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Maturation-associated gene expression profiles along normal human bone marrow monopoiesis

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Summary

Human monopoiesis is a tightly coordinated process which starts in the bone marrow (BM) haematopoietic stem cell (HSC) compartment and leads to the production of circulating blood mature monocytes. Although mature monocytes/macrophages have been extensively studied in both normal or inflammatory conditions, monopoiesis has only been assessed *in vitro* and *in vivo* animal models, due to low frequency of the monocytic precursors in the normal human BM. Here we investigated the transcriptional profile along normal human BM monopoiesis. Five distinct maturation-associated stages of monocytic precursors were identified and isolated from (fresh) normal human BM through fluorescence-activated cell sorting, and the gene expression profile (GEP) of each monocytic precursor subset was analysed by DNA-oligonucleotide microarrays. Overall, >6000 genes (18% of the genes investigated) were expressed in ≥1 stage of BM monopoiesis at stable or variable amounts, showing early decrease in cell proliferation with increased levels of expression of genes linked with cell differentiation. The here-defined GEP of normal human BM monopoiesis might contribute to better understand monocytic differentiation and the identification of novel monocytic candidate markers, while also providing a frame of reference for the study of monocytic maturation in both neoplastic and non-neoplastic disease conditions involving monocytic precursor cells.

Keywords: normal human monopoiesis, bone marrow monocytic maturation, gene expression profile, transcriptome, microarray.

Monocytes are haematopoietic cells that play an important role in innate immune responses, with the potential to differentiate to macrophages and myeloid dendritic cells (DC) (Tussiwand & Gautier, 2015). Monopoiesis is a complex multistep process which starts in the bone marrow (BM) haematopoietic stem cell (HSC) compartment and leads to the production of BM-derived circulating blood monocytes through several tightly regulated proliferation and differentiation cycles (De Kleer *et al*, 2014; Huber *et al*, 2014). Thus, *in vitro* studies have shown that multipotent self-renewing HSCs may give rise to 'multipotent progenitors' (common progenitors of lymphoid and myeloid cell lineages), which subsequently differentiate into 'common myeloid progenitors', granulocyte/monocyte progenitors and 'monocyte/macrophage and dendritic cells progenitors' (MDP). Further differentiation of MDP cells will give rise to monoblasts, promonocytes, monocytes and either macrophages or myeloid DC (De Kleer *et al*, 2014; Huber *et al*, 2014).

Despite this knowledge, direct identification of early monocytic precursors in primary (e.g. freshly obtained, non-cultured) human BM samples has remained a challenge for decades. This is mainly due to the low frequency at which

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monocytic precursors (e.g. monoblasts and promonocytes) are present in human BM samples (<5% of the whole sample cellularity) and the lack of specific phenotypic markers and phenotypes for clear-cut dissection of the earliest steps of monocytic differentiation (Huber *et al*, 2014). Altogether, this contributes to explain, at least in part, why most research studies on monocytic differentiation have mainly focused on the final stages of maturation that occur outside the BM (Auffray *et al*, 2009; Hoeffel & Ginhoux, 2015).

The EuroFlow consortium has recently delineated the distinct stages of monocytic maturation in normal human BM, based on the combined assessment of a limited number (e.g. 8) of markers (van Lochem *et al*, 2004; van Dongen *et al*, 2012; Kalina *et al*, 2012; Matarraz *et al*, 2015). Although this approach provided an invaluable tool for the identification of different maturation-associated compartments of monocytic precursor cells in the BM, it did not provide much insight or detailed information about the specific changes occurring in the overall gene expression profile (GEP) of precursor cells along the normal BM monocytic maturation pathway, that could validate the well-known *in vitro* culture findings and increase our knowledge about the production of monocytic cells in human BM (Cohn *et al*, 1966; Daigneault *et al*, 2010).

Here we investigated the overall GEP of five distinct maturation-associated compartments of BM monocytic precursors and monocytes, and the specific maturation-associated changes in their transcriptome, via detailed analysis of highly-purified human monocytic BM cells at different stages of maturation, using the specific maturation-associated proteins CD64 (FCGR1A), CD14 and CD300E, from early CD64$^+$ CD14$^-$ CD300E$^-$ and CD64$^+$ CD14$^+$ CD300E$^-$ precursors to the more mature CD64$^+$ CD14$^+$ CD300E$^+$ monocytes. Our goal was to better understand the dynamic changes that occur in the GEPs of monocytic BM cells during *in vivo* monopoiesis. Such detailed knowledge further allowed us to establish a frame of reference of a normal monocytic GEP, for better identification and understanding of the alterations that occur in inflammatory and autoimmunity disorders and/or distinct neoplastic disease conditions – e.g. myelodysplastic syndromes, myeloproliferative neoplasms and acute monoblastic and monocytic leukaemia.

Materials and methods

Samples

A total of five BM samples from five healthy donor volunteers (two males and three females; mean age 30 years, range 11–53 years), who gave their informed consent to participate, were studied. BM samples were from BM transplantation donors (n = 3) and subjects undergoing orthopaedic surgery for bone fractures (n = 2) at the University Hospital of Salamanca, (Salamanca, Spain). The study was approved by the local Ethics Committee.

