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Peroxisome Proliferator-activated Receptor γ-regulated ABCG2 Expression Confers Cytoprotection to Human Dendritic Cells*[S]

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Istvan Szatmari[‡], György Vámosi[§], Peter Brazda[‡], Balint L. Balint[‡], Szilvia Benko[¶], Lajos Széles[‡], Viktoria Jeney[‖], Csilla Özvegy-Laczka[**], Attila Szántó[‡], Endre Barta[‡‡], József Balla[‖], Balazs Sarkadi[**], and Laszlo Nagy[‡1]

From the [‡]Department of Biochemistry and Molecular Biology, the [§]Cell Biophysics Research Group of the Hungarian Academy of Sciences, the [¶]Department of Immunology, and the [‖]Department of Internal Medicine, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, Egyetem ter 1., Debrecen, H-4032, Hungary, the [**]Membrane Research Group of the Hungarian Academy of Sciences, Institute of Haematology and Immunology, National Medical Center, H-1113 Budapest, Hungary, and the [‡‡]Agricultural Biotechnology Center, Gödöllő, Szent-Györgyi Albert u. 4, H-2100, Hungary

ABCG2, a member of the ATP-binding cassette transporters has been identified as a protective pump against endogenous and exogenous toxic agents. ABCG2 was shown to be expressed at high levels in stem cells and variably regulated during cell differentiation. Here we demonstrate that functional ABCG2 is expressed in human monocyte-derived dendritic cells by the activation of a nuclear hormone receptor, PPARγ. We identified and characterized a 150-base pair long conserved enhancer region, containing three functional PPAR response elements (PPARE), upstream of the human *ABCG2* gene. We confirmed the binding of the PPARγ·RXR heterodimer to this enhancer region, suggesting that PPARγ directly regulates the transcription of ABCG2. Consistent with these results, elevated expression of ABCG2 mRNA was coupled to enhanced protein production, resulting in increased xenobiotic extrusion capacity via ABCG2 in PPARγ-activated cells. Furthermore PPARγ instructed dendritic cells showed increased Hoechst dye extrusion and resistance to mitoxantrone. Collectively, these results uncovered a mechanism by which up-regulation of functional ABCG2 expression can be achieved via exogenous or endogenous activation of the lipid-activated transcription factor, PPARγ. The increased expression of the promiscuous ABCG2 transporter can significantly modify the xenobiotic and drug resistance of human myeloid dendritic cells.

The ATP-binding cassette (ABC)[2] transporter G2 (ABCG2), also known as breast cancer resistance protein (BCRP), mitoxantrone resistance protein (MXR), and ATP-binding cassette placenta (ABCP), belongs to a transmembrane protein superfamily that mediates the ATP-dependent translocation of a variety of lipophilic substrates (1–3). Within the human ABC superfamily, ABCG2 belongs to a group of half-transporters that consist of six transmembrane spanning domains that homodimerize to form the active membrane transporter (4). The *ABCG2* gene is highly expressed in the plasma membrane of several drug-resistant cell lines, where it has been shown to transport antitumor drugs including mitoxantrone, topotecan, daunorubicin, and doxorubicin (3, 5, 6). In normal tissues, high level expression of ABCG2 is found in the placenta and small intestine, and this protein was shown to play a role in the protection of the organism against toxic xenobiotics (7). ABCG2 is also abundantly expressed in various stem cells, characterized by an increased Hoechst dye efflux capacity (side population, SP) (8). During hematopoietic differentiation, ABCG2 level is initially down-regulated, but the protein is expressed again in some mature cell types *e.g.* of the erythroid lineage (9). ABCG2 was shown to reduce the accumulation of toxic heme metabolites, thus the pump expression is part of cell survival strategy under hypoxic conditions (10).

Despite its apparently tight regulation, the molecular details of *ABCG2* gene expression control are poorly defined and not well understood. A recent report suggested that ABCG2 expression is induced by hypoxia, and this regulation involves the hypoxia-inducible transcription factor complex HIF-1 (10).

The expression of several ABC transporters is known to be regulated by nuclear hormone receptors. It was described that the increased expression of both ABCA1 and ABCG1 occurs via the oxysterol-activated nuclear receptor, the liver X receptor (LXR), in macrophages (11). In addition, in this cell type, ABCA1 expression is also induced by PPARγ activators, through the activation of LXRα receptor (12, 13).

As part of our gene expression profiling efforts of nuclear receptor-mediated changes in human dendritic cell differentiation and subtype specification, we have identified ABCG2 as a tightly regulated gene by one of the receptors. Here we report

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

[1] An International Scholar of the Howard Hughes Medical Institute and recipient of a Wellcome Trust Senior Research Fellowship in Biomedical Sciences in Central Europe. To whom correspondence should be addressed. Tel.: 36-52-416-432; Fax: 36-52-314-989; E-mail: lnagy@indi.biochem.dote.hu.

[2] The abbreviations used are: ABC, ATP-binding cassette; ABCG2, ABC transporter G2; PPAR, peroxisome proliferator-activated receptor; FACS, fluorescent-activated cell sorting; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide; DC, dendritic cell; RSG, rosiglitazone; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; IL, interleukin; MX, mitoxantrone; siRNA, small interfering RNA.

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that PPARγ directly and transcriptionally induces ABCG2 expression in a cell type of the human myeloid lineage, monocyte-derived dendritic cells. We have identified and characterized the regions of the gene involved in this transcriptional activation. We also document that dendritic cells activated by PPARγ express high levels of functional ABCG2 protein, and gain an enhanced capacity to extrude xenobiotics. These features may have important consequences to the survival and drug resistance of human dendritic cells during immune regulation and also have therapeutic ramifications.

EXPERIMENTAL PROCEDURES

Ligands—Rosiglitazone, troglitazone, and T0901317 were obtained from Alexis Biochemicals, GW9662 and GW347845X were provided by T. M. Willson (GlaxoSmithKline, Research Triangle Park, NC). KO143 was kindly provided by Dr. Gerit-Jan Koomen, Amsterdam.

Plasmids—Mammalian expression vectors coding human retinoid X receptor α (RXRα), mouse PPARγ, β-galactosidase, and thymidine kinase (TK)-Luc were described previously (14). The identified 150-bp fragment of the genomic region of the ABCG2 promoter containing 3 response elements was cloned into TK-Luc to obtain enhancer trap construct.

Transient Transfections and Reporter Gene Assays—TK-Luc enhancer were transfected along with the indicated receptors. All transfection experiments were performed with COS1 and 293T cells using jetPEI reagent (Qbiogene) according to the manufacturer's instructions. Cells were lysed and assayed for reporter expression 24 h after transfection. The luciferase assay system (Promega) was used according to the manufacturer's instruction. The β-galactosidase activity was determined as described previously (15). Measurements were made using a Wallac Victor-2, multilabel counter. Luciferase activity of each sample was normalized to the β-galactosidase activity.

