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PPARδ is a ligand-dependent negative regulator of vitamin D3-induced monocyte differentiation

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A number of reports indicate that peroxisome proliferator-activated receptor (PPAR) δ is involved in the molecular control of monocyte–macrophage differentiation. In this regard, the recent demonstration that PPARδ is a primary response gene of 1α,25-dihydroxyvitamin D3 (VD), i.e. a powerful inducer of such process, allowed us to hypothesize the existence of a cross talk between PPARδ and VD receptor pathways. To address this issue, we analyzed the effects promoted by stimulation with PPARδ ligands and by overexpression of this nuclear receptor in monoblastic cell lines undergoing exposure to VD. The results obtained evidenced that, although promoting a weak differentiation effect by themselves, PPARδ ligands efficiently co-operated with VD treatment. In spite of this, PPARδ overexpression exerted a remarkable inhibitory effect on monocyte–macrophage differentiation induced by VD that was, at least partly, reverted by stimulation with a highly specific PPARδ ligand. These data indicate that, although acting through a ligand-dependent modality, PPARδ is a negative regulator of VD-mediated monocyte differentiation, allowing us to hypothesize a role of the investigated nuclear receptor in the differentiation block of M5 type (monoblastic) acute myeloid leukemias (AMLs). Bioinformatic analysis of a microarray database, containing the expression profiles of 285 AML cases, further supported this hypothesis demonstrating the existence of a subset of M5 type (monoblastic) AMLs that overexpress PPARδ gene.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors defining a subfamily of the wider nuclear receptor family (1). To date, three distinct PPARs have been described, named α, δ (also called β) and γ, that are encoded by separate genes and exhibit different tissue distribution and ligand preference. PPARα and γ display a prevalent expression in liver and adipose tissue, respectively, whereas PPARδ is ubiquitous (2). In general, natural ligands for these transcription factors are represented by fatty acids and prostaglandins, although a considerable number of synthetic agonists have been also developed in recent years and are currently under investigation (3). Both categories of compounds have been demonstrated to act, in the majority of cases, at micromolar concentrations (2,4). All PPARs have a common structural organization that is shared with other members of the nuclear receptor family and is characterized by the presence of a N-terminal DNA-binding domain and C-terminal ligand-binding and transactivation domains (3). All members of the PPAR family heterodimerize with retinoid X receptor and recognize direct repeat spaced by one nucleotide elements, known as PPAR response elements, which are localized in the promoter region of target genes. Similar to other nuclear receptors, PPARs are able to bind corepressors and inhibit transcription in the unliganded state, promoting the opposite effect when they interact with the ligand (1,3). This bimodal regulatory mechanism is believed to occur as consequence of a conformational change resulting in release of corepressors and recruitment of coactivators. In this regard, it is worth consider that PPARδ distinguishes itself for a repression activity that is remarkably powerful in comparison with the other PPAR family members (5). Initially involved in lipid metabolism and glucose homeostasis (6), these receptors have been subsequently implicated in the regulation of important biological processes such as proliferation, differentiation and apoptosis (3). Inside the hematopoietic system, PPARs have been implicated in monocyte/macrophage biology. In this regard, a number of reports have demonstrated an upregulated expression of these receptors in monocyte–macrophage activation where they inhibit macrophage functions by downregulating expression of inflammatory cytokines and chemokines (1,2). Some observations also exist indicating a role of such transcription factors in the regulation of monocyte–macrophage differentiation. In fact, ligands for the α and γ members of the PPAR family have been shown to induce the monocyte differentiation of HL60 and U937 hematopoietic cell lines (7,8), whereas stimulation of the δ member has been demonstrated to co-operate in macrophage differentiation of THP1 cells induced by phorbol l2-myristate l3-acetate (PMA) (9). Further investigation, carried out in primary cell models, has led to the demonstration that PPARδ is significantly more expressed in CD34+-derived monocyte precursors, in comparison with granulocyte precursors obtained by the same cells (10), and is a target gene of the MafB transcription factor, recently proposed as one of the main regulators of monocyte commitment (11). Evidence has also emerged that PPARδ gene is a primary response gene of 1α,25-dihydroxyvitamin D3 (VD), a distinct and physiological inducer of monocyte–macrophage differentiation exerting its activity through the interaction with the vitamin D nuclear receptor (VDR) (4). Taken together, these findings allowed us to hypothesize the existence of a cross talk between PPARδ and VDR receptor pathways, regulating the considered differentiation process. To clarify this issue, we planned a number of experiments in order to: (i) analyze PPARδ expression in different hematopoietic cell populations by reverse transcription (RT)–polymerase chain reaction (PCR) and western blot; (ii) assess the effects elicited on monoblastic cell lines by stimulation with natural and synthetic ligands provided with high [carbaprostacyclin (cPGI), GW501516] or low [arachidonic acid (AA) and linoleic acid (LA)] specificity for PPARδ (12,13); (iii) evaluate the effects exerted on these cells by retroviral vector-mediated overexpression of PPARδ in absence or presence of the ligand. The results of these experiments showed that PPARδ expression is closely associated with the monocyte cell context and sensibly upregulated by stimulation of hematopoietic cells with inducers of monocyte–macrophage differentiation such as PMA and VD. Exposure to PPARδ ligands, although promoting a weak differentiation effect by itself, efficiently co-operated with VD treatment. Unexpectedly, PPARδ overexpression exerted a remarkable inhibitory effect on monocyte–macrophage differentiation induced by VD that was, at least in part, reverted by stimulation with the highly specific GW501516 PPARδ ligand. These findings suggested that PPARδ is a negative regulator of VD-mediated

Abbreviations: AA, arachidonic acid; AML, acute myeloid leukemia; cDNA, complementary DNA; cPGI, carbaprostacyclin; FAB, French-American-British classification; mRNA, messenger RNA; NGFR, nerve growth factor receptor; PCR, polymerase chain reaction; PMA, phorbol l2-myristate l3-acetate; PPAR, peroxisome proliferator-activated receptor; QRT, quantitative reverse transcription; RT, reverse transcription; siRNA, small interference RNA; VD, 1α,25-dihydroxyvitamin D3; VDR, vitamin D receptor.

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monocyte differentiation exerting its action through a ligand-dependent modality. Bioinformatic analysis of a microarray database, containing the expression profiles of 285 acute myeloid leukemias (AMLs) characterized by different French-American-British classification (FAB) cytotypes, confirmed the association between PPARδ expression and the monocyte phenotype and demonstrated the existence of a subset of M5 type (monoblastic) AMLs exhibiting an evident overexpression of PPARδ gene. Taken together, these observations allow us to hypothesize a leukemogenic role of the investigated nuclear receptor.