Isolation of monocytic precursors at different stages of maturation

Each BM sample was stained with an identical 8-colour combination of fluorochrome-conjugated monoclonal antibodies – fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanin7 (PECy7), peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5), allophycocyanin (APC), APC-hilite7 (APC-H7), pacific blue (PacB) and pacific orange (PacO) – for the dissection of BM monocytic maturation, as follows: surface membrane (Sm) CD36, CD64 (FCGR1A), CD117 (KIT), CD34, CD300E, CD14, HLA-DR and CD45 (PTPRC). Staining of BM cells was performed as previously described in detail, following the standard operating procedures proposed by the EuroFlow consortium (van Lochem *et al*, 2004; van Dongen *et al*, 2012; Kalina *et al*, 2012). Immediately after staining, cells were acquired and sorted in a FACSAriaI or a FACSAriaIII flow cytometer [Becton/Dickinson Biosciences (BD), San José, CA, USA] using the FACSDiva software (BD). For data analysis, the INFINICYT™ software (Cytognos SL, Salamanca, Spain) was used. Prior to sorting the cells, a total of 10^5 cellular events were measured per tube and appropriate gating regions were set for the identification of five maturation-associated subsets of monocytic precursors and monocytes, to be subsequently applied for fluorescence-activated cell sorting (FACS). Thus the following subsets of monocytic precursors were isolated from each BM sample: (i) stage 1 (2·9% ± 0·3% of all monocytic precursors) consisted of CD34$^+$ HLADR^{high} CD117$^+$ CD64^{low} CD45^{low} CD36$^-$ CD14$^-$ CD300E$^-$ early monocytic precursors; (ii) stage 2 (7·7% ± 1% of all monocytic precursors) was defined by CD34$^-$ HLADR^{high} CD117$^-$ CD64^{high} CD45$^+$ CD36$^{-/+}$ CD14$^-$ CD300E$^-$ monoblasts; (iii) stage 3 (10·6% ± 0·3% of all monocytic precursors) included CD34$^-$ HLADR^{high} CD117$^-$ CD64^{high} CD45$^+$ CD36^{low} CD14^{int} CD300E$^-$ monoblasts; (iv) stage 4 (22·9% ± 2% of all monocytic precursors) consisted of CD34$^-$ HLADR^{high} CD117$^-$ CD64^{high} CD45$^+$ CD36^{high} CD14^{high} CD300E$^-$ monocytic precursors and (v) stage 5 (56% ± 3% of all monocytic precursors) was composed of CD34$^-$ HLADR$^+$ CD117$^-$ CD64^{high} CD45$^+$ CD36^{high} CD14^{high} CD300E$^+$ matured monocytes (Fig 1).

From each population, a total of $5{\cdot}6 \times 10^4$–5×10^5 cells were FACS-sorted directly into phosphate-buffered saline. Purified cells were immediately centrifuged and resuspended in 100 μl of RA1 buffer – NucleoSpin® RNA XS kit [Macherey-Nagel (MN), Düren, Germany]. In parallel, the purity of the isolated BM monocytic precursors cell populations was systematically confirmed to be >97%, by re-running approximately 500 sorted cells in the flow cytometer.

RNA extraction and gene expression profiling

GEPs were assessed on mRNA extracted from each of the purified FACS-sorted cell populations placed in RA1 buffer

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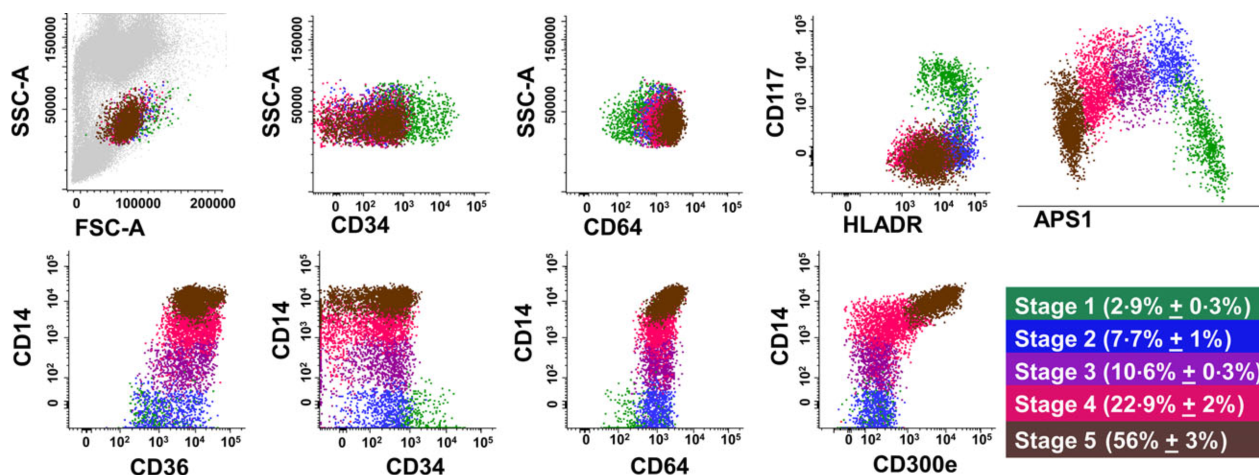

Fig 1. Immunophenotypic characteristics of human bone marrow monocytic cell populations at different stages of maturation. The following five different monocytic cell subsets (mean % from all monocytic cells) were identified by flow cytometry (and sorted by fluorescence-activated cell sorting): Immature stage 1 monocytic precursors were identified as CD34^{+} HLADR^{high} CD117^{+} CD64^{low} CD45^{low} CD36^{-} CD14^{-} CD300E^{-} cells (green dots); stage 2 monoblasts were CD34^{-} HLADR^{high} CD117^{-} CD64^{high} CD45^{+} CD36$^{-/+}$ CD14^{-} CD300E^{-} (blue dots); stage 3 monocytic precursors showed a CD34^{-} HLADR^{high} CD117^{-} CD64^{high} CD45^{+} CD36^{low} CD14^{int} CD300E^{-} phenotype (purple dots); stage 4 monoblasts had a CD34^{-} HLADR^{high} CD117^{-} CD64^{high} CD45^{+} CD36^{high} CD14^{high} CD300E^{-} phenotypic profile (pink dots), and; mature monocytes (stage 5) were CD34^{-} HLADR^{high} CD117^{-} CD64^{high} CD45^{+} CD36^{high} CD14^{high} CD300E^{+} (brown dots).