RNA Interference—For siRNA delivery the AMAXA Nucleofection technology was applied. MM6 cells (1×10^6) were harvested and electroporated with 1.5 μg of siRNA using the cell line nucleofector kit V (AMAXA), program v-001. We used a previously described PPARγ-specific siRNA sequence (16): GCCCTTCACTACTGTTGAC-d(TT). As a negative control, Silencer Negative Control 1 siRNA (Ambion) was used.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was carried out as described previously (14, 17). Immunoprecipitation was performed with the following monoclonal antibodies: anti-RXRα (2ZK8508H; Perseus Proteomics), anti-PPARγ (2ZK8713; Perseus Proteomics).

Electrophoretic Mobility Shift Assays (EMSAs)—Electrophoretic mobility shift assays were performed as described earlier (14, 18). In brief, full-length, RXRα and PPARγ receptors were produced by using the T7 Quick TNT *in vitro* transcription-translation kit (Promega). For supershift experiments, the receptors were preincubated with the indicated monoclonal antibodies: anti-RXRα (2ZK8508H), anti-PPARγ (2ZK8713). The sequences of oligonucleotides that were used are presented in the supplemental Table S1.

Cell Culture and Ligand Treatment—Monocytes (98% CD14+) were obtained from buffy coats by Ficoll gradient centrifugation and immunomagnetic cell separation using anti-CD14-conjugated microbeads (VarioMACS, Miltenyi Biotec). DCs were prepared as described previously (19). In brief, monocytes were resuspended into 6-well culture dishes at a density of 1.5×10^6 cells/ml and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen), containing 800 units/ml GM-CSF (Leucomax) and 500 units/ml IL-4 (Peprotech). Cells were cultured for 5 or 6 days (unless otherwise indicated), and the IL-4 and GM-CSF addition was repeated at day 3. Ligands or vehicle control (50% Me_2SO/ethanol) were added to the cell culture from the first day on. MonoMac-6 (MM6) cells were a kind gift of E. Duda (Biological Research Center, Szeged, Hungary). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Western Blot Analysis—50 μg of protein from whole cell extract were separated by electrophoresis in 10% polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were probed with anti-ABCG2 (BXP-21) antibody (Alexis Biochemicals), then stripped and reprobed with anti-GAPDH antibody (ab8245-100, Abcam).

FACS Analysis—Cell staining was performed using FITC- or PE-conjugated monoclonal antibodies: Labeled antibodies for flow cytometry included anti-CD1a-PE, CD1d-PE, CD80-PE, CD86-PE, and isotype-matched controls (BD PharMingen). For the ABCG2 detection cells were fixed with 1% paraformaldehyde for 5 min, then incubated with unlabeled anti-ABCG2 (5D3) or isotype-matched control (R and D system) antibody for 30 min at 37 °C, followed by staining with FITC-labeled anti-mouse antibody (BD PharMingen). The fluorescence of labeled cells was measured using a FACSCalibur flow cytometer (Beckton Dickinson).

Real-time RT-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen). Reverse transcription was performed at 42 °C for 1 h and 72 °C for 5 min from 100–200 ng of total RNA using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using real-time PCR (ABI PRISM 7900, Applied Biosystems): 40 cycles at 95 °C for 12 s and 60 °C for 30 s using Taqman assays. All PCR reactions were done in triplicate with one control reaction containing cDNA that was reverse transcribed without RT enzyme. The comparative Ct method was used to quantify transcripts, and the expression level was normalized to 36B4 or cyclophilin A. The sequences of the primers and probes are presented in supplemental Table S1.

Measurement of Hoechst Retention by Confocal Laser-scanning Microscopy and FACS— DCs were plated on Lab-Tek coverslip chambers. Cells were incubated with 0.5 μg/ml Hoechst 33342 for 1 h at 37 °C with or without KO143 (1 μM), then subjected to microscopic analysis. Hoechst fluorescence was measured on a Zeiss LSM 510 confocal microscope with a Plan-Apochromat ×60 (NA 1.4) oil immersion objective using maximal detection pinhole diameter (1 mm) to get signal from the whole depth of the nucleus. Hoechst was excited by the 351 and 364 nm lines of an Argon ion laser, emission was measured through a 385–470-nm bandpass filter.

Hoechst content was also measured on a Becton Dickinson FACSVantage flow cytometer. Hoechst fluorescence was excited by 351- and 364-nm lines, and the blue and red Hoechst signals were detected through 402–446-nm and >650-nm filters, respectively.

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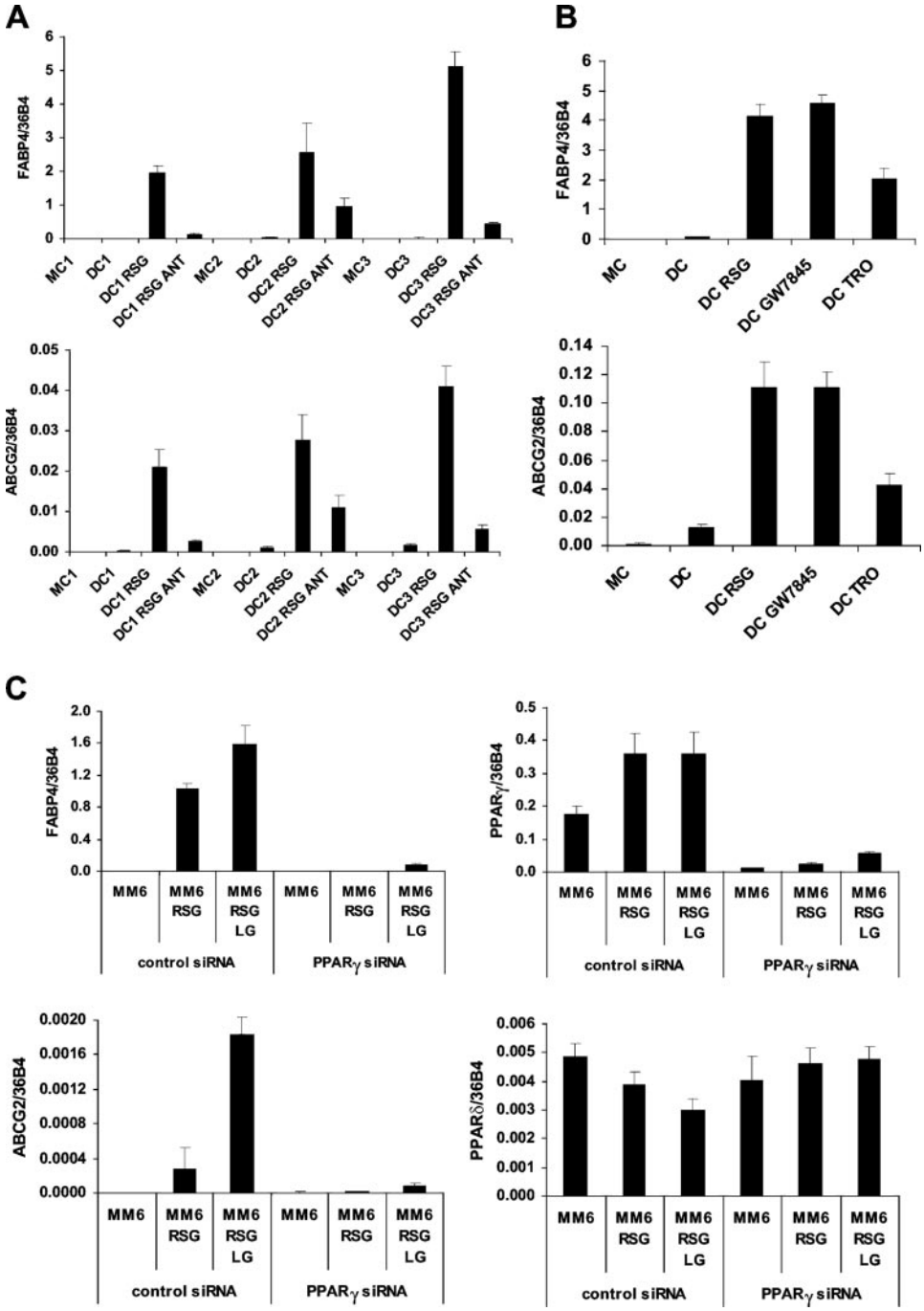

