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Retinoids potentiate PPARγ action in differentiation, gene expression and lipid metabolic processes in developing myeloid cells

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Running title: Retinoids enhance PPARγ responses

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Abbreviations: PPAR: Peroxisome Proliferator Activated Receptor, RAR: Retinoic Acid Receptor, RXR: Retinoid X Receptor

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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 gamma (PPARγ) appears to be a key component of lipid uptake by inducing the scavenger receptor CD36 that mediates oxidized low-density lipoprotein (oxLDL) uptake in macrophages. Retinoic Acid Receptors (RAR) on the other hand were also shown to play important roles in myeloid cell differentiation. Here we present evidence for a crosstalk 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). Interestingly, 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.

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Introduction

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 (CFU-GM, CFU-G and CFU-M). Monocyte differentiation proceeds from CFU-M 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 in addition nuclear receptors 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 were 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, keratinization (Goyette et al., 2000) and also in the developing nervous system (Sucov and Evans, 1995). RARs are expressed in nearly all hematopoietic lineages (Tsai et al., 1992) and play 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 granulocyte/monocyte differentiation to basophil/mast cell development (Tsai et al., 1992). RAR was reported to act as a

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differentiation checkpoint switch at the promyelocytic stage of granulopoiesis resulting in granulocytic differentiation (Zhu and Emerson, 2002). Following induction of myelomonocytic differentiation an induction of RARα was observed (Zhu et al., 2001). Retinoic acid (RA) 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 (APL) generates a PML-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 RXR (Retinoid X Receptor). 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), (Rosen et al., 1999), (Kubota et al., 1999) and was shown to influence myeloid development (Nagy et al., 1998), (Tontonoz et al., 1998). PPARγ has not appeared 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 (TZDs)) results in inhibition of proinflammatory cytokine production (Ricote et al., 1998). Interestingly, the anti-inflammatory effects of TZDs are receptor independent effects, do not require functional PPARγ (Chawla et al., 2001a), (Moore et al., 2001). Activation of PPARγ also leads to the induction of a

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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. In order to explore the role for PPARγ during myelopoiesis, the effects and the 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 crosstalk between retinoid and PPARγ signaling: retinoids potentiate developing cells' response to PPARγ activators. This crosstalk represents a novel convergence of the two signal pathways important in the maturation of myeloid precursors and suggests new opportunity for regulating PPARγ-related metabolic processes.

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Materials and methods

Cell culture

KG-1, HL-60, THP-1 cells were obtained from ATCC (Manassas, VA). MonoMac-6 cells were kind gift of E. Duda (Biological Research Center, Szeged, Hungary). The cells were grown in RPMI 1640 (Sigma-St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen-Carlsbad, CA), 2mM glutamine, penicillin and streptomycin (Sigma). Cells were treated with vehicle (ethanol-dimethyl-sulfoxide), AM580 (Biomol-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), phorbol-12-myristate 13-acetate (PMA), respectively. All other reagents were purchased from Sigma (St. Louis, MO) 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 peripherial 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 volunteer's buffy coat according to the manufacturer's instructions using CD14 MicroBeads

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(Miltenyi Biotec-Bergisch Galdbach, Germany). Monocytes were differentiated during attachment for 2-4 days. Primary human cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2mM glutamine, penicillin and streptomycin.

RNA extraction and quantitation

Total RNA was isolated according to the instructions with Trizol Reagent (Invitrogen) from cells after appropriate treatment. Transcript quantitation was performed via quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using Taqman probes. Every sample was assayed in triplicates. For RT reaction 20-100 ng total RNA, specific reverse primer and Superscript II Reverse Transcriptase (Invitrogen) were used performing 42°C for 30 min and 72°C for 5 min. Real-time monitoring was carried out using an ABI Prism 7900 (Perkin Elmer Life Sciences-Wellesley, MA) performing 40 cycles of 95°C for 12 sec 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 quantitation are listed in Supplemental Table 1.

Flow cytometry

Analysis of cell surface expression of proteins was performed on a Coulter Flow Cytometer (Beckman Coulter Inc.-Fullerton, CA). Briefly, cells were washed in PBS (phosphate buffered saline) pH7.4 supplemented with 0.5% (BSA) bovine serum albumin

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then were incubated with antiCD14-RPE (Dako-Glostrup, Denmark), antiCD36-FITC (Beckton-Dickinson-Franklin Lakes, NJ) antibody, respectively for 1 hour at 4°C, finally cells were washed in PBS-BSA and 10000 cells were counted on the cytometer.

Western blot

Cells were treated for two days as indicated and were washed in PBS then lysed in buffer A (Tris-HCl pH7.5, 1mM EDTA, 15mM beta-mercapto-ethanol, 0.1% Triton X 100, 0.5mM PMSF (phenyl-methyl-sulfonyl fluoride). Protein concentration was determined with Bradford reagent (Bio-Rad Laboratories-Hercules, CA) and 25μg protein was separated by 10% SDS-PAGE (polyacrylamid gel electrophoresis) and transferred to PVDF membrane (Bio-Rad Laboratories). 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 Biotechnology Inc.-Rockford, IL) was used for signal detection.

Microarray analysis

Total RNA was isolated using Trizol Reagent (Invitrogen) and further purified by using the RNeasy kit (Quiagen). 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). 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 EMBL in Heidelberg. Further analysis was performed using GeneSpring 7.0 (Silicon Genetics, Redwood City, CA).

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These analyses provided a signal for each specific transcript that was subsequently normalized by comparing to the median signal (arbitrary value of 1.0) obtained from the whole array. Genes associated with macrophages were chosen by using Gene Ontology (GO) in GeneSpring 7.0 software.

OxLDL uptake

For oxLDL uptake experiments we used diI-labeled oxidized human LDL (Frederick, MD). Following two days of treatment with the indicated ligands cells were treated with 5µg protein/ml diI-oxLDL for 6 hours at 37°C. Cells were washed and 10000 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 ±SD. 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 and results were considered significant with $p<0.01$.

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Results

First we compared the expression of PPARα, γ and δ in primary human CD34 positive cells after isolation and following differentiation with M-CSF for eight days to primary human macrophages. Surprisingly, we found a significant and consistent change in the mRNA expression profile under the various conditions (Figure 1A). During the maturation of stem cells we could detect a 7-fold induction of PPARγ along with minor increase in PPARα and δ levels. Similarly, 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 (Figure 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 to the CD34 positive cells while we could detect a 2.6-fold and a 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 below 2-fold. RARβ and RXRγ could not be detected in these cells.