Materials and methods

Culture, purification and ligand stimulation of hematopoietic cells

KG1a, KG1, KASUMI1, HL60, NB4, THP1 and U937 cell lines were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI1640 medium (Euroclone, Devon, UK), supplemented with 10% heat-inactivated fetal bovine serum (Biowhittaker, Walkersville, MD) and 1 mM L-glutamine (Euroclone). Cord blood CD34+ stem/ progenitor cells and adult peripheral blood neutrophils and monocytes were immunoselected as described (11) using the MACS magnetic cell sorting procedure (MiniMacs, Miltenyi Biotec, Auburn, CA). Purity of freshly separated cells, determined by flow cytometry and morphological analysis (May-Grunwald–Giemsa staining), always exceeded 95%. Myeloblasts and monoblasts were generated by *in vitro* culture of cord blood CD34+ hematopoietic progenitors as already described (10). Stimulation of primary CD34+ hematopoietic progenitors, normal myeloblasts and monoblasts, THP1 cells, U937 cells and HL60 cells with differentiation inducers was carried out by incubation with PMA (Sigma Chemical Co, St Louis, MO), VD (Hoffman-Laroche, Basel, Switzerland) and/or the following PPARδ ligands: GW501516 (GW), cPGI, AA and LA (Cayman Chemicals, Ann Arbor, MI). Treatment modalities varied among the different experiments and are described in detail in the Results.

Semiquantitative and quantitative RT–PCR analysis

Total cellular RNA from hematopoietic cell lines and primary cells was isolated by means of the guanidinium–cesium chloride centrifugation technique as described previously (14). RNA integrity and concentration was then assessed by the Bio-Analyzer technique (Applied Biosystems, Foster City, CA). Semiquantitative RT–PCR analysis of PPARδ messenger RNA (mRNA) expression was performed, as already reported (14), using the following oligonucleotide primers: direct, 5′-ATCGATATGGAGCAGCCACAGGAGG-3′ and reverse, 5′-ATCGATCGCCGTTAGTACATGTCCTTG-3′. The glyceraldehyde-3-phosphate dehydrogenase mRNA was also amplified to normalize RNA amounts of the different samples (see ref. 11 for primer sequences). Quantitative reverse transcription (QRT)–PCR analysis was performed, as already described, using oligonucleotides primers and probes designed and provided by Applied Biosystems company (11).

Western blot analysis

Nuclear and cytoplasmatic extracts of control untreated and ligand-stimulated THP1 and U937 cells were obtained according to the Dignam procedure with minor modifications (15). Expression of PPARδ protein was then assessed by western blot analysis, performed as described (15), using a primary anti-PPARδ rabbit polyclonal antibody and a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG, both obtained from Cayman Chemicals. Detection of immunoreactive bands was carried out using the BM Chemiluminescence Blotting Substrate (Roche Diagnostics, Mannheim, Germany).

Retroviral vector construction and packaging

A PPARδ full-length complementary DNA (cDNA) was obtained by RT–PCR performed on total RNA extracted from human normal monocytes using the oligonucleotide primers described above. The amplified fragment was initially inserted in the pCR TOPO XL T/A cloning vector (Invitrogen, Carlsbad, CA), where it was fully sequenced to exclude DNA polymerase-induced mutations, and subsequently cloned in the LXIΔN retroviral vector (11) obtaining the LPδIΔN construct. Packaging of the LXIΔN and LPδIΔN retroviral vectors was performed by transient transfection of the Phoenix amphotropic cell line.

Hematopoietic cell transduction and purification

THP1 cells were transduced by two to three cycles of infection (4 h each) with viral supernatant in the presence of polybrene (8 μg/ml). Infected cells were subsequently nerve growth factor receptor (NGFR) immunoselected using a purified mouse anti-human p75–NGFR monoclonal antibody (BD Biosciences, San Diego, CA) and M-450 goat anti-mouse immunomagnetic beads (Dynal, Oslo, Norway).

Immunophenotypic and morphological analysis

Flow cytometry analysis of monocyte differentiation in THP1, U937 and HL60 cells was carried out using a mouse anti-human CD14 monoclonal antibody conjugated to fluorescein isothiocyanate (BD Biosciences). Transduction efficiency of THP1 cells was monitored using a primary purified murine anti-human p75–NGFR monoclonal antibody (BD Biosciences) and a secondary rabbit anti-mouse polyclonal antibody conjugated to fluorescein isothiocyanate (DAKO A/S, Copenhagen, Denmark). Antibodies' incubations were carried out as described (16). Stained cells were analyzed by flow cytometry using a Coulter Epics XL flow cytometer. Morphological examination was accomplished on cytocentrifuged specimens performed upon staining with May–Grunwald–Giemsa.

Oligonucleotide siRNA-mediated inactivation of PPARδ gene

PPARδ gene silencing was conducted on THP1 cells using a mix of three predesigned oligonucleotide small interference RNA (siRNA) duplexes provided by Applied Biosystem (Cat. # 4390824, siRNA ID#: s10883, s10884 and s10885). Oligonucleotide siRNA transfection was achieved by electroporation performed using the Amaxa nucleofector technology according to the manufacturer's instructions. For this purpose, 2×10^6 THP1 cells were nucleofected with a mix containing 3 μg of each siRNA. In order to exclude non-specific effects of nucleofected siRNA, every experiment included a negative control represented by a non-targeting siRNA synthesized by Dharmacon (Lafayette, CO). Treatment of the GW501516 PPARδ ligand was carried out 12 h after nucleofection adding of a 10 μM concentration of this compound. The efficiency of PPARδ silencing and the extent of monocyte differentiation were evaluated 72 h later by immunophenotypic and QRT–PCR analysis.

Analysis of publicly available AML gene expression profiles

The publicly available dataset by Valk *et al.* (17) was retrieved from the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo, accession number GSE1159). The dataset comprises 293 gene expression profiles obtained with Affymetrix GeneChips HG-U133A, including 285 AML cases exhibiting different FAB phenotypes. Among the three available probe sets for PPARδ, we selected the probe set giving the highest expression levels, as confirmed by the analysis of different datasets, i.e. 37152_at. Nevertheless, the other PPARδ probe sets showed similar distribution of expression levels across samples.