at identical concentration. Total mRNA was extracted using the NucleoSpin RNA XS reagent kit (MN), according to the instructions of the manufacturer. The quality and amount of the obtained mRNA was evaluated using the RNA6000 Nanochips or Picochips and the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). GEPs were assessed through the Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA), using the hybridization and washing buffers and procedures recommended by the manufacturer.

For data analysis, GEP raw data (Affymetrix.CEL files) was normalized using the Robust Multi-array Average (RMA) algorithm, which sequentially included background correction, quantile normalization, log2 transformation, and probe set summarization (Irizarry et al, 2003). In order to further decrease the risk for false-positive results, a filter was used to discriminate between positive and negative control probe sets ('X_mad_residual_mean' and 'Pos-vs-Neg-AUC' Affymetrix quality control, respectively), after correcting for differences in the overall quality of the array hybridization. For this purpose, those values obtained for the 1195 positive gene controls and the 2904 negative gene controls included in the Affymetrix GeneChip Human Gene 1.0 ST Array, were used. Based on such controls, a cut-off was established for positive expression signals, which was set at the first lower quartile of the positive control gene values.

Genes differentially expressed among different stages of maturation of BM monocytic precursors were identified using the 'gene set enrichment analysis' (GSEA) algorithm. Differentially expressed genes were ranked based on the existence of statistically significant differences in their expression levels between two distinct, but subsequent, stages of maturation (Subramanian et al, 2005). Subsequently, the GeneCodis (Gene Annotation Co-occurrence Discovery, Madrid, Spain) (Carmona-Saez et al, 2007; Nogales-Cadenas et al, 2009; Tabas-Madrid et al, 2012) functional annotation tool was used to define gene ontology and to perform functional enrichment analyses of those genes that were differentially expressed along the distinct monocytic maturation stages evaluated.

Evaluation of the purity of the FACS-sorted populations of BM monocytic precursor cells and characterization of their GEP

In order to assess the purity and quality of the mRNA extracted from the distinct BM monocytic cell populations analysed, the expression of genes that are highly characteristic of other haematopoietic cell lineages was evaluated. For this purpose *TFRC* (transferrin receptor, highly expressed in erythroid precursors, also termed *CD71*), *CD3* (T-cell receptor) and *CD19* (B-cell receptor) mRNA gene expression levels were evaluated for each of the distinct fractions of purified maturation-associated BM monocytic cell populations. In addition, expression of genes that are known to show distinct expression levels along BM monopoiesis [e.g. *PTPRC* (previously termed *CD45*), *KIT* (also termed *CD117*), *CD34*, *HLA-DR* genes, *CD36*, *FCGR1A* (also termed *CD64*), *CD14* and *CD300E*] was also evaluated in parallel to the corresponding protein levels, for each of the purified BM maturation-associated monocytic precursor cell populations.

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Results

Identification of monocytic precursors and their different maturation stages in human BM

Overall, five different stages of maturation of monocytic cells were defined and identified in all BM samples analysed (Fig 1); cells included in each of these maturation stages were subsequently purified. The earliest stage of monocytic maturation included BM CD34^{+} precursor cells that showed progressively greater CD64 expression levels. Subsequently, stage 2 monocytic precursors showed strong CD64 (CD64^{high}) and HLA-DR expression, they already had downregulated CD34 and CD117 expression, and lacked expression of CD14, CD36 and CD300E. Maturation toward stage 3 monocytic precursors was associated with upregulation of CD36 and CD14 expression, while retaining the strong positivity for CD64. Both stages 4 and 5 showed a CD14^{high} CD64^{high} CD36^{high} phenotype, but could be distinguished by the absence (stage 4) *vs.* presence (stage 5) of CD300E expression (Fig 1).

Protein expression data obtained by flow cytometry for the above listed phenotypic markers was compared with mRNA expression levels for the corresponding genes, with fully consistent results (Fig 2). Thus, both *CD34* gene and protein expression were restricted to the first stage of maturation, while *CD300E* expression measured at both protein and mRNA levels, was only found among the more mature (stage 5) monocytes. In turn, *FCGR1A*, *CD14*, *CD36* and *PTPRC* showed parallel, progressively greater amounts in both their protein and mRNA expression levels from stage 3 precursors to mature (stage 5) monocytes (Fig 2). Of note, the increase in mRNA levels frequently preceded that observed in the amount of cell surface membrane protein (e.g. *CD14*/CD14 in Fig 2). Other classical pan monocyte-associated genes such as *LYZ* (lysozyme), *ITGAM* (previously termed *CD11b*) and *ITGAX* (previously termed *CD11c*) were also expressed from the earliest stages throughout the whole monocytic maturation pathway.

GEP along the maturation of BM monocytic precursors cells

Overall, 6067 genes out of 33 927 genes evaluated (18%) were expressed in one or more subsets of purified maturation-associated BM monocytic precursors. Of note, around half of these genes (3334/6607; 55%) were expressed across all monocytic precursor cell populations, whereas the other half showed expression restricted to one – stage 1 (247/6067; 4·1%), stage 2 (68/6067; 1·1%), stage 3 (141/6067; 2·3%), stage 4 (91/6067; 1·5%) or stage 5 (113/6067; 1·9%) – or frequently ≥2 (2073/6067; 34·1%) of the five distinct monocytic precursor cell subsets identified (Fig S1).