As a model for the different stages of myeloid differentiation we chose myeloid leukemia cell lines: KG-1 representing acute myelogenous leukemia, HL-60 representing acute promyelocytic leukemia (FAB M3), 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

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markers (Ziegler-Heitbrock et al., 1988). We determined the absolute mRNA copy numbers of PPARs, RARs and RXRs expressed in these cells and compared to the levels of monocytes and macrophages (Figure 1C and D). PPARs are expressed at high levels in these cell types except 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 matured MonoMac-6 cells reaching the level of that of the macrophage's (Figure 1C). RARα, RXRα and β are expressed in the two differentiated cell lines (HL-60 and THP-1) and in monocytes-macrophages at the highest level and interestingly, 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 or 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 due to the very low level of the receptor expressed in these cells. In

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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, CD36 differentiation markers on the cell surface (Figure 2A, B). In Figure 2C 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 previously reported findings in THP-1 cells (Nagy et al., 1998), (Tontonoz et al., 1998) with one important difference: in the case of MonoMac-6 cells phorbol-12-myristate 13-acetate (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 (Figure 1C), higher than in THP-1, to ensure optimal target gene expression. These data suggested that activation of PPARγ:RXR heterodimer enhanced 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 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 in order 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 one hand we found that RAR agonists caused maturation only in the

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least differentiated cells (KG-1, HL-60) examined directing them towards 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 as PPARγ activators (Figure 2C), suggesting that RXR specific ligands might activate and function through PPARγ:RXR heterodimers. Very surprisingly, 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 (Figure 3A and B), the latter one is a direct target gene of PPARγ:RXR heterodimers. We could show that this phenomenon was present in both THP-1 and MonoMac-6 cells (Figure 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 hours followed by a vehicle or Rosiglitazone treatment for an additional 48 hours. 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) (Figure 4A, B), CD36 (Nagy et al., 1998), (Tontonoz et al., 1998) (Figure 4C, D), adipose differentiation-related protein (ADRP) (Gupta et al., 2001), (Vosper et al., 2001) (Figure 4E, F) and PPARγ angiopoietin-related protein

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(PGAR) (Yoon et al., 2000) (Figure 4G, H). Rosiglitazone readily induced (see bars C/Rosigl. in the figure) target gene expression in MonoMac-6 cells in the cases of FABP4 (x42), CD36 (x5) and PGAR (x150) and in THP-1 cells in the case of FABP4 (x43) and PGAR (x3). We also noted that all retinoids induced CD36 expression in both cell lines (Figure 4C and D). ATRA caused potentiation in both cell lines of all the measured target genes: the fold inductions are x213, x19, x3, x150 compared to x42, x5, x0.7, x150 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, H). The effect of 9-*cis* RA was similar to ATRA with even higher inductions. AM580 was less effective than ATRA but also showed enhanced induction with Rosiglitazone (e.g. see (A) x102 compared to x42, (C) x20 compared to x5, (D) x9 compared to x0.4, (H) x17 compared to x3). LG268 pretreatment potentiated FABP4, ADRP, PGAR and CD36 expression induced by Rosiglitazone. Synergy was particularly striking in MonoMac-6 cells for FABP4 and PGAR probably due to 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 (C and D) (x20 compared to x5 and x9 compared to x0.4), FABP4 (A) (x102 compared to x42) induction in MonoMac-6 and PGAR (H) (x17 compared to x3) induction in THP-1 cells. Significantly, pretreatment of cells with either 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

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cell types for all four target genes. These results clearly indicated that retinoid pretreatment although 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 hours of pretreatment was sufficient for the enhancement of PPARγ response (data not shown). Obviously, there are many potential mechanisms to account for this effect ranging from epigenetic changes through changes in cofactor, co-activator 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 whilst PPARα and δ mRNA levels were unaffected in either THP-1 or MonoMac-6 cells (Figure 5A and B). In order 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, while Rosiglitazone had no effect on PPARγ mRNA level (Figure 5C). We also found that this transcriptional activation of PPARγ resulted in an elevation in the protein level (Figure 5D). Interestingly, retinoids proved to be as effective as PMA in the induction of PPARγ. The next question was whether 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 (Figure 6A) in THP-1 cells. The PPARγ mRNA level remains unchanged for 12 hours after retinoid treatment while a *bona fide*

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RAR and RXR-regulated target gene, CYP27's mRNA shows a marked induction after 2 and 4 hours already (Szanto et al., 2004). Both the induction of PPARγ (Figure 6C) and CYP27 (Figure 6D) appeared to be dose dependent. To test if retinoids that to what extent retinoids induced macrophage maturation on the THP-1 monocytic leukemia we carried out microarray analysis on retinoid (AM580) treated THP-1 cells (Table 1). Part A shows the induction of PPARs while C and D show the expression of macrophage-associated genes based on 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 if C/EBPs are induced by retinoids and found a three-fold induction in C/EBPβ level while 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.

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 (Figure 7). Interestingly and 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

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RXR agonist in control cells while 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 agonists 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.

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Discussion

After the initial discovery of PPARγ in myeloid cells (Greene et al., 1995), (Kliewer et al., 1994) there have been a large number of studies trying to define its biological role. Many studies including some of our own has 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 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

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at day 10 of embryonic development (Barak et al., 1999), (Rosen et al., 1999), (Kubota 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 adipogensis, i.e. fat storage (Barak et al., 1999). Although PPARγ-null embryonic stem cells were capable to in vitro differentiate into macrophages (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 PPARγ gene in mice resulted in reduced total plasma and HDL cholesterol levels. The lack of the PPARγ gene in macrophages is therefore likely to be pro-atherogenic. Based on 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. Previously, 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). Recently, it was reported that retinoids have a role earlier in myelogenesis affecting the common granulocyte/monocyte precursors (Johnson et al., 2002). This observation is

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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.