Results

RT–PCR analysis of PPARδ mRNA expression in hematopoietic cells

To characterize PPARδ expression pattern inside the hematopoietic system, we carried out a preliminary survey on different cell populations. In this regard, results of semiquantitative RT–PCR analysis, performed on a panel of seven myeloid cell lines, revealed that, although detected in all the analyzed cell samples, PPARδ transcript exhibited the highest levels in Kasumi1, THP1 and U937 cells, i.e. cell contexts characterized by a monoblastic phenotype (Figure 1A, left panel). Similarly, among the analyzed primary cell samples, this transcript displayed the strongest expression in monocytes, while it was less represented in granulocytes and barely detectable in CD34+ cells (Figure 1A, left panel). The use of a more accurate analysis procedure, such as QRT–PCR, confirmed the relative differences of PPARδ mRNA levels observed with semiquantitative RT–PCR (Figure 1A, right panel).

A previous report has already demonstrated the capacity of PMA to upregulate PPARδ expression in THP1 cells (9). Nevertheless, to confirm this observation and to extend its validity to other myeloid cell contexts, we examined the effect of PMA stimulation on HL60 cells, THP1 cells and their normal counterparts i.e. primary CD34+-derived myeloblasts and monoblasts, respectively. These cells were exposed to a 16 nM concentration of PMA for 72 h and then analyzed for PPARδ mRNA expression using the QRT–PCR. The results of this analysis demonstrated that, with different extents, PMA treatment upregulated PPARδ transcript in all the analyzed cell samples, giving rise to an induction that ranged 16- to 83-fold in HL60/myeloblasts and 3- to 10-fold in THP1/monoblasts (Figure 1B, left and right panel).

Although the PPARδ gene has been recently reported to be a direct target of VD (4), it is worth consider that this observation was achieved in epithelial cells and the capacity of VD to upregulate PPARδ expression in hematopoietic cells still remains to be verified.

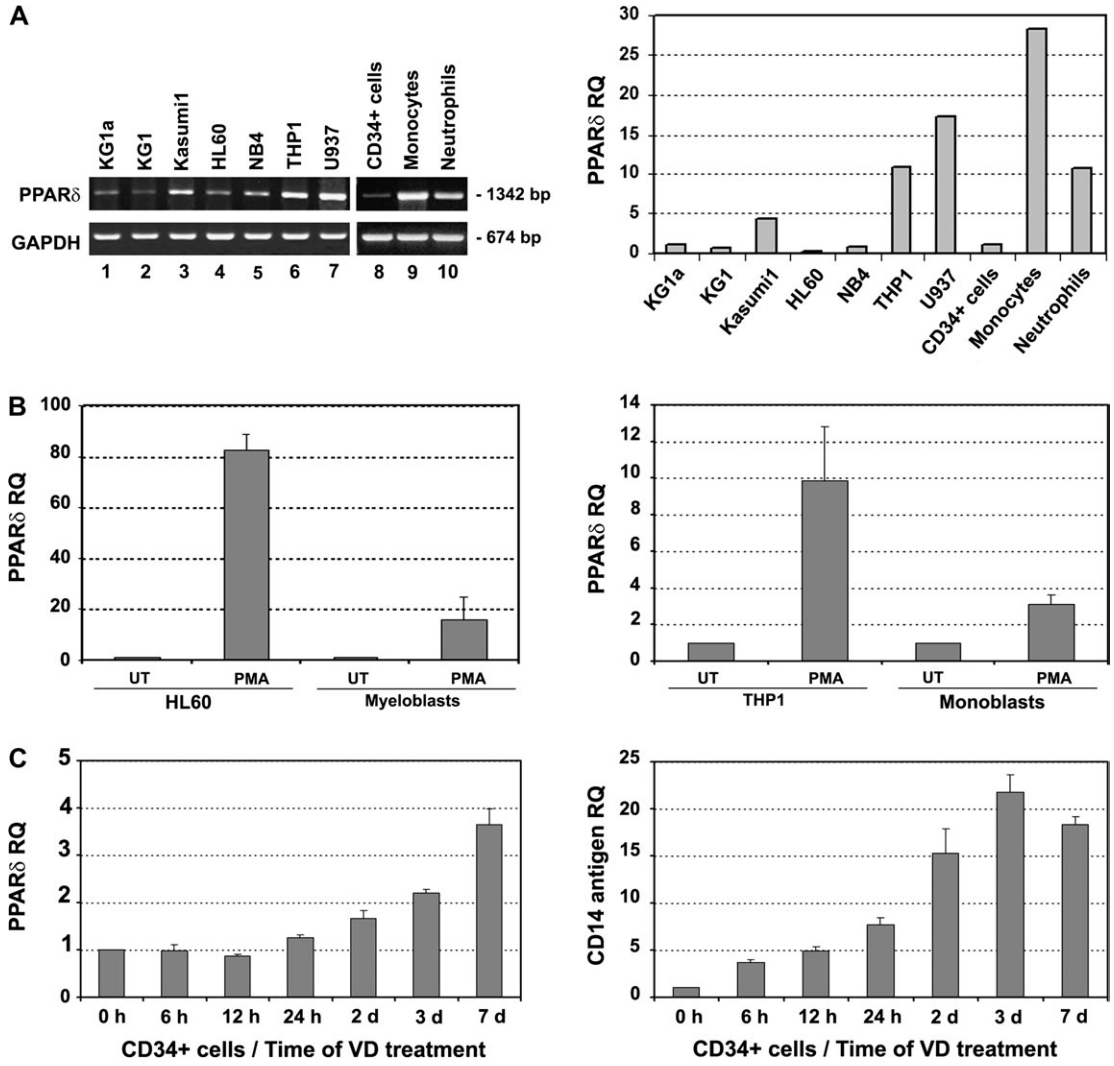

Fig. 1. RT–PCR analysis of PPARδ mRNA expression in different human hematopoietic cell populations. Panel (**A**): Analysis of PPARδ mRNA expression, performed by semiquantitative and QRT–PCR, on leukemic myeloid cell lines, cord blood CD34+ hematopoietic progenitors, peripheral blood monocytes and neutrophils. Semiquantitative RT–PCR results are shown on the left as agarose gels stained with ethidium bromide. Analyzed cell populations are indicated on the top, the size of amplified gene fragments is reported on the right. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also amplified to normalize RNA amounts of the different samples. QRT–PCR results are instead reported on the right as bar histogram. Differences in expression levels are reported on *y*-axis as relative quantity (RQ) normalized using KG1a signal as calibrator. Analyzed cell populations are indicated on *x*-axis. Panel (**B**): QRT–PCR analysis of PPARδ mRNA expression performed on myeloid cell populations untreated (UT) and treated with PMA. This analysis was performed on HL60 cells, normal primary myeloblasts (left), THP1 cells and normal primary monoblasts (right). The results obtained are presented as in panel (A). Analyzed cell populations and treatment conditions are reported on the *x*-axis of each histogram. Data are expressed as mean ± SD values deriving from a triplicate experiment. Panel (**C**) shows the results of QRT–PCR analysis of PPARδ (left) and CD14 antigen (right) mRNA expression performed on CD34+ hematopoietic stem/progenitor cells undergoing stimulation with VD. The data obtained are shown as described in panel (A and B). VD treatment times are reported on the *x*-axis of each histogram.

