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Retinoids Potentiate Peroxisome Proliferator-Activated Receptor γ Action in Differentiation, Gene Expression, and Lipid Metabolic Processes in Developing Myeloid Cells[S]

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ABSTRACT

Nuclear hormone receptors have been shown to be important transcription factors for regulating lipid metabolism in myeloid cells and were also implicated in differentiation processes of the myeloid lineage and macrophages. Peroxisome proliferator-activated receptor γ (PPARγ) seems to be a key component of lipid uptake by inducing the scavenger receptor CD36 that mediates oxidized low-density lipoprotein uptake in macrophages. Retinoic acid receptors, on the other hand, were also shown to play important roles in myeloid cell differentiation. In this study, we present evidence for a cross-talk between these two nuclear receptor pathways in myeloid cells. We show that expression level of PPARγ increases with the degree of monocyte/macrophage commitment during maturation. Activation of PPARγ leads to the increased expression of maturation markers (e.g., CD14, CD36). It is interesting that retinoid treatment potentiates PPARγ's ability to induce transcription of its target genes. Retinoid-increased PPARγ response is sufficient for enhancing lipid uptake. Our data, taken together, indicate that the expression level of PPARγ increases during monocyte/macrophage development. PPARγ activity can be enhanced by retinoids at least in part via increasing PPARγ expression level. These observations can be exploited to enhance therapeutically beneficial PPAR responses in myeloid cells.

Monocytes and polymorphonuclear phagocytes develop from pluripotent stem cells characterized by the expression of CD34. These cells give rise to granulocyte-monocyte, granulocyte, and monocyte colony-forming units. Monocyte differentiation proceeds from monocyte colony-forming units through monoblast to circulating monocytes, which mature to macrophages in various tissues, leading to the formation of multiple types of tissue-specific macrophages (Friedman, 2002). Markers for this lineage are M-CSF receptor, lysozyme, macrosialin, and cell-surface proteins (e.g., CD36, CD14, CD11b, and CD18). The differentiation of myeloid cells is principally regulated by cytokines, but nuclear receptors also have been implicated in these process.

Nuclear hormone receptors are ligand-activated transcription factors that regulate gene expression. One group of these receptors, the retinoid receptors, was reported to participate in developmental processes (Chambon, 1993). RARs have been implicated in embryonic (Mendelsohn et al., 1994), skeletal (Lohnes et al., 1994), myeloid development (Kastner et al., 2001; Friedman, 2002), wound healing, and keratinization (Goyette et al., 2000) and in the developing nervous system (Sucov and Evans, 1995). RARs are expressed in nearly all hematopoietic lineages (Tsai et al., 1992) and play a critical role in hematopoiesis (Kastner et al., 2001; Friedman, 2002). RARα and -γ knockout mice display a block in granulocytic differentiation (Labrecque et al., 1998). Dominant-negative RAR blocks neutrophil development at promyelocyte stage (Tsai and Collins, 1993) and switches normal

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ABBREVIATIONS: M-CSF, macrophage colony-stimulating factor; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; oxLDL, oxidized low-density lipoprotein; 9-*cis* RA, 9-*cis* retinoic acid; ATRA, all-*trans* retinoic acid; PMA, phorbol-12-myristate 13-acetate; PBS, phosphate-buffered saline; ADRP, adipose differentiation-related protein; PGAR, angiopoietin-related protein; RT, reverse transcriptase; PCR, polymerase chain reaction; Q, quantitative; FABP4, fatty acid-binding protein 4; AM580, 4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbonyl)aminobenzoic acid; LG268, 6-[-1(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-cyclopropyl]-pyridine-3-carboxylic acid; C/EBP, CCAAT/enhancer-binding protein; dil, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.

granulocyte/monocyte differentiation to basophil/mast cell development (Tsai et al., 1992). RAR was reported to act as a differentiation checkpoint switch at the promyelocytic stage of granulopoiesis, resulting in granulocytic differentiation (Zhu and Emerson, 2002). After the induction of myelomonocytic differentiation, an induction of RARα was observed (Zhu et al., 2001). Retinoic acid stimulates the maturation of myeloid precursors in cytokine-stimulated CD34-positive cells (Johnson et al., 2002). The 15;17 chromosome translocation in acute promyelocytic leukemia generates a promyelocytic leukemia-RARα fusion protein that inhibits RARs, resulting in a block of terminal differentiation of granulocytes (Kakizuka et al., 1991; Wang et al., 1998; Johnson et al., 2002).

RARs, like other nuclear receptors, function as heterodimers. The common dimerization partner is the retinoid X receptor (RXR). RXRs influence the retinoid pathways (Kastner et al., 1994) and all other metabolic pathways being regulated by RXR heterodimers.

The PPAR family of nuclear receptors consists of three receptors: PPARα, -γ, and -δ. All have been implicated in lipid metabolic processes (Kliewer et al., 1999; Kersten et al., 2000). PPARγ is essential for adipogenesis (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999) and was shown to influence myeloid development (Nagy et al., 1998; Tontonoz et al., 1998). PPARγ has not seemed to regulate the formation of the monocytic lineage but modulates differentiation and metabolic functions of macrophages (Chawla et al., 2001a; Moore et al., 2001). Treatment of activated macrophages with PPARγ ligands (e.g., thiazolidinediones) results in the inhibition of proinflammatory cytokine production (Ricote et al., 1998). It is interesting that the anti-inflammatory effects of thiazolidinediones are receptor-independent effects and do not require functional PPARγ (Chawla et al., 2001a; Moore et al., 2001). Activation of PPARγ also leads to the induction of a scavenger receptor, CD36, that potentiates the uptake of oxidized lipids from oxLDL (Nagy et al., 1998; Tontonoz et al., 1998).

Recent observations suggest that although PPARγ is not necessary for monocyte differentiation (Chawla et al., 2001a; Moore et al., 2001), modulation of the level and activity of PPARγ has critical consequences in the fate and metabolism of a macrophage. The mechanism by which PPARγ affects differentiation is still unknown, and the possible interconnection between retinoid and PPARγ signaling in the maturation process has not been studied in detail yet. To explore the role of PPARγ during myelopoiesis and the effects and consequences of PPARγ activation on differentiation, we studied human hematopoietic stem cells, monocytes, macrophages, and several myeloid leukemia cell lines representing various stages of development. Here, we show that the expression of PPARγ is highly induced in differentiating myeloid cells, and it increases parallel with the degree of maturation. Moreover, activation of PPARγ contributes to subsequent differentiation in the monocyte/macrophage pathway. We have also found evidence for cross-talk between retinoid and PPARγ signaling: retinoids potentiate developing cells' response to PPARγ activators. This cross-talk represents a novel convergence of the two signal pathways significant in the maturation of myeloid precursors and suggests a new opportunity for regulating PPARγ-related metabolic processes.