Crosstalk between PPARγ and retinoid signaling

This is not the first example of a crosstalk between retinoid and PPARγ signaling during a differentiation process. A crosstalk 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 crosstalk is cell-type specific. We have recently identified a link between RAR, PPARγ and LXR signaling in macrophages, where 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 previously believed and that retinoid signaling may have a larger role in macrophage gene expression and metabolism as previously suspected. 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 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

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this effect our microarray analysis shows the 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, 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 only *in vivo* experiments can be tested. Needless to say that a pharmaceutically amenable pathway to modulate PPARγ responsiveness can be utilized to boost the anti-atherogenic 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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References

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A and Evans RM (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell* **4**:585-95.

Chambon P (1993) The molecular and genetic dissection of the retinoid signalling pathway. *Gene* **135**:223-8.

Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P and Evans RM (2001a) PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med* **7**:48-52.

Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM and Tontonoz P (2001b) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell* **7**:161-71.

Friedman AD (2002) Transcriptional regulation of granulocyte and monocyte development. *Oncogene* **21**:3377-90.

Goyette P, Feng Chen C, Wang W, Seguin F and Lohnes D (2000) Characterization of retinoic acid receptor-deficient keratinocytes. *J Biol Chem* **275**:16497-505.

Graves RA, Tontonoz P and Spiegelman BM (1992) Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. *Mol Cell Biol* **12**:1202-8.

Greene ME, Blumberg B, McBride OW, Yi HF, Kronquist K, Kwan K, Hsieh L, Greene G and Nimer SD (1995) Isolation of the human peroxisome proliferator activated receptor gamma cDNA: expression in hematopoietic cells and chromosomal mapping. *Gene Expr* **4**:281-99.

Gupta RA, Brockman JA, Sarraf P, Willson TM and DuBois RN (2001) Target genes of peroxisome proliferator-activated receptor gamma in colorectal cancer cells. *J Biol Chem* **276**:29681-7.

Johnson BS, Mueller L, Si J and Collins SJ (2002) The cytokines IL-3 and GM-CSF regulate the transcriptional activity of retinoic acid receptors in different in vitro models of myeloid differentiation. *Blood* **99**:746-53.

Kakizuka A, Miller WH, Jr., Umesono K, Warrell RP, Jr., Frankel SR, Murty VV, Dmitrovsky E and Evans RM (1991) Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* **66**:663-74.

Kastner P, Grondona JM, Mark M, Gansmuller A, LeMeur M, Decimo D, Vonesch JL, Dolle P and Chambon P (1994) Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**:987-1003.

Kastner P, Lawrence HJ, Waltzinger C, Ghyselinck NB, Chambon P and Chan S (2001) Positive and negative regulation of granulopoiesis by endogenous RARalpha. *Blood* **97**:1314-20.

Kersten S, Desvergne B and Wahli W (2000) Roles of PPARs in health and disease. *Nature* **405**:421-4.

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Molecular Pharmacology Fast Forward. Published on March 1, 2005 as DOI: 10.1124/mol.104.006445
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Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K and Evans RM (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A* **91**:7355-9.

Kliewer SA, Lehmann JM and Willson TM (1999) Orphan nuclear receptors: shifting endocrinology into reverse. *Science* **284**:757-60.

Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S, Kadowaki T and et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* **4**:597-609.

Labrecque J, Allan D, Chambon P, Iscove NN, Lohnes D and Hoang T (1998) Impaired granulocytic differentiation in vitro in hematopoietic cells lacking retinoic acid receptors alpha1 and gamma. *Blood* **92**:607-15.

Lohnes D, Mark M, Mendelsohn C, Dolle P, Dierich A, Gorry P, Gansmuller A and Chambon P (1994) Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120**:2723-48.

Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P and Mark M (1994) Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120**:2749-71.

Moore KJ, Rosen ED, Fitzgerald ML, Randow F, Andersson LP, Altshuler D, Milstone DS, Mortensen RM, Spiegelman BM and Freeman MW (2001) The role of PPAR-gamma in macrophage differentiation and cholesterol uptake. *Nat Med* **7**:41-7.

Nagy L, Tontonoz P, Alvarez JG, Chen H and Evans RM (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **93**:229-40.

Ricote M, Li AC, Willson TM, Kelly CJ and Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* **391**:79-82.

Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ and Spiegelman BM (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev* **16**:22-6.

Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM and Mortensen RM (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* **4**:611-7.

Schwarz EJ, Reginato MJ, Shao D, Krakow SL and Lazar MA (1997) Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription. *Mol Cell Biol* **17**:1552-61.

Sucov HM and Evans RM (1995) Retinoic acid and retinoic acid receptors in development. *Mol Neurobiol* **10**:169-84.

Szanto A, Benko S, Szatmari I, Balint LB, Furtos I, Rühl R, Molnar S, Csiba L, Garuti R, Calandra S, Larsson H, Diczfalusy U and Nagy L (2004) Transcriptional

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regulation of human CYP27 integrates retinoid, PPAR and LXR signaling leading to cholesterol efflux from macrophages. *Mol Cell Biol.*

Tontonoz P, Nagy L, Alvarez JG, Thomazy VA and Evans RM (1998) PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* **93**:241-52.

Tsai S, Bartelmez S, Heyman R, Damm K, Evans R and Collins SJ (1992) A mutated retinoic acid receptor-alpha exhibiting dominant-negative activity alters the lineage development of a multipotent hematopoietic cell line. *Genes Dev* **6**:2258-69.

Tsai S and Collins SJ (1993) A dominant negative retinoic acid receptor blocks neutrophil differentiation at the promyelocyte stage. *Proc Natl Acad Sci U S A* **90**:7153-7.

Vosper H, Patel L, Graham TL, Khoudoli GA, Hill A, Macphee CH, Pinto I, Smith SA, Suckling KE, Wolf CR and Palmer CN (2001) The peroxisome proliferator-activated receptor delta promotes lipid accumulation in human macrophages. *J Biol Chem* **276**:44258-65.

Wang ZG, Delva L, Gaboli M, Rivi R, Giorgio M, Cordon-Cardo C, Grosveld F and Pandolfi PP (1998) Role of PML in cell growth and the retinoic acid pathway. *Science* **279**:1547-51.

Wu Z, Xie Y, Bucher NL and Farmer SR (1995) Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. *Genes Dev* **9**:2350-63.

Xue JC, Schwarz EJ, Chawla A and Lazar MA (1996) Distinct stages in adipogenesis revealed by retinoid inhibition of differentiation after induction of PPARgamma. *Mol Cell Biol* **16**:1567-75.