To clarify this issue, we planned a set of experiments in which primary CD34+ hematopoietic stem/progenitor cells were stimulated for up to 1 week with a 10^{-8} M concentration of VD and subsequently analyzed by QRT–PCR to estimate the variations of PPARδ mRNA levels. This analysis was also conducted on the CD14 antigen gene due to the evidence that, besides coding for a monocyte-specific marker, it has been recently included among the direct targets of VD (18). The results obtained indicated a gradual upregulation of PPARδ expression peaking 7 days post-stimulation with VD (Figure 1C, left panel) and, at the same time, they confirmed that the PPARδ gene is a weak target of VD (4-fold induction) (4) as compared with the CD14 antigen (22-fold induction) (Figure 1C, right panel).

Expression studies performed in our laboratory, for the most part obtained on normal primary hematopoietic progenitors and precursors, thus indicate that, in spite of its ubiquity, PPARδ expression is strictly related to monocyte–macrophage differentiation. This evidence was supported by the observation that PPARδ is preferentially expressed in monocyte cell contexts and clearly upregulated in response to inducers of monocyte–macrophage differentiation such as PMA and VD.

Western blot analysis of PPARδ expression in monoblastic cell lines

To assess whether the THP1 and U937 monoblastic cell lines were suitable experimental models for subsequent investigation on PPARδ, the expression of this nuclear receptor was verified at the protein level by western blot analysis. The data obtained evidenced that PPARδ protein was comparably expressed in both cytoplasmic and nuclear extracts of analyzed cells and the levels of detected protein were not significantly modified by preincubation of cells with PPARδ ligands (Figure 2). These results substantially underline that PPARδ protein is constitutively localized in the nuclear compartment of analyzed cells,

as expected for a nuclear receptor acting with a bimodal regulatory mechanism.

Ligand stimulation of myeloid cell lines

Based on these results and due to the sensitivity of THP1 and U937 cells to monocyte differentiation induced by VD, they were adopted as experimental models to investigate the relationship between the PPARδ- and VDR-signaling pathways. This aim was initially pursued by means of stimulation experiments in which THP1 cells were treated with VD and PPARδ ligands, used individually or in combination. The former was added once to the culture at concentrations ranging from 10^{-10} to 10^{-8} M, whereas the latter were administered 10 μM daily. This treatment condition was decided on the base of a preliminary dose–response experiment in which the differentiation effect induced by the GW501516 and cPGI compounds, i.e. the more specific among the used PPARδ ligands, was absent at 0.1 μM, half maximal at 1 μM and maximal at 10 μM. Higher concentrations of these compounds resulted in a significant cell mortality and were consequently avoided in subsequent stimulation experiments. Other ligands employed in these experiments were represented by AA and LA, characterized by a lower specificity for PPARδ. The extent of differentiation was monitored 72 h after exposure to the analyzed inducers by flow cytometry analysis of the monocyte-specific CD14 antigen. Results of this assay showed that, although PPARδ ligands promoted a weak monocyte differentiation by themselves, they clearly co-operated with the differentiation effect of VD, especially at suboptimal concentrations of this nuclear hormone ($<10^{-8}$ M) (Figure 3). In fact, stimulation with cPGI resulted in a 19% monocyte differentiation when used alone, whereas addition of this compound to cells treated with 10^{-9} M VD upregulated the percentage of CD14+ cells from 53 to 89%, allowing to achieve a biological effect comparable with that observed with maximal (10^{-8} M) VD concentrations (99%) (Figure 3). Similar results were observed using 10^{-10} M VD in combination with cPGI or other PPARδ agonists such as the GW501516 compound and AA (Figure 3). It is worth notice that costimulation with two PPARδ ligands, such as cPGI and GW501516 or cPGI and AA, together with 10^{-10} M VD promoting only a 4% differentiation by itself resulted, respectively, in a 54 and a 50% of CD14 positivity (Figure 3). These findings clearly indicate that ligand activation of PPARδ co-operates monocyte–macrophage differentiation induced by exposure to VD, exerting an additive effect on such process. Stimulation of U937 and HL60 cells with the same compounds resulted in a comparable effect, allowing us to extend the validity of our observations to virtually all hematopoietic cell lines responsive to VD treatment (data not shown).

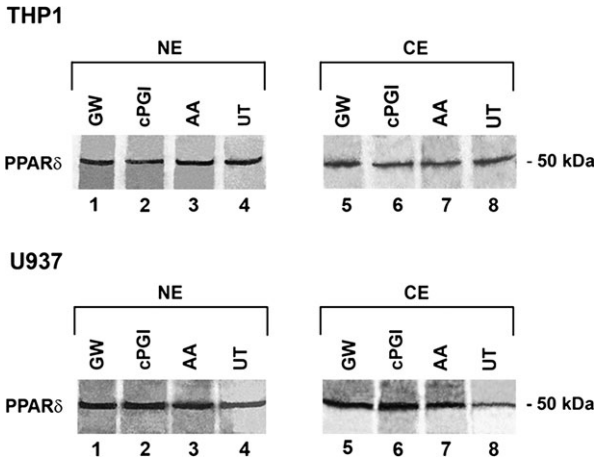

Fig. 2. Western blot analysis of PPARδ protein expression in nuclear and cytoplasmic extracts of untreated and ligand-stimulated THP1 and U937 cell lines. Analyzed cell lines were incubated for 24 h with a 10 μM concentration of the following PPARδ ligands: GW501516 (GW), cPGI, AA and LA. Untreated cells (UT) were also analyzed as a control. The size of the detected immunoreactive band and treatment conditions are, respectively, reported on the right side and on the top of each panel. NE, nuclear extract; CE, cytoplasmic extract.