Materials and Methods

Cell Culture. KG-1, HL-60, and THP-1 cells were obtained from American Type Culture Collection (Manassas, VA). MonoMac-6 cells were a kind gift from E. Duda (Biological Research Center, Szeged, Hungary). The cells were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM glutamine, penicillin, and streptomycin (Sigma-Aldrich). Cells were treated with vehicle (ethanol-dimethyl sulfoxide), AM580 (BIOMOL Research Laboratories, Plymouth Meeting, PA), LG268, a gift from R. Heyman (Ligand Pharmaceuticals, San Diego, CA), rosiglitazone (Alexis Biochemicals, San Diego, CA), all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid (9-*cis* RA), and phorbol-12-myristate 13-acetate (PMA), respectively. All other reagents were purchased from Sigma-Aldrich or as indicated.

Isolation of Human Stem Cells and Monocytes. Human CD34-positive stem cells were isolated with CliniMax (AmCell GmbH, Bergisch Gladbach, Germany) from peripheral blood of granulocyte colony-stimulating factor-treated patients according to the protocol. Stem cells were expanded with recombinant human Flt-3L (25 ng/ml), stem cell factor (20 ng/ml), interleukin-6 (20 ng/ml), and interleukin-3 (20 ng/ml) for 10 days and then differentiated to macrophages with recombinant human M-CSF (10 ng/ml) for 8 days. All cytokines were purchased from Peprotech Inc. (Rocky Hill, NJ). Human monocytes were isolated from healthy volunteers' buffy coat according to the manufacturer's instructions using CD14 MicroBeads (Miltenyi Biotec, Bergisch Galdbach, Germany). Monocytes were differentiated during attachment for 2 to 4 days. Primary human cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, penicillin, and streptomycin.

RNA Extraction and Quantification. Total RNA was isolated according to the manufacturer's instructions with TRIzol reagent (Invitrogen) from cells after appropriate treatment. Transcript quantification was performed via quantitative real-time reverse-transcriptase (RT) polymerase chain reaction (PCR) using Taqman probes. Every sample was assayed in triplicate. For RT reaction, 20 to 100 ng of total RNA, specific reverse primer, and Superscript II reverse transcriptase (Invitrogen) were used performing at 42°C for 30 min and 72°C for 5 min. Real-time monitoring was carried out using an ABI Prism 7900 (Applied Biosystems, Foster City, CA) performing 40 cycles at 95°C for 12 s and 60°C for 1 min. Values of transcripts in unknown samples were calculated from standard curve derived from transcript-specific oligonucleotides. Transcript levels were normalized to the level of cyclophilin D and 36B4. Sequences of primers and Taqman probes used in transcript quantification are listed in Supplemental Table S1.

Flow Cytometry. Analysis of cell-surface expression of proteins was performed on a Coulter flow cytometer (Beckman Coulter Inc., Fullerton, CA). In brief, cells were washed in phosphate-buffered saline (PBS), pH 7.4, supplemented with 0.5% bovine serum albumin and then were incubated with anti-CD14-retinal pigment epithelial (DakoCytomation Denmark A/S, Glostrup, Denmark), anti-CD36-fluorescein isothiocyanate (BD Biosciences, San Jose, CA) antibody, respectively, for 1 h at 4°C; finally, cells were washed in PBS-bovine serum albumin, and 10,000 cells were counted on the cytometer.

Western Blot. Cells were treated for 2 days as indicated, were washed in PBS, and then lysed in buffer A (Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM β-mercaptoethanol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration was determined with Bradford reagent (Bio-Rad, Hercules, CA), and 25 μg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad). After blocking in 5% dry milk, the membrane was probed with anti-PPARγ antibody (BIOMOL) and subsequently with peroxidase-conjugated secondary antibody. ECL detection kit (Pierce Chemical, Rockford, IL) was used for signal detection.

TABLE 1

Retinoids induce PPARγ, C/EBPβ, and some macrophage-associated genes but do not induce monocyte/macrophage development per se

Affymetrix GeneChip analysis was carried out from AM580 (100 nM)-treated THP-1 cells. After 96 hours of treatment, total RNA was extracted and labeled as described under *Materials and Methods*. The fold changes in PPAR and C/EBP mRNAs, in the induction of CD markers, and in genes from Gene Ontology database associated with macrophages are shown in the table. Genes with inductions of at least 2-fold are shown in bold.

Gene Name	Fold Change
PPARs	
PPAR α	0.714
PPAR γ	**6.862**
PPAR δ	1.561
C/EBPs	
C/EBP α	1.105
C/EBP β	**3.005**
C/EBP γ	1.296
C/EBP δ	0.932
C/EBP ϵ	Absent
C/EBP ζ	0.721
CD markers	
CD14	**121.943**
CD1D	**5.161**
CD209	**4.334**
CD36	**7.369**
CD74	**2.656**
CD86	**2.620**
CDW52	**4.379**
CDW92	**3.799**
CD163	Absent
CD164	1.323
CD164L1	0.783
CD1A	Absent
CD1B	Absent
CD1C	Absent
CD1E	Absent
CD209L	0.862
CD33	0.686
CD48	Absent
CD63	1.092
CD68	1.172
CD69	Absent
CD84	1.113
Macrophage-associated genes	
COL8A1	**2.236**
CSF1	**2.308**
CSF1R	**3.409**
CSF2RA	**3.324**
IL18	**2.220**
IL31RA	**124.907**
ITGAM	**5.084**
ITGB2	**4.593**
MPEG1	**8.584**
SLAMF8	**6.755**
TLR4	**2.014**
AZU1	1.076
CCL15	Absent
CCL23	1.096
CD80	1.321
CES1	0.815
CKLF	0.772
CLECSF14	1.346
CNR2	Absent
CSF2	1.438
ELA2	0.503
IL31RA	Absent
INHA	1.431
INHBA	Absent
LOC221938	Absent
MAEA	0.695
Magmas	0.847
MARCO	Absent
MIF	0.544
MMD	1.258
MMP12	0.837
MMRP19	1.327
MSR1	Absent
MST1	Absent
NOS2A	Absent
PTPNS1	1.147
SAA2	Absent
SCARA3	Absent
SFTPD	Absent
TLR6	Absent
ZBP1	Absent