Yeh WC, Cao Z, Classon M and McKnight SL (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev* **9**:168-81.

Yoon JC, Chickering TW, Rosen ED, Dussault B, Qin Y, Soukas A, Friedman JM, Holmes WE and Spiegelman BM (2000) Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. *Mol Cell Biol* **20**:5343-9.

Zhu J and Emerson SG (2002) Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* **21**:3295-313.

Zhu J, Heyworth CM, Glasow A, Huang QH, Petrie K, Lanotte M, Benoit G, Gallagher R, Waxman S, Enver T and Zelent A (2001) Lineage restriction of the RARalpha gene expression in myeloid differentiation. *Blood* **98**:2563-7.

Ziegler-Heitbrock HW, Thiel E, Futterer A, Herzog V, Wirtz A and Riethmuller G (1988) Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int J Cancer* **41**:456-61.

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Figure legends

Figure 1

Expression of PPARγ is linked to the differentiation of macrophage. (A) and (B) CD34 positive cells and monocytes were isolated and differentiated as in Materials and methods. RNA was isolated from stem cells, monocytes and after 8 days of differentiation with M-CSF (10 ng/ml) from CD34 positive cell-derived macrophages and after two 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 to the CD34 positive stem cells or to the monocytes, respectively (C) and (D) RNA was isolated from KG-1, HL-60, THP-1 and MonoMac-6 cells, monocytes and monocyte-derived macrophages than real-time RT Q-PCR was used to determine nuclear receptor mRNA levels. All measurements were performed in triplicates and the results normalized to cyclophilin D are shown ±SD.

Figure 2

Activation of PPARγ induces differentiation of myeloid cells. MonoMac-6 cells were treated with LG268 (100 nM), Rosiglitazone (1 μM), both or vehicle for two days. Cells labeled with anti-CD14-RPE (A) and with anti-CD36-FITC (B) were analyzed with flow cytometer and 10000 cells were counted. Total RNA isolated from the same experiment was used to quantitate the mRNA levels of CD14 (C) and CD36 (D) with RT Q-PCR. All measurements were performed in triplicates and the results normalized to 36B4 are shown ±SD.

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Figure 3

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

Figure 4

Retinoid pretreatment enhances the expression of PPARγ-induced target genes. MonoMac-6 (A, C, E, G) and THP-1 (B, D, F, 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 two days. Cell were washed three times in PBS, plated out and treated with Rosiglitazone (Rosigl.) (1 μM) or vehicle (C as control) for additional two days. RNA was extracted and PPARγ target gene mRNA levels were quantitated 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 in the figure compared to the vehicle-treated cells (C/C). All measurements were performed in triplicates and the results normalized to cyclophilin D are shown ±SD.

Figure 5

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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 two days. Cell were washed three times in PBS, plated out and treated with Rosiglitazone (1 μM) or vehicle for additional two days. RNA was extracted and PPARα, γ and δ mRNA levels were quantitated using RT Q-PCR. All measurements were performed in triplicates and the results normalized to cyclophilin D are shown ±SD. (C) - Primary human monocytes were differentiated and treated with 9-*cis* RA (1 μM), Rosiglitazone (1 μM) or vehicle for two days then RNA was extracted and PPARγ mRNA levels were measured with RT Q-PCR. Measurements were performed in triplicates and the results normalized to cyclophilin D are shown ±SD. (D) MonoMac-6 cells were treated with AM580 (100 nM), 9-*cis* RA (1 μM), PMA (50 ng/ml) or vehicle for two days. After cell lysis Western blot was performed as described in Materials and methods with anti-PPARγ antibody.

Figure 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 triplicates and the results normalized to cyclophilin D are shown ±SD.

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Figure 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 two days. Cell were washed three times in PBS, plated out and treated with Rosiglitazone (1 μM), Rosiglitazone and LG268 (100 nM) or vehicle for additional two days. DiI-labeled oxLDL (5 μg/ml) was added to cells for six hours and 10000 cells were counted by flow cytometer and the fold changes are shown. All of these values are compared to 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.

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 in Materials and Methods. (A) shows the fold changes of PPAR, (B) of C/EBP mRNA levels. The fold changes in the induction of CD markers (C) and genes from Gene Ontology database associated with macrophages are shown. Genes showing at least two-fold inductions are in gray.

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Table 1

A PPARs

Gene name	Fold change
PPAR alpha	0.714
PPAR gamma	**6.862**
PPAR delta	1.561

B C/EBPs

Gene name	Fold change
C/EBP alpha	1.105
C/EBP beta	**3.005**
C/EBP gamma	1.296
C/EBP delta	0.932
C/EBP epsilon	Absent
C/EBP zeta	0.721