In order to identify differentiation-associated markers, subsequent analyses were focused on those 50 'top' genes that were found to be expressed at significantly different levels between two sequential stages of maturation (e.g. stage 1 *vs.* 2, stage 2 *vs.* 3, stage 3 *vs.* 4 and stage 4 *vs.* 5), as summarized in Table I. Of note, most of those 50 genes with greater differences in their expression levels between stage 1 and 2 monocytic precursors were upregulated (n = 41/50 genes); only a minority (n = 9/50 genes) were downregulated. Similarly, almost all genes that appear to modify significantly their expression between the subsequent stages 2 and 3 were also upregulated (n = 48/50 genes), with only two genes – *MCM5* and *DTL* – decreasing their expression between these maturation stages. In contrast, from those 'top' 50 genes differentially expressed in the transition between stages 3 and 4 of BM monocytic maturation, most (n = 45/50 genes) had decreased expression levels at stage 4 *vs.* 3, with only a minority being upregulated (n = 5/50 genes) genes. Finally, the transition between stage 4 monocytic precursors and mature (stage 5) BM monocytes was associated with downregulation of 30 of the 'top' 50 differentially expressed genes and upregulation of the other 20/50 genes (Table I).

Transcriptional profile of monocytic precursors along human BM maturation

Overall, the 6067 genes that were found to be expressed during monopoiesis could be classified into 12 distinct gene groups according to their biological function. These included genes involved in: (i) apoptosis, (ii) cell differentiation, (iii) cell response, (iv) cell signalling, (v) cell cycle, (vi) DNA/RNA processing, (vii) immune response, (viii) intracellular response, (ix) metabolic processes, (x) mitochondrial function, (xi) protein processing and (xii) structural cell organization.

Interestingly, a high number of the genes expressed in BM monocytic precursors that were functionally related with DNA/RNA processing and/or cell signalling were constantly expressed along the different stages of BM monopoiesis, although the number of expressed genes from the later functional group slightly increased from stage 2 onwards (Fig 3). Genes related to apoptosis and intracellular responses had a similar pattern of expression, but lower numbers of both groups of genes were expressed. In contrast, the number of genes associated with cellular response and differentiation, progressively increased from the earliest to the intermediate stages (stage 3) of monocytic maturation, remaining relatively constant (with minor variations) thereafter, until the stage of mature monocytes (stage 5). Finally, genes involved in structural cell organization and mitochondrial processes were almost constantly expressed at all distinct maturation stages, whereas those genes associated with cell cycle, immune response, metabolic processes and protein

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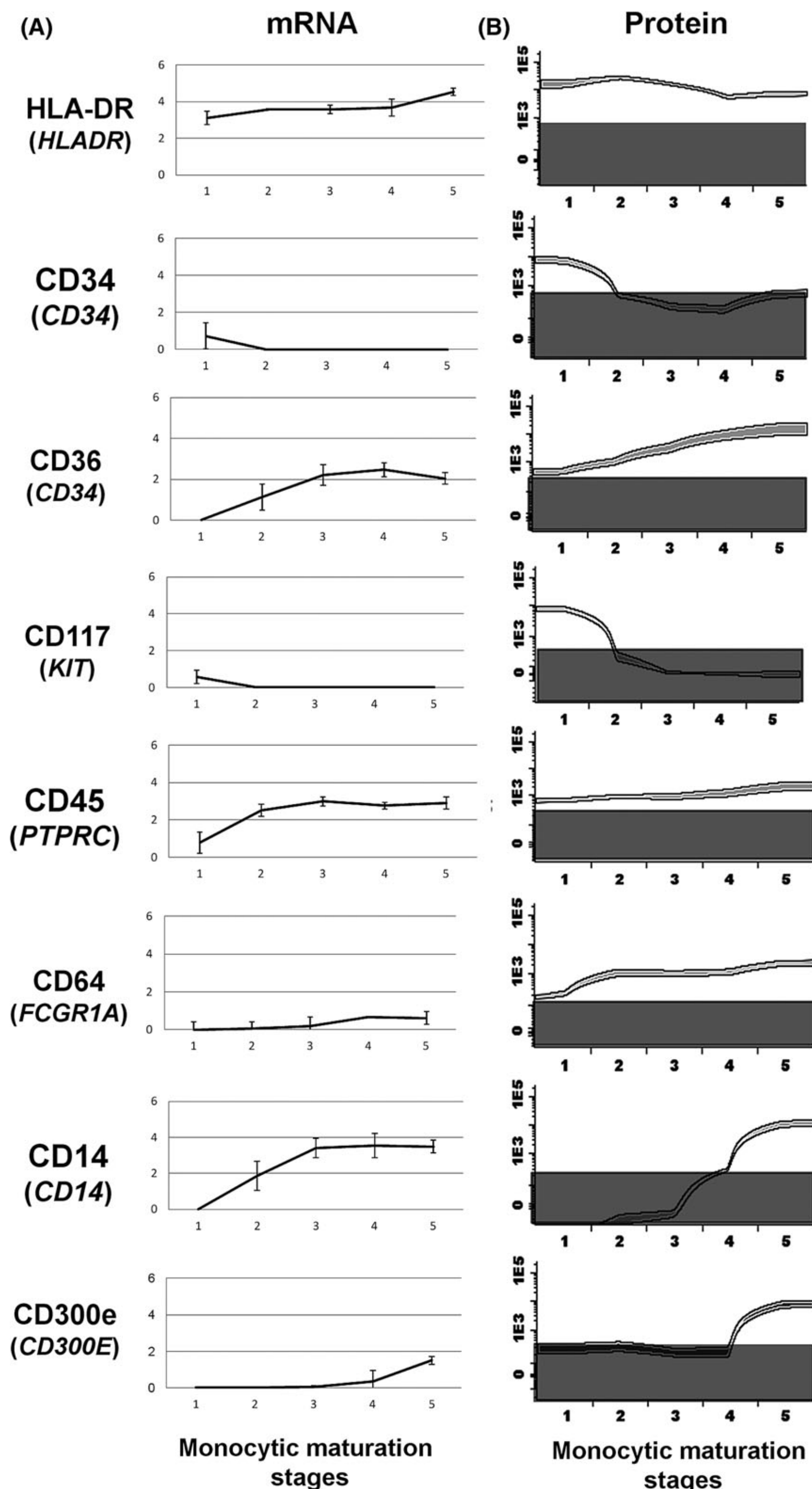

