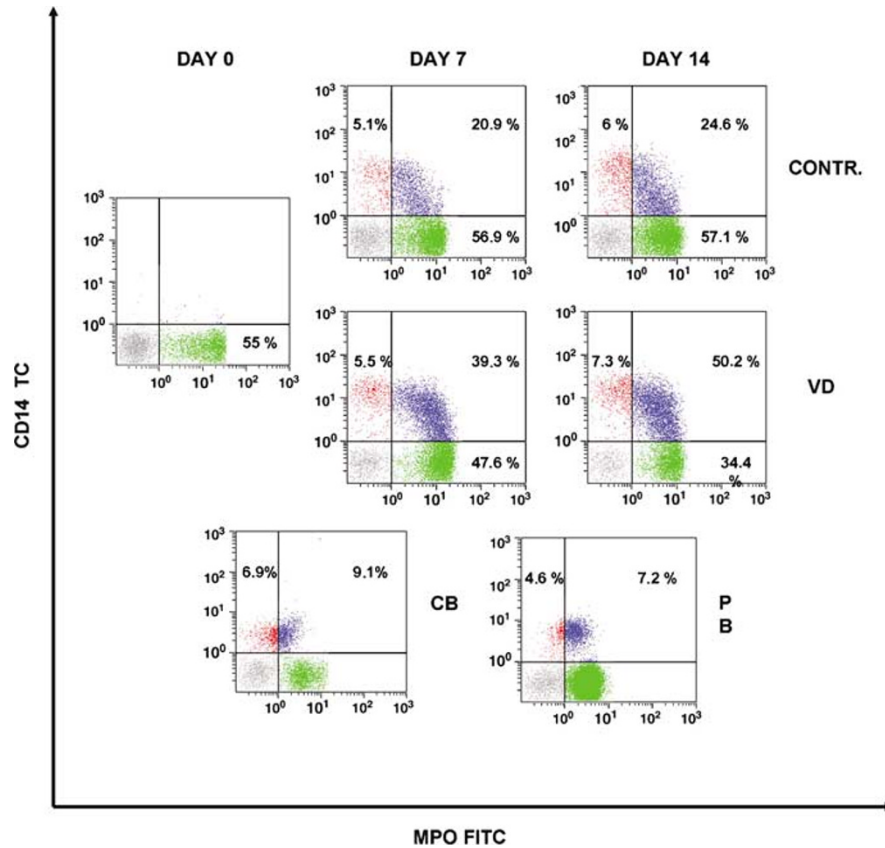
MPO is a bactericidal enzyme localized in the primary granules of neutrophilic granulocytes and their immediate

precursors.<sup>24</sup> By using a multi-parameter flow cytometry approach, it has been clearly demonstrated that the MPO protein is not expressed in CB CD34<sup>+</sup> progenitors, whereas it specifically appears at the promyelocyte stage of granulocytic maturation.<sup>25</sup> In other terms, MPO can be virtually considered as a marker of azurophilic/primary granules of granulocytic precursors, although a low-level expression of this enzyme has been also detected in PB monocytes using flow cytometry.<sup>26</sup> Based on this observation, we decided to monitor the trans-differentiation of (CD14<sup>-</sup>) myeloblasts to monocytes by a protocol of MPO/CD14 biparametric flow cytometry analysis optimized in our laboratory (see Materials and Methods). MPO expression in freshly separated CD14<sup>-</sup> myeloblasts averaged  $55 \pm 5\%$ . In a representative experiment, 26 and 30% of total analyzed cells became CD14<sup>+</sup>, respectively, at days 7 and 14 post-separation in the presence of hematopoietic cytokines (Figure 4, CONTR.). VD treatment resulted in about a two-fold increase of positivity: 26 to 44% at day 7 and 30 to 58% at day 14 post-purification (Figure 4, VD). Regardless of the considered cell population (control or VD treated), virtually all CD14<sup>+</sup> cells co-expressed MPO and CD14, enforcing the observation that trans-differentiating monocytes are directly generated by CD14<sup>-</sup>/MPO<sup>+</sup> late myeloblasts/promyelocytes. Interestingly, acquisition of CD14 antigen was accompanied by a parallel decline of MPO mean fluorescence intensity (MFI), resulting in an MPO/CD14 expression pattern that is very similar to that of both neonatal and adult PB monocytes (Figure 4, CB and PB).



**Figure 3** Differentiation capacity of CD14<sup>+</sup> myeloid precursors upon stimulation with nuclear hormones and CSFs. (a) Flow cytometry evaluation of CD14 positivity, performed after a 7-day stimulation of CD14<sup>+</sup> cells with VD, ATRA, G-CSF and M-CSF. Results were reported as mean ± S.E.M. and compared with control untreated cells (CONTR.). (b) Morphological assessment of control (CONTR.), VD-treated (VD) and ATRA-stimulated (ATRA) CD14<sup>+</sup> cells





**Figure 4** Flow cytometry analysis of CD14 and MPO lineage markers in unstimulated and VD-treated CD14<sup>+</sup> granulocyte precursors. Multi-parameter flow cytometry was used to assess CD14 and MPO expression in unstimulated control (CONTR.) and VD-treated (VD) CD14<sup>+</sup> cells at different times of liquid culture. Days of stimulation are indicated on the top. Circulating monocytes purified by CB and PB from adult donors were also analyzed