C CD markers

Gene name	Fold change
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

D Macrophage associated genes

Gene name	Fold change
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

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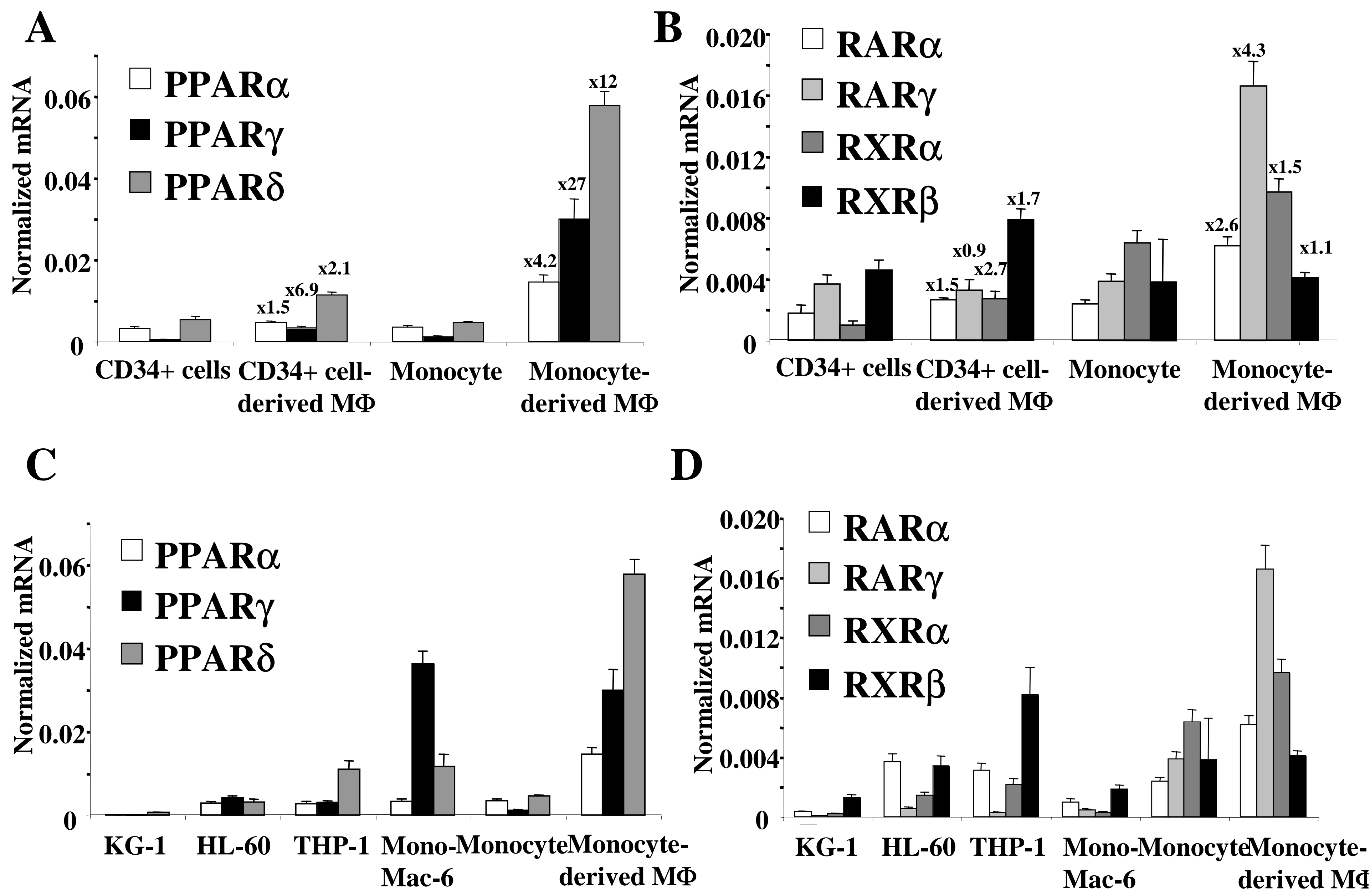

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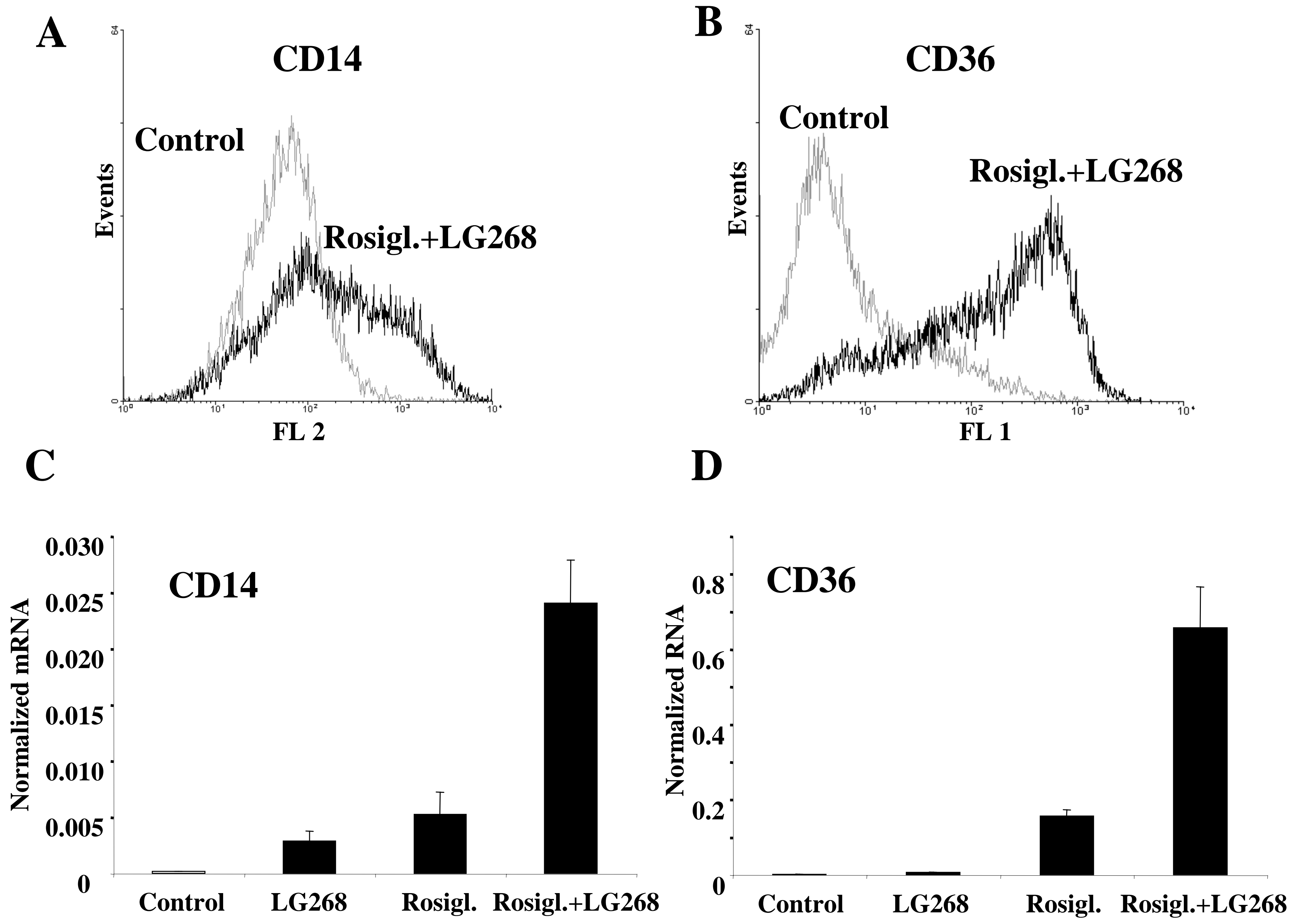