Fig 2. Pattern of expression of those proteins (and their corresponding mRNA levels) used to delineate the different stages of maturation of monocytic lineage cells in human bone marrow. (A) The intensity signal obtained by microarray analysis of gene expression profile mRNA levels of the seven immunophenotypic markers used to sort bone marrow monocytic cell precursors. (B) Median fluorescence intensity protein expression values, as assessed by multiparameter flow cytometry. The grey areas highlight regions defined as having no protein expression by flow cytometry.

processing displayed a bell curve with maximal expression at intermediate monocytic precursor cells – stage 3 – (Fig 3).

In order to better characterize the gene expression patterns found throughout monocytic precursors to mature monocytes, we investigated the GEP of the 50 genes that showed greater differences in their expression levels between adjacent stages of monocytic maturation in the BM. In addition, we specifically focused on the 150 genes that were associated with cell signalling, cell cycle, cell differentiation, immune response and DNA/RNA processing (Fig 4). Twenty-eight of these 150 genes (19%) were associated with cell differentiation; some of them (e.g. *PHGDH* and

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Table I. Top 50 differentially expressed genes throughout human bone marrow monocytic maturation by gene set enrichment analysis. Genes are grouped according to the precise stage of maturation at which they were upregulated (panel A) or downregulated (panel B) in the transition between two consecutive stages of monocytic maturation.

↑Stage 1–stage 2↓	↑Stage 2–stage 3↓	↑Stage 3–stage 4↓		↑Stage 4–stage 5↓	
SLC17A9	*MCM5*	*SAE1*	*MCFD2*	*MOSPD3*	*HIST1H2BM*
POLD2	*DTL*	*MCM5*	*XPOT*	*LGALS12*	*RNF24*
SPPL2B		*SPPL3*	*VDAC2*	*DYSF*	*PDSS1*
PRSS57		*MCM7*	*CASC4*	*MARC1*	*STIM1*
DNPH1		*TYMS*	*IMPDH2*	*GYS1*	*ZNF281*
CDK6		*MAP3K4*	*ARL5A*	*F5*	*YDJC*
ITM2C		*BABAM1*	*SF3B3*	*DGCR2*	*PADI4*
CXXC5		*SPTAN1*	*ANLN*	*SH3RF3*	
CTSW		*RAN*	*SLC17A9*	*HLX*	
		DHRS7	*UFD1L*	*ANO10*	
		AURKB	*SERINC2*	*SLCO4C1*	
		RFWD3	*STIP1*	*NCAPD2*	
		SMC1A	*RANBP3*	*SLC22A15*	
		CDK4	*KANSL3*	*VCL*	
		TBL1XR1	*SLC25A39*	*MAPK14*	
		IL27RA	*UHRF1*	*PSTPIP1*	
		TAF1	*PGGT1B*	*HIST1H2BH*	
		GALNT10	*CNOT6L*	*CA5BP1*	
		FAF1	*NNT*	*DNTTIP1*	
		ABCB10	*VPS13D*	*SLC2A9*	
		SMARCC1	*TESC*	*QPCT*	
		HIST1H3B	*PCED1A*	*HAL*	
		RETN		*CR1*	

↓Stage 1–stage 2↑		↓Stage 2–stage 3↑		↓Stage 3–stage 4↑	↓Stage 4–stage 5↑
SCPEP1	*PPP1R18*	*ACAA1*	*SIGIRR*	*SECTM1*	*SECTM1*
CTSB	*ITPK1*	*CD52*	*PPP1R3D*	*MAFB*	*MUSTN1*
SQSTM1	*CAPN2*	*GLTP*	*FURIN*	*TTYH2*	*ADGRE2*
CDC42EP3	*RHOB*	*PCED1A*	*MAPK8IP3*	*SGK1*	*FAM110A*
FBP1	*CKAP4*	*MAFB*	*MARCH1*	*SLC24A4*	*CPVL*
ZDHHC3	*TIMP1*	*ZNF710*	*CAMTA2*		*HIC1*
KRT18	*PTPN18*	*CXXC5*	*ICAM1*		*LGALS2*
MS4A6A	*MBD2*	*GRINA*	*FAM214B*		*HPS1*
NINJ1	*CD68*	*SIRT2*	*BCL3*		*HLADQA1*
GABARAPL1	*USP3*	*RHOF*	*ZFAND2B*		*CASC4*
CFP	*BRI3*	*REC8*	*CR1*		*HLADPB1*
HLX	*CES1*	*TNIP1*	*C5AR1*		*HSBP1*
C10ORF54	*RNF166*	*G0S2*	*ECE1*		*SLC881*
ARHGEF1	*IFI30*	*ERN1*	*SETD1B*		*AGPAT3*
MCOLN1	*TGFB1*	*FOXJ2*	*FHL3*		*RUNX3*
IQSEC1	*HEXB*	*ZNF385A*	*RARA*		*GPR77*
CSTA	*FGR*	*SGSH*	*BCR*		*LMF2*
ELF4		*GAA*	*ADAM8*		*TMEM110*
CAPG		*AES*	*TOLLIP*		*ALDH1A1*
INPPL1		*LFNG*	*C19ORF22*		*ZFP36L1*
MYO1G		*RIN2*	*KDM6B*		
USF1		*LPPR2*	*C19ORF60*		
RIOK3		*ZNF687*	*LPAR2*		
RNPEPL1		*SERPINA1*	*SELENOK*		

