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Differential gene expression patterns coupled to commitment and acquisition of phenotypic hallmarks during neutrophil differentiation of human leukaemia HL-60 cells

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ABSTRACT

We have identified sequential changes in the gene expression pattern of human myeloid leukaemia HL-60 cells during their neutrophil differentiation induced by dimethyl sulfoxide (DMSO) that account for the acquisition of mature neutrophil phenotypic hallmarks. Following 1-day DMSO treatment, HL-60 cells were committed to neutrophil differentiation with loss of their proliferative capacity, and gene expression changes were mostly related to transcription and cell cycle, including up-regulation of inhibitor of DNA binding (Id) genes and down-regulation of cyclins, CDC kinases and MCM proteins. After 3–4-day DMSO treatment, zinc finger proteins 266 and 51 (BCL-6) were dramatically up-regulated, and additional gene expression changes, involving functional and signalling proteins as well as genes involved in RNA processing, apoptosis, and protein degradation, correlated with acquisition of the mature neutrophil phenotype. Our data define changes in the gene expression pattern of a rather restricted number of genes during the differentiation process that seem to regulate neutrophil maturation, providing a molecular basis for the acquisition of neutrophil phenotype. Peripheral blood mature neutrophils showed a qualitatively similar expression profile for these selected genes. These results show changes in gene expression during the transformation of a proliferating leukaemic cell into an end apoptotic-prone cell, which might be of importance to get a molecular insight for the use of differentiation therapy in leukaemia.

1. Introduction

The genetic lesions of leukaemia result in a blockage of cell differentiation that allows leukaemic cells to continue to proliferate and prevents the terminal differentiation and apoptosis of normal leukocytes. Differentiation therapy is an anti-neoplastic strategy that tries to overcome the maturation arrest of leukaemic cells and it has received increasing attention due to the remarkable activity of all-*trans* retinoic acid as a differentiation-inducing drug in patients with acute promyelocytic leukaemia (APL) (Huang et al., 1988; Tallman, 1996; Chen et al., 2007). Myeloid leukaemia cells established from patients with either acute myeloid leukaemia (AML) or the blastic phase of chronic myeloid leukaemia (CML) are arrested at different stages of cell maturation, and have been widely used as models of myeloid differentiation. The human acute myeloid leukaemia HL-60 cell line was derived from a patient initially considered to have acute promyelocytic leukaemia (APL) (Collins et al., 1977), but showed atypical clinical features, including lack of the t(15:17) chromosomal translocation that represents a cytogenetic hallmark for APL. Further studies reclassified the leukaemia from which the HL-60 cells were derived as an AML with maturation (Dalton et al., 1988). HL-60 cells can be induced to differentiate *in vitro* towards the polymorphonuclear neutrophil lineage following dimethyl sulfoxide (DMSO) treatment (Collins, 1987; Mollinedo et al., 1998). Neutrophil maturation of HL-60 cells is somewhat defective, shown by failure in forming specific/secondary granules, and by qualitative and quantitative differences in gene expression between neutrophil-differentiated HL-60 cells and peripheral blood neutrophils (Collins, 1987; Mollinedo et al., 1992; Itoh et al., 1998). However, despite these drawbacks, differentiated HL-60 cells share many functional features with peripheral blood mature neutrophils, and therefore HL-60 cells are an invaluable tool in different areas of neutrophil biology, including

Abbreviations: DMSO, dimethyl sulfoxide; C/EBP, CCAAT/enhancer binding protein; HSP, heat shock protein; HSP70B, heat shock 70 kD protein 6; Id, inhibitor of DNA binding; SLR, signal log ratio; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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neutrophil differentiation and function (Gahmberg et al., 1979; Newburger et al., 1979; Collins, 1987; Collado-Escobar and Mollinedo, 1994; Mollinedo et al., 1998; Hauert et al., 2002).

A number of studies have analysed the modulation and role of one or few genes at a time in the neutrophil differentiation process. In this regard, transcription factor C/EBP (CCAAT/enhancer binding protein) has been shown to regulate neutrophil maturation (Chih et al., 1997; Lekstrom-Himes et al., 1999; Lekstrom-Himes, 2001). However, our understanding of the global mechanism occurring during neutrophil differentiation is still sketchy, and the precise mechanism of neutrophil differentiation as well as the major underlying gene expression changes remains uncertain. The complexity and diversity of the processes involved, and the existence of mechanisms still unknown, make it difficult to identify critical factors in neutrophil differentiation and lead to the need to use a high-throughput technology able to evaluate global gene expression patterns. Previous cDNA microarray analyses during 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation towards monocytic lineage (Seo et al., 2000; Zheng et al., 2002) and all-*trans* retinoic acid-induced differentiation towards neutrophils (Lee et al., 2002) of HL-60 cells, as well as an oligonucleotide microarray analysis during monocytic TPA-induced HL-60 differentiation (Tamayo et al., 1999) have highlighted the complexity of the differentiation process. However, microarray studies using DMSO-induced HL-60 differentiation and analyses correlating gene expression patterns with acquisition of neutrophil phenotypic hallmarks have not yet been performed. Treatment of HL-60 cells with DMSO leads to a gradual neutrophil differentiation process that entails longer incubation times than retinoic acid-induced differentiation (Mollinedo et al., 1998), and thereby allows a more precise differential analysis of gene expression patterns coupled to the acquisition of distinct neutrophil features at different neutrophil differentiation stages. Here, we have analysed the differential gene expression patterns during DMSO-induced neutrophil differentiation of HL-60 cells using oligonucleotide microarrays, and we have coupled changes in gene expression profiles with the distinct phenotypic characteristics acquired by neutrophil differentiating HL-60 cells.

2. Materials and methods

2.1. Cell culture and neutrophil isolation

HL-60 cells were cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. HL-60 cells were induced to differentiate towards the neutrophil lineage by incubation with 1.3% (v/v) DMSO (Mollinedo et al., 1998). Neutrophils were obtained from fresh human peripheral blood by dextran sedimentation and Ficoll-Hypaque centrifugation, followed by hypotonic lysis of residual erythrocytes as previously described (Santos-Beneit and Mollinedo, 2000). The final cell preparation contained more than 99% neutrophils, as assessed by May–Grünwald–Giemsa staining.

2.2. Immunofluorescence flow cytometry

Cell surface expression of CD11b antigen was analysed by immunofluorescence flow cytometry as previously described (Mollinedo et al., 1992), using the anti-CD11b Bear-1 monoclonal antibody and the P3X63 myeloma culture supernatant, as a negative control (kindly provided by Dr. F. Sánchez-Madrid, Hospital de la Princesa, Madrid, Spain).

2.3. Proliferation assay

Cells (10^5/ml) were incubated in 96-well plates with 250 μl of culture medium in the absence and in the presence of DMSO, and pulsed with 0.5 μCi of [^{3}H]thymidine/ml for 12 h, before harvesting in glass-fibre filters, and [^{3}H]thymidine incorporation was measured in a liquid-scintillation counter (Collado-Escobar and Mollinedo, 1994). Results were expressed as percentage of proliferation with respect to control values for untreated cells (100%).