Figure 2

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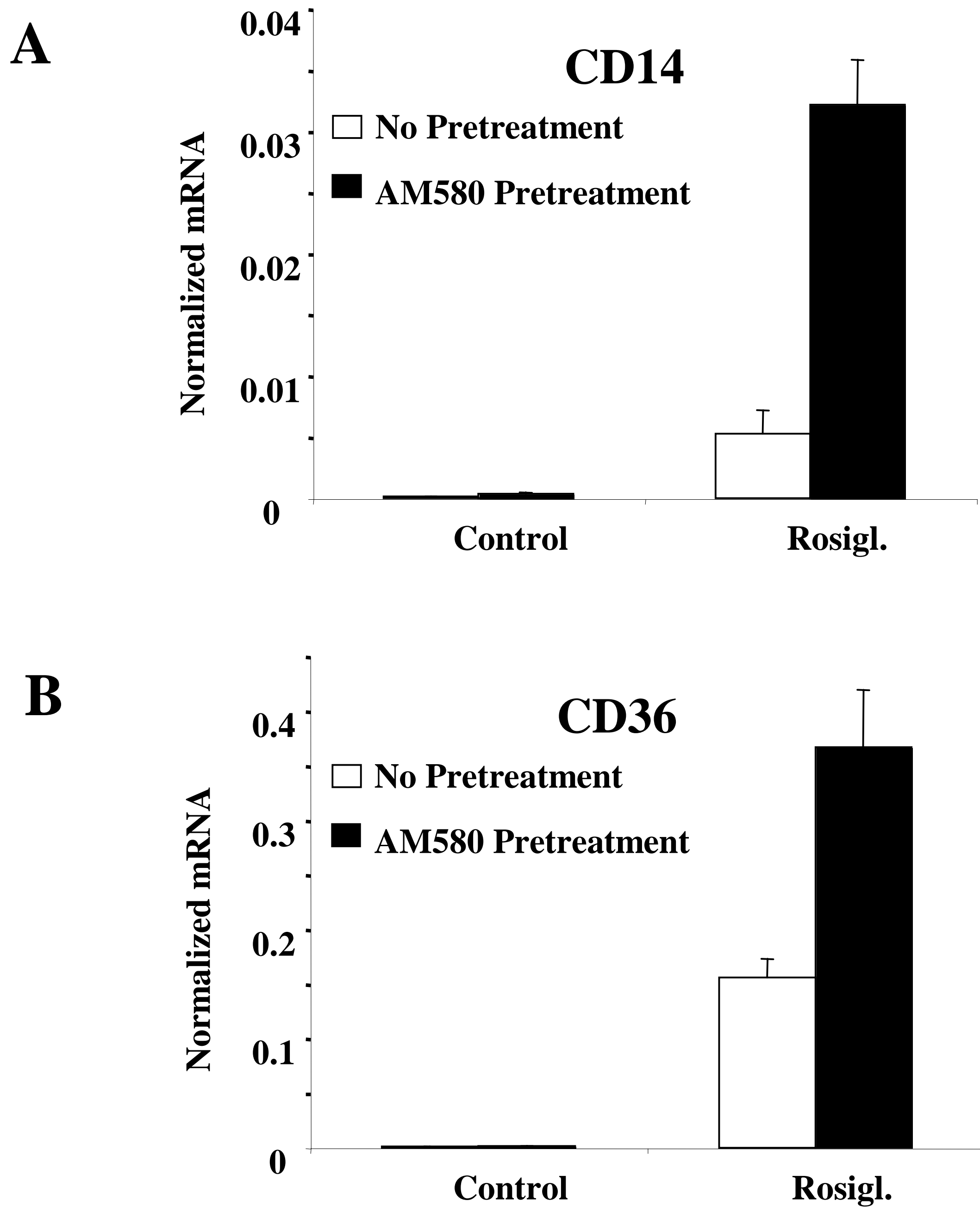

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Figure 3

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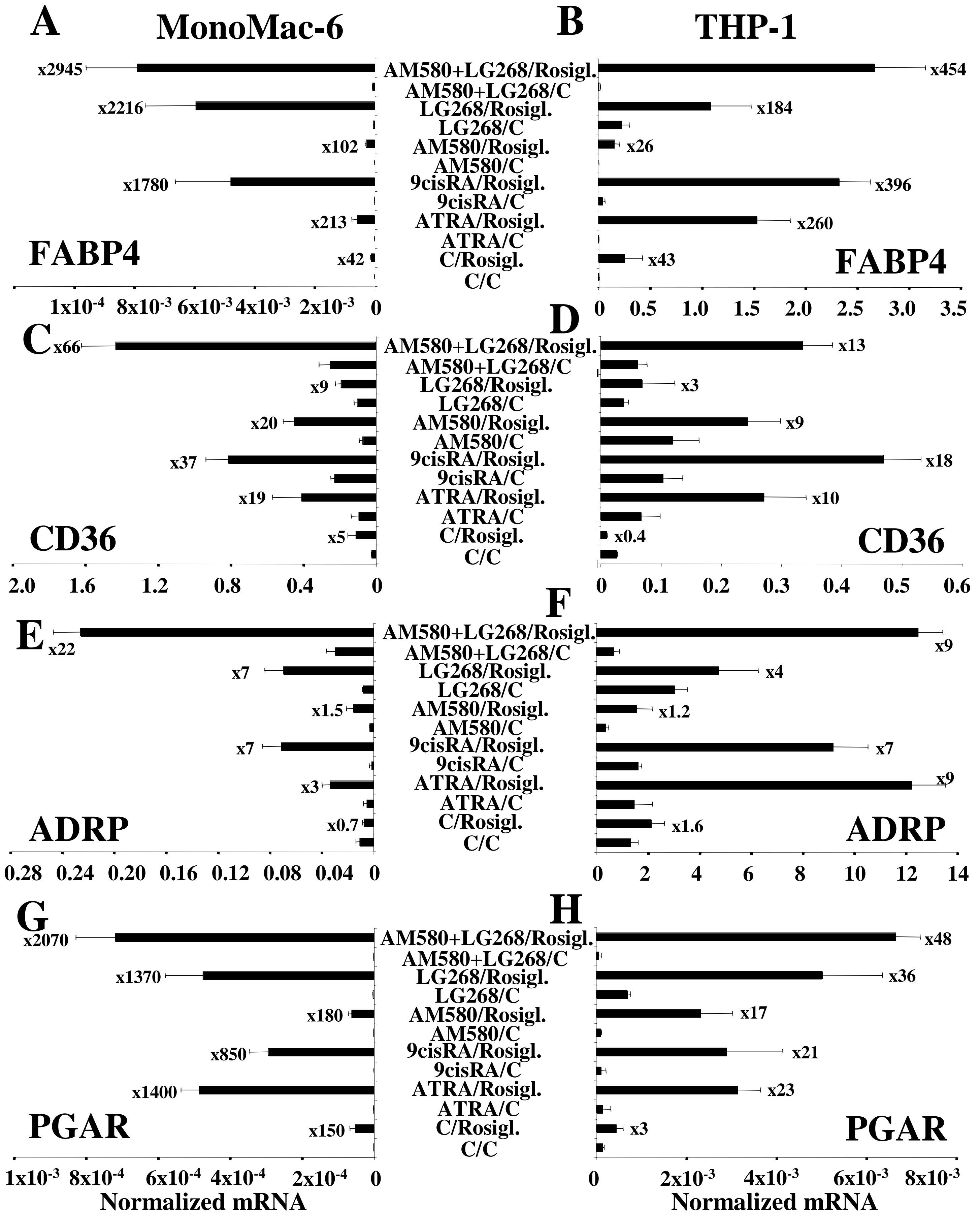