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ANPEP) were expressed only among the more immature monocytic precursors, while others [e.g. *C5AR2* (previously termed *GPR77*)] were detected only among mature monocytes, and a third, larger group of genes was upregulated their expression from stage 2 onwards (e.g. *VCAN*, *FGR*, *BCL6*).

Another 27% of these 150 genes (41/150) were associated with cell signalling pathways and showed a more heterogeneous GEP (Fig 4). Thus, some genes were upregulated at stage 2 and their expression remained similar until the more mature stage 5 monocytes (e.g. *IFI30*, *CD68* and the *HLA-DP* gene family), other genes were only expressed in stage 1 and/or stage 2 precursors (e.g. *SLC43A1*, *STMN1*, *RFWD3*, *SMARCC1*), a third group peaked at stage 3 (e.g. *ARL5A*) and a fourth group of genes showed expression restricted to the more mature stage 5 monocytes (e.g. *TTYH2*).

Of note, the above patterns are in contrast with that of cell cycle-related genes (34/150 genes; 23%) which presented two clearly distinct profiles: (i) one group of cell cycle-related genes were upregulated in the more immature BM monocytic precursors and their expression decreased thereafter (21/34 genes; 62%), including *MCM7*, *CDK4* and *CDK6*, while (ii) a second group of genes were upregulated at the more mature BM monocytic precursors (13/34 genes; 38%), including genes such as *G0S2*, *FURIN* and *TGFBI*. In turn, most of the genes associated with DNA/RNA processing (14/150 genes; 9%) were upregulated at different stages of monocytic maturation, increasing their levels thereafter (e.g. *MBD2*, *MAFB*), with only the *TBL1XR1* gene showing the opposite pattern (it was upregulated among the most immature monocytic precursors), and the expression of *PADI4* and *CAMTA2* peaking at the intermediate stage of maturation (stage 3) (Fig 4).

Regarding those genes associated with the immune response (33/150 genes; 22%), a heterogeneous GEP was observed. Thus, some genes were expressed throughout the monocytic maturation at variable levels (e.g. *HLA-DPA1*, *HLA-DPB1*, *MAPK14*), others were downregulated during monopoiesis (e.g. *CTSW*, *LRP8*, *STIP1*) and another group of genes showed expression restricted to a single maturation stage, typically an intermediate stage of maturation (e.g. *PSTPIP1*, *SIGIRR*, *CR1*).

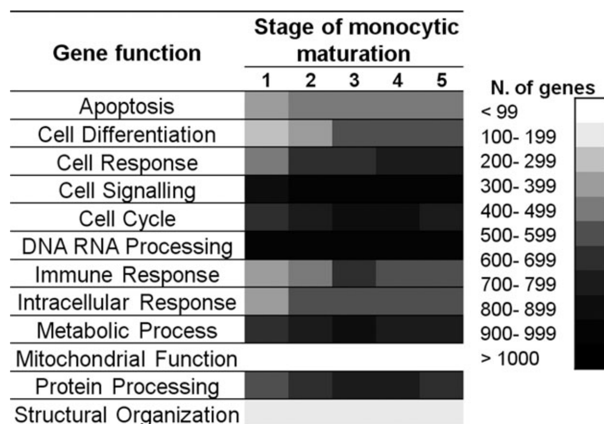

Fig 3. Overall gene expression profile of human bone marrow monocytic precursors at different stages of maturation according to the number of genes expressed, classified into distinct functional groups. In the right panel, colour codes indicate the distinct numbers of genes expressed per maturation stages and group of genes.

Discussion

Here we describe for the first time the GEP of normal human BM monocytic precursors at different stages of maturation. For this purpose we dissected the maturation of monocytic cells into five distinct maturation stages, based on the unique and distinct pattern of expression of 8 cell membrane protein markers, from CD34^{+} CD64^{high} monoblasts to CD14^{high} CD300E^{+} mature BM monocytes, following the criteria previously defined by the EuroFlow consortium (van Dongen *et al*, 2012; Kalina *et al*, 2012). Of note, the purity and identity of the distinct maturation-associated monocytic cell populations was also confirmed through parallel evaluation of the mRNA expression levels and the protein-based phenotypes for those markers that showed differential expression levels between distinct stages of maturation (e.g. *CD14*, *CD36*, *CD300E* and the *HLA-DR* gene family). The purity of the FACS-sorted monocytic populations was further confirmed by the lack of expression of markers (CD19, CD3 and TFRC) that are highly expressed in other non-monocytic BM cell compartments such as B, T and erythroid cells, respectively. Overall 6067 genes were expressed in one or more maturation-associated BM monocytic precursor subsets, such genes being functionally associated with 12 different major biological functions.