Microarray Analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) and further purified by using the RNeasy kit (QIAGEN, Valencia, CA). cRNA was generated from 5 μg of total RNA by using the SuperScript Choice Kit (Invitrogen) and the High-Yield RNA transcription-labeling kit (Enzo Diagnostics, New York, NY). Fragmented cRNA was hybridized to Affymetrix (Santa Clara, CA) arrays (HU133 Plus 2.0) according to Affymetrix standard protocols. Preliminary data analysis was performed by the Microarray Core Facility of the European Molecular Biology Laboratory (Heidelberg, Germany). Further analysis was performed using GeneSpring 7.0 (Silicon Genetics, Redwood City, CA). These analyses provided a signal for each specific transcript that was subsequently normalized by comparing with the median signal (arbitrary value of 1.0) obtained from the whole array. Genes associated with macrophages were chosen by using Gene Ontology in GeneSpring 7.0 software.

oxLDL Uptake. For oxLDL uptake experiments, we used diI-labeled oxidized human LDL (Intracel, Frederick, MD). After 2 days of treatment with the indicated ligands, cells were treated with 5 μg of protein/ml diI-oxLDL for 6 h at 37°C. Cells were washed, and 10,000 cells were counted by flow cytometer. Median values of the fluorescence intensities are shown. The experiment was repeated three times with similar results.

Statistical Analysis. Data are presented as means ± S.D. In real-time quantitative PCR experiments, the mean and standard deviation were calculated for both the normalized and the normalizer values. To incorporate the random errors of the measurements, we used the propagation of errors to determine the standard deviation of the normalized values. For all experiments, we made at least four biological replicates, and on the fold changes, we performed an F test followed by an unpaired (two-tail) t test; results were considered significant at $p < 0.01$.

Results

First, we compared the expression of PPARα, -γ, and -δ in primary human CD34-positive cells after isolation and after differentiation with M-CSF for 8 days to primary human macrophages. We were surprised to find a significant and consistent change in the mRNA expression profile under the various conditions (Fig. 1A). During the maturation of stem cells, we could detect a 7-fold induction of PPARγ along with a minor increase in PPARα and -δ levels. Likewise, in the monocyte/macrophage transition, PPARγ induction was the highest (27-fold), and PPARα and -δ were induced to a lesser degree; however, these changes were greater than those observed in stem cells. We also measured the expression levels of RARs and RXRs (Fig. 1B). All of these receptors were expressed at levels lower than PPARs. RXRα showed the biggest (almost 3-fold) change in the CD34-positive cell-derived macrophages compared with the CD34-positive cells, whereas we could detect 2.6- and 4.3-fold induction of RARα and RARγ, respectively, during monocyte/macrophage transition. The levels of other retinoid receptors were not changed, or the changes were lower than 2-fold. RARβ and RXRγ could not be detected in these cells.

As a model for the different stages of myeloid differentia-

tion, we chose myeloid leukemia cell lines: KG-1 representing acute myelogenous leukemia, HL-60 representing acute promyelocytic leukemia (FAB M3), and THP-1 (FAB M5) and MonoMac-6 (FAB M5) representing two monocytic leukemia cell lines. MonoMac-6 proved to be most committed to the monocytic lineage, characterized by its increased phagocytic capacity, chemotactic potential, cytokine production, and cell-surface expression of monocytic markers (Ziegler-Heitbrock et al., 1988). We determined the absolute mRNA copy numbers of PPARs, RARs, and RXRs expressed in these cells and compared with the levels of monocytes and macrophages (Fig. 1, C and D). PPARs are expressed at high levels in these cell types except in the least matured KG-1 cells. PPARα level is similar in the other three cell lines, PPARδ is higher in the two monocytic leukemia cell lines, and PPARγ mRNA is the most abundant in the most mature MonoMac-6 cells, reaching the level of that of the macrophage (Fig. 1C). RARα, RXRα, and RXRβ are expressed in the two differentiated cell lines (HL-60 and THP-1) and in monocytes/macrophages at the highest level and only at low levels in the least differentiated KG-1 cells. RARγ level in the cell lines is lower than that in monocytes. These data suggested that monocyte/macrophage differentiation was accompanied by an induction of PPARγ and at a lesser degree of PPARδ levels. The significant induction in PPARγ levels raised the possibility that activation of the receptor may be part of or may contribute to the maturation process. Expression of retinoid receptors did not show such a change during maturation, but their presence indicated possible roles for these receptors in macrophage development. These data also suggested that the leukemia cell lines are likely to serve as an appropriate and representative model for studying the contribution of varying PPARγ levels to myeloid cell differentiation and function.

To characterize the role of PPARγ in these cells, we activated them with a synthetic agonist, rosiglitazone, and analyzed the effects on monocytic differentiation and on activation of target gene transcription. In the least differentiated cell line (KG-1), activation resulted in no significant changes in cell-surface markers (data not shown), which was probably because of the very low level of the receptor expressed in these cells. In the most matured MonoMac-6 cells activation of the PPARγ/RXR heterodimer with synthetic agonists, rosiglitazone for PPARγ and LG268 for RXR induced the expression of CD14 and CD36 differentiation markers on the cell surface (Fig. 2, A and B). In Fig. 2, C and D, we show that the induction was caused by changes in mRNA levels, and a synergy between Rosiglitazone and LG268 could also be observed at the mRNA expression level. These results are in agreement with our findings reported previously in THP-1 cells (Nagy et al., 1998; Tontonoz et al., 1998) with one important difference: in the case of MonoMac-6 cells, PMA pretreatment was not necessary to obtain PPARγ responses, unlike in the case of THP-1 cells. This phenomenon can be explained by the fact that PPARγ level, which is induced upon PMA treatment (data not shown) (Nagy et al., 1998; Tontonoz et al., 1998), was high enough in the MonoMac-6 cells (Fig. 1C), higher than in THP-1, to ensure optimal target gene expression. These data suggested that activation of PPARγ/RXR heterodimer enhanced the differentiation of cells in the monocytic pathway and also supported the notion that this process could occur only if the cells were in a permissive stage of their maturation (cells need to be committed in the monocytic pathway but not fully differentiated yet) and expressed a sufficient amount of PPARγ. Our next question was how one could prime the cells to enter this final differentiation stage and regulate PPARγ expression levels to become matured cells and acquire a full capacity to induce transcription of PPARγ target genes that regulate lipid homeostasis. There is extensive evidence suggesting that retinoids play important roles in the early developmental processes of hematopoietic cells (Kastner et al., 2001; Friedman, 2002). We tested whether they induced myeloid differentiation. On the one hand, we found that RAR agonists caused maturation only in the least differentiated cells (KG-1, HL-60) examined, directing them toward the granulocytic lineage, but we have found no evidence that retinoids could induce monocyte/macrophage markers in these cell lines. On the other hand, RXR-specific agonists caused similar effects