### Microarray analysis of CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors

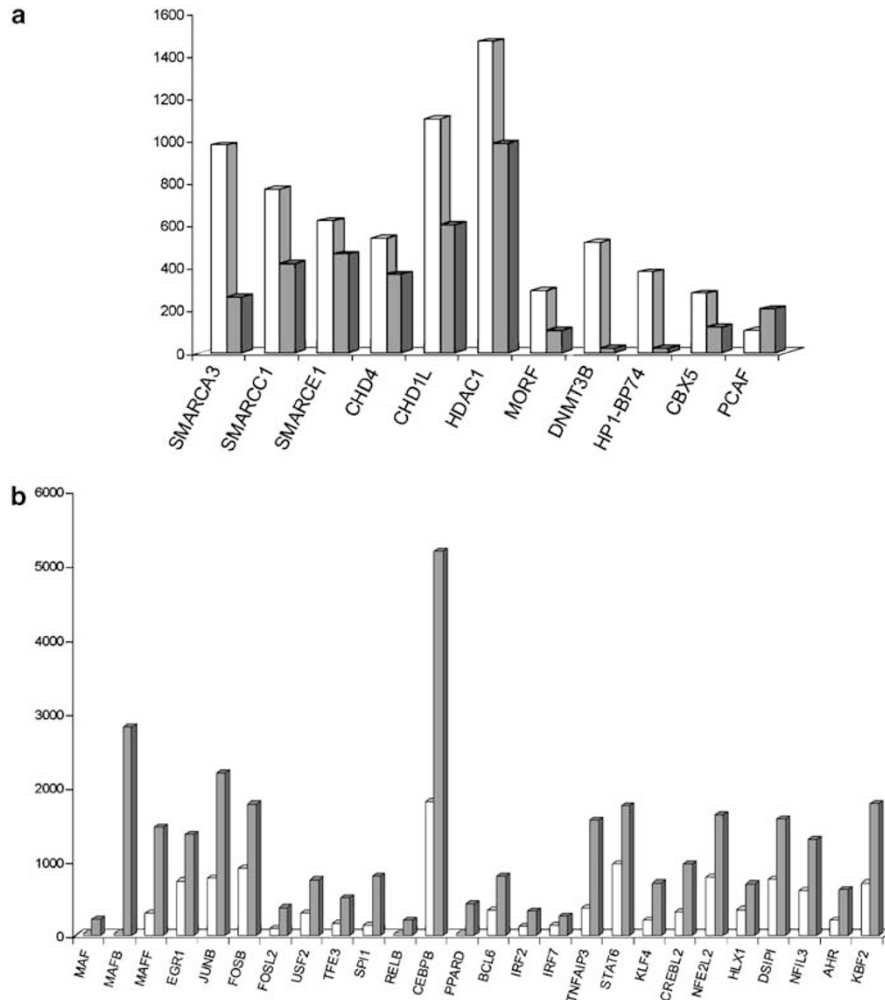
Data obtained so far indicate that CD14<sup>-</sup> granulocytic precursors, deriving from CB CD34<sup>+</sup> hematopoietic progenitors, are able to trans-differentiate to CD14<sup>+</sup> monocytes maintaining, at the same time, the capacity to activate a granulocytic differentiation program. CD14<sup>+</sup> monocytic precursors, on the other hand, probably represent a cell population that is irreversibly committed to a terminal monocyte/macrophage differentiation and is unable to convert to a distinct maturation lineage. To clarify the molecular mechanisms underlying this differentiation plasticity and to better characterize the transcriptional regulation of monocyte/macrophage commitment, we analyzed mRNA expression profiles of CD34<sup>+</sup>-derived CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors, recovered at day 7 of liquid culture, by cDNA microarray that was performed using the GeneChip Affymetrix methodology. Comparative analysis was then performed according to the Affymetrix guidelines (see Materials and Methods) in order to identify lists of differentially expressed genes that were subsequently classified into defined functional categories, that is, chromatin remodeling complexes, transcription factors, myeloid differentiation markers (CD antigens, cytoplasmic granules), cytokines/chemokines and receptors.

### Chromatin remodeling complexes

It is generally believed that chromatin remodeling is a crucial process for the activation of genetic programs underlying the differentiation potentiality of hematopoietic progenitor/precursor cells.<sup>6,27,28</sup> Based on this premise, we assessed whether the expression pattern of the main chromatin remodeling complexes<sup>27-29</sup> could explain the different maturation plasticity of analyzed cell populations. As reported in Figure 5, panel a, five different chromatin regulators belonging to the SWI/SNF ATPase family (SMARCA3, SMARCC1, SMARCE1, CHD4, CHD1L), the HDAC1 deacetylase, the MORF acetyl-transferase, two methyl-lysine binding proteins (HP1, CBX5) and the DNA methyl-transferase 3 B exhibited a downregulated expression in CD14<sup>+</sup> monocyte precursors. Conversely, with the only exception of PCAF acetyl-transferase, no upregulated chromatin remodeling complexes were detected in these cells.

### Transcription factors

Upregulation of transcription factors is a widely recognized mechanism to induce the lineage commitment of hematopoietic stem/progenitor cells.<sup>3,5,30</sup> Based on this premise and to better characterize the transcriptional regulation of monocyte/macrophage differentiation, an important phase of microarray