The dynamic equilibrium between proliferation/differentiation processes did not result in a difference in the number of cell cycle-associated genes expressed during different stages of BM monopoiesis and the overall amount of genes related to the cell cycle was maintained at rather stable quantities along the whole monocytic maturation. However, more detailed analysis of the specific genes expressed at each individual maturation stage showed that cell cycle inducer genes (e.g. *CDK4* and *CDK6*) were expressed during the earliest stages of maturation, whereas at later stages they were replaced by genes associated with cell cycle arrest [e.g. *CDKN1A* (also known as p21, *CIP1*, *WAF1*) and *CDKN1B* (also known as p27, *KIP1*)] that bind to *CDK4* and *CDK6*, respectively, leading to a reduced activity of both cyclins (Lee *et al*, 2007), and the *G0S2* and *MBD2* genes that are associated with repression/activation of transcription and regulation of cell proliferation and differentiation (Heckmann *et al*, 2013; Menafra & Stunnenberg, 2014) that showed increased levels expression along monopoiesis. Overall, a similar pattern of expression of these genes has been previously described during granulopoiesis (Klausen *et al*, 2004), suggesting that highly similar mechanisms regulate the expression and differentiation of monocytic and neutrophil

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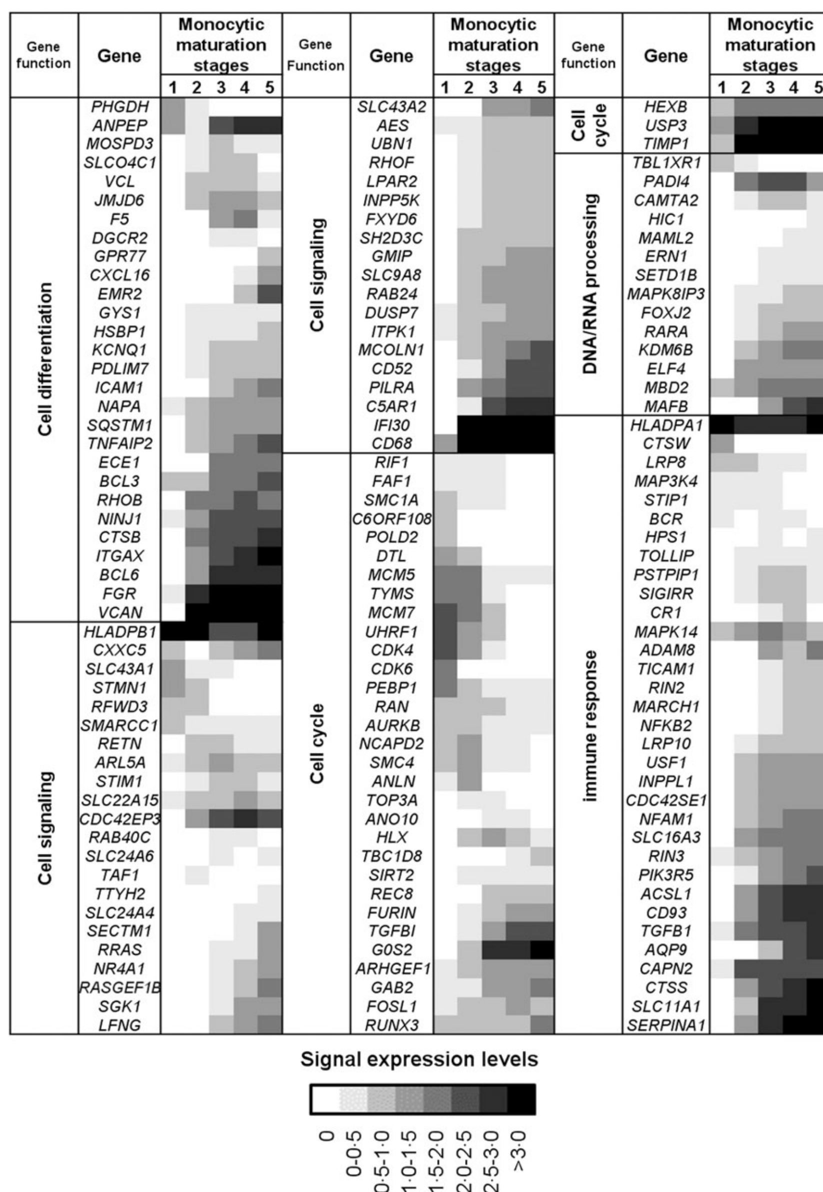

Fig 4. Gene expression profile observed per gene functional subgroup for the top 50 differentially expressed genes during monopoiesis, including gene associated with cell cycle- cell differentiation, cell signalling, DNA/RNA processing and immune response. The intensity of mRNA expression for individual genes per each stage of maturation is colour coded from the greatest expressing levels (black), to intermediate (grey) and lower (white) expression levels. Only those genes associated with five major biological functions (cell cycle, cell differentiation, cell signaling, DNA/RNA processing and immune response) are shown.

precursors during normal human BM haematopoiesis. In line with previous data from our group (Matarraz *et al*, 2015) the above results also suggest that proliferation of monocytic precursors would rapidly decrease after commitment of CD34$^+$ precursors to the earlier stages of monocytic maturation, allowing for further differentiation of monoblasts toward mature monocytes.