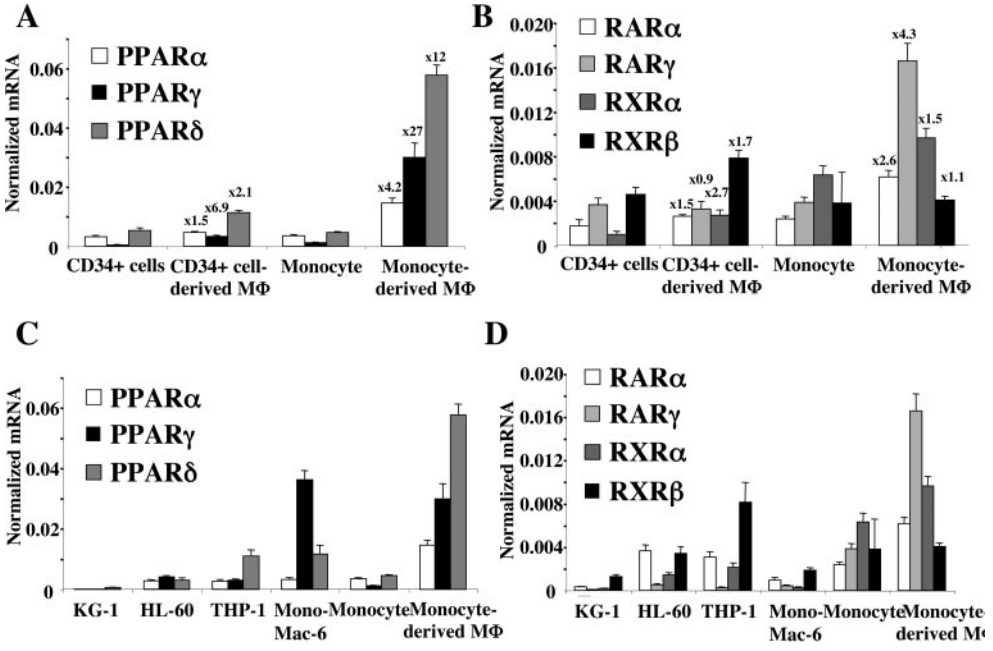

Fig. 1. Expression of PPARγ is linked to the differentiation of macrophage. A and B, CD34-positive cells and monocytes were isolated and differentiated as described under *Materials and Methods*. RNA was isolated from stem cells and monocytes after 8 days of differentiation with M-CSF (10 ng/ml) from CD34-positive cell-derived macrophages and after 2 days from monocyte-derived macrophages (MΦ). Real-time RT Q-PCR was performed to measure nuclear receptor mRNA levels. The fold changes are shown compared with the CD34-positive stem cells or with the monocytes, respectively. C and D, RNA was isolated from KG-1, HL-60, THP-1, and MonoMac-6 cells and monocytes and monocyte-derived macrophages, and then real-time RT Q-PCR was used to determine nuclear receptor mRNA levels. All measurements were performed in triplicate, and the results normalized to cyclophilin D are shown ± S.D.

as PPARγ activators (Fig. 2C), suggesting that RXR-specific ligands might activate and function through PPARγ/RXR heterodimers. A combination of RAR and PPARγ activators resulted in an unexpected synergy on PPARγ-induced gene expression. In the experiments, we used sequential treatment (retinoids followed by PPARγ activators), because we reasoned that it probably represented a more physiological setting (i.e., previous reports propose a role for retinoids in the earlier steps of myeloid cell differentiation) (Kastner et al., 2001; Friedman, 2002). When we treated the MonoMac-6 cells sequentially first with RAR agonists and then with PPARγ agonists, we observed an increased effect of PPARγ-specific ligands on gene expression of CD14 and CD36 (Fig. 3, A and B), the latter being a direct target gene of PPARγ/RXR heterodimers. We could show that this phenomenon was present in both THP-1 and MonoMac-6 cells (Fig. 4) but not in the least matured KG-1 and HL-60 cells (data not shown). The cells were treated first with a receptor-selective retinoid (RARα or RXR-specific agonists), the combination of both, or natural retinoids (ATRA or 9-*cis* RA) for 48 h, followed by a vehicle or rosiglitazone treatment for an additional 48 h. We characterized this retinoid-evoked potentiation of PPARγ response by measuring the induction of various PPARγ target genes: fatty acid-binding protein 4 (FABP4) (Graves et al., 1992) (Fig. 4, A and B), CD36 (Nagy et al., 1998; Tontonoz et al., 1998) (Fig. 4, C and D), adipose differentiation-related protein (ADRP) (Gupta et al., 2001; Vosper et al., 2001) (Fig. 4, E and F), and PPARγ angiopoietin-related protein (PGAR) (Yoon et al., 2000) (Fig. 4, G and H). Rosiglitazone readily induced (see bars C/Rosigl. in Fig. 4) target gene expression in MonoMac-6 cells in the cases of FABP4 (42-fold), CD36 (5-fold), and PGAR (150-fold) and in THP-1 cells in the case of FABP4 (43) and PGAR (3). We also noted that all retinoids induced CD36 expression in both cell lines (Fig. 4, C and D). ATRA caused potentiation in both cell lines of all of the measured target genes: the inductions are 213-, 19-, 3-, and 150-fold compared with 42-, 5-, 0.7-, and 150-fold, respectively, in MonoMac-6 cells in the case of FABP4 (A), CD36 (C), ADRP (E), and PGAR (G). THP-1 cells behaved similarly (B, D, F, and H). The effect of 9-*cis* RA was similar to that of ATRA, with even higher inductions. AM580 was less effective than ATRA but also showed enhanced induction with rosiglitazone (e.g., Fig. 4, A, 102-fold compared with 42-fold, C, 20-fold compared with 5-fold, D, 9-fold compared with 0.4-fold, and H, 17-fold compared with 3-fold). LG268 pretreatment potentiated FABP4, ADRP, PGAR, and CD36 expres-