**Figure 5** Histogram showing the comparative mRNA expression profile of chromatin remodeling complexes and transcription factors in CD14<sup>-</sup> and CD14<sup>+</sup> myeloid precursors. Microarray analysis was performed on freshly purified CD14<sup>-</sup> (white bars) and CD14<sup>+</sup> (grey bars) cells deriving from CD34<sup>+</sup> hematopoietic progenitors cultured for 7 days in liquid culture. The extent of mRNA expression of each analyzed gene is indicated on the y-axis as a signal value assigned by the MAS 5.0 software based on the observed probe set hybridization pattern. All genes are indicated on the x-axis using gene symbols (complete designation and description of each gene is available in the OMIM website). See Materials and Methods for details

analysis was focused on transcription factors exhibiting an increased expression in the CD14<sup>+</sup> cell context. Among these genes, we identified three distinct members of the large Maf family (MafB, MafF, c-Maf) (Figure 5, panel b), one of which (MafB) is highly and specifically expressed in CD14<sup>+</sup> cells, confirming previous observations reporting induction of mono-macrophage differentiation following vector-mediated overexpression in a variety of hematopoietic cells.<sup>12,31</sup> Other transcription factors displayed a less pronounced upregulation in the CD14<sup>+</sup> cell context (Figure 5, panel b). For the majority of these genes, described below in more detail, an involvement in mono-macrophage differentiation has been already reported. Egr1 was demonstrated to induce the macrophage differentiation of the M1 murine myeloid cell line.<sup>32</sup> Jun and Fos family proteins are induced during the mono-macrophage differentiation of several hematopoietic cell lines.<sup>33,34</sup> TFE3 and USF are helix-loop-helix transcription factors forming a dimeric complex that transactivates the macrophage-related Fc $\gamma$ RIII antigen (CD16) by binding an E-

box-like element (MyE) placed inside its promoter region.<sup>35,36</sup> USF is also responsible for the macrophage-specific expression of mannose receptor, a surface molecule involved in antigen phagocytosis.<sup>37</sup> Both USF activities are cooperated by the PU.1 (Spi1) myeloid-related transcription factor.<sup>36,37</sup> RELB participate in the formation of NF $\kappa$ B complex regulating dendritic cell (DC) differentiation and antigen-presenting cell (APC) activities.<sup>38,39</sup> C/EBP family transcription factors are important regulators of myeloid differentiation and the beta member was shown to be involved in monocyte differentiation and activation.<sup>40-43</sup> Recently, PPAR $\delta$  and BCL-6 have been demonstrated to regulate MCP1 (CCL2) monocyte chemokine transcription.<sup>44,45</sup> IRFs,<sup>46-48</sup> TNFAIP3<sup>49</sup> and Stat6<sup>50-52</sup> are intracellular targets of macrophage-activating cytokines (IFNs, TNF $\alpha$  and IL-4, respectively). For all the other transcription factors reported in Figure 5, panel b, a possible role in the regulation of mono-macrophage differentiation and/or activation remains to be established.

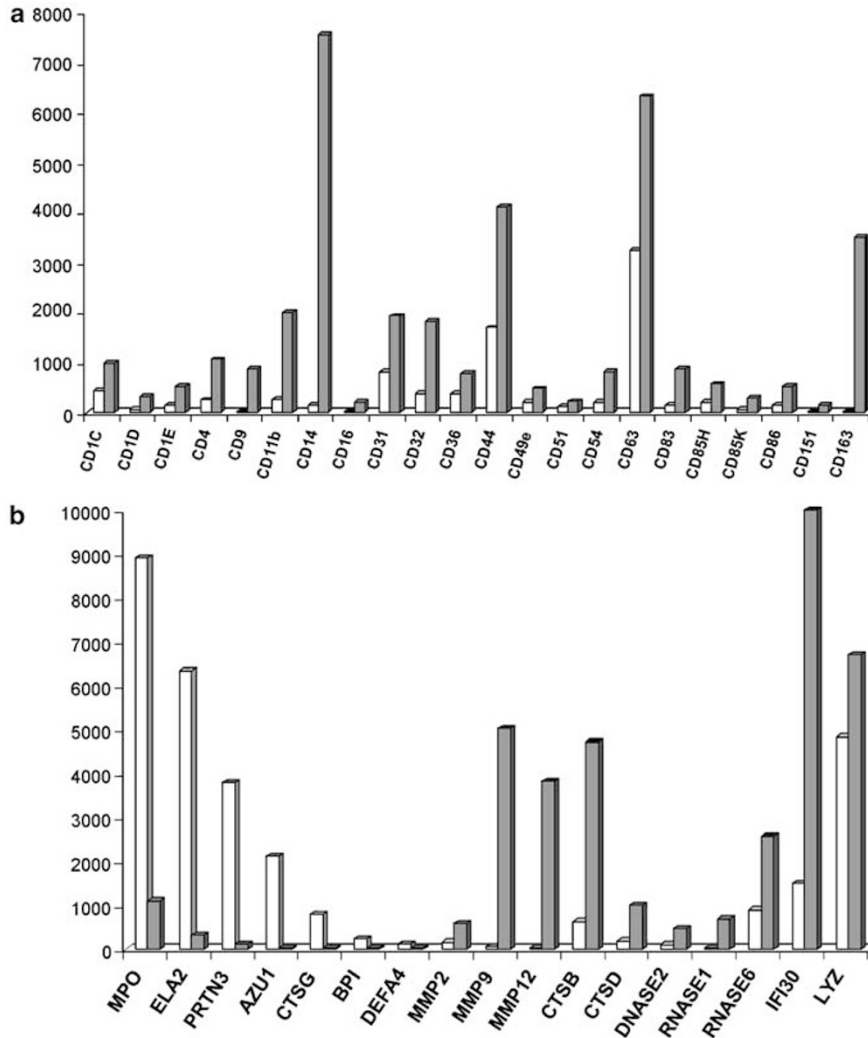
**Surface antigens**

Virtually all CD antigens characterized by a preferential expression in the CD14+ cell context (Figure 6, panel a) have been currently detected on cells belonging to the mononuclear phagocytic system (monocytes, macrophages, DCs), where they mediate a variety of biological functions related to macrophage activation, such as cell adhesion (CD9, CD11b, CD31, CD44, CD49e, CD54), recognition of bacterial components (CD14), opsonin-mediated phagocytosis (CD11b, CD16, CD32), antigen presentation (CD1cde, CD74, CD83) and scavenging of inflammatory or toxic molecules (CD163). Interestingly, some of these surface molecules, such as CD163 (hemoglobin-haptoglobin scavenger receptor) and CD9 (adhesion molecule), were specifically expressed by CD14+ cells. A limited number of CD antigens (CD38, CD24, CD69, CD71) displayed a remarkably higher expression in CD14- cells (not shown). (For a detailed description of the expression pattern and biological functions of the