2.4. Secretion assay

Cells (10^6/ml) were preincubated with 5 μg/ml cytochalasin B in HEPES/glucose buffer (150 mM NaCl, 10 mM HEPES, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM glucose, pH 7.5) for 5 min at 37 °C, transferred to tubes containing 20 ng/ml TPA and incubated for a further 10 min at 37 °C. Then, the tubes were placed on ice and centrifuged at 300 g for 8 min. Supernatants were saved and assayed for *N*-acetyl-β-glucosaminidase activity, as previously described (Mollinedo, 1986; Collado-Escobar and Mollinedo, 1994). Results were expressed as percentage of enzyme release with respect to total enzyme content, and corrected for enzyme release from unstimulated cells.

2.5. Apoptosis assay

The induction of apoptosis was monitored as the appearance of the sub-G_1 peak in cell cycle analysis (Gajate et al., 2000), using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA). Quantitation of apoptotic cells was calculated as the percentage of cells in the sub-G_1 region (hypodiploidy) in cell cycle analysis.

2.6. Respiratory burst activity

The generation of superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* in a discontinuous assay upon TPA stimulation (Mollinedo et al., 1991a). Superoxide anion generation is expressed as nmol of cytochrome *c* reduced per 10^6 cells per 10 min.

2.7. Affymetrix microarray analysis

We analysed RNA samples prepared from 3 independent experiments for each experimental condition, namely resting peripheral blood human neutrophils, untreated HL-60 cells as well as HL-60 cells treated with 1.3% (v/v) DMSO for 1, 2, 3, and 4 days. Total RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA). RNA integrity was assessed with an AGILENT 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using a RNA Nano LabChip (Agilent Technologies). In this way, we found that our RNA preparations were free of DNA contamination. Double-stranded cDNA was synthesized from 15 μg of total RNA by means of the SuperScript™ double-stranded cDNA synthesis kit (Invitrogen, San Diego, CA) with oligo$(dT)_{24}$ primer containing T7 RNA polymerase promoter. *In vitro* transcription was carried out using the Bioarray High Yield RNA Transcript Labelling kit (Enzo Diagnostics, Farmingdale, NY) with biotinylated cytidine triphosphate and uridine triphosphate. The biotin-labelled complementary RNA (cRNA) was purified with an RNeasy column and fragmented at 94 °C for 35 min in fragmentation buffer (40 mM Tris acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). Integrity of cDNA, cRNA, and fragmented cRNA was assessed by electrophoresis of the samples on 1% agarose gels.

Microarray RNA analysis was performed according to the manufacturer's protocol using Human Genome U95Av2 GeneChip oligonucleotide arrays (Affymetrix, Santa Clara, CA), that contain 12,625 sets of oligonucleotide probes, representing about 12,000 full-length genes. Additional microarray analyses were conducted with Human Genome U133A arrays (Affymetrix, Santa Clara, CA), that contain more than 22,000 probe sets, representing 18,400 transcripts, including 14,500 well-characterized human genes. Chips were stained and washed using an Affymetrix fluids station and following the

manufacturer's protocol. Chips were scanned with the HP Agilent GeneArray Scanner and values were normalized and analysed with the Affymetrix Microarrays Suite 5.0 (MAS5.0) software (U95Av2 chips) or the dChip1.3 software (U133A chips). Using MAS5.0 software, arrays were scaled to an average intensity of 100 using 1 as normalization factor and analysed independently, using the untreated control samples as baseline. We compared average "signal" values (representative values of absolute intensity) from samples treated with 1.3% (v/v) DMSO for 1, 2, 3 and 4 days in triplicates using as baseline the average value of day 0 (untreated), and obtained a log number (base 2) called "signal log ratio" (SLR) algorithm, which is a quantitative measure of relative change in transcript abundance. The SLR algorithm measures the magnitude and direction of change between transcript levels of the experimental and baseline chips. The use of logs in the analysis between the probe sets eliminates difficulties caused by one very high data point in the set masking information from lower valued data points. SLR is related to the fold change by the following formula:

$$\text{Fold change} = 2^{SLR} \text{ for SLR} \geq 0$$

$$\text{Fold change} = (-1) \times 2^{-SLR} \text{ for SLR} < 0.$$

Using the Data Mining Tool 3.0 software (Affymetrix), genes with a "Signal" value higher than 30 were considered up- or down-regulated if the expression was changed at least two-fold (SLR values higher than 1 or lower than −1) from the control. Data with a low signal intensity (<30) and high variability among experiments (>15%) were eliminated. The selected genes were subjected to cluster analysis according to their expression levels in the different incubation times using a hierarchical clustering with average linkage method and uncentred correlation through Cluster and TreeView software programs (Eisen et al., 1998), and analysed using the Gene Ontology Mining Tool. When dChip1.3 software was used, a stringent requirement included a gene's expression level within replicate arrays to be consistent, in terms of median "standard deviation/mean" ratio of all replicate groups where this gene is called "present". Only values with a 0 < median (standard deviation / mean) < 0.025 variation within triplicate assays were considered. This filtering excludes genes that are inherently variable during sample amplification and hybridization stages, as manifested in inconsistent expression values between replicate arrays.

2.8. RT-PCR, cDNA cloning and sequencing

Total RNA was prepared from cells using a RNeasy kit and RNA integrity was assessed as above with an AGILENT 2100 Bioanalyzer using a RNA Nano LabChip. For the RT reactions, total RNA (10 μg) was primed with oligo(dT) and reverse-transcribed into cDNA with 30 units of M-MLV reverse transcriptase (Promega, Madison, WI), according to the manufacturer's instructions, in a final volume of 20 μl. The mixture was incubated at 37 °C for 2 h. The generated cDNA was used for the semiquantitative RT-PCR analysis to assess mRNA expression as previously described (Mollinedo et al., 1997; Santos-Beneit and Mollinedo, 2000), and the β-actin gene was used as an internal control. A 25-μl PCR mixture contained 1 μl of the RT reaction, 10 pmol of each primer, each dNTP (0.2 mM), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of EcoTaq DNA polymerase derived from *Thermus aquaticus*. PCR reactions were performed in a GeneAmp PCR System model 9600 (Perkin Elmer, Norwalk, CT). The primers used are listed below, where the nucleotide numbers indicate the primer location in the corresponding sequences of human origin obtained from the GeneBank/EMBL database:

β-actin (accession number: X00351)
(forward; nt 936–955) 5′-CTGTCTGGCGGCACCACCAT-3′
(reverse; nt 1170–1189) 5′-GCAACTAAGTCATAGTCCGC-3′
A1 (accession number: U27467)
(forward; nt 65–86) 5′-AGGCTGGCTCAGGACTCTCTGC-3′
(reverse; nt 544–568) 5′-TTCTGGTCAACAGTATTGCTTCAGG-3′
caspase-1 (accession number: U13700)
(forward; nt 6–25) 5′-CGACAAGGTCCTGAAGGAGA-3′
(reverse; nt 1062–1081) 5′-CATCTGGCTGCTCAAATGAA-3′
caspase-3 (accession number: U26943)
(forward; nt 340–361) 5′-TTTGTTTGTGTGCTTCTGAGCC-3′
(reverse; nt 720–739) 5′-ATTCTGTTGCCACCTTTCGG-3′
VAMP-4 (accession number: AF044310)
(forward; nt 182–201): 5′-ATGCCTCCCAAGTTTAAGCG-3′
(reverse; nt 587–607): 5′-TCAAGTACGGTATTTCATGAC-3′
VAMP-5 (accession number: NM_006634)
(forward; nt 58–78) 5′-ATGGCAGGAATAGAGTTGGAG-3′
(reverse; nt 389–399) 5′-GTCAGTTCCCAGGCCCTGAG-3′