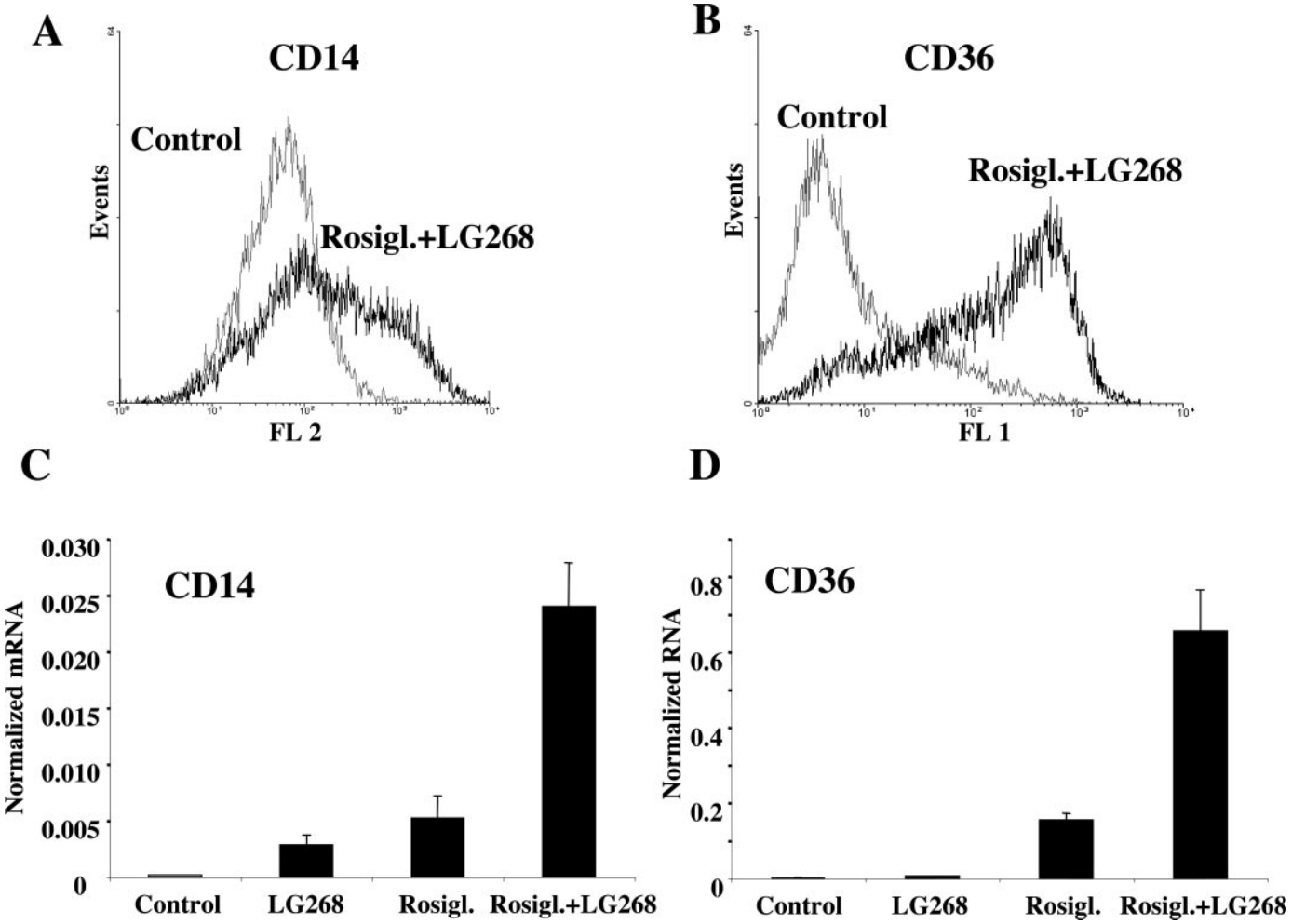

Fig. 2. Activation of PPARγ induces the differentiation of myeloid cells. MonoMac-6 cells were treated with LG268 (100 nM), rosiglitazone (1 μM), or both, or vehicle for 2 days. Cells labeled with anti-CD14-retinal pigment epithelial (A) and anti-CD36-fluorescein isothiocyanate (B) were analyzed with flow cytometry, and 10,000 cells were counted. Total RNA isolated from the same experiment was used to quantify the mRNA levels of CD14 (C) and CD36 (D) with RT Q-PCR. All measurements were performed in triplicate, and the results normalized to 36B4 are shown ± S.D.

sion induced by rosiglitazone. Synergy was particularly striking in MonoMac-6 cells for FABP4 and PGAR probably because of the fact that these genes are not induced by LG268 alone. Some of the effects may be explained by remnant LG268 binding to RXR in heterodimers and synergy with rosiglitazone. The highest inductions could be observed in the case of AM580 + LG268 pretreatments. Probably the most striking and unexpected effect was that AM580, an RARα-selective compound, could readily potentiate rosiglitazone-induced CD36 expression in both cell lines (Fig. 4, C and D) (20-fold compared with 5-fold, and 9-fold compared with 0.4-fold), FABP4 (Fig. 4A) (102-fold compared with 42-fold) induction in MonoMac-6, and PGAR (Fig. 4H) (17-fold compared with 3-fold) induction in THP-1 cells. It was significant that pretreatment of cells with the pan-retinoid receptor agonist 9-*cis* RA or a combination of AM580 and LG268 followed by rosiglitazone produced the highest level of target gene expression in both cell types for all four target genes. These results clearly indicated that retinoid pretreatment differentially affected the two cell types and the four target genes by potentiating PPARγ responses. A significant component of this potentiation is the induction by RARα-selective retinoids. Next, we wanted to know how long the pretreatment was necessary for the potentiation to develop. We found that 6 h of pretreatment was sufficient for the enhancement of PPARγ response (data not shown).

There are many potential mechanisms to account for this effect, ranging from epigenetic changes through changes in cofactor/coactivator levels to the direct or indirect induction of the receptor levels. We tested the most obvious mechanism, the induction of PPARs. We measured PPAR mRNA levels and found that both natural and synthetic RAR and RXR agonists induced PPARγ mRNA levels, whereas PPARα and δ mRNA levels were unaffected in either THP-1 or MonoMac-6 cells (Fig. 5, A and B). To obtain data from cells of ex vivo origin, we examined monocyte-derived macrophages. We found a similar induction of PPARγ transcription in primary human monocyte-derived macrophages when treated with 9-*cis* RA, whereas rosiglitazone had no effect on PPARγ mRNA level (Fig. 5C). We also found that this transcriptional activation of PPARγ resulted in an elevation in the protein level (Fig. 5D). It is interesting that retinoids proved to be as effective as PMA in the induction of PPARγ.