above-mentioned CD antigens, see [www.ncbi.nlm.nih.gov/prov](http://www.ncbi.nlm.nih.gov/prov), CD Index.)

**Cytoplasmic granules**

The messenger RNA expression profile of genes coding for granule proteins is reported in Figure 6, panel b. As expected, primary azurophilic granule proteins (MPO, elastase 2, proteinase 3, azurocidin 1, cathepsin G) were highly expressed by CD14- late myeloblasts/promyelocytes, while secondary specific granule proteins (lactoferrin, histaminase) and secretory vesicle proteins<sup>24</sup> (alkaline phosphatase, albumin, tetranectin) were undetectable (not shown). All these genes resulted clearly downregulated in CD14+ monoblasts where MPO maintained the highest mRNA expression levels as compared to the others. This finding confirms the observation that, although at lower levels, MPO is also expressed in the monocyte maturation lineage, as demonstrated by flow cytometry data reported in this paper



**Figure 6** Histogram comparing the mRNA expression of CD antigens (a) and granule proteins (b) in CD14- (white bars) and CD14+ (grey bars) myeloid precursors. Experimental procedure and modality of data analysis and presentation have been described in more detail in the legend of Figure 5 and in the Materials and Methods section

(see Figure 4) and published by other authors.<sup>26</sup> On the other hand, expression of typical monocyte granule proteins<sup>53</sup> (matrix metalloproteinase 2, 9, 12, DNase 2, RNase A 1 and 6, cathepsin B and D, IFI30) was remarkably increased in CD14+ as compared to CD14- cells.

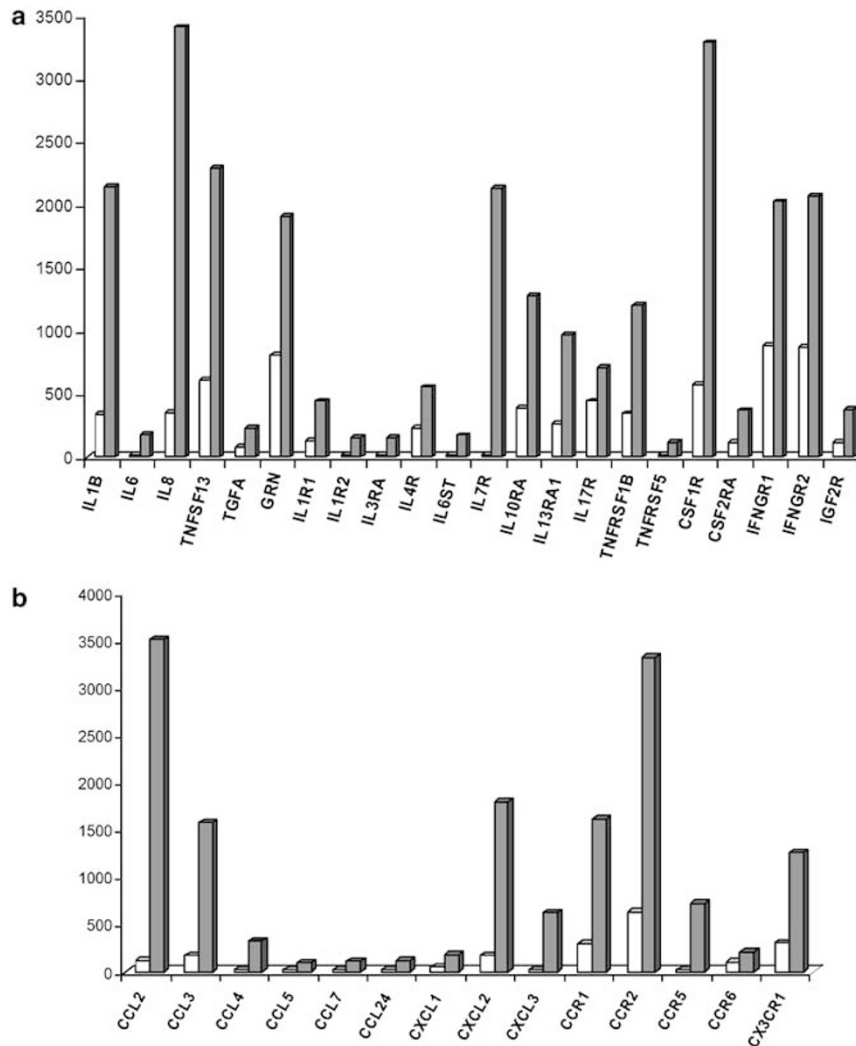
### Cytokines/chemokines and their receptors