Primers were designed using the Primer3 program (S. Roze and H.J. Skaletsky, Whitehead Institute for Biomedical Research, MIT Center for Genome Research, Boston, MA). The conditions for PCR amplification of cDNA were as follows: 1 cycle at 95 °C for 5 min as an initial denaturation step; then, denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 90 s (the number of cycles was 26 to 30, depending on the linear range); followed by further incubation for 15 min at 72 °C (1 cycle). The annealing step was carried out at 60 °C (caspase-1), 64 °C (caspase-3), 65 °C (VAMP-4, VAMP-5, β-actin), or 66 °C (A1). These experimental conditions were shown to be at the linear phase of amplification for each gene. After amplification, an aliquot of the PCR reaction was size fractionated onto a 2% agarose gel in 1×TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) and checked for the expected PCR products. The PCR products were cloned into the pCR™ 2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA), and DNA sequencing was performed on both strands from 10 independent cDNA clones using a PE Applied Biosystems 377 DNA sequencer (Perkin Elmer).

3. Results

3.1. Acquisition of phenotypic neutrophil hallmarks during DMSO treatment of HL-60 cells

The human myeloid leukaemic HL-60 cell line can be differentiated to cells showing many of the features of mature neutrophils following treatment with 1.3% (v/v) DMSO. This differentiation process was characterized by an increase in CD11b cell surface expression, loss of the proliferative capacity, and acquisition of a variety of functional characteristics of mature neutrophils, including generation of superoxide anion and secretion of lysosomal enzymes and (Fig. 1). Fully neutrophil-differentiated HL-60 cells after treatment with DMSO for 3–4 days acquired most of the functional features of mature neutrophils and were ready to undergo spontaneous apoptosis (Fig. 1), like their physiologic mature neutrophil counterparts (Santos-Beneit and Mollinedo, 2000). In addition, previous reports have shown that DMSO-induced progressive morphologic neutrophil differentiation as well, with the highest proportion of metamyelocyte and neutrophil morphology after 4-day DMSO treatment (Gahmberg et al., 1979). HL-60 cells treated with DMSO for 24 h followed by exhaustive washing, and then cultured in DMSO-free culture medium for additional 48 h, showed the acquisition of the same neutrophil differentiation markers as those HL-60 cells treated with DMSO for 72 h (data not shown), corroborating our previous finding that 1-day DMSO treatment is sufficient to commit HL-60 cells to neutrophil differentiation (Mollinedo et al., 1993). Thus, neutrophil differentiation of HL-60 cells following DMSO treatment follows a series of stages that include: commitment (1-day treatment), loss of proliferative capacity (1- and 2-day treatment), acquisition of mature neutrophil functions (3- and 4-day treatment), and cell death by apoptosis (5-day treatment).

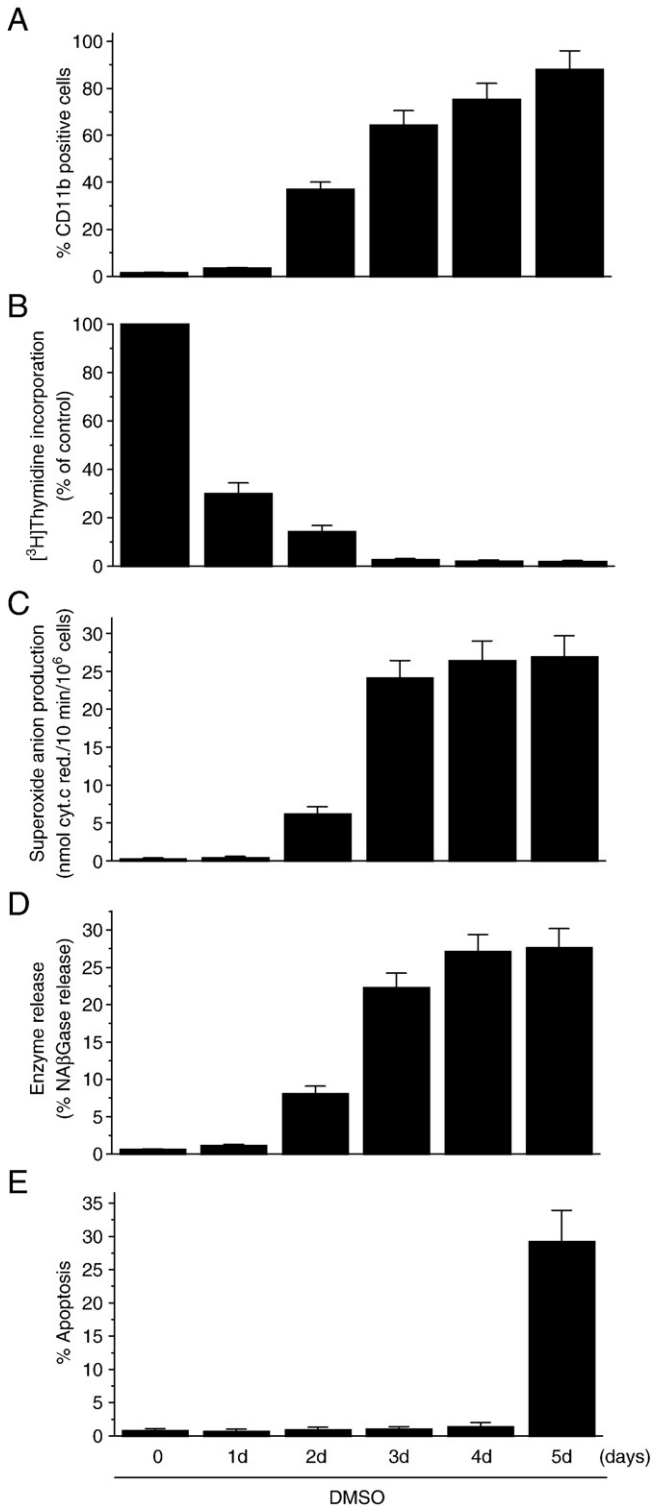

Fig. 1. Functional analysis of HL-60 cell differentiation towards the neutrophil lineage induced by 1.3% (v/v) DMSO. The following parameters were examined every day during the 5-day DMSO treatment of HL-60 cells: CD11b cell surface expression (A), cell proliferation (B), superoxide anion generation (C), secretion (D) and apoptosis (E). Values in A–D referred to viable cells. Untreated control cells were run in parallel. Data are shown as the means of three independent experiments ± S.D.