The next question was whether the induction of PPARγ is a direct or indirect effect. First, we analyzed the promoters of PPARγ1, -2, and -3 in a transient transfection experiment and found that retinoids had no effect on PPARγ promoter (data not shown). Next, we performed a time course of PPARγ induction (Fig. 6A) in THP-1 cells. The PPARγ mRNA level remains unchanged for 12 h after retinoid treatment, whereas the mRNA of a bona fide RAR- and RXR-regulated target gene, CYP27, shows a marked induction after 2 and 4 h (Szanto et al., 2004). Both the induction of PPARγ (Fig. 6C) and CYP27 (Fig. 6D) seemed to be dose-dependent. To test whether and to what extent retinoids induced macrophage maturation on the THP-1 monocytic leukemia, we carried out microarray analysis on retinoid (AM580)-treated

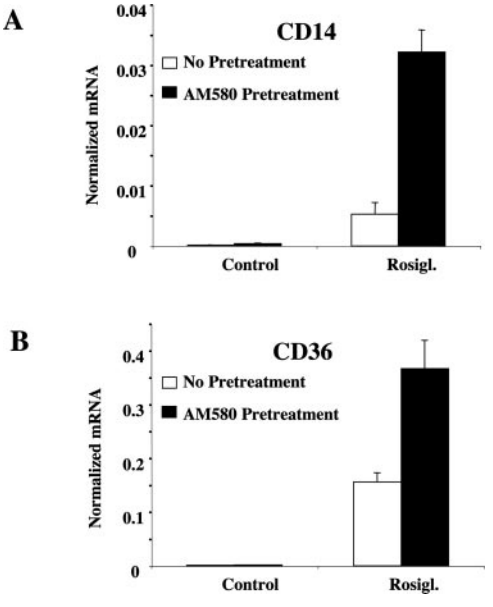

Fig. 3. Retinoid pretreatment enhances PPARγ-induced differentiation. MonoMac-6 cells were pretreated with AM580 (100 nM) or vehicle for 2 days, washed three times in PBS and plated out again, and finally treated with rosiglitazone (1 μM) or vehicle for an additional 2 days. RNA was isolated, and mRNA levels of CD14 (A) and CD36 (B) were measured with RT Q-PCR. All measurements were performed in triplicate, and the results normalized to 36B4 are shown ± S.D.

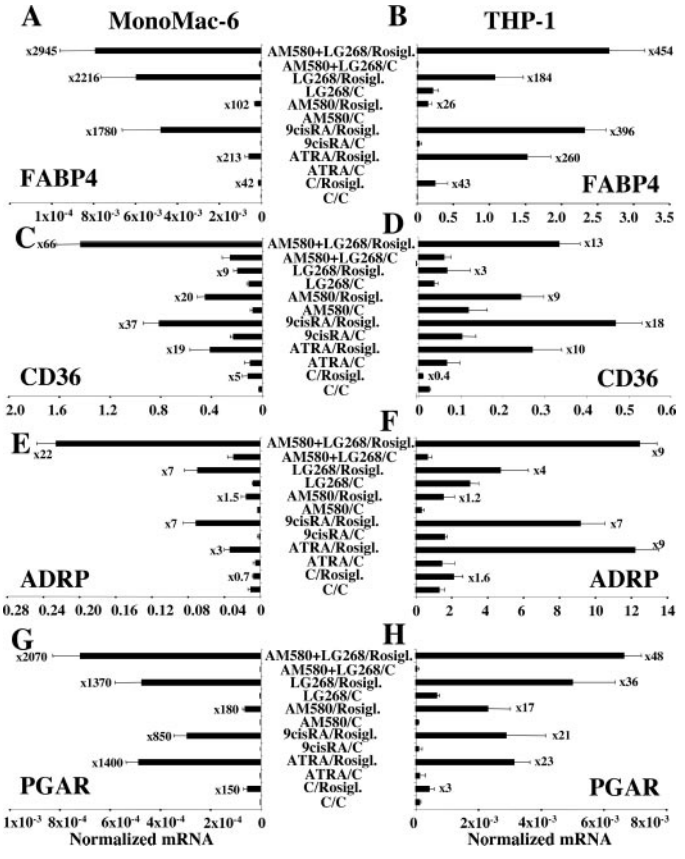

Fig. 4. Retinoid pretreatment enhances the expression of PPARγ-induced target genes. MonoMac-6 (A, C, E, and G) and THP-1 (B, D, F, and H) cells were pretreated with ATRA (1 μM), 9-*cis* RA (1 μM), AM580 (100 nM), LG268 (100 nM), AM580 + LG268, or vehicle (C as control) for 2 days. Cell were washed three times in PBS, plated out, and treated with rosiglitazone (Rosigl.) (1 μM) or vehicle (C as control) for an additional 2 days. RNA was extracted, and PPARγ target-gene mRNA levels were quantified by RT Q-PCR: FABP4 in A and B, CD36 in C and D, ADRP in E and F, and PGAR in G and H. The fold inductions by rosiglitazone are shown compared with the vehicle-treated cells (C/C). All measurements were performed in triplicate, and the results normalized to cyclophilin D are shown ± S.D.

THP-1 cells (Supplemental Table S1). Part A shows the induction of PPARs, whereas C and D show the expression of macrophage-associated genes on the basis of CD markers (C) and gene ontology classifications (D). Some markers are induced as shown in gray, but most of these genes do not change after retinoid treatment. We concluded from this analysis that unlike PMA, retinoids do not induce cellular differentiation per se in these cells; therefore, this effect is unlikely to be strictly differentiation-related. The microarray analysis allowed us to examine the expression changes of transcriptional regulators of PPARγ. PPARγ and its induction are known to be related to another differentiation process, adipogenesis. In this well-characterized process, C/EBPs induce the expression of PPARγ (Wu et al., 1995; Yeh et al., 1995; Rosen et al., 2002). We tested whether C/EBPs are induced by retinoids and found a 3-fold induction in C/EBPβ level, whereas other C/EBPs remained unchanged. These results show that the induction of PPARγ is a late, indirect, dose-dependent mechanism accompanied by the induction of only a few macrophage-associated genes and C/EBPβ.