Analysis of genes belonging to the cytokine category revealed an increased mRNA expression of inflammatory (TNFsf13, IL-1 $\beta$ , IL-6, IL-8),<sup>54</sup> fibrogenic<sup>55</sup> (TGF $\alpha$ ) and osteogenic<sup>56</sup> (BMP15) cytokines in CD14+ monoblasts (Figure 7, panel a). Similarly, these cells exhibited an upregulated expression of growth factor receptors that have been previously demonstrated to mediate monocyte production<sup>22</sup> (GM-CSFR, M-CSFR), activation<sup>57</sup> (M-CSFR, IFN $\gamma$ R, IL-1R, TNFRs), secretion of inflammatory cytokines<sup>58</sup> (IL-7R), differentiation to DC<sup>59</sup> (GM-CSFR, IL-4R, IL-13R) and to osteoclasts<sup>60</sup> (IL-17R). In addition, a large number of chemokines<sup>61,62</sup> (CC

ligand 2, 3, 4, 5, 7, 24; CXC ligand 1, 2, 3) and chemokine receptors<sup>61,62</sup> (CCR1, 2, 5, 6 and CX3CR1) underwent 'de novo' induction or upregulation in CD14+ cells (Figure 7, panel b). These results, together with the above-mentioned transcription factor, CD antigen and granule protein expression data, strongly suggest that monocyte precursors are already set to play a role in defense/immunity mechanisms that will be accomplished by the subsequent terminal differentiation to the macrophage stage. These considerations were also confirmed by the GO mapping procedure performed using the biological process modality and revealing a clear upregulation of defense/immunity-related genes in CD14+ monoblasts as compared to CD14- myeloblasts/promyelocytes (not shown).

### Cell cycle

Analysis of cell-cycle-related gene expression evidenced a clear upregulation of growth arrest genes in the CD14+ cell



**Figure 7** Histogram reporting a comparison microarray analysis of cytokines, chemokines and their receptors in CD14- and CD14+ myeloid precursors. Messenger RNA expression profile of cytokines (a), chemokines (b) and the corresponding receptors was evaluated by microarray in CD14- granulocyte precursors (white bars) and CD14+ monocyte precursors (grey bars). Data were analyzed and presented as described in the legend of Figure 5 and in Materials and Methods



context such as p21, Mad, Rb1 and the p53 targets ATF3<sup>63</sup> and BTG2<sup>64,65</sup> transcription factors. In addition, proliferation markers such as Myc<sup>66</sup> and Cdc2<sup>67</sup> were consistently down-regulated in these cells, confirming their postmitotic state (Figure 1, Supplementary material).

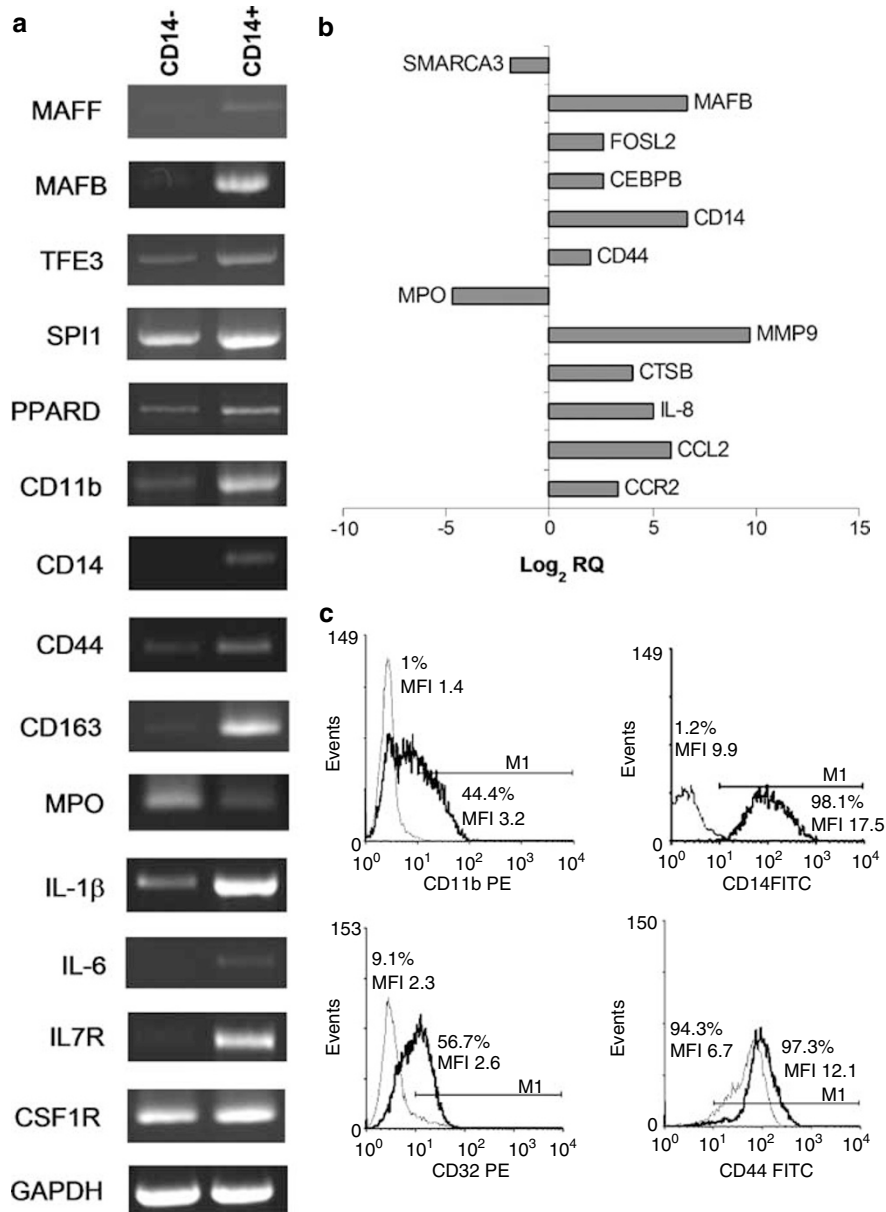
### Validation of differentially expressed genes