3.2. Changes in gene expression related to transcription, cell cycle, proliferation and RNA processing during neutrophil differentiation of HL-60 cells

We next analysed changes in gene expression patterns during the whole neutrophil differentiation process by oligonucleotide microarrays in order to couple gene expression profiles with the acquisition of the above-mentioned neutrophil phenotypic hallmarks. We searched for those genes that showed over a two-fold change in their expression in at least one incubation time with DMSO compared to untreated control cells. We have found that 962 out of the 12,625 gene probes were up- or down-regulated during neutrophil differentiation of HL-60 cells (Supplementary Fig. S1). Most dramatic gene expression changes occurred during the irreversible commitment stage towards neutrophil differentiation (1-day DMSO treatment). Major gene expression changes during DMSO-induced neutrophil differentiation of HL-60 cells corresponded to genes involved in transcription, cell cycle and proliferation, RNA processing, antigens and receptors, superoxide anion generation, exocytosis, function and signalling, cytokines, and apoptosis (Figs. 2 and 3).

Table 1 shows a list of genes whose expression change is ≥8-fold during neutrophil differentiation of HL-60 cells. A more complete list of genes, whose expression change is at least 2-fold in most of the DMSO incubation times, is displayed in Supplementary Table S1.

A large number of transcription-related genes were up- and down-regulated during neutrophil differentiation of HL-60 cells (Fig. 2, Table 1, and Supplementary Table S1). We found a relatively higher number of up-regulated genes, including distinct inhibitor of DNA binding (Id) genes, zinc finger proteins, jun B, and ring finger protein 13. The inhibitor of DNA binding 1 gene (Id1) was dramatically up-regulated (15-fold) during the first day of DMSO treatment, and thereafter its relative expression was highly diminished. Id2 was up-regulated during the whole neutrophil differentiation process, its highest expression occurring after a 3-day DMSO treatment (8-fold). A number of zinc finger proteins resulted up-regulated during neutrophil differentiation, especially during the third day of DMSO treatment, suggesting a role for these genes in the acquisition of the mature neutrophil phenotype. Zinc finger protein 266 and zinc finger protein 51 (BCL6), showed the highest up-regulation levels (40 and 20-fold, respectively at 3-day DMSO incubation) among all the transcription-related genes analysed. In contrast, c-myc was strongly down-regulated (13-fold) since the first day of DMSO treatment, in agreement with previous data (Watanabe et al., 1985). In our microarray study, we detected a high increase in C/EBPδ (5-fold) and C/EBPε (6-fold) after a 3-day DMSO treatment, supporting a role of these transcription factors in the late events of neutrophil differentiation, as previously reported (Lekstrom-Himes, 2001). Interestingly, we found that RNA polymerase I was dramatically down-regulated by day 3 of DMSO treatment (11-fold). Because RNA polymerase I transcribes 18S and 28S genes, its down-regulation could explain the low macromolecule biosynthetic capacity of neutrophils. Concerning the expression of genes coding for transcription factors, we can distinguish several time-dependent gene expression changes that could be relevant for the whole neutrophil differentiation process (Supplementary Table S1), namely: a) transcription factors whose expression was altered in the commitment stage of HL-60 differentiation towards neutrophils (1-day DMSO treatment), especially Id1 and Id2 genes; b) those whose expression was altered in the late stages of neutrophil maturation (3- and 4-day DMSO treatment), such as Id2, zinc finger protein 266, BCL-6, C/EBPδ and C/EBPε; and c) those whose expression was modified along the whole neutrophil differentiation process, such as jun B and ring finger protein 13.

Most of the genes related to cell cycle, DNA replication and cell proliferation were down-regulated during DMSO-induced neutrophil differentiation of HL-60 cells, including cyclins, CDC kinases, and MCM proteins (Fig. 2, Table 1, and Supplementary Table S1). In contrast, genes

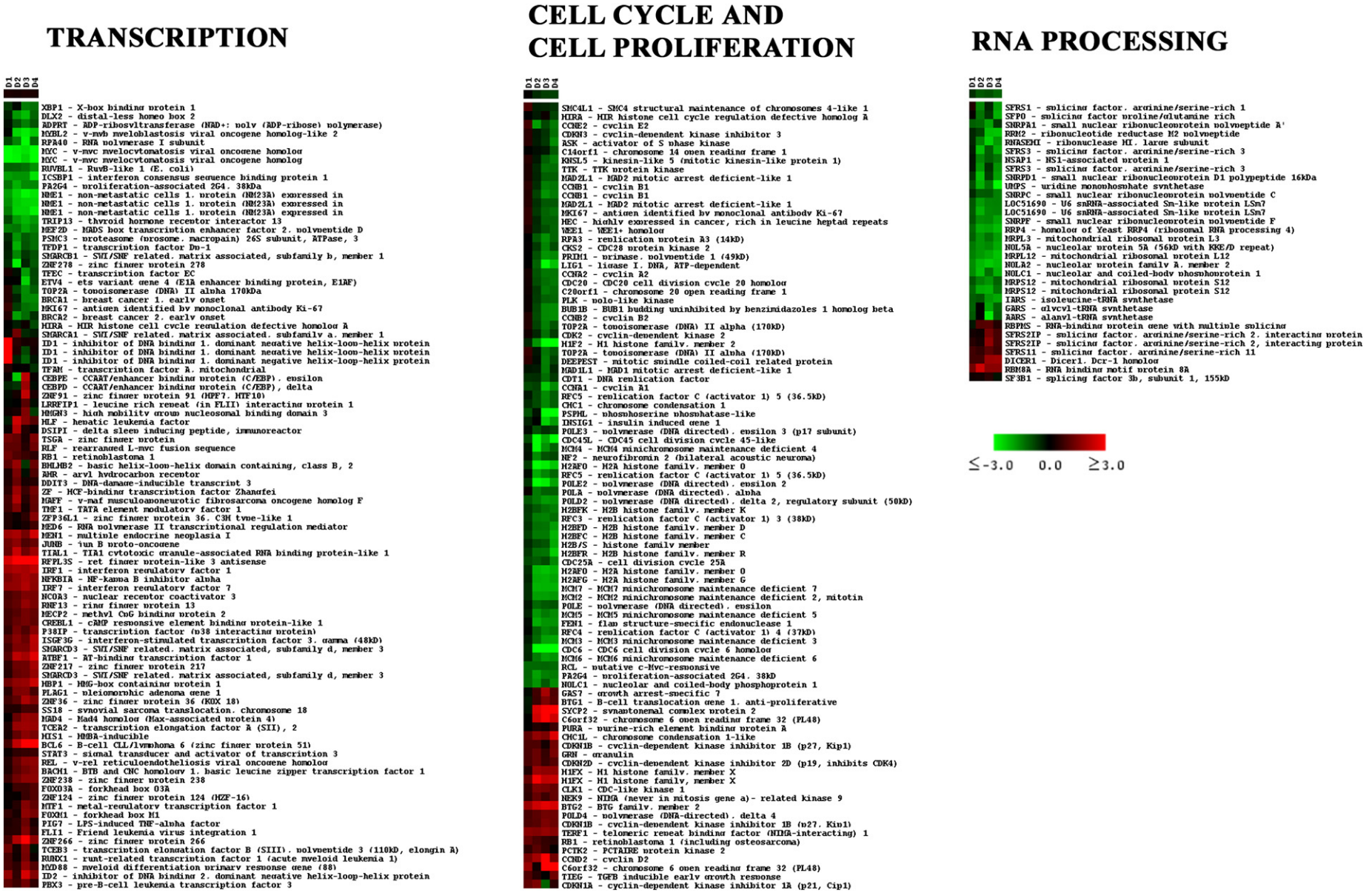

Fig. 2. Functional classification of genes modulated in DMSO-treated HL-60 cells. Genes were clustered hierarchically into three groups according to their respective cellular role: transcription, cell cycle and cell proliferation, and RNA processing. For each gene, the ratio of mRNA levels in HL-60 cells at the indicated time after DMSO treatment (D1–4, days 1–4) to mRNA levels in untreated HL-60 cells is represented by color and ranges from highly increased (intense red) to highly decreased (intense green), according to the color scale at the bottom right.