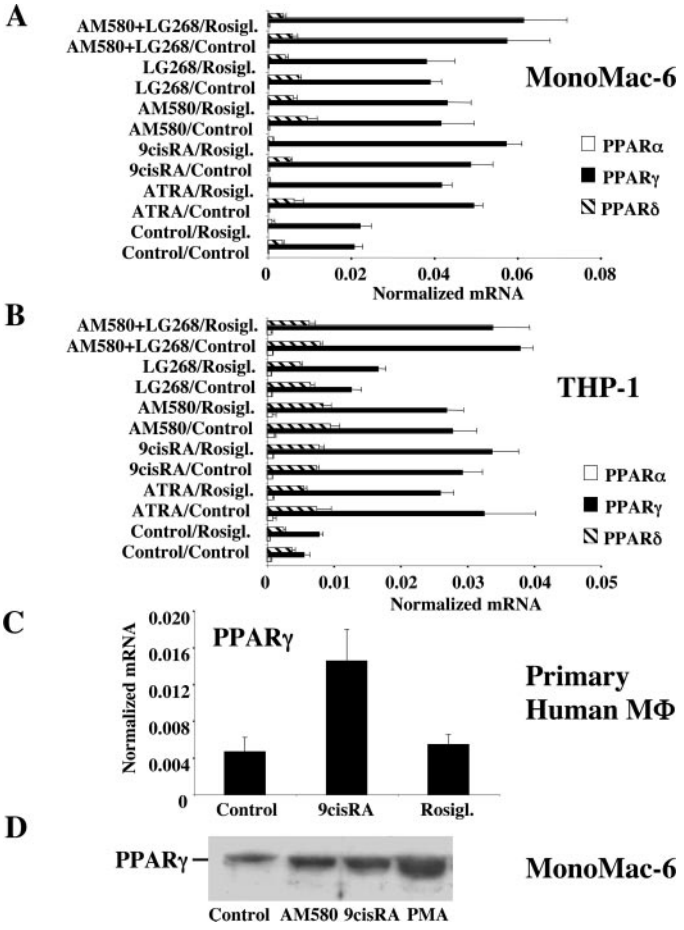

Fig. 5. Retinoids induce the expression of PPARγ. MonoMac-6 (A) and THP-1 (B) cells were pretreated with ATRA (1 μM), 9-*cis* RA (1 μM), AM580 (100 nM), LG268 (100 nM), AM580 + LG268, or vehicle for 2 days. Cell were washed three times in PBS, plated out, and treated with rosiglitazone (1 μM) or vehicle for an additional 2 days. RNA was extracted, and PPARα, -γ, and -δ mRNA levels were quantified using RT Q-PCR. All measurements were performed in triplicate, and the results normalized to cyclophilin D are shown ± S.D. C, primary human monocytes were differentiated and treated with 9-*cis* RA (1 μM), rosiglitazone (1 μM), or vehicle for 2 days; then, RNA was extracted, and PPARγ mRNA levels were measured with RT Q-PCR. Measurements were performed in triplicate, and the results normalized to cyclophilin D are shown ± S.D. D, MonoMac-6 cells were treated with AM580 (100 nM), 9-*cis* RA (1 μM), PMA (50 ng/ml), or vehicle for 2 days. After cell lysis, Western blot was performed as described under *Materials and Methods* with anti-PPARγ antibody.

Finally, we sought to obtain evidence for the biological consequence of retinoid-enhanced PPARγ response. Therefore, we measured the uptake of diI-labeled oxLDL in retinoid-pretreated MonoMac-6 cells after activation with PPARγ agonists (Fig. 7). Similarly to our previous report on THP-1 cells (Nagy et al., 1998; Tontonoz et al., 1998), rosiglitazone induced oxLDL uptake only in the presence of an RXR agonist in control cells, whereas retinoid-treated cells and only those that were treated with RAR agonists became capable of taking up oxLDL after PPARγ agonist treatment alone. These data suggest that the efficacy of low-affinity or partial agonists of PPARγ could be substantially increased with retinoid pretreatment. The PPARγ and RXR agonist-induced uptake was also increased in retinoid-pretreated cells. These results showed that retinoid pretreatment not only induced increased transcription of PPARγ target genes but also facilitated oxLDL uptake into the cells, providing a potentially new target for the modulation of cholesterol uptake and metabolism in macrophages.

Discussion

Since the initial discovery of PPARγ in myeloid cells (Kliewer et al., 1994; Greene et al., 1995), a large number of studies have sought to define its biological role. Many studies, including some of our own, have established PPARγ as a differentiation-related transcription factor in myeloid cells (Nagy et al., 1998; Ricote et al., 1998). However, no systematic analysis of its expression and the regulation of the PPARγ response have been carried out on myeloid cells of human origin.

In this study, we have attempted to systematically characterize the level of PPARγ in human normal and leukemia myeloid cells. We have established a role for nuclear receptors in myelogenous differentiation: we showed that PPARγ expression is tightly linked to the differentiation stage of myeloid cells in the monocytic lineage and that promotion of differentiation induces the expression level of this gene. Moreover, activation of this nuclear receptor results in the induction of differentiation markers of macrophages. We also showed that retinoids potentiate the effects of PPARγ activators by inducing the transcription of PPARγ itself. We provided evidence that retinoids may contribute to the physiological/pathophysiological function of PPARγ by increasing the uptake of oxLDL particles. These findings establish a link between retinoid receptors and PPARγ in myeloid differentiation and implicate RAR as a potential "jump-starter" of PPARγ signaling pathway in macrophages.

The Role of PPARγ Signaling in Monocytes/Macrophages. PPARγ has been implicated in numerous developmental processes. Disruption of the PPARγ gene in mice is lethal during early development: it is required for differentiation of the trophoblast and placental vascularization, and homozygous PPARγ-deficient embryos die at day 10 of embryonic development (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999). The PPARγ-null mice that survived to term were deficient in all forms of fat, substantiating the

fundamental role for PPARγ in adipogenesis (i.e., fat storage) (Barak et al., 1999). Although PPARγ-null embryonic stem cells were capable to differentiate into macrophages in vitro (Chawla et al., 2001a), it was shown that PPARγ activators enhance monocytic development (Nagy et al., 1998; Tontonoz et al., 1998), and PPARγ-specific roles have been identified in macrophage lipid metabolism (Nagy et al., 1998; Tontonoz et al., 1998; Chawla et al., 2001b). These were the regulation of oxidized LDL uptake and the transcriptional activation of LXRα. Via this latter pathway, PPARγ can also contribute to cholesterol efflux by indirectly inducing ABCA1 transcription. Myeloid-specific disruption of the PPARγ gene in mice resulted in reduced total plasma and high-density lipoprotein cholesterol levels. The lack of the PPARγ gene in macrophages is therefore likely to be proatherogenic. From this evidence, it is not difficult to see that understanding the regulation of PPARγ responsiveness in myeloid cells and macrophages is important and may prove to be therapeutically relevant. We have used myeloid leukemia cell lines blocked at different stages of differentiation and also normal human myeloid cells to study the regulation of PPARγ responsiveness during myeloid maturation. The expression level of PPARγ showed remarkable correlation with the differentiation stage of myeloid cells. Retinoids have been implicated in myeloid differentiation on multiple levels but mainly in the granulocytic pathway (Tsai et al., 1992; Tsai and Collins, 1993; Labrecque et al., 1998; Zhu and Emerson, 2002). It has been reported that retinoids have a role earlier in myelogenesis affecting the common granulocyte/monocyte precursors (Johnson et al., 2002). This observation is consistent with our findings and suggests that it is likely that retinoid action precedes the appearance of PPARγ responsiveness and may contribute to its development during macrophage differentiation.