A common pattern of expression was found during monopoiesis for many cell differentiation-associated genes. Thus, a relatively high number of such genes showed increased expression from the intermediate stages of monocytic maturation (e.g. stages 2 and 3) onwards, suggesting that stage 2–3 monocytic precursors acquire a marked GEP of differentiation towards the monocytic lineage. Among other genes, *VCAN* and *FGR*, which play a role in the regulation of cell motility, growth and differentiation, as well as in cytoskeleton remodelling in response to extracellular stimuli, phagocytosis, cell adhesion and migration, respectively (Hatakeyama *et al*, 1994; Potter-Perigo *et al*, 2010), were strongly upregulated during monopoiesis, from stage 3 onwards. Of note, only a few of all strongly differentially expressed genes appeared to be reliable monocytic differentiation-associated markers such as *CD68* and the *HLA-DP* gene family; this further confirms the utility of those markers together with *FCGR1A*, *LYZ*, *ITGAM* and *ITGAX* for the sequential identification of monocytic lineage cells, already at the earliest stages of monopoiesis (Njoroge *et al*, 2001; Nicholson *et al*, 2007; Nuutila *et al*, 2007; Georgakopoulos *et al*, 2008; Iqbal *et al*, 2014).

As should be expected, many genes associated with the immune response that were expressed during monopoiesis were upregulated during the course of BM monocytic maturation. An example of these genes is *ADAM8* (*CD156*), a gene that codes for a membrane anchored cell surface adhesion

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protein, involved in cell–cell interactions and diapedesis, which are key functions of mature monocytes/macrophages (Higuchi *et al*, 2002). Despite this, another group of genes associated with the immune response, such as *CTSW* and *LRP8*, were expressed already at the earliest stages of maturation but decreased their expression levels afterwards.

Overall, the above results indicate that the earlier stages of human BM monocytic precursors show a proliferation-associated GEP with multiple genes involved in promoting cell cycle and potentially also the expansion of immature progenitor cells, whereas subsequent maturation was progressively associated with cell cycle arrest and increasing differentiation-associated monocytic GEP. Of note, genes involved in DNA/RNA processing, mitochondrial function and structural cell organization, were constantly expressed along monopoiesis indicating that they play a role in key cellular functions required at any stage of maturation.

Despite the above, it should be noted that the genes associated with DNA/RNA processing also showed some differential patterns of expression. Thus, the *TBL1XR1* gene was expressed at the earliest stages of monocytic maturation. The protein coded by this gene forms a complex with the endogenous Nuclear receptor corepressor 1, and this complex represses the transcription of factors such as *RARA* (retinoic acid receptor alpha (RARalpha) (Tomita *et al*, 2003). In contrast, expression of the *MAFB* gene only became detectable in monocytic stage 3 precursors, remaining upregulated thereafter. Of note, *MAFB* acts as a transcription factor that plays an important role in inducing differentiation of human $CD34^{+}$ cells into monocytic precursors (Gemelli *et al*, 2008), in line with our findings.

Some genes associated with cell signalling functions were expressed early during monocytic maturation, such as *CD68*. As mentioned above, *CD68* has long been used to identified monocyte/macrophage cells by e.g. immunohistochemistry in different normal and altered tissues (Cojocaru *et al*, 2012); CD68 is a member of the lysosome-associated membrane protein (LAMP) family and plays a role in phagocytic activities associated with cell–pathogen interactions at, for example, the intracellular lysosomal level (Holness & Simmons, 1993). Another gene which showed a similar pattern of expression to *CD68* was *IFI30*. Previous studies have shown that *IFI30* is expressed by haematopoietic cells and that it is involved in the generation of major histocompatibility complex class II-restricted peptides, suggesting it would play a critical role in antigen-presentation by monocytic cells (Cresswell *et al*, 1999) and early induction of these receptors, as also found here. In contrast to *CD68* and *IFI30*, the *TTYH2* gene was only expressed in matured BM monocytes. *TTYH2* has been shown to play a role in calcium signal transduction (He *et al*, 2008), in different cell types, its increased expression being associated with cell proliferation and cell aggregation (Toiyama *et al*, 2007); further studies are required to investigate its role in mature monocytes.

Overall, the information provided here regarding the GEP associated with the normal human BM monocytic maturation provides also a frame of reference for a better understanding and classification of monocytic disorders. Thus, they might contribute to a better definition of distinct subtypes of monocytic leukaemias (e.g. acute monoblastic vs monocytic leukaemia vs chronic myelomonocytic leukaemia) (Kern *et al*, 2011; Arber *et al*, 2016) and to better understand the impact of genetic alterations in monocytes from patients with primary immunodeficiencies (e.g. chronic granulomatous disease) (Leiding & Holland, 2012; Roos *et al*, 2010) and lysosomal storage diseases involving the monocyte-macrophage system (e.g. Gaucher disease) (Florena *et al*, 1996; Lisi *et al*, 2016).

In summary, we provide for the first time, detailed information about the GEP of human monocytic precursor cells during their maturation in normal BM. This information contributes to a better understanding of monocytic differentiation, and might also contribute to the identification of novel monocytic candidate markers, providing a frame of reference for the study of monopoiesis in both neoplastic and non-neoplastic disease conditions involving monocytic cells.

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Conflicts of interest

The authors declare no conflicts of interest.

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Author contributions

FVM, ESC, LRA and AO conceived and designed the experiments. FVM, MLS and PB performed the flow cytometry cell sorting experiments. FVM and CT performed all others experiments. FVM, RTP, CEP and MGPL analysed the data. FVM, LRA, ESC and AO wrote the paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Scatterplot graphical representation of the pairwise comparison of the gene expression levels detected between two consecutive stages of maturation of human BM monocytic precursor cells.

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