Retroviral transduction of THP1 monoblasts with a full-length PPARδ cDNA

To better characterize the co-operation between the VD-signaling pathway and the investigated nuclear receptor, we constructed the

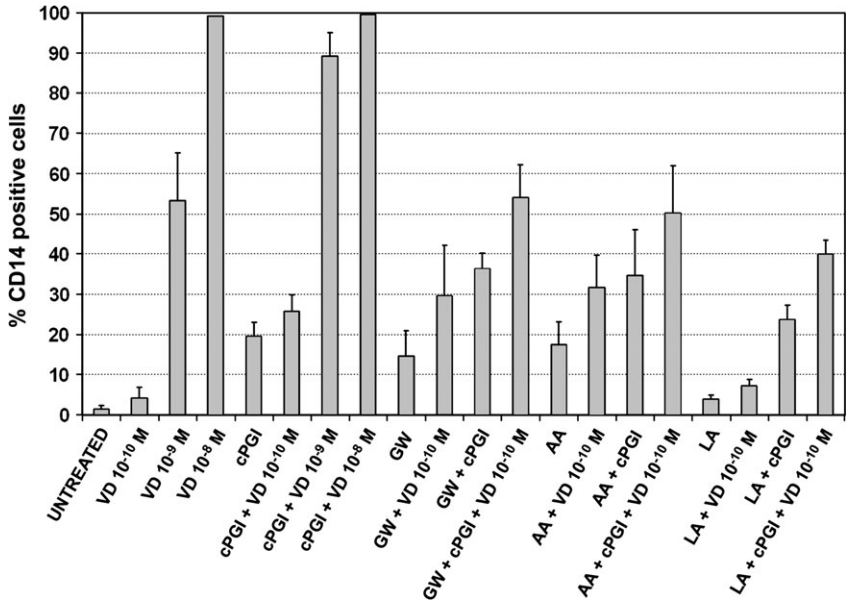

Fig. 3. Differentiation effect promoted on THP1 cells by PPARδ ligand stimulation. THP1 cells were incubated for 72 h with the indicated combinations of differentiation inducers: VD, GW501516 (GW), cPGI, AA and LA (see Results for details). Unstimulated cells were also tested as a control (UT). Monocyte differentiation was monitored by flow cytometry analysis of the monocyte-specific CD14 antigen. Results are presented as bar histogram reporting the analyzed treatment conditions on *x*-axis and the percentage of CD14-positive cells on *y*-axis. Data are expressed as mean ± SD values deriving from a triplicate experiment.

LPδIΔN retroviral vector, expressing a PPARδ full-length cDNA and a truncated version of low-affinity NGFR (ΔlNGFR), used as reporter gene, in the context of a bicistronic transcript driven by the viral long terminal repeat. In this vector, the PPARδ and ΔlNGFR cDNAs are, respectively, localized upstream and downstream in an internal ribosomal entry site sequence. The 'empty' version of the same vector, named LXIΔN and expressing only the reporter gene, was used as control. The described retroviral vectors were subsequently used to transduce THP1 cells that, in turn, were NGFR purified in order to obtain a cell population homogeneously expressing the transgene. RT–PCR and western blot analysis, performed on transduced/purified THP1 cells, confirmed the expected overexpression of the transgene (data not shown), allowing us to consequently assess the effects exerted in these cells by stimulation with VD and with the highly specific GW501516 PPARδ ligand. This analysis was performed under the treatment conditions of the previous experiment using a 10^{-8} M VD concentration. A representative experiment, reported in Figure 4, showed that the CD14 antigen, assessed by flow cytometry, was substantially not expressed in unstimulated cells regardless of the considered vector. Treatment with VD resulted in a significantly lower percentage of CD14+ cells in PPARδ-overexpressing cells (28%) as compared with empty vector-transduced cells (60%), implying that overexpression of the investigated nuclear receptor in absence of the corresponding ligand leads to a paradoxical inhibition of VD-induced monocyte differentiation (Figure 4A). This inhibitory effect was reproducibly observed in three independent experiments averaging a 5 ± 2-fold reduction of CD14 positivity. Addition of GW501516 to these cells apparently restored the differentiation activity of VD, allowing us to obtain a 58% CD14 antigen expression (Figure 4A). In spite of this, PPARδ overexpression failed to enhance the differentiation effect promoted by costimulation with VD and GW501516, maintaining on the contrary a residual inhibition on such process (81 versus 58% of CD14 antigen expression) (Figure 4A). As expected, treatment with GW501516 increased the extent of CD14 positivity in THP1 cells transduced with the analyzed nuclear receptor (9 versus 4% of control LXIΔN-transduced cells), confirming that the differentiation effect promoted by this ligand is PPARδ mediated (Figure 4A).

Morphological examination, performed upon May–Grunwald–Giemsa staining of cytocentrifuged specimens, confirmed the results of flow cytometry analysis. In fact, empty vector-transduced THP1 cells, treated with VD, assumed the expected monocyte–macrophage morphology characterized by loss of basophilia and acquisition of enlarged and vacuolated cytoplasms, whereas PPARδ-overexpressing THP1 cells, undergoing the same treatment, exhibited an immature monoblast morphology that appeared absolutely comparable with that observed in untreated THP1 cells (both empty and PPARδ vector transduced) (Figure 4B).

Daily cell counts demonstrated that, consistently with data deriving from immunophenotypic and morphological analysis of differentiation, PPARδ overexpression was also able to restore the proliferation activity of VD-treated THP1 cells (Figure 5, supplementary Figure 1 is available at *Carcinogenesis* Online).