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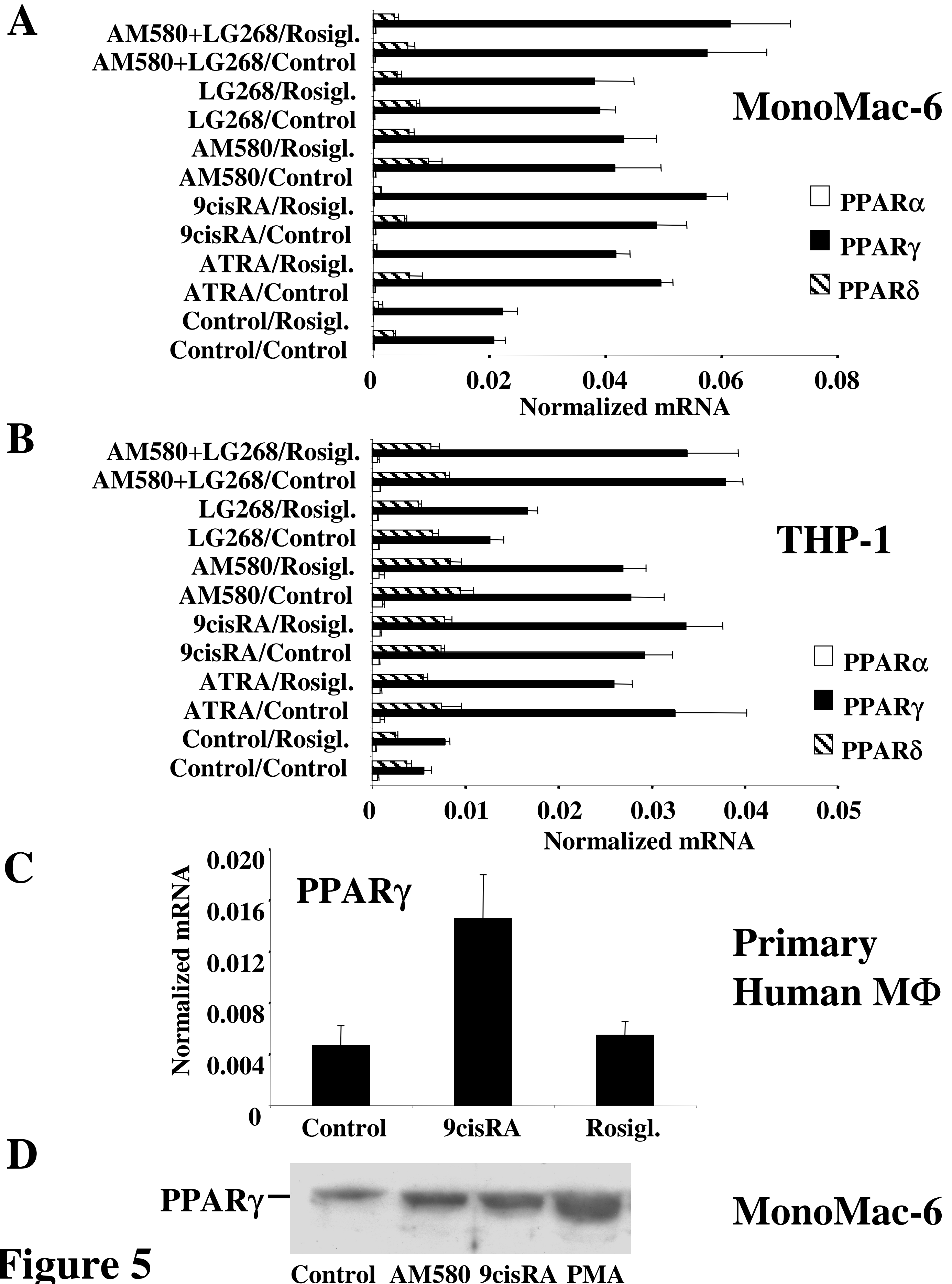