To confirm microarray data, a number of differentially expressed genes belonging to the above-mentioned functional categories and mainly exhibiting an upregulated expression in the CD14+ cell context were validated at the mRNA and/or protein level using semiquantitative RT-PCR, quantitative real-time RT-PCR or flow cytometry analysis (Figure 8, panels a, b and c, respectively). All genes analyzed by semiquantitative and quantitative RT-PCR (transcription factors, CD antigens, cytokines and receptors) displayed a higher expression in the CD14+ cell context, the only exception being represented by the MPO and the SMARCA3 genes that, as expected, presented an inverted expression pattern (Figure 8, panels a and b). Similarly, flow cytometry analysis confirmed the increased expression of a defined set of CD antigens (CD11b, CD14, CD32, CD44) in terms of positivity percentage and/or MFI (Figure 8, panel c). Interestingly, five out of 23 genes selected for this evaluation were validated by at least two different approaches underlining the reliability of the used analysis methods.

### Discussion

In spite of their apparent limited differentiation potential, hematopoietic precursors are plastic cells able to trans-differentiate from a maturation lineage to another.<sup>7,8</sup> This property, referred to as 'lineage switching' or 'intra-hematopoietic plasticity', was initially based on the observation that a number of leukemic cell lines undergo phenotypic changes 'in vitro' as a result of specific manipulations such as pharmacological treatments<sup>9,10</sup> or transcription factor overexpression.<sup>11,12</sup> Further reports have subsequently indicated that trans-differentiation processes can be reproduced by using normal CD34+ -derived myeloid precursors. The capacity of primary myeloblasts/promyelocytes to undergo mono-macrophage differentiation upon treatment with VD represents a typical example of this plasticity.<sup>13,14</sup> To better characterize this process and to gain more insight into the transcriptional regulation of monocyte commitment, we purified CD14- and CD14+ myeloid precursors obtained by expansion/differentiation in liquid culture of cord blood CD34+ hematopoietic progenitors. A preliminary assessment of morphology and proliferation capacity, subsequently confirmed by microarray analysis, evidenced that CD14- cells were represented by cycling granulocyte precursors at the late myeloblast/promyelocyte differentiation stage, whereas CD14+ cells appeared as postmitotic monoblast/promonocyte elements. Treatment with differentiation inducers, and particularly nuclear hormones, revealed that CD14- granulocyte precursors are plastic cells able to undergo either granulocyte or monocyte differentiation when treated with ATRA or VD, respectively, while CD14+ cells rapidly underwent macro-

phage differentiation regardless of the used stimulation modalities. To a lesser extent, activation of CD14 antigen expression in CD14- granulocyte precursors was also observed by simply culturing them in the presence of early-acting hematopoietic cytokines, though these cells remained morphologically immature. Interestingly, the analyzed trans-differentiation capacity was restricted to the late myeloblast/promyelocyte stage of granulocyte maturation, since CD34+ -derived myelocytes/neutrophils and PB granulocytes were unable to convert to the mono-macrophage phenotype in response to VD stimulation. By using a flow cytometry protocol optimized in our laboratory, we demonstrated that trans-differentiation of late myeloblasts/promyelocytes to monocytes occurs through a maturation transition in which markers of granulocyte (MPO) and monocyte (CD14) lineages are co-expressed. Upregulation of CD14 expression in CD14- granulocyte precursors was accompanied by a parallel decline of MPO expression, resulting in a flow cytometry pattern clearly reminding that of mature PB monocytes. In addition, stimulation with the VD monocyte differentiation inducer<sup>14</sup> remarkably enhanced the entity of MPO+/CD14+ cell population, underlining its crucial importance in the activation of the investigated trans-differentiation process. Recently, the trans-differentiation of mature neutrophils to macrophages upon treatment with a combination of monocytopoietic cytokines (GM-CSF, M-CSF, IL-4, IFN $\gamma$ , TNF $\alpha$ ) has been reported,<sup>68</sup> even if MPO underwent a complete downregulation and CD14/MPO co-expression was not observed in these experimental conditions. In spite of differences concerning target cells, stimulation modalities and the observed phenotype changes, all these findings clearly indicate that monocytopoiesis is a plastic process and, besides the conventional differentiation cascade arising from the monocyte-committed progenitor (CFU-M), alternative maturation pathways can be generated, involving a lineage switching of cycling granulocyte precursors or even mature postmitotic neutrophils. Microarray analysis of myeloid differentiation and activation markers (CD antigens, granule proteins, cytokines, chemokines, receptors) provided further evidence that CD14- cells were represented by late myeloblast/promyelocyte elements, whereas CD14+ cells clearly exhibited a mono-macrophage mRNA expression profile indicating that, in spite of their immature morphology, they are already set to play a role in biological functions as innate immunity, inflammatory processes and tissue repair. Consistently with these data, CD14+ precursors displayed an upregulated expression of several transcription factors related to monocyte differentiation and activation. Although Maf family proteins have been previously demonstrated to promote monocyte commitment in Myb-Ets transformed avian progenitors (MafB)<sup>31</sup> and in HL60 leukemic cells (c-Maf),<sup>12</sup> our data represent the first demonstration supporting this possibility in a model of normal human hematopoiesis. Furthermore, based on its peculiar expression pattern, characterized by selective and high-level expression in the CD14+ cell context, the MafB member of this family appears as a strong candidate for the role of master regulator transcription factor of monocyte differentiation. Experiments of viral vector-mediated expression of this transcription factor in CD34+ hematopoietic stem/progenitor cells, performed in