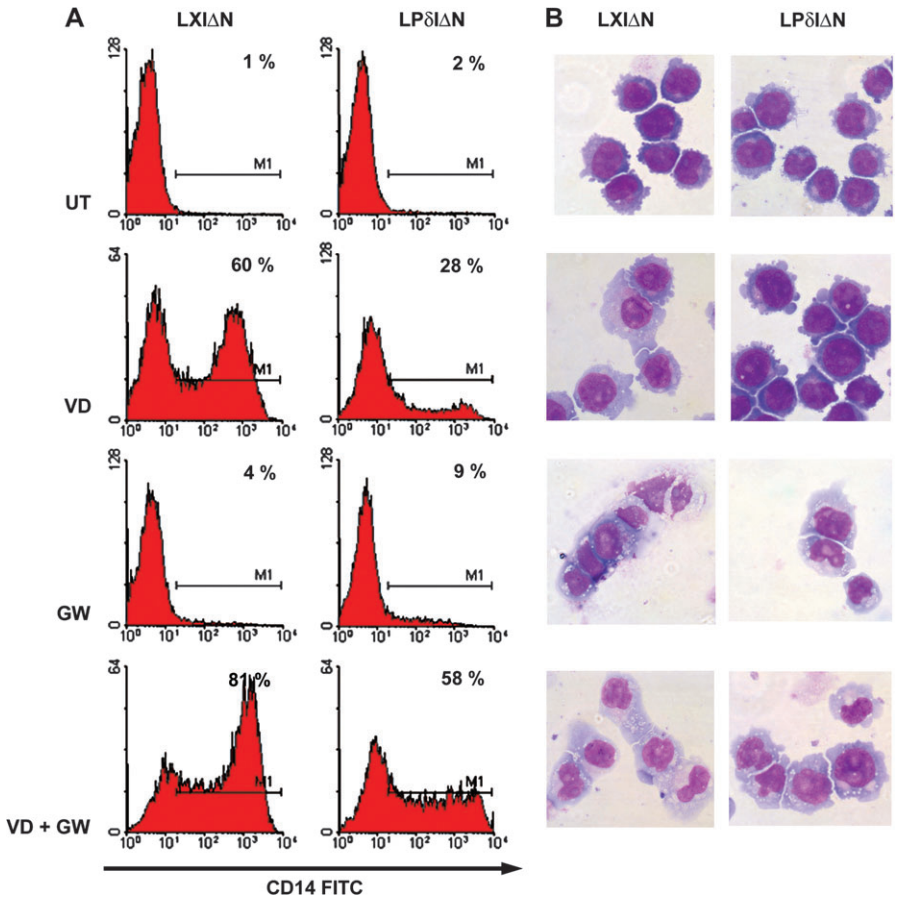

Fig. 4. Biological effects promoted by retrovirally mediated PPARδ overexpression in THP1 cells. THP1 cells were retrovirally transduced with the LPδIΔN vector, containing a PPARδ full-length cDNA, and with the empty LXIΔN control vector. Transduced cells were then NGFR purified and stimulated for 72 h with VD and/or GW501516 (GW). The effect promoted by these compounds was compared with that of untreated cells (UT). The extent of monocyte differentiation was assessed by flow cytometry analysis of the monocyte-specific CD14 antigen (panel **A**) and by morphological examination carried out on cytocentrifuged cells stained with May–Grunwald–Giemsa (panel **B**). In panel (A), results are presented as flow cytometry histograms in which *x*- and *y*-axis, respectively, indicate CD14 expression and number of analyzed events. Analyzed treatments are reported on the left, and retroviral vectors used to infect THP1 cells are indicated on the top.

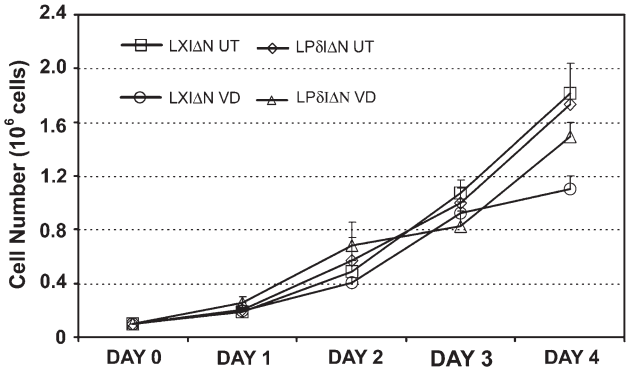

Fig. 5. Growth curves calculated on THP1 cells transduced with the empty and PPARδ retroviral vectors and subsequently treated with the VD nuclear hormone. THP1 cells under the experimental conditions of Figure 4 were cultured for 4 days and counted daily to assess the effect of PPARδ overexpression and VD treatment on cell proliferation. Retroviral vectors and treatment conditions are indicated as in Figure 4. Results are reported as line histogram in which cell number and treatment times are, respectively, indicated on *y*- and *x*-axis. Data are presented as mean ± SD values deriving from a triplicate experiment.

To assess whether the inhibitory effect of PPARδ overexpression was also able to interfere with the activation of VDR-dependent genetic program, we analyzed by QRT–PCR the expression of two VD direct targets, i.e. the CD14 antigen and p21^{waf1} genes (18), in transduced THP1 cells under the treatment conditions described above. The results of this analysis revealed that, again, retroviral transduction of PPARδ, followed by VD stimulation, sensibly reduced mRNA levels of both the studied genes (Figure 6, upper and lower panel, compare LPδIΔN VD with LXIΔN VD). Furthermore, addition of GW501516 to PPARδ-transduced/VD-treated THP1 cells limited the inhibitory effect observed on CD14 antigen gene (Figure 6, upper panel, see LPδIΔN VD + GW) and completely reverted the same effect on p21^{waf1} gene (Figure 6, lower panel, see LPδIΔN VD + GW). It is also worth to underline that combined treatment of THP1 cells with VD and GW501516 resulted in a synergistic upregulation of the analyzed VD target genes (especially evident comparing LXIΔN VD + GW with LXIΔN VD and LXIΔN GW in Figure 6, upper and lower panels).

These experiments globally allow one to conclude that overexpression of PPARδ in the absence of its ligand inhibits the monocyte–macrophage differentiation of THP1 cells induced by VD interfering, at the same time, with genes that require VDR function for their transcriptional activation. In addition, although to a variable extent and depending on the considered effect, this inhibitory activity is efficiently reverted by stimulation with the highly specific GW501516 ligand.

Oligonucleotide siRNA-mediated inactivation of PPARδ gene in THP1 cells treated with the GW501516 ligand

The observation that PPARδ overexpression enhances the effect exerted by the GW501516 ligand on CD14 antigen expression (see Figure 4A) suggests that the differentiation activity of this compound is actually mediated by the studied nuclear receptor. To further verify this finding, we designed an oligonucleotide siRNA-based strategy to inactivate PPARδ gene expression in THP1 cells undergoing treatment with GW501516. For this purpose, THP1 cells were nucleofected with anti-PPARδ and with control oligonucleotide siRNA and stimulated for 72 h with 10 μM GW501516. As shown in Figure 7, treatment with the anti-PPARδ siRNA resulted in ∼86% downregulation of PPARδ transcript as assessed by QRT–PCR, demonstrating the efficacy of the inactivation strategy (right panel). The same analysis also evidenced a decreased mRNA expression of the CD14, chemokine 2 and interleukin-8 monocyte–macrophage differentiation markers. Flow cytometry analysis confirmed the downregulation of the CD14 antigen at the protein level (left panel).