Figure 5

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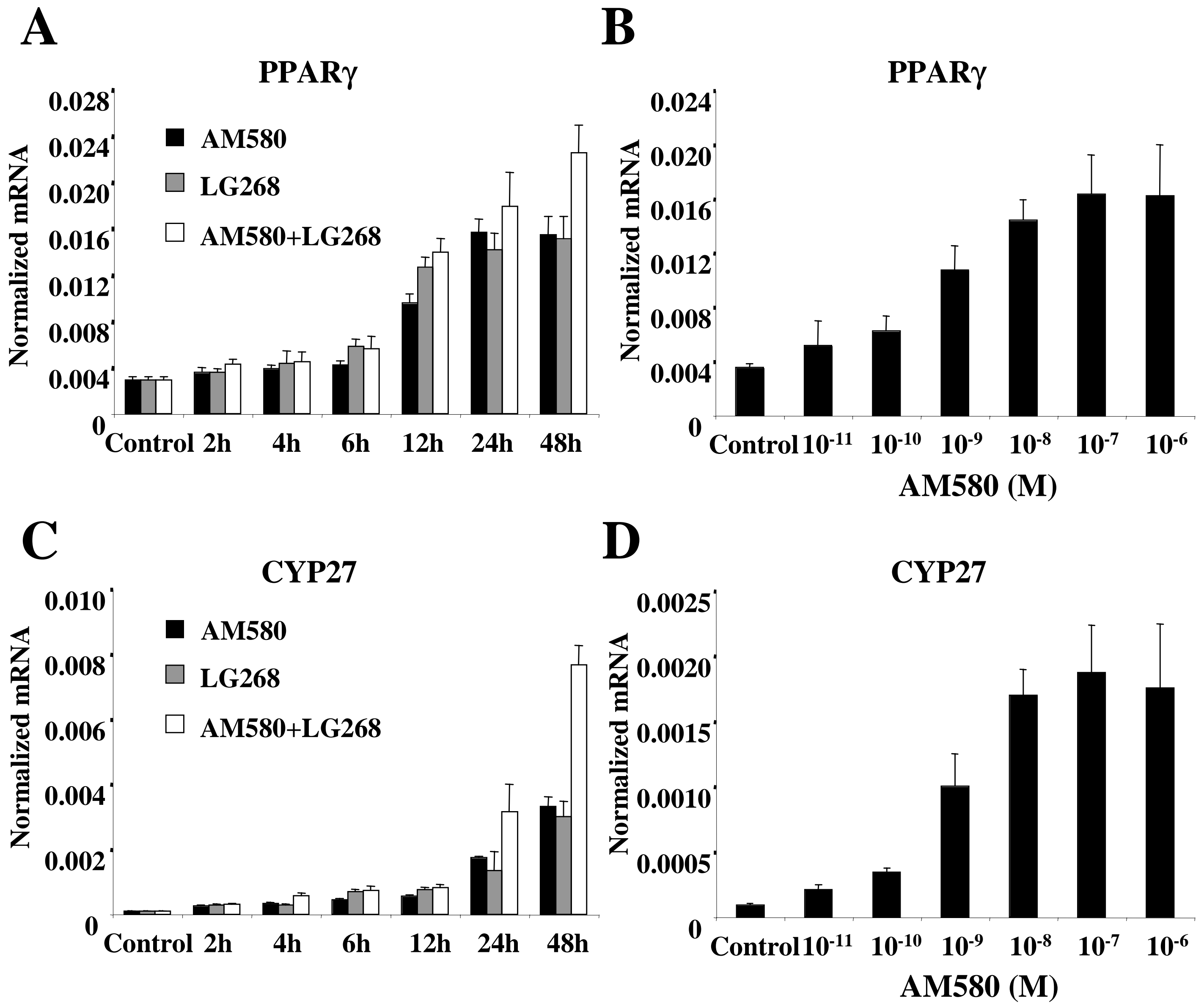

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Figure 6

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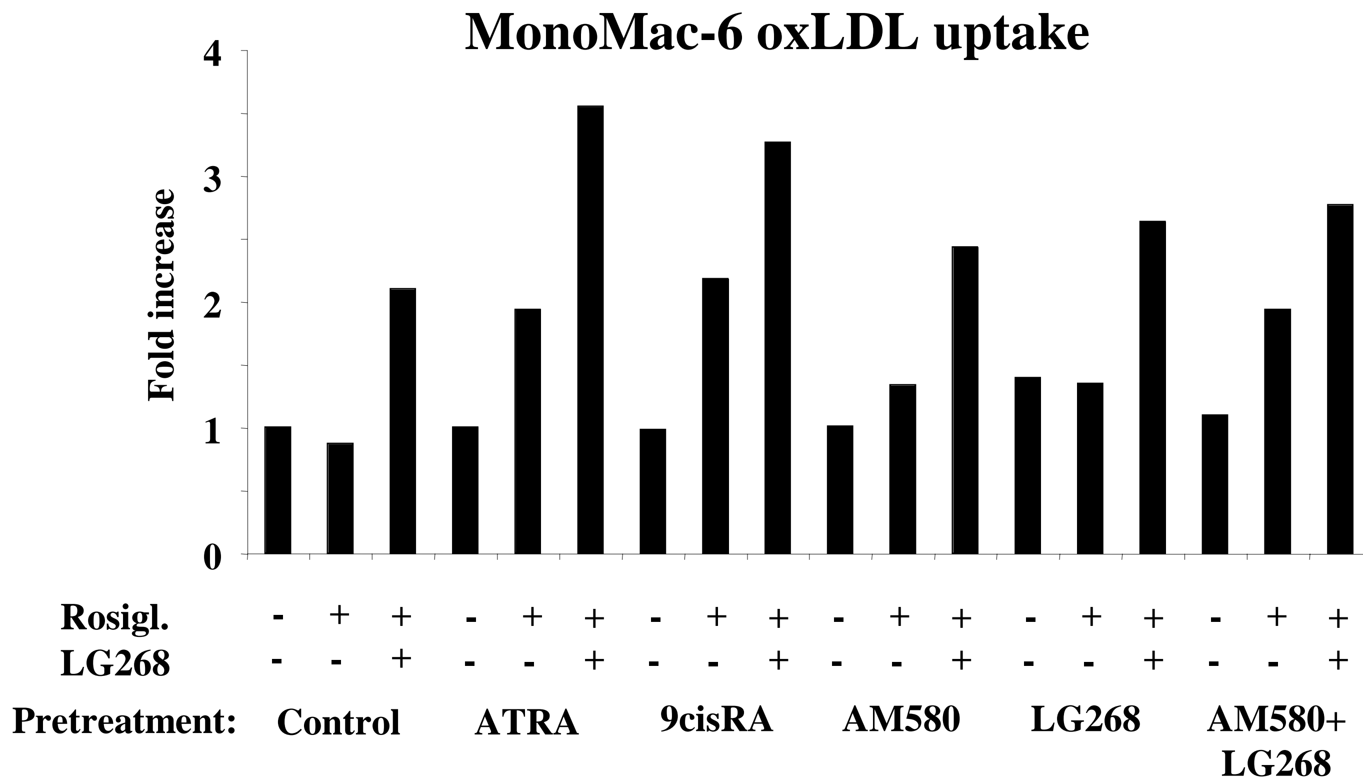

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Figure 7