Fig. 3. Functional classification of genes modulated in DMSO-treated HL-60 cells. Genes were clustered hierarchically into six groups according to their respective cellular role: function and signalling, apoptosis, antigens and receptors, superoxide anion generation, exocytosis, and cytokines. For each gene, the ratio of mRNA levels in HL-60 cells at the indicated time after DMSO treatment (D1-4, days 1–4) to mRNA levels in untreated HL-60 cells is represented by color as in Fig. 2.

Table 1
Table 1 Major gene expression changes in DMSO-induced differentiation of HL-60 cells and peripheral blood human neutrophils

Gene name	Gene symbol	SLR over uHL-60				Relative change over uHL-60	GenBank accession no.	Biological role
		HL-60+DMSO						
		1 d	2 d	3 d	4 d	PMN		
B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	0.95	2.69	4.31	3.87	↑↑↑	U00115	Transcription, inflammatory response
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	3.89	0.22	−0.67	0.12	≈	X77956	Transcription
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	2.04	0.71	3.00	1.85	↑	D13891	Transcription
Interferon consensus sequence binding protein 1	ICSBP1	−2.2	−2.1	−2.09	−3.19	↓↓	M91196	Repression of transcription
RNA polymerase I subunit	RPA40	−1.03	−1.87	−3.51	−1.44	↓	AF008442	Transcription
v-myc myelocytomatosis viral oncogene homolog	MYC	−3.64	−3.38	−3.18	−3.88	↓↓↓	V00568	Transcription
Zinc finger protein 266	ZNF266	0.91	2.48	5.31	1.99	≈	AA868898	Transcription
BTG family, member 2	BTG2	2.34	3.15	3.49	3.30	↑↑↑	U72649	Negative control of cell proliferation, DNA repair
CDC45 cell division cycle 45-like	CDC45L	−1.16	−5.09	−2.64	−4.43	↓↓	AJ223728	DNA replication
CDC6 cell division cycle 6 homolog	CDC6	−0.69	−4.16	−5.05	−5.59	↓	U77949	Cell cycle, DNA replication
Chromosome 6 open reading frame 32 (PL48)	C6orf32	1.17	1.73	5.28	3.83	↑↑↑	U49187	Cell cycle
Chromosome condensation 1-like	CHC1L	1.41	2.24	1.90	3.42	↑	AF060219	Chromosome condensation
Cyclin A1	CCNA1	−1.17	−1.00	−3.09	−2.13	≈	U66838	Cell cycle
MCM4 minichromosome maintenance deficient 4	MCM4	−0.43	−4.68	−2.25	−1.51	↓↓↓	X74794	DNA replication, cell cycle
Polymerase (DNA directed), epsilon 2	POLE2	−0.81	−4.01	−3.69	−2.53	↓↓	AF025840	DNA replication
Synaptonemal complex protein 2	SYCP2	−0.31	3.39	4.37	4.16	↑	Y08982	Chromosome condensation
Uridine monophosphate synthetase	UMPS	−0.94	−2.13	−1.42	−3.00	↓↓	J03626	Nucleic acid biosynthesis
Adenosine a2a receptor	ADORA2A	1.57	2.24	3.92	3.53	↑↑	S46950	Cellular defense response, inflammatory response
CD11b antigen (integrin, alpha M)	ITGAM	4.07	5.23	5.26	5.87	↑↑↑	J03925	Cell adhesion
CD11c antigen (integrin, alpha X)	ITGAX	1.72	3.77	2.48	3.49	↑↑	Y00093	Cell adhesion
Complement component (3b/4b) receptor 1 (CD35)	CR1	−0.59	0.30	3.15	4.83	↑↑↑	X14362	Complement activation
Complement component (3d/Epstein Barr virus) receptor 2 (CD21)	CR2	−0.29	3.17	3.57	2.14	↑↑↑	M26004	Complement activation
Fc fragment of IgG, low affinity IIb, receptor for (CD32)	FCGR2B	2.63	3.4	0.11	4.22	↑↑	M31933	Immune response
Toll-like receptor 1	TLR1	2.58	1.45	3.44	3.26	↑↑	AL050262	Immune response
Cytochrome b-245, beta polypeptide (cytochrome b558) (gp91phox)	CYBB	−1.63	−1.54	3.11	0.95	≈	X04011	Superoxide anion generation
Neutrophil cytosolic factor 1 (47 kD) (p47phox)	NCF1	1.63	1.40	2.33	3.00	↑↑	M55067	Superoxide anion generation
Neutrophil cytosolic factor 2 (65 kD) (p67phox)	NCF2	1.54	1.83	3.29	2.89	↑↑↑	M32011	Superoxide anion generation
Vesicle-associated membrane protein 5 (myobrevin)	VAMP5	1.68	3.06	2.39	2.73	↑	AF054825	Vesicle transport
Aquaporin 9	AQP9	−0.13	−0.63	3.20	2.93	↑↑↑	AB008775	Water transport, cell motility
Ninjurin 1	NINJ1	1.30	1.79	2.63	3.12	↑	U91512	Cell adhesion
S100 calcium-binding protein a12 (calgranulin C)	S100A12	1.23	1.52	6.11	5.61	↑↑↑	D83664	Calcium binding, inflammatory response
S100 calcium-binding protein A8 (calgranulin A) (MRP-8)	S100A8	0.83	1.00	3.20	3.30	↑↑	AI126134	Calcium binding, inflammatory response
S100 calcium-binding protein A9 (calgranulin B) (MRP-14)	S100A9	0.98	1.26	3.43	3.57	↑↑	W72424	Calcium binding, inflammatory response
Serine (or cysteine) proteinase inhibitor, clade A, member 1	SERPINA1	0.46	1.44	3.17	3.19	↑↑↑	X01683	Protease inhibition
TYRO protein tyrosine kinase binding protein (DAP12)	TYROBP	1.93	2.68	3.72	4.03	↑↑	W60864	Signal transduction
Vanin-2	VNN2	−0.35	0.41	6.43	5.92	↑↑↑	D89974	Cell motility and adhesion
Chemokine (C–C-motif) ligand 2 (small inducible cytokine A2)	CCL2	0.01	−0.96	3.17	1.64	↑↑	M26683	Cell–cell signalling, signal transduction
Chemokine (C–C motif) ligand 3	CCL3	1.69	3.15	−0.72	1.71	↑↑	D90144	Cell–cell signalling, chemotaxis, inflammatory response
Interleukin 1, beta	IL1B	3.35	3.97	3.39	3.37	↑↑	X04500	Signal transduction
BCL2-related protein A1	BCL2A1	0.59	1.42	2.90	3.51	↑↑↑	U27467	Anti-apoptosis
Heat shock 27 kD protein 1	HSPB1	−1.30	−1.38	−4.56	−1.93	↓↓↓	Z23090	Anti-apoptosis
Heat shock 70 kD protein 6 (HSP70b)	HSPA6	2.38	3.83	1.13	5.12	↑↑↑	X51757	Anti-apoptosis