**Figure 8** Validation of microarray data. To confirm microarray data, 23 differentially expressed genes belonging to several functional categories were validated at the mRNA and/or protein level by different methods. **(a)** Semi-quantitative RT-PCR analysis. Agarose gels stained with ethidium bromide are shown. Cell populations are indicated on the top and analyzed genes on the left. Normalization of amplified RNA amounts was achieved by the GAPDH house-keeping gene. **(b)** Histogram showing the results of quantitative real-time RT-PCR. Genes analyzed by this approach are reported near single bars. Modulation of mRNA levels in CD14+ cells as compared to CD14- cells (relative quantity (RQ)) were assessed and are indicated on the x-axis as  $\text{log}_2$  value. **(c)** Flow cytometry analysis. Analyzed CD antigens are indicated below histograms. The entities of surface antigen expression, measured as positivity percentage and MFI, are shown inside the histograms. Grey line, CD14- cells; black line, CD14+ cells

our laboratory, have confirmed this hypothesis (data presented in a distinct manuscript), although it is worth considering that the molecular control of monocyte commitment is a redundant process in which other transcription factors, such as HOX-A10 and VDR, have been demonstrated to play a fundamental role in human primary cell models.<sup>14,69</sup> The massive induction of the mono-macrophage differentiation program in CD14+ precursors represents a possible explanation for the complete loss of differentiation plasticity observed in these cells. Downregulation of chromatin remo-

deling complexes could also play a role in this regard although functional assays, such as treatment with deacetylation and methylation inhibitors, are needed to address this issue. As recently reviewed, 'polymerase accessibility to chromatin is a limiting step for both RNA and DNA synthesis'.<sup>28</sup> For this reason, it is generally believed that chromatin remodeling, RNA transcription, DNA replication and, consequently, cell cycle control are strictly coordinated processes sharing molecular components and probably subnuclear localization. According to the Quesenberry model of hematopoiesis,<sup>28</sup>

'chromatin remodeling associated with cell cycle transit' is responsible for the differentiation potentiality of hematopoietic stem/progenitor cells. Based on these considerations, it is conceivable that cell cycle arrest and downregulation of chromatin remodeling complexes, occurring in CD14+ monocyte precursors, are related phenomena leading to restriction of differentiation potentiality.

## Materials and Methods

### CD34+ stem/progenitor and CD14- /CD14+ precursor cell purification, culture and differentiation

Human CD34+ cells were purified from umbilical cord blood samples, collected after normal deliveries, according to the institutional guidelines for discarded material. Mononuclear cells were isolated by Ficoll-Hypaque (Lymphoprep; Nycomed Pharma, Oslo, Norway) gradient separation, washed twice with PBS, and then CD34+ cells separated using a MACS magnetic cell sorting procedure (MiniMacs; Miltenyi Biotec, Auburn, CA, USA), normally yielding a purity higher than 95%. To achieve an optimal expansion and differentiation of the primary CD34+ hematopoietic cells, a liquid culture was performed by seeding CD34+ cells in six-well plates at a density of  $5-10 \times 10^4$ /ml (3 ml/well) in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY, USA), added with 20% FCS (Bio-Whittaker, Walkersville, MD, USA), in the presence of human (hu) hematopoietic cytokines such as: SCF (50 ng/ml), Flt3-ligand (Flt3-l) (50 ng/ml), IL-11 (50 ng/ml), IL-6 (10 ng/ml), IL-3 (10 ng/ml) and G-CSF (10 ng/ml) (all from R&D Systems, Minneapolis, MN, USA). After the first week of culture, hematopoietic cells were analyzed, by flow cytometry, for CD14 antigen expression, estimated at about 25-30% of the entire cell population. CD14+ and CD14- cell fractions, obtained by immunomagnetic separation using the MACS technology (Miltenyi), were seeded in liquid culture as reported above and stimulated weekly with a number of differentiation agents:  $10^{-8}$  M VD (Hoffman-Laroche, Basel, Switzerland),  $10^{-6}$  M ATRA (Sigma Chemical Co., St. Louis, MO, USA), 50 ng/ml G-CSF and 50 ng/ml M-CSF (both from R&D Systems).

### Morphological and immunophenotypic analysis

Differentiation of CD34+ cells was monitored by morphological analysis of MGG-stained cytopins and by flow-cytometric analysis of CD34, CD38 and CD14 surface antigen expression, performed at days 0, 7 and 14 postpurification. CD11b, CD32 and CD44 surface antigen expression was also analyzed by flow cytometry on freshly separated CD14- and CD14+ cells. The following monoclonal antibodies (MoAbs) were used for labeling cell samples: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 MoAb, phycoerythrin (PE)-conjugated mouse anti-human CD38 MoAb, PE-conjugated mouse anti-human CD11b MoAb, PE-conjugated mouse anti-human CD32 MoAb (all from Becton Dickinson Systems, Mountain View, CA, USA), FITC-conjugated mouse anti-human CD44 MoAb (Sigma-Aldrich, St. Louis, MO, USA) and FITC-conjugated mouse anti-human CD14 MoAb (Miltenyi). Negative controls were also performed by staining cells with isotype-matched nonspecific antibodies (Becton Dickinson). Briefly, cell samples were incubated in the presence of the indicated MoAbs, at the proper dilution, in PBS containing 5% FCS and 1% Fc receptor (FcR) blocking reagent (Miltenyi) for 30 min at 4°C. Cells were then washed twice, resuspended with PBS and analyzed by a Coulter Epics XL (Coulter Electronics Inc., Hialeah, FL, USA) flow cytometer. MPO intracellular staining was performed using a FIX & PERM

cell permeabilization kit (Caltag laboratories, Burlingame, CA, USA) in dual-color immunofluorescence using FITC-conjugated mouse anti-human MPO MoAb and tri-colour (TC)-conjugated mouse anti-human CD14 MoAb (both from Caltag laboratories, Burlingame, CA, USA). After washing, cells were resuspended in PBS, added with 1% paraformaldehyde and analyzed as described. At least 10 000 events were counted for each sample to ensure statistical relevance. Analysis was performed in terms of positivity percentage or MFI.