FIGURE 1. **PPARγ ligand treatment up-regulates the expression of ABCG2.** *A,* FABP4 and ABCG2 transcript is highly up-regulated in PPARγ ligand-treated DCs. Transcript levels were determined from monocytes (*MC*), vehicle or PPARγ ligand-treated DCs (2.5 μM RSG) alone or with a PPARγ-specific antagonist (*ANT*): 5 μM GW9662 by RT-Q-PCR. Gene expression data are expressed as a ratio of FABP4 or ABCG2 transcripts relative to 36B4 expression ± S.D. Data were obtained from three individuals. *B,* transcript levels of ABCG2 and FABP4 were determined in DC treated with various ligands: 2.5 μM RSG, 100 nM GW347845X (abbreviated as GW7845), 1 μM troglitazone (*TRO*). *C,* expression of FABP4 and ABCG2 is impaired in PPARγ knock-down MM6 cells. MM6 cells were electroporated with a control or PPARγ-specific siRNA as described under "Experimental Procedures." After electroporation MM6 cells were cultured for 12 h; thereafter cells were treated with 2.5 μM RSG alone or along with an RXR agonist (*LG*, 100 nM LG268) and cultured for 24 h. Transcript levels of FABP4, ABCG2, PPARγ, and PPARδ were determined by RT-Q-PCR.

Measurement of Mitoxantrone Uptake by FACS Analyses—Cells were resuspended in phenol red-free HPMI medium containing the desired fluorescent compound (5 μM mitoxantrone) with or without KO143 (1 μM) and incubated at 37 °C for 30 min, Thereafter, the cells were washed with cold HPMI and subjected to flow cytometric analyses. The cells were assessed for fluorescence intensity with a FACSCalibur cytometer (Beckton Dickinson).

Cytotoxicity Assay—Cytotoxicity assays were carried out using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide) assay. Briefly, cells were plated in 96-well plates at a density of 10^5 cells per well. Mitoxantrone at various concentrations with or without KO143 was added to DCs, and cells were incubated at 37 °C for 48 h. After incubation, cytotoxicity was assessed by using the MTT assay. The MTT assay was performed as described previously (20) and as specified by ATCC.

Statistical Analyses—Values are expressed as mean ± S.D. of the mean. Significant differences between mean values were evaluated using two-tailed, unpaired Student's *t* test.

RESULTS

ABCG2 Is Highly and Selectively Induced by PPARγ Agonists in Human Dendritic Cells—Previously we have characterized the role of PPARγ nuclear receptor in monocyte-derived dendritic cell (DC) differentiation (19). We observed that PPARγ was acutely and rapidly up-regulated during DC differentiation and acted as a modifier of DC gene expression and function. This cellular system is particularly suitable to identify PPARγ responsive genes because the *bona fide* PPARγ target, the fatty acid-binding protein 4 (FABP4 also known as aP2) was highly induced (50–100-fold) upon PPARγ agonist treatment. We sought to identify direct targets of PPARγ with expression pattern similar to that of FABP4 using global gene expression analysis (Affymetrix Microarrays). One of the candidate genes matching these criteria was an ABC half-transporter, ABCG2. To validate the microarray data, the transcript level of ABCG2 was determined by real-time quantitative RT-PCR (RT-Q-PCR) from RNAs obtained from monocytes and monocyte-derived human DCs. When compared the expression of FABP4 to ABCG2 a similar

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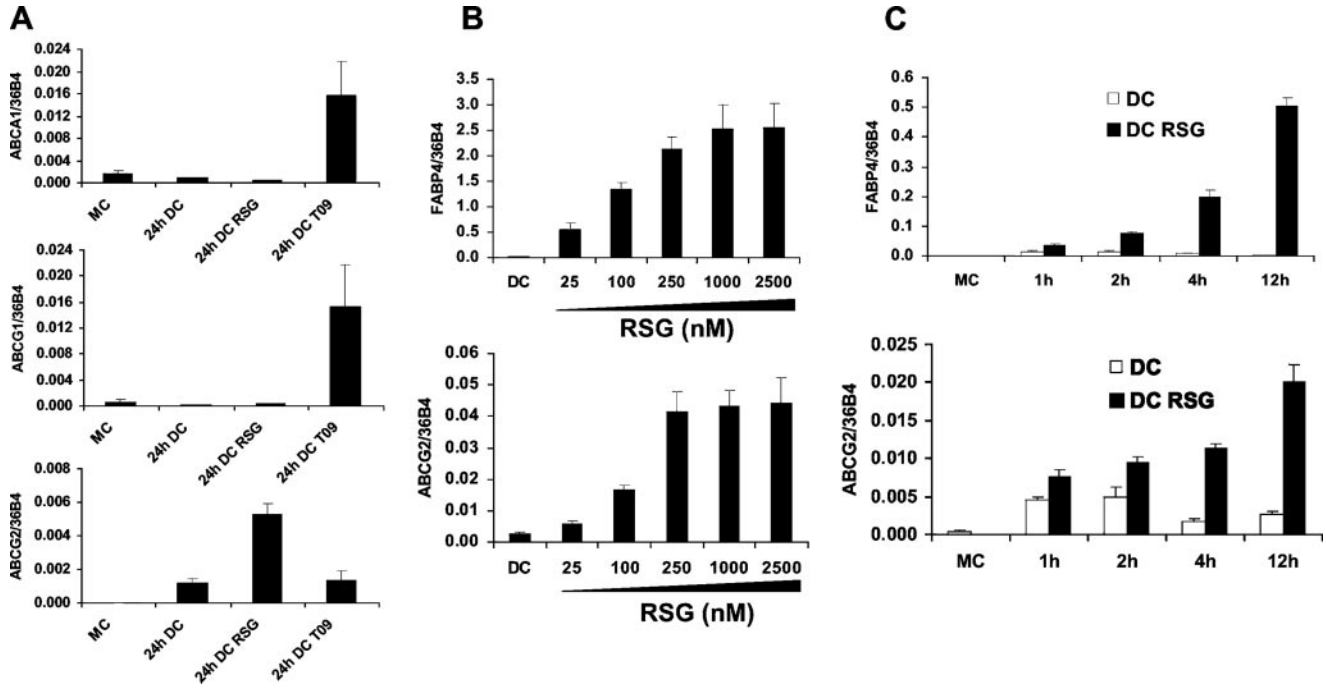