List of genes selected from Supplementary Fig. S1 and Supplementary Table S1 with a ≥8-fold change in expression during the neutrophil differentiation of HL-60 cells with DMSO. The relative changes in gene expression values during DMSO-induced HL-60 cell differentiation compared to untreated control HL-60 cells were obtained using U95Av2 arrays and MAS5.0 software and are shown as "signal log ratio" (SLR). The relative changes in gene expression values between peripheral blood human polymorphonuclear neutrophils (PMN) and untreated HL-60 cells were obtained using U133A arrays and dChip1.3 software. ↑↑↑/↓↓↓, over 100-fold change; ↑↑/↓↓, fold change between 15–100-fold; ↑/↓ , fold change between 2–14-fold; ≈, no change.

negatively controlling cell proliferation (p19, $p21^{CIP1}$, $p27^{KIP1}$, BTG1 and BTG2) were up-regulated (Table 1, and Supplementary Table S1). These changes were generally detected since the first two days of DMSO treatment, coincident with the drastic loss of proliferative capacity of DMSO-treated HL-60 cells. BUB and MAD genes, major components of the mitotic checkpoint, and polo-like kinase, which plays an essential role for mitosis progression, were down-regulated since day 2 of DMSO incubation. We also found that a gene known as PL48 behaved as a marker for HL-60 neutrophils and was highly up-regulated during the third (39-fold) and fourth (14-fold) day of DMSO treatment, when neutrophil-like HL-60 cells were achieved. Expression of this gene has been previously found to be dramatically increased by Northern blot in

HL-60 cells treated with DMSO for 3 days, and has been suggested to function as a tumor suppressor, arresting cells in G_0 or G_1 (Dakour et al., 1997). Our data suggest that the loss of cell division capacity during neutrophil differentiation is due to both down-regulation of mitotic proteins and up-regulation of proliferation inhibitors.

Some genes involved in chromosome condensation, including chromosome condensation 1-like and synaptonemal complex protein 2, were up-regulated during neutrophil differentiation of HL-60 cells (Table 1). We also found that a wide number of genes involved in RNA processing resulted down-regulated (Supplementary Table S1), that could explain the low macromolecule biosynthesis capacity of mature neutrophils (Jack and Fearon, 1988; Mollinedo et al., 1991b). Taken together, these data show a general down-regulation of genes related to cell cycle, proliferation and RNA processing, whereas there is a net up-regulation in transcription-related genes (Fig. 2).

3.3. Changes in gene expression related to cell surface antigens and receptors during neutrophil differentiation of HL-60 cells

The myeloid differentiation marker CD11b was dramatically increased during neutrophil differentiation (59-fold after full neutrophil differentiation). Other cell surface proteins that were highly up-regulated in fully differentiated HL-60 neutrophils include CD35 (28-fold), adenosine A2a receptor (15-fold), CD11c (11-fold), and ICAM-3 (7-fold) (Fig. 3, Table 1, and Supplementary Table S1). Toll-like receptor 1 (TLR1) and TLR2, key elements in pathogen recognition, were also highly up-regulated (11- and 5-fold, respectively) during HL-60 differentiation.

3.4. Changes in gene expression related to neutrophil functions, signalling and cytokines during neutrophil differentiation of HL-60 cells

Expression of $gp91^{phox}$, responsible for superoxide anion generation, was highly increased (9-fold) at day 3 of DMSO treatment (Table 1), when an efficient respiratory burst is detected in fully differentiated HL-60 neutrophils (Fig. 1). $p47^{phox}$ and $p67^{phox}$, required for the stabilization and full activation of the NADPH oxidase complex, were also up-regulated (≅8-fold) after 3- and 4-day of DMSO treatment (Fig. 3, Table 1).

The secretory capacity of HL-60 cells was greatly increased along the differentiation process (Fig. 1), and several exocytosis-related genes were up-regulated in parallel (Fig. 3, Supplementary Table S1). A number of SNARE proteins have been shown to be involved in neutrophil exocytosis (Martin-Martin et al., 2000; Mollinedo et al., 2003; Mollinedo et al., 2006), and some SNARE proteins, including SNAP-23, syntaxin 3A, VAMP-3, and VAMP-5 were up-regulated during HL-60 neutrophil differentiation (Supplementary Table S1).

We also found significant changes in the expression of genes coupled to the acquisition of additional functions during neutrophil differentiation, especially a predominant up-regulation during the third and fourth day of DMSO treatment, when cells acquired functional features of mature neutrophils (Fig. 1). This gene up-regulation pattern included stannin (7-fold), a stress response gene that has been involved in vascular endothelial damage (Horrevoets et al., 1999); ADAM8 (CD156) (4-fold), involved in regulating leukocyte infiltration (Higuchi et al., 2002; Gomez-Gaviro et al., 2007); aquaporin-9 (9-fold), a water transporting protein involved in neutrophil motility (Loitto et al., 2002); and vanin-2 (86-fold), a glycosylphosphatidyl inositol-anchored protein involved in cell motility and adhesion (Suzuki et al., 1999) (Table 1, and Supplementary Table S1).

We found that the expression of ornithine decarboxylase (ODC), a molecule that is overexpressed in various cancers and is associated with promoting tumor growth, resulted down-regulated (6-fold) during neutrophil differentiation of HL-60 cells (Supplementary Table S1). ODC is a physiological transcriptional target of c-Myc in association with induction of cell proliferation and transformation (Auvinen et al., 2003), and is the rate-controlling enzyme of spermine biosynthesis, which together with other polyamines are essential for cell survival and proliferation (Nitta et al., 2002).

The expression levels of calgranulins A (S100A8, MRP-8) (10-fold increase), B (S100A9, MRP-14) (12-fold increase) and C (S100A12) (69-fold increase) were dramatically up-regulated during the late stages of neutrophil differentiation (Table 1). These proteins are members of the S100 multigene family of calcium-binding proteins implicated in the Ca^{2+}-dependent regulation of a variety of intracellular activities, including the dynamics of cytoskeletal rearrangement and structural organization of membranes, intracellular Ca^{2+} homeostasis, inflammation and protection from oxidative cell damage (Santamaria-Kisiel et al., 2006). Inhibitors of neutral serine proteinases (serpins) (10-fold), and the adhesion protein ninjurin (9-fold) were up-regulated in fully neutrophil-like differentiated HL-60 cells (Table 1). DAP12, an immunoreceptor tyrosine-based activation motifs-bearing transmembrane adapter molecule expressed on the surface of NK and myeloid cells and involved in both innate and adaptive immune responses (Aoki et al., 2003), was also up-regulated (16-fold) during neutrophil differentiation of HL-60 cells (Table 1). Genes involved in proteasome-dependent protein degradation, such as proteasome subunits and ubiquitin carrier protein, were down-regulated during HL-60 differentiation (Supplementary Table S1). This could lead to a prolonged protein half-life that could compensate for the poor macromolecule biosynthesis capacity exerted by neutrophils.