### RNA extraction

Total cellular RNA was extracted from  $0.5-1 \times 10^6$  cells of each analyzed sample, by means of the guanidinium-cesium chloride centrifugation technique,<sup>70</sup> in four different experiments. RNA evaluation was assessed by formaldehyde-agarose gel and quantified by Bio-Analyzer technique (Applied Biosystem).

### Biotin-labeled transcription, Genechip hybridization and microarray analysis

Biotin-labeled target sequences were synthesized according to the manufacturer's guidelines (Affymetrix, Santa Clara, CA, USA). Briefly, 5 µg of RNA, obtained as a pool of four different samples, were converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (SuperScript Choice System, Invitrogen, Paisley, UK) based on a T7-(dT)<sub>24</sub> primer (MWG-Biotech, Ebersberg, Germany). After the second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription reaction (Enzo bio array HY RNA transcript labeling kit, Enzo, Farmingdale, NY, USA) supplemented with biotin-11-cytidine 5'-triphosphate (CTP) and biotin-16-uridine 5'-triphosphate (UTP) as described. The labeled cRNA was purified using RNeasy spin columns (Qiagen, Valencia, CA, USA) and concentration was determined using the Bio-Analyzer system. In all cases, 15 µg of each biotinylated cRNA preparation were fragmented, checked by agarose gel electrophoresis, and added to the hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD and Cre). Samples were then hybridized for 16 h to Affymetrix HG-U133A GeneChip arrays. GeneChips were washed and stained using the instrument's standard Eukaryotic GE WS2 protocol based on an antibody-mediated signal amplification. Images from the scanned chips were processed using Affymetrix Microarray Analysis Suite 5.0 (MAS 5.0) (Affymetrix, www.affymetrix.com). Based on statistical analysis algorithms, this software allowed to perform an absolute analysis, estimating expression levels (signal) and assigning an 'absolute call', 'present' (P), 'marginal' (M) or 'absent' (A), to each transcript. Subsequently, MAS 5.0 algorithm was used to perform a comparison analysis between CD14- and CD14+ myeloid precursors. The analysis employs the scaling technique to minimize differences in overall signal intensity between the arrays. The data were comparatively analyzed to generate different calls indicating whether the expression level of a given transcript was 'increased' (I), 'decreased' (D) or 'not changed' (NC) in CD14+ (sample) *versus* CD14- cells (baseline). The relative change of each transcript between the sample and the baseline is expressed as 'fold change', which is usually quantified as log<sub>2</sub> value (signal log ratio). Gene lists created by the MAS5.0 comparison analysis were then transferred to Microsoft Excel, linked to internet genome databases (i.e. NetAffx, GenBank, online Mendelian inheritance in man (OMIM)) and filtered by key words and probe list to assess for specific categories of genes.

The Gene Ontology Mining Tool Affymetrix (www.affymetrix.com/netaffex) database was used to cluster annotate U133A genes for

functional categories. Gene categories were chosen by browsing the three main terms of the ontology vocabulary: molecular function, biological process and cellular component.

### Semiquantitative RT-PCR analysis

Semiquantitative RT-PCR reaction was carried out using 1  $\mu$ g of total RNA, obtained from CD14<sup>-</sup> myeloblasts and CD14<sup>+</sup> monoblasts, as previously described,<sup>71</sup> using oligonucleotide primers reported in Table 1, Supplementary material. Normalization of the amplified samples was obtained by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 'housekeeping' gene<sup>72</sup> as previously described.<sup>14</sup>

### Quantitative real-time RT-PCR

Total RNA (100 ng) was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time quantitative PCR was then performed with an ABI PRISM 7900 sequence detection system (Applied Biosystems) to quantify in CD14<sup>+</sup> monoblasts and in CD14<sup>-</sup> myeloblasts samples the relative levels of the following mRNAs: SMARCA3, MAFB, FOSL2, CEBPB, CD14, CD44, MPO, MMP9, CTSS, IL-8, CCL2 and CCR2. Quantitation was performed by amplifying GAPDH mRNA as endogenous control. All the primers and probes have been designed by Applied Biosystems. Each cDNA sample was run in triplicate for every gene (target) and GAPDH (endogenous control) in 50  $\mu$ l reaction volume using Taqman Universal PCR Master Mix (Applied Biosystems). Thermal cycling was started with an initial denaturation at 50°C for 2 min and 95°C for 10 min, followed by 40 thermal cycles of 15 s at 95°C and 1 min at 60°C. Statistical analysis of the quantitative real-time PCR was obtained using the ( $2^{-\Delta\Delta Ct}$ ) method<sup>73,74</sup> which calculates relative changes in gene expression of the target gene, normalized to the endogenous control, and referred to a calibrator sample (CD14<sup>-</sup> cells).

### Acknowledgements

This work has been supported by a grant from MURST-Cofin 2002/2003 and AIRC 2003/2004. We thank also A.I.L. (Italian Association against Leukemias and Lymphomas) and Transfusion Center of Modena for their support.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)