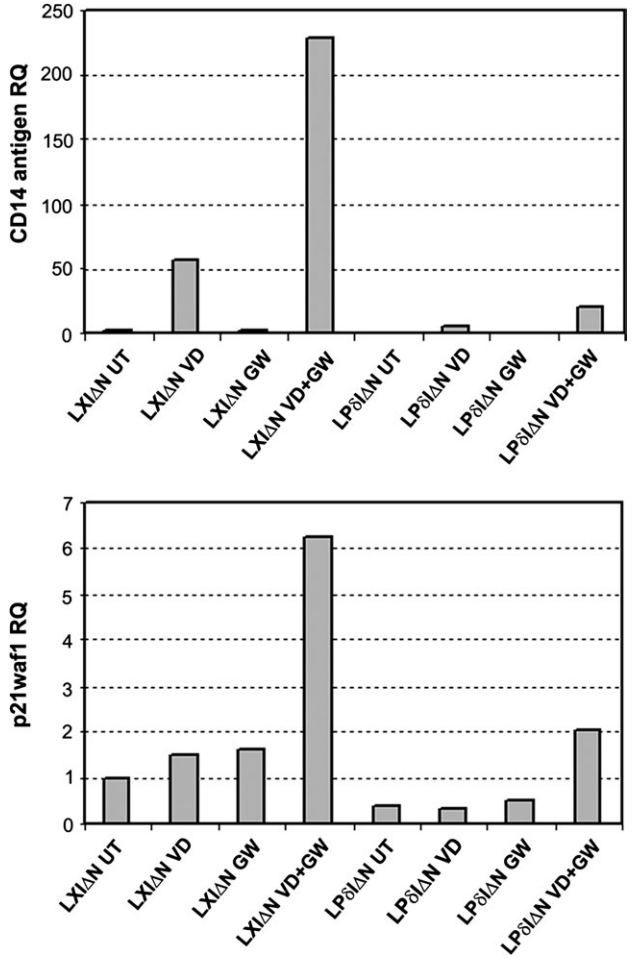

Fig. 6. QRT–PCR analysis of CD14 antigen and p21^{waf1} mRNA expression. CD14 antigen and p21waf1 mRNA expression were analyzed by QRT–PCR performed on THP1 cells under the experimental conditions described in Figure 4. Results of this analysis are shown as bar histogram in upper and lower panel of the figure, respectively. Differences in expression levels are reported on *y*-axis of each histogram as relative quantity (RQ) normalized using signal of LXIΔN-transduced/ligand-untreated cells (LXIΔN UT) as calibrator. Analyzed cell populations are indicated on *x*-axis of the two histograms.

These data clearly indicate that GW501516 stimulates the monocyte–macrophage differentiation of THP1 cells acting through the PPARδ nuclear receptor.

Microarray analysis of PPARδ expression in AML cases

Data presented so far overall indicate that overexpression of PPARδ in the absence of its ligand lead to a remarkable inhibition of monocyte–macrophage differentiation. In light of this finding, it is plausible to hypothesize that this nuclear receptor may play a role in the differentiation block of at least some cases of AML. To clarify this issue, we took advantage of the Valk database containing the microarray expression profiles of 285 AML cases characterized by different FAB cytotypes. Bioinformatic analysis of this database evidenced that the increase of PPARδ expression level percentile is accompanied by a parallel increase of the percentage of M5 type (monoblastic) AMLs (Figure 8A). This enrichment of the M5 phenotype in PPARδ-hyperexpressing AMLs became especially evident over the 50th percentile, peaking at the 99th percentile, in which two of three AML cases resulted to be classified as M5 type (i.e. 67 versus 23% of the whole database) (Figure 8B). Although these last samples

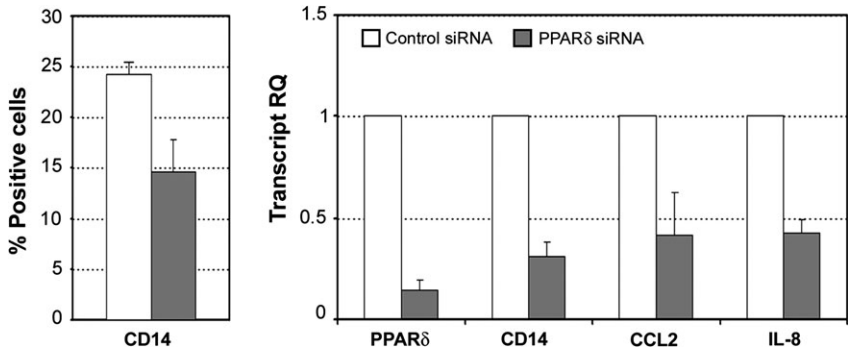

Fig. 7. Oligonucleotide siRNA inactivation of PPARδ gene expression in THP1 cells undergoing stimulation with the GW501516 ligand. THP1 cells were nucleofected with anti-PPARδ and with control oligonucleotide siRNA and stimulated for 72 h with GW501516. Effect of siRNA treatment was then assessed by flow cytometry analysis of CD14 expression, reported as bar histogram on the left, and by QRT–PCR analysis of PPARδ, CD14 antigen, CCL2 and interleukin-8 mRNA expression, reported as bar histogram on the right. Data are presented as mean ± SD values of a triplicate experiment.