Furthermore, we found that neutrophil differentiating HL-60 cells up-regulated the expression of a number of cytokines and cytokine receptors (Fig. 3, Table 1, and Supplementary Table S1), including small inducible cytokine A2, chemokine (C-C motif) ligand 3, chemokine (C-X-C) receptor 4, interleukins (IL) 1β, 8 and 18, IL-10 receptor, and transforming growth factor beta receptor. These data support the ability of human neutrophils to modulate immune responses (Kobayashi, 2008).

3.5. Changes in gene expression related to apoptosis during neutrophil differentiation of HL-60 cells

A remarkable hallmark of peripheral blood mature neutrophils is their ability to undergo spontaneous apoptosis. In agreement with our previous semiquantitative RT-PCR data (Santos-Beneit and Mollinedo, 2000), we found a dramatic up-regulation of A1 (11-fold), and a slight decrease in caspase-3 expression during neutrophil differentiation of HL-60 cells (Fig. 3, Table 1, and Supplementary Table S1). Chuang et al. (1998) also found by Northern blot analysis a progressive A1 mRNA accumulation in HL-

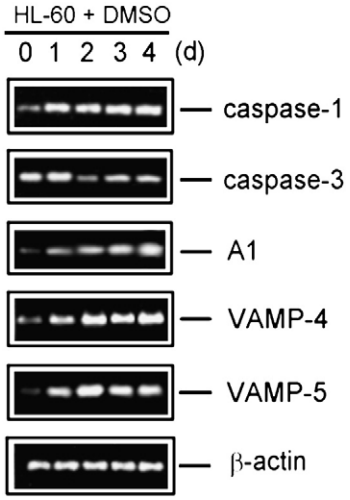

Fig. 4. RT-PCR validation of the gene expression profiling data. The pattern of expression of the indicated genes during DMSO-induced neutrophil differentiation of HL-60 cells was assessed by semiquantitative RT-PCR. Representative gels of three performed are shown.

60 cells during all-trans retinoic acid-driven neutrophilic differentiation. Some pro-apoptotic genes were up-regulated during neutrophil differentiation, including genes potentiating apoptotic signalling, such as BNIP3L, caspases 1 and 8, dual specificity phosphatases, NF-kB inhibitor alpha, and serine/threonine kinases (Fig. 3, Supplementary Table S1). Although some heat shock proteins (HSPs) were down-regulated during neutrophil development, including HSP10 and HSP27, a dramatic increase in heat shock 70 kD protein 6 (HSP70B) was detected (35-fold) after a 4-day DMSO treatment (Table 1, and Supplementary Table S1). In this regard, the Bcl-2-associated anthanogene 2 (BAG2) as well as the HSP70-interacting protein HSPBP1 that function as negative regulators of HSP70 chaperone activity (Nollen et al., 2000) were down-regulated (Supplementary Table S1). Thus, the HSP70B up-regulation, together with the down-regulation of two major negative regulators of its chaperone activity, suggests a role for HSP70B in neutrophil survival.

3.6 . Validation of gene expression profiles detected with oligonucleotide microarrays

We performed semiquantitative RT-PCR with a subset of differentially expressed genes to confirm the observed gene expression patterns (Fig. 4). The results of this RT-PCR analysis showed a very similar expression pattern to that of the microarray analysis, namely caspase 1, A1, VAMP-4 and VAMP-5 were up-regulated during DMSO-induced differentiation of HL-60 cells, whereas caspase 3 was slightly down-regulated with a higher decrease at 2-day of DMSO incubation (cf. Figs. 3 and 4, and Supplementary Table S1). In addition, our present microarray results fit very well with previous Northern blot analyses of certain genes during HL-60 cell differentiation towards neutrophil lineage, such as the up-regulation of *jun* B (Mollinedo and Naranjo, 1991; Mollinedo et al., 1991b) and SNAP-23 (Mollinedo and Lazo, 1997) during the differentiation process.

3.7. Comparison of gene expression profiles between undifferentiated HL-60 cells and peripheral blood human neutrophils

In order to compare the above data from the HL-60 cell line, used as a neutrophil-like cell culture model, with the corresponding human mature neutrophil counterparts, we next analysed and compared the relative abundance of mRNA transcripts in undifferentiated HL-60 cells and peripheral blood human neutrophils. We used U133A oligonucleotide arrays, that contained 22,283 sets of oligonucleotide probes representing about 18,000 full-length human sequences, and the dChip1.3 software for analysis. A comparison of the gene expression level in the U133A arrays showed 23% similarity between the gene expression profile of undifferentiated HL-60 cells and mature human neutrophils. Interestingly, we found that practically all major gene expression changes observed during DMSO-induced neutrophil differentiation of HL-60 cells were correlated with a qualitatively similar expression level in mature neutrophils (Table 1, and Supplementary Table S1). This suggests that the regulation of, at least, part of this set of selected genes could be enough and critical to differentiate HL-60 cells into a neutrophil-like cell type, or that is to say, to turn a