Cross-Talk between PPARγ and Retinoid Signaling. This is not the first example of a cross-talk between retinoid and PPARγ signaling during a differentiation process. A cross-talk between retinoid and PPARγ signaling during fat cell differentiation has been extensively characterized by the Lazar group. They found that retinoic acid blocks adipogenesis by inhibiting C/EBPβ-mediated transcription (Xue et al., 1996; Schwarz et al., 1997). In myeloid cells, the situation is clearly different. Retinoids promote PPARγ expression and responsiveness, indicating that the pathways are interrelated, but the consequence of the cross-talk is cell-type–specific. We have recently identified a link between RAR, PPARγ, and LXR signaling in macrophages, in which PPARγ- and/or RAR-mediated activation of a P450 enzyme, CYP27, leads to LXR activation (Szanto et al., 2004). Our data presented here suggest that the interrelatedness of the three nuclear receptor-mediated pathways are more complex than believed previously and that retinoid signaling may have a larger role in macrophage gene expression and

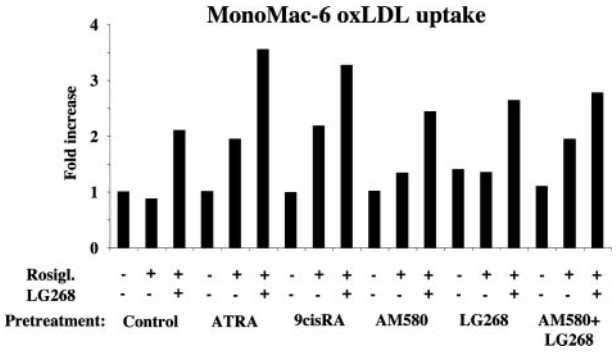

Fig. 7. Retinoid pretreatment enhances oxLDL uptake induced by PPARγ activators. MonoMac-6 cells were pretreated with ATRA (1 μM), 9-*cis* RA (1 μM), AM580 (100 nM), LG268 (100 nM), AM580 + LG268, or vehicle for 2 days. Cell were washed three times in PBS, plated out, and treated with rosiglitazone (1 μM), rosiglitazone and LG268 (100 nM), or vehicle for an additional 2 days. DiI-labeled oxLDL (5 μg/ml) was added to cells for 6 h, and 10,000 cells were counted by flow cytometry; the fold changes are shown. All of these values are compared with the value of the sample without any ligand treatments. The experiment was repeated three times with similar results. From these, one representative experiment is shown.

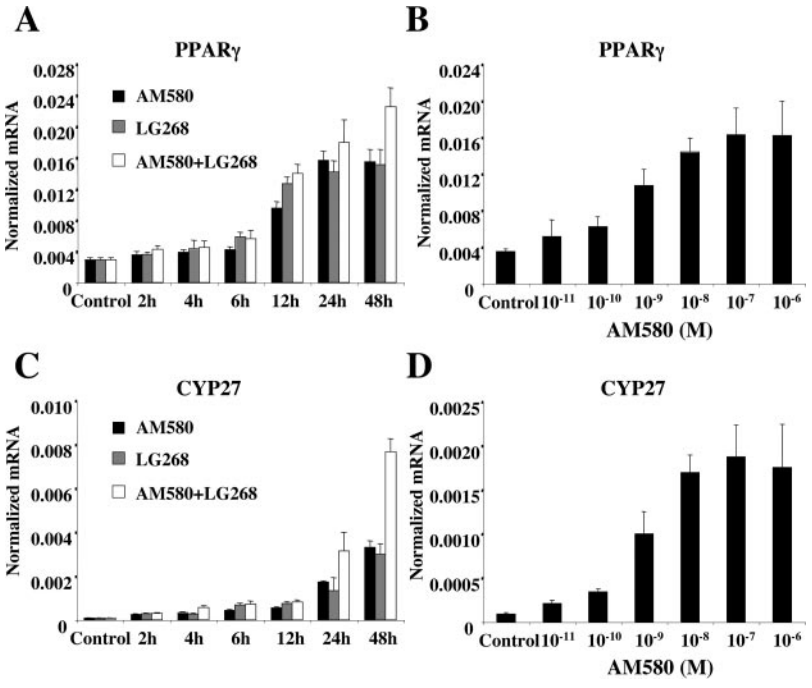

Fig. 6. Induction of PPARγ is time- and dose-dependent. A and B, THP-1 cells were treated with AM580 (100 nM), LG268 (100 nM), and both for the indicated times. Total RNA was isolated from the cells, and PPARγ (A) and CYP27 (B) mRNA levels were determined using RT Q-PCR. C and D, THP-1 cells were treated with AM580 at the indicated concentrations for 2 days; then, RNA was extracted from the cells, and PPARγ (C) and CYP27 (D) mRNA levels were determined using RT Q-PCR. All measurements were performed in triplicate, and the results normalized to cyclophilin D are shown ± S.D.

metabolism than suspected previously. The fact that retinoids potentiate PPARγ responses seems to be part of a complex differentiation pathway. Retinoids are known to induce differentiation of myeloblastic leukemia cells, resulting in granulocytes, and also are known not to induce maturation of monocytic leukemia cells. Our results indicate that retinoids have some early maturation effect also on these cells without committing the cells per se to the macrophage lineage. As for the potential mechanism of this effect, our microarray analysis shows that retinoids also induce the expression of C/EBPβ, which is known to induce PPARγ transcription during adipogenesis. This may also contribute to the increase in PPARγ levels. These mechanisms only explain the induction of PPARγ, but the potentiation in PPARγ responses could be an even more complicated phenomenon, and one possible contribution to this is the induction of the receptor. Other mechanisms, like epigenetic changes and induction of cofactors, cannot be excluded, and dissecting the complexity of these interrelated processes are far beyond this study. One may speculate that sequential effects of retinoids followed by PPARγ activators are coordinately regulating myeloid maturation and gene expression, a proposition that can be tested only in in vivo experiments. Needless to say, a pharmaceutically amenable pathway to modulate PPARγ responsiveness can be used to boost the antiatherogenic effects of PPARγ-regulated gene expression. It clearly requires further studies before retinoid-regulated PPARγ responsiveness becomes a valid clinical target.

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