FIGURE 2. **ABCG2 is regulated by PPARγ via a mechanism independent of LXR.** *A,* mRNA levels of ABCA1, ABCG1, and ABCG2 were determined with RT-Q-PCR. Cells were cultured for 24 h and treated with 1 μM RSG or with an LXR-specific ligand: 1 μM T0901317 (T09). *B*, dose-dependent induction of FABP4 and ABCG2. Cells were treated with the indicated concentration of RSG; the mRNA expression was determined by RT-Q-PCR. *C*, kinetics of FABP4 and ABCG2 induction. Cells were cultured for 12 h; thereafter cells were treated with 1 μM RSG and harvested at the indicated time points and the mRNA expression was determined by RT-Q-PCR.

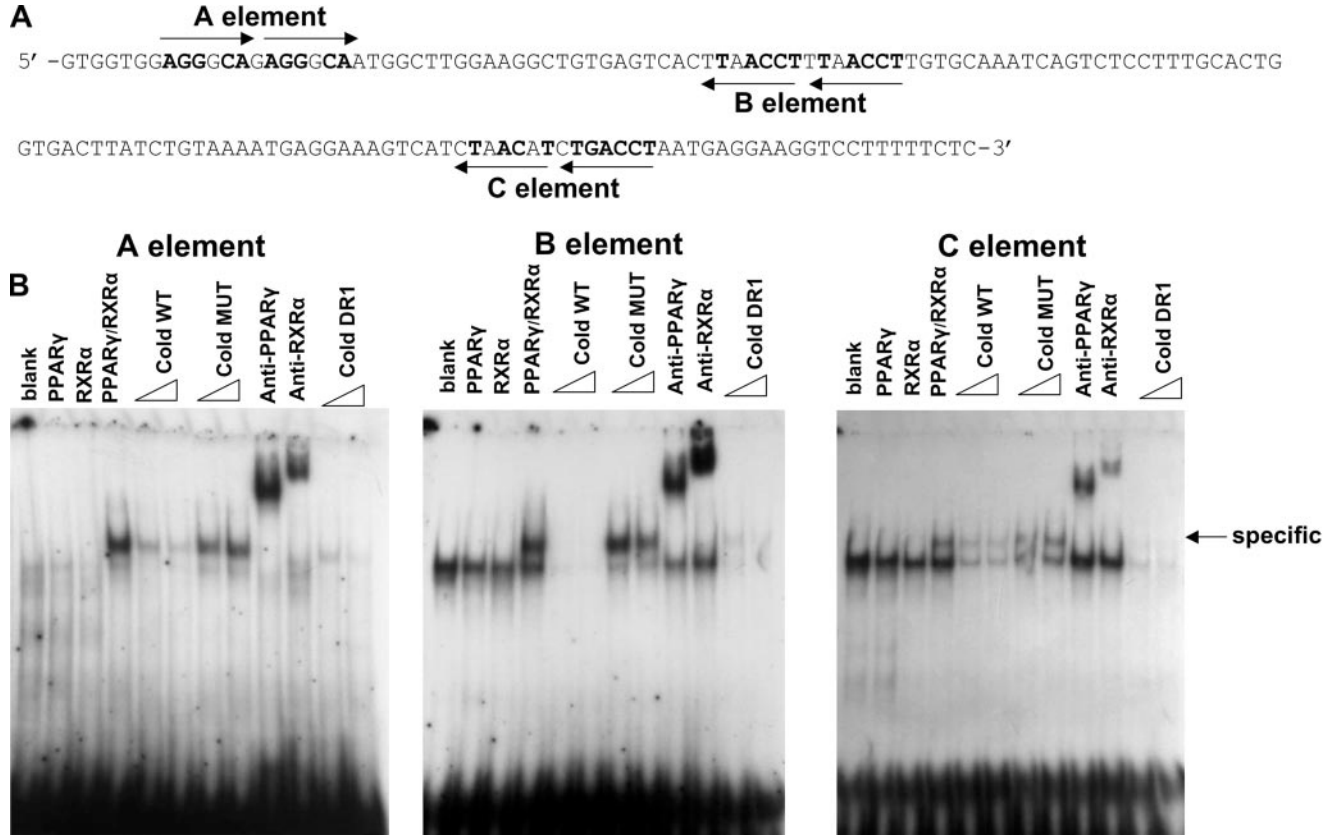

FIGURE 3. **Identification of PPAR·RXR binding sites in the human *ABCG2* gene.** *A,* sequence of a portion of the human ABCG2 upstream region (−3946/−3796 bp), highlighting the three putative PPAR responsive elements (*A*, *B*, and *C elements*). *B*, EMSA was carried out by using *in vitro*-translated receptors of PPARγ, RXRα, and α-^{32}P-labeled oligonucleotides as described under "Experimental Procedures." For competition experiments, cold (*Cold WT*), mutated (*Cold MUT*) or consensus DR1 oligonucleotides were used at ×10 and ×20 concentrations. For supershift experiments, the receptors were preincubated with the indicated antibodies prior to the binding reaction.

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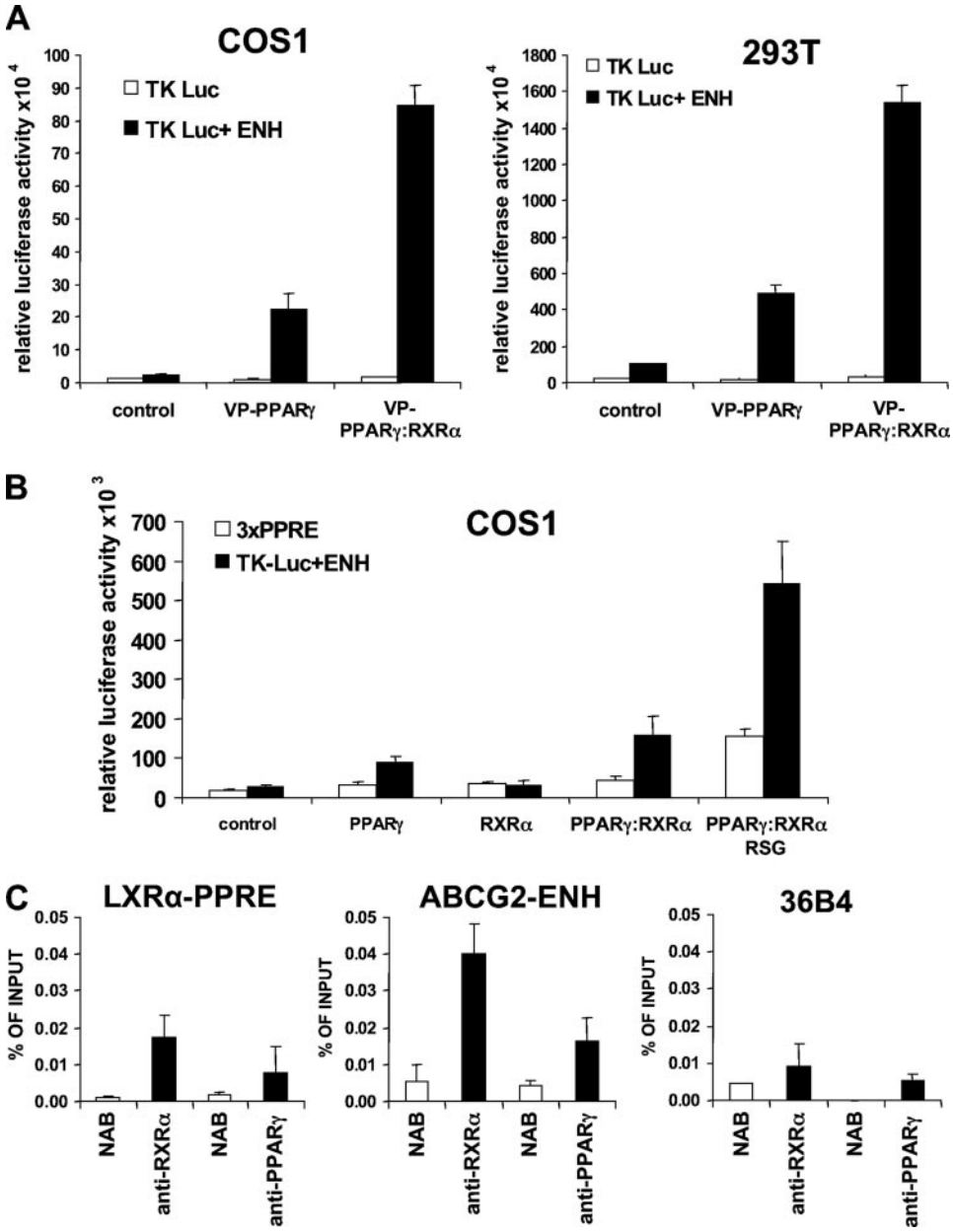

FIGURE 4. **An upstream sequence of the human *ABCG2* gene, containing three DR1s, is able to promote transcription in a PPARγ-dependent manner in a cell based binding assay.** *A,* COS1 or 293T cells were cotransfected with TK-Luc or with TK-Luc enhancer, the VP16 fusion protein of PPARγ with or without RXRα, and β-galactosidase. Cells were lysed after 24 h, and the luciferase and β-galactosidase activity were assayed as described under "Experimental Procedures." All experiments were done in triplicates ± S.D. *B*, COS1 cells were cotransfected with the indicated nuclear receptors, TK-Luc enhancer or with luciferase reporter construct containing 3 PPAR responsive elements (*3×PPRE*) and β-galactosidase. The indicated cells were treated with 1 μM RSG. *C*, ChIP was performed with anti-RXR and anti-PPARγ antibodies using chromatin obtained from RXRα- or PPARγ-transduced 293T cells, respectively. DNA content was analyzed with RT-Q-PCR assays specific for the genomic region of LXRα containing a PPRE, the identified ABCG2 enhancer region and a genomic region of the *36B4* gene. The results are shown as percentages of input DNA. All measurements were done in triplicate. As a negative control no-antibody control (*NAB*) was used.

pattern of mRNA levels was observed (Fig. 1*A*). Consistent with the microarray data, PPARγ agonist (rosiglitazone, abbreviated as RSG) treatment highly enhanced the expression of both genes. Although rosiglitazone is a specific PPARγ agonist, its ability to induce ABCG2 in developing DCs still may be related to other pharmacologic activities. To address this issue a PPARγ antagonist (GW9662) was simultaneously added to the cells. As shown in Fig. 1*A*, administration of the antagonist (ANT) abolished the induction of ABCG2 suggesting that this regulation is indeed PPARγ receptor-dependent. We used other chemically distinct synthetic PPARγ agonists to further establish the receptor specificity of ABCG2 induction. Cells were treated with troglitazone (TRO) or with GW347845X (abbreviated as GW7845). As shown in Fig. 1*B*, all of these PPARγ activators induced FABP4 and ABCG2 expression.

Up-regulation of ABCG2 was also detected in a monocytic/macrophage leukemia cell line (Monomac 6, abbreviated as MM6) by the activation of the PPARγ·RXR heterodimer (Fig. 1*C*). PPARγ forms a heterodimer with the RXR receptor. Interestingly, in MM6 cells both components of the heterodimer had to be activated to elicit a profound ABCG2 expression induction (MM6 cells were simultaneous treated with 2.5 μM RSG and 100 nM LG268, an RXR agonist). To further establish the role of PPARγ in ABCG2 regulation, we used RNA interference to suppress the expression of PPARγ in MM6 cells. We used a previously described (16) PPARγ-specific siRNA that effectively inhibited the expression of endogenous PPARγ but it did not interfere with expression of PPARδ (Fig. 1*C*). Significantly, PPARγ-specific siRNA-treated cells showed impaired induction of FABP4 and ABCG2, indicating that the transcriptional induction of these genes are receptor-dependent. Taken together, these results demonstrate that activation of PPARγ induces elevated ABCG2 mRNA expression both in DCs and in the myeloid leukemia cell line MM6. Moreover, this regulation appears to be selectively PPARγ receptor dependent in both cell types.

ABCG2 Is Regulated by PPARγ via a Mechanism Independent of LXR—It has been documented that, under certain conditions, PPARγ can indirectly activate gene expression by the induction of its direct target receptor, LXRα. For example, ABCA1 expression is regulated via such a mechanism (12, 13). A previous study has also established that ABCA1 and ABCG1 are direct target genes of LXRs (11). We also observed that a PPARγ ligand enhances the expression of LXRα in DCs (data

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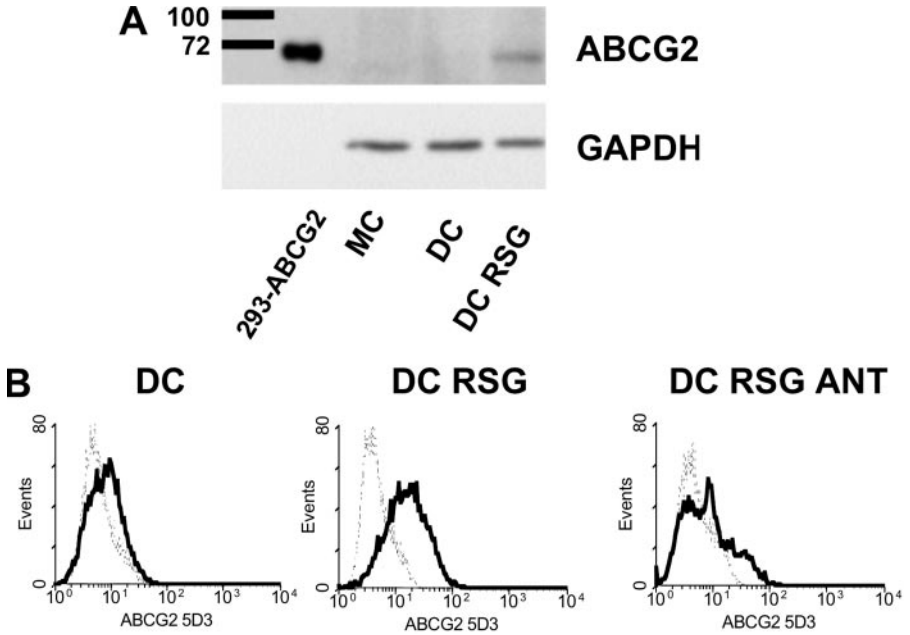

FIGURE 5. **ABCG2 protein is expressed in PPARγ-instructed DCs.** *A*, Western blot analysis of ABCG2 protein expression in differentiating DC. 50 μg of cell extract (monocyte-MC, the indicated cells were treated with 2.5 μM RSG) were subjected to Western blot analysis, the identity of the 70-kDa bands was confirmed by co-migration with a band seen in the extract (2 μg) of ABCG2-transfected 293T cells. *GAPDH* was used as a loading control. *B*, determination of the cell surface expression of ABCG2 in DCs. Control or ligand-treated cells were fixed and stained with an unlabeled anti-ABCG2 (5D3) or isotype-matched control and after 30 min of incubation with anti-mouse FITC-labeled antibody. Cell surface molecule specific antibodies indicated (*solid line*) *versus* isotype-matched control (*dotted line*). Cells were treated with 2.5 μM RSG alone or RSG plus a PPARγ antagonist (ANT, GW9662).

not shown) suggesting that an LXR-dependent pathway may be responsible for the up-regulation of certain ABC transporters.

To examine if the activation of PPARγ enhances ABCG2 transcription via the induction of LXRα, we compared the effects of PPARγ and LXR agonists on the mRNA levels of ABCA1, ABCG1, and ABCG2 by RT-Q-PCR. As shown in Fig. 2*A*, we found that in this cell type the activation of PPARγ failed to induce ABCA1 and ABCG1 expression, whereas we detected a profound up-regulation of these transporter mRNAs if cells were treated with an LXR-specific agonist (T0901317). In contrast to ABCA1 and ABCG1, T0901317 did not affect the expression of ABCG2. These data indicate that in human DCs ABCG2 regulation is independent from LXRα induction/activation, and in these cells the selective activation of nuclear hormone receptors results in a non-overlapping ABCA1-ABCG1 *versus* ABCG2 expression pattern.

PPARγ Directly Regulates the Transcription of the ABCG2 Gene—Our results demonstrated that the mRNA expression of ABCG2 is positively regulated by PPARγ-specific ligands in a receptor-dependent manner. Next, we have examined the dose and time dependence of ABCG2 induction. PPARγ activators (for example rosiglitazone) belong to a class of antidiabetic compounds (thiazolidinediones), that are used in the clinic to treat type II diabetes (21). The plasma concentration of RSG in patients treated with 4 mg of RSG is between 50–350 ng/ml (100–1000 nM) (22). To examine whether ABCG2 can be activated *ex vivo* in this concentration range, we performed dose response experiments. As shown in Fig. 2*B*, 100 nM RSG readily induced the expression of ABCG2, suggesting that this gene is likely to be induced in DCs *in vivo* upon RSG treatment.

Next we asked whether the activation of PPARγ directly elicits the accumulation of ABCG2 transcripts. Time course experiments (Fig. 2*C*) revealed that ABCG2 similarly to FABP4 were rapidly up-regulated upon PPARγ ligand administration. Although this finding is not a proof in itself, it strongly suggests that PPARγ directly regulates the expression of ABCG2.

To further test this possibility, we analyzed the promoter sequence of the *ABCG2* gene to find putative PPAR responsive elements. The sequence analysis of the previously reported promoter region (−1285/+362) of the human *ABCG2* gene (10) did not reveal any canonical PPAR response elements. Furthermore transient transfection assays carried out to assess the activity of this promoter region (−1285/+362) failed to indicate any PPARγ-dependent transcriptional response (data not shown). Therefore we decided to use a bioinformatics approach for the identification of conserved promoter regions and putative PPAREs. The ABCG2 protein is relatively well conserved in evolutionary distant animals. However the completely untranslated first exon shows very little similarity between species, thus the identification of the promoter region is rather difficult. In addition several species such as the mouse and rat contain paralogous *ABCG2* genes with alternatively spliced 5′-untranslated region (UTR) exon(s). Using cDNA sequences to identify the first UTR exons we have succeeded in finding putative promoter regions of bovine and dog *ABCG2* genes beside the almost identical primate sequences. Comparison of 5000 base pairs of human, dog, and bovine ABCG2 promoter regions (counting from the position of the beginning of the 5′ longest cDNA sequence) revealed a well conserved region (supplemental Fig. S1). We have identified a 150-bp portion (−3946/−3796) of this conserved region, which contains three potential PPAR response elements in the human and one in bovine and dog (Fig. 3*A* and supplemental Fig. S1). To test the ability of these elements for binding PPARγ·RXR *in vitro*, we carried out EMSA. The EMSA analysis revealed that all three putative elements, containing direct repeat 1 (DR1, AGGTCANAGGTCA) motifs, were able to specifically bind PPARγ·RXR heterodimers (Fig. 3*B*). In these experiments we also found that if the two receptors were added together, a stronger, specific binding was observed. This binding was effectively competed by either unlabeled (cold) oligos, or a canonical DR1 oligonucleotide, but not with oligonucleotides containing mutations. The specificity of the binding was further demonstrated by the use of antibodies against RXRα and PPARγ, which induced marked supershifts (Fig. 3*B*). Next an enhancer trap method was employed to assess the binding and transcription activation capacity of these elements. We introduced the identified element

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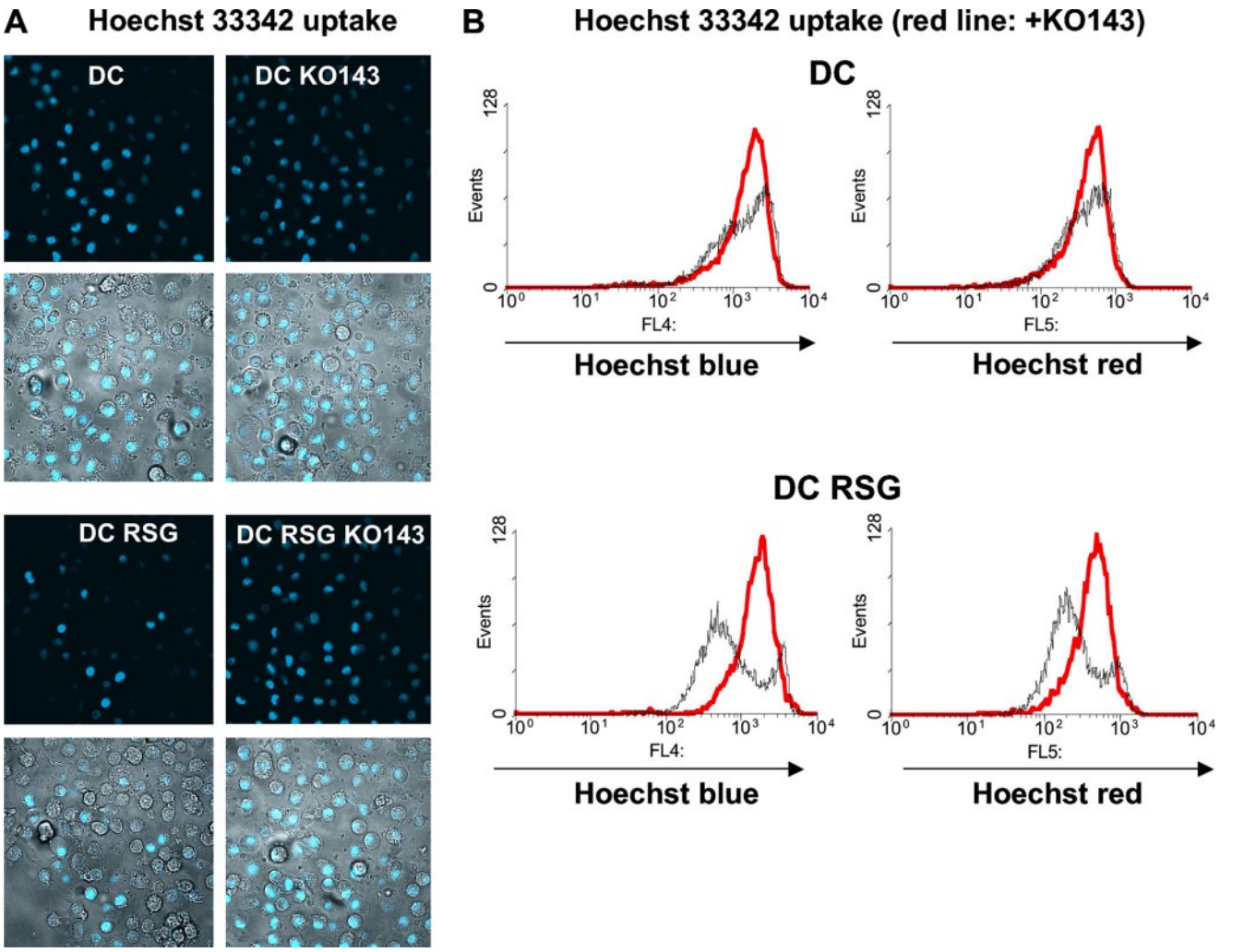

FIGURE 6. **Enhanced efflux of Hoechst 33342 in DCs upon PPARγ activation.** *A*, cells were incubated with 0.5 μg/ml Hoechst dye for 1 h at 37 °C with or without KO143 (1 μM), then subjected to microscopic analysis as described under "Experimental Procedures." *B*, same samples were quantitatively analyzed with a Becton Dickinson FACSVantage flow cytometer.

(−3946/−3796) into a reporter plasmid upstream of the minimal TK-luciferase promoter, generating the heterologous promoter construct termed ABCG2enhancer-TK-Luc (TK-Luc ENH). To determine the receptor binding capacity of the enhancer region in a cellular system, we co-transfected the ABCG2enhancer-TK-Luc reporter construct with the expression vector, containing constitutively active PPARγ (VP16-PPARγ) alone or VP16-PPARγ along with RXRα. We obtained a robust induction of reporter gene activity when the cells were cotransfected with VP16-PPARγ, and, as anticipated, the induction was further increased in the presence of RXRα (Fig. 4*A*). We obtained similar results in two independent cell lines (COS1 and 293T). In a specificity control experiment, the TK-Luc dependent transcription was not modified by receptor expression in the absence of the enhancer region. These data provided evidence for a possible *in vivo* binding of the receptor heterodimer to the identified enhancer elements in the *ABCG2* gene.

To test this role directly, COS1 cells were co-transfected with the ABCG2enhancer-TK-Luc reporter construct, along with expression vectors coding for PPARγ or RXRα in various combinations (Fig. 4*B*). We observed that the presence of the PPARγ·RXRα heterodimer induced a major transcription response, and this was further elevated by the administration of a PPARγ-specific ligand, RSG. We also included a promoter element containing canonical PPAR responsive elements (3×PPRE) as a positive control (Fig. 4*B*). Finally, we analyzed if PPARγ and RXRα protein was bound to the ABCG2 enhancer region in cells using chromatin immunoprecipitation (ChIP) combined with real-time PCR. Chromatin was obtained from PPARγ- or RXRα-transduced 293T cells. A genomic region of the *LXRα* gene containing a PPRE element was used as a positive control and a genomic region of the *36B4* gene was tested as a negative control. Consistent with EMSA and the transient transfection results, our analyses demonstrated that both PPARγ and RXRα were present on the ABCG2 enhancer region also in the chromatin context (Fig. 4*C*).

Taken together, all these data strongly suggest that the genomic region examined, upstream of the *ABCG2* coding sequence, can bind PPARγ·RXR heterodimers, and this enhancer region is able to confer a PPARγ-dependent transcriptional response. Thus this genomic region most likely participates in the PPARγ-dependent regulation of ABCG2 expression.

ABCG2 Protein Expression Is Up-regulated in PPARγ-activated Cells—Our results described above suggest that PPARγ directly and specifically induces the mRNA expression of

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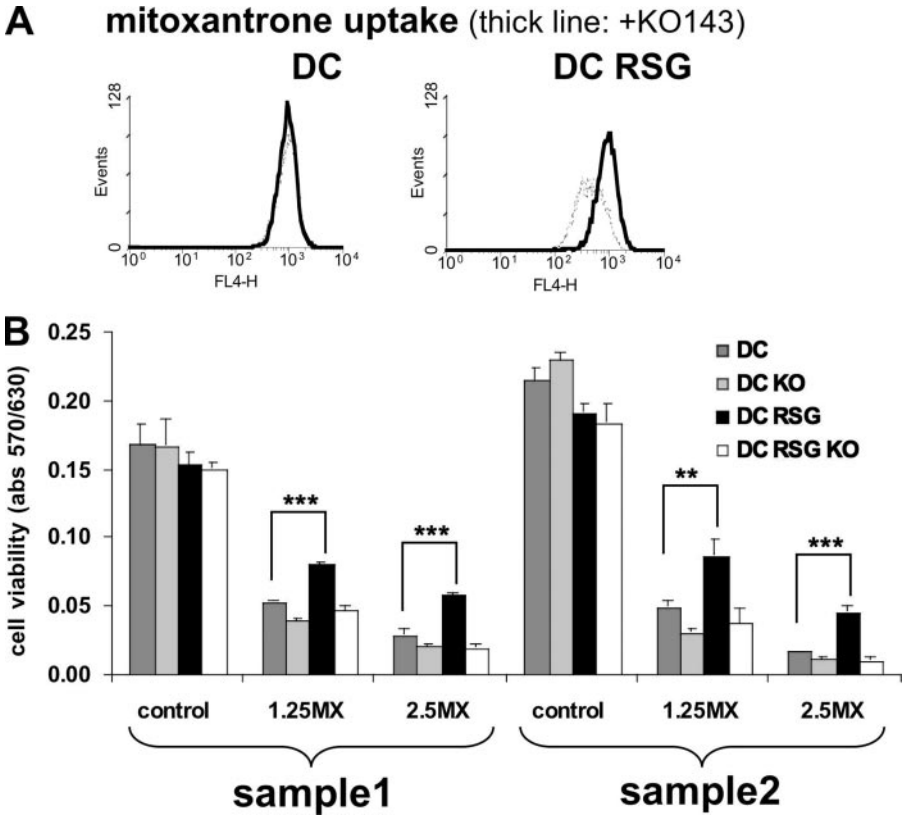

FIGURE 7. **PPARγ-activated DCs have an enhanced viability upon mitoxantrone exposure.** *A*, efflux of mitoxantrone by ABCG2 in DCs. Cells were incubated with 5 μM mitoxantrone in the presence or absence of 1 μM ABCG2 inhibitor (KO143) for 30 min and washed with ice-cold phosphate-buffered saline, and finally the intracellular accumulation of mitoxantrone was assessed by flow cytometry. *B*, control or PPARγ-activated (2.5 μM RSG) DCs were cultured for 48 h and treated with 1.25 or with 2.5 μg/ml mitoxantrone. Cell cytotoxicity was assessed with the MTT assay as described under "Experimental Procedures." **, $p < 0.01$; ***, $p < 0.001$.

ABCG2 in monocyte-derived DCs. To estimate the changes in the protein level and the cell surface expression of ABCG2 in DCs, we performed antibody staining in Western blot and in flow cytometry experiments. By using the BXP 21, ABCG2-specific monoclonal antibody in Western blotting, we could not detect the ABCG2 protein either in human monocytes or untreated DCs. However, a specific 70-kDa protein product appeared on the immunoblot when human DCs were pretreated with a PPARγ ligand, RSG (Fig. 5*A*). We have also determined the cell surface expression of the ABCG2 protein by flow cytometry (Fig. 5*B*), using the 5D3 monoclonal antibody, which reacts with an extracellular epitope of the transporter. Consistent with the mRNA expression and Western blot data, significant cell surface expression of the ABCG2 protein was detected only in the RSG-treated DCs, whereas this expression was reduced if the cells were treated with a PPARγ antagonist (GW9662). All these data clearly indicate that PPARγ-dependent activation of transcription results in an enhanced ABCG2 protein production, which is correctly processed to reach the cell membrane surface in DCs. Next we investigated the functional consequences of the elevated level of this xenobiotic transporter on DCs.

PPARγ-instructed DCs Have an Enhanced Capacity to Extrude Xenobiotics and Acquire Resistance against Anticancer Agents—The ABCG2 transporter has a wide substrate recognition and transport capacity and, by performing an efficient extrusion of the Hoechst 33342 fluorescent dye, the expression of ABCG2 has been implicated in the so called side population phenotype of stem cells (8, 23). With the aim of correlating ABCG2 expression level and the function of this transporter, Hoechst 33342 dye accumulation was compared in normal and PPARγ agonist-treated DCs, respectively. We have monitored the nuclear Hoechst dye fluorescence with confocal microscopy and flow cytometry analyses. As shown in Fig. 6, *A* and *B*, most of the PPARγ-activated DCs accumulated less Hoechst dye than the non-stimulated control cells, indicating an enhanced pump activity of ABCG2. To assess the specificity of the assay, KO143, a specific and selective inhibitor of ABCG2, was added to block the transporter activity. We found that the reduced accumulation of the fluorescent dye in the stimulated DCs was ABCG2-dependent, because the administration of KO143 abolished the effect of the PPARγ ligand treatment. Next we assessed the mitoxantrone (MX) efflux capacity of the PPARγ-activated DCs. As shown in Fig. 7*A*, mitoxantrone accumulation was rapid in the control DCs, whereas after the addition of the PPARγ activator RSG, the cells accumulated less mitoxantrone than the control DCs. Similarly to that found in the Hoechst dye uptake studies, mitoxantrone extrusion by ABCG2 in the activated cells was fully inhibited by the specific inhibitor of the pump, KO143. Next we examined whether the presence of ABCG2 in RSG treated DCs conferred a protective effect against the cytotoxic drug, mitoxantrone. DCs were treated with different doses of mitoxantrone for 48 h and then cell viability was assessed by MTT assay. As shown in Fig. 7*B*, whereas low doses of MX-induced cell death in the control DCs, the PPARγ ligand-treated DCs were significantly less sensitive to this cytotoxic agent. The addition of ABCG2 inhibitor KO143 removed this protective effect. In agreement with the enhanced expression of ABCG2 after PPARγ activation of DCs, the observed reduced Hoechst dye and drug accumulation, as well as the reduced sensitivity to MX in these cells indicate that ABCG2 is fully functional, and activated DCs acquire a protection against cytotoxic agents.

Blocking of ABCG2 Only Mildly Modified the DC Phenotype—Our results suggest that PPARγ-activated DCs have an enhanced transport capacity to extrude various chemically synthesized drugs. It is an intriguing question, what is the physiological role of this transporter on DCs. To further assess the functional consequence of the elevated expression of ABCG2, including its effect on gene expression, we cultured the PPARγ-

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