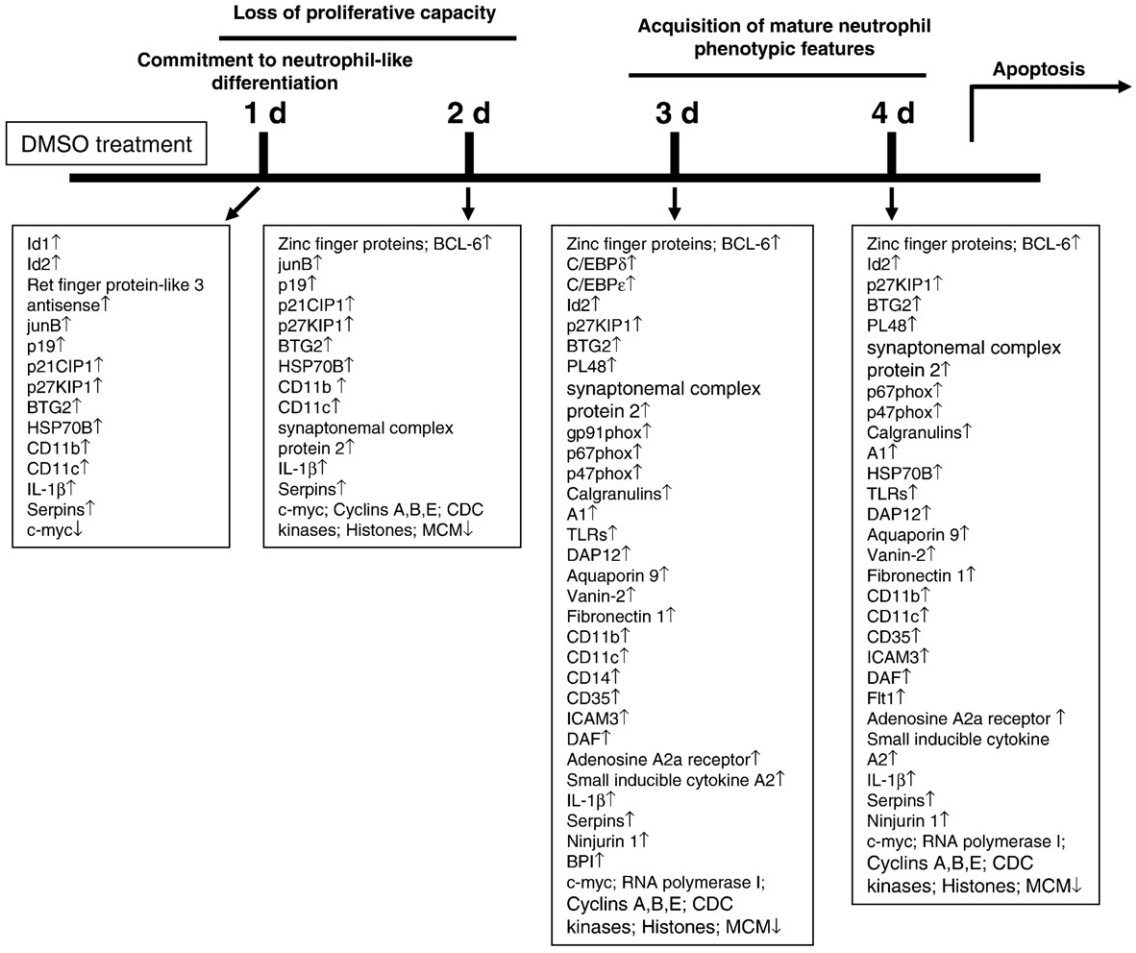

Fig. 5. Major gene expression changes during DMSO-induced HL-60 cell differentiation into the neutrophil lineage. This is a schematic diagram designed to portray some remarkable changes in gene expression coupled to the acquisition of neutrophil hallmarks in DMSO-induced HL-60 cell differentiation. Genes shown here were selected on both a high fold change expression and their putative involvement in the granulocytic maturation process. Timings (1–4 days) for the functional and gene expression changes elicited by DMSO incubation are indicated. See text for further details.

leukemic and proliferating cell into a non-proliferating and terminal cell type that undergoes spontaneous apoptosis.

4. Discussion

The results reported here point out that a rather large number of gene expression changes account for the acquisition of the neutrophil phenotype during neutrophil differentiation of HL-60 cells. Our data support the notion that neutrophil differentiation occurs via coordinated, complex and successive changes in gene expression that are modelling the final functionally active neutrophil. Fig. 5 depicts a schematic diagram of some of the major gene expression changes herein detected during DMSO-induced neutrophil differentiation of HL-60 cells coupled to the acquisition of neutrophil hallmarks. First, differentiating HL-60 cells are arrested at G1 during the commitment phase of HL-60 cell differentiation into the neutrophil lineage, which correlates with a general down-regulation of genes related with cell cycle and proliferation and remarkable changes in the expression of a number of transcription factors. Differentiating HL-60 cells show also defects in the RNA processing machinery that can be a regulatory pathway to modulate the amount and activity of certain gene products, and might also explain the rather high expression of novel alternative splicing-derived isoforms in neutrophils (Mollinedo and Lazo, 1997; Martin-Martin et al., 1999; Eckhart et al., 2001; Herrero-Turrion et al., 2006). Then, a dramatic change occurs in the expression of genes that account for the functional traits of human mature neutrophils.

Lee et al. (2002) did not previously find a common gene expression profile regulated by all-*trans* retinoic acid in NB4 and HL-60 cells, even though this agent induced granulocytic differentiation in both cell types. Thus, these data suggested that the molecular mechanism and genes involved in retinoic acid-induced differentiation of leukemic cells may be cell-type specific (Lee et al., 2002). Our present gene expression profile data of DMSO-induced differentiating HL-60 cells partially differ from the gene expression pattern achieved by retinoic acid, but a number of genes, including Id2, BTG1, c-*myc*, interferon regulatory factor 1, cyclin A1, CDK4, small inducible cytokine A2, calgranulin B, and ninjurin 1, are similarly regulated (Lee et al., 2002; Song et al., 2003). These data suggest that different inducers of HL-60 cell differentiation towards granulocytes can render differential gene expression changes. In addition, part of the differences between our present gene expression data and those previously reported during HL-60 cell differentiation may lie in the use of different microarrays. Earlier microarray studies with retinoic acid-induced differentiation of HL-60 cells involved the use of cDNA microarrays, namely 872 and 8353 cDNAs (Lee et al., 2002; Song et al., 2003), whereas we have used here oligonucleotide microarrays, analysing the expression of about 12,000 full-length genes through the use of 12,625 sets of oligonucleotide probes.

HL-60 cells acquire the capacity to undergo spontaneous apoptosis after full neutrophil differentiation (Fig. 1). However, we have found that the highest up-regulation levels in apoptosis-related gene expression were detected in anti-apoptotic genes, namely HSP70B (35-fold) and A1 (11-fold) (Table 1). BTG2, identified as an anti-proliferative gene (Sasajima et al., 2002) and involved in the survival of differentiated cells (el-Ghissassi et al., 2002), was also highly up-regulated (11-fold) (Table 1). Thus, BTG2, A1 and HSP70B could also play a role in maintaining the short neutrophil lifespan. HSP70B up-regulation together with the down-regulation of two negative regulators of HSP70 chaperone activity, such as BAG2 and HSPBP1 (Supplementary Table S1), further supports a role for HSP70B in preserving the lifespan of human neutrophils. On the other hand, IL-1β acts in an autocrine manner to inhibit spontaneous neutrophil apoptosis through its processing by caspase-1 (Watson et al., 1998). We have found here that IL-1β and caspase-1 were up-regulated during neutrophil development of HL-60 cells (Table 1, and Supplementary Table S1). Thus, caspase-1-dependent activation of IL-1β represents another additional mechanism by which neutrophils might delay its constitutive apoptosis. These data lead us to postulate that: a) fully differentiated neutrophils acquire, during their development, a gene expression pattern that drives cells into apoptosis by default; b) neutrophil lifespan is regulated by the action of anti-apoptotic genes that delay the apoptotic fate of mature neutrophils. In this regard, inhibition of protein synthesis accelerates neutrophil cell death (Sakamoto et al., 2003), suggesting that *de novo* synthesis of anti-apoptotic proteins might regulate neutrophil lifespan. Thus, neutrophils are an excellent example of programmed cell death that acquire their deadly genetic program during development.

The implications of the results reported here are 3-fold: a) to unravel the gene expression changes that underlie the acquisition of neutrophil hallmarks during granulocytic differentiation; b) to gain insight into the transcriptional processes that regulate the short neutrophil lifespan; c) to identify global gene expression changes that turn a proliferating leukaemic cell into a non-dividing and terminal apoptosis-programmed cell type. Definition of the gene expression patterns reported here during neutrophil differentiation of HL-60 cells could be of interest to get further insight into the molecular basis of "differentiation therapy", which is part of standard treatment for patients with acute promyelocytic leukaemia (Parmar and Tallman, 2003). Subsequent knockdown and overexpression studies will be required to unravel the role of the distinct genes, either individually or in combination, in neutrophil differentiation. It could be envisaged, in analogy with structure-activity relationship studies with home-made drugs, that the functionality and phenotypic features of these leukocytes could be modulated by appropriate modification of the pattern or timing of gene expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2008.04.015.

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