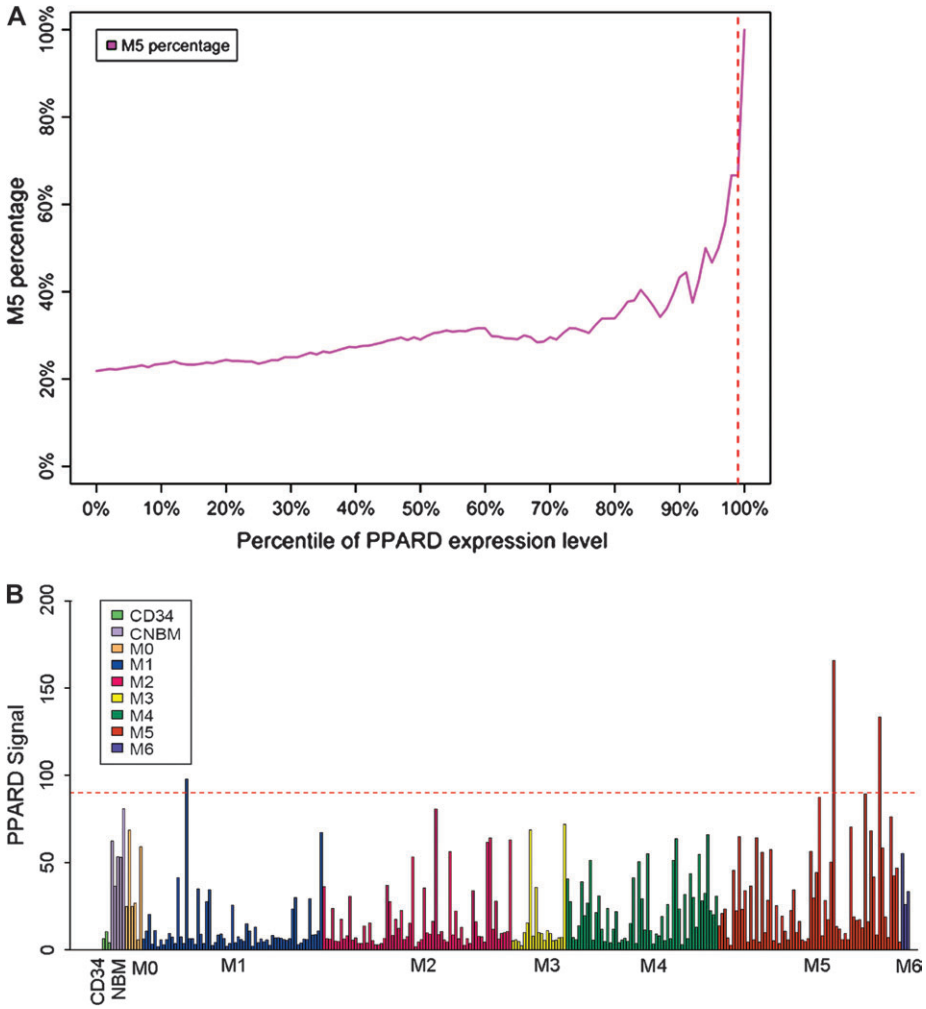

Fig. 8. Bioinformatic analysis of PPARδ mRNA expression in the Valk microarray database. PPARδ mRNA expression was analyzed in the Valk microarray database containing the expression profiles of 285 AML cases exhibiting different FAB phenotypes. Panel (**A**): Percentiles of PPARδ (PPARD) expression levels across the whole set of samples (*x*-axis) were analyzed and the percentage of M5 AMLs among samples expressing PPARδ above each specific percentile was plotted (*y*-axis). The vertical dashed red line indicates the 99th percentile of PPARδ expression levels: the samples expressing PPARδ above this threshold include two M5 of three AMLs detected (67%). Panel (**B**): PPARδ expression levels in AML cases characterized by different FAB phenotypes. Expression levels of PPARδ gene are represented as signal and reported on *y*-axis. Analyzed cell populations included: normal CD34+ cells (CD34), normal bone marrow cells (NBM) and AMLs exhibiting different FAB phenotypes (M0–M6), all grouped based on their cell type and reported on *x*-axis. The horizontal dashed red line indicates the threshold corresponding to the 99th percentile of PPARδ expression levels across the whole dataset.

represented only a limited subset (3%) of the M5 type AMLs contained in the analyzed database, they exhibited a significant overexpression of the PPARδ gene with a signal ± SEM value of 132.4 ± 19.7 versus 20.1 ± 1.2 of the general database (*P* value 0.0016). These results enforce the previously underlined association existing between PPARδ expression and the monoblastic phenotype

and further support the possibility of a leukemogenic role of this nuclear receptor.

Discussion

Our results evidenced that, in spite of their modest inducing activity, PPARδ ligands significantly potentiated the monocyte differentiation effect promoted on myeloid cell lines by stimulation with VD. Apparently, the GW501516 compound, generally recognized as a highly specific PPARδ ligand, determined this response at concentrations ∼100 times higher than those previously used by other authors to obtain different effects, usually metabolic. This aspect of our experimental observation is not surprising since compounds such as all trans retinoic acid and VD, sharing a number of properties with the considered PPARδ ligand, induce the differentiation of hematopoietic cell lines (the same used in our study) at concentrations even 10 000 higher than those required to elicit their metabolic and/or physiological effects (15,19,20). The maturation arrest secondary to the leukemic nature of these cells is the mechanism universally invoked to explain their relative resistance to the stimulation with inducing agents. It is therefore conceivable that PPARδ ligands in general, and the GW501516 in particular, exert their differentiation activity according to this rule. Paradoxically, PPARδ overexpression determined a remarkable inhibitory effect on monocyte–macrophage differentiation induced by VD that was, at least partly, reverted by stimulation with the highly specific GW501516 PPARδ ligand. In this regard, gene silencing experiments performed in our laboratory provided a definitive confirmation indicating that the monocyte differentiation activity of GW501516 is actually mediated by its interaction with the PPARδ nuclear receptor. These findings are in perfect agreement with observations obtained by other authors using distinct experimental models and supporting the existence of a bimodal and ligand-dependent activity of PPARδ (5,21). Standing on the proposed mechanism, the balance between the unliganded and liganded forms of PPARδ is responsible for the final effect, becoming inhibitory when the former is prevalent on the latter and stimulatory in the opposite case. The mechanism underlying our results could reside in a direct repression of VDR target genes, exerted by unliganded PPARδ, and in the sequestration of the common retinoid X receptor dimerization partner, leading to a reduced availability of VDR/retinoid X receptor heterodimers necessary to activate VD-dependent differentiation response. Addition of the ligand is expected to counteract the first mechanism but to leave unaffected or even potentiate the second. This interpretation would consequently explain why addition of GW501516 reverts only partially the inhibition exerted by PPARδ overexpression on VD-induced monocyte differentiation. It is worth considering that a quantitatively and/or qualitatively inefficient ligand stimulation could also account for this observation. Altogether, our data indicate that PPARδ is a negative regulator of VD-mediated monocyte differentiation acting through a ligand-dependent modality. This observation implies a possible role of the investigated nuclear receptor in leukemogenesis where it may participate in the differentiation block of AMLs. Bioinformatic analysis of the Valk microarray database, containing the mRNA expression profiles of 285 AML cases exhibiting different FAB cytotypes, provided support to this hypothesis demonstrating the existence of a subset of M5 type AMLs that overexpress PPARδ gene. Further experimental work is needed to identify similar AML cases in a perspective study and to assess their response to treatment with PPARδ ligands. More in general, the sensitivity of leukemic monoblasts to the differentiation activity of these compounds indicate them as potential therapeutic agents for M5 type AML, a condition in which PPARδ ligands could be administered together with VD allowing to reduce the dosage and to limit the undesired hypercalcemic effect of this nuclear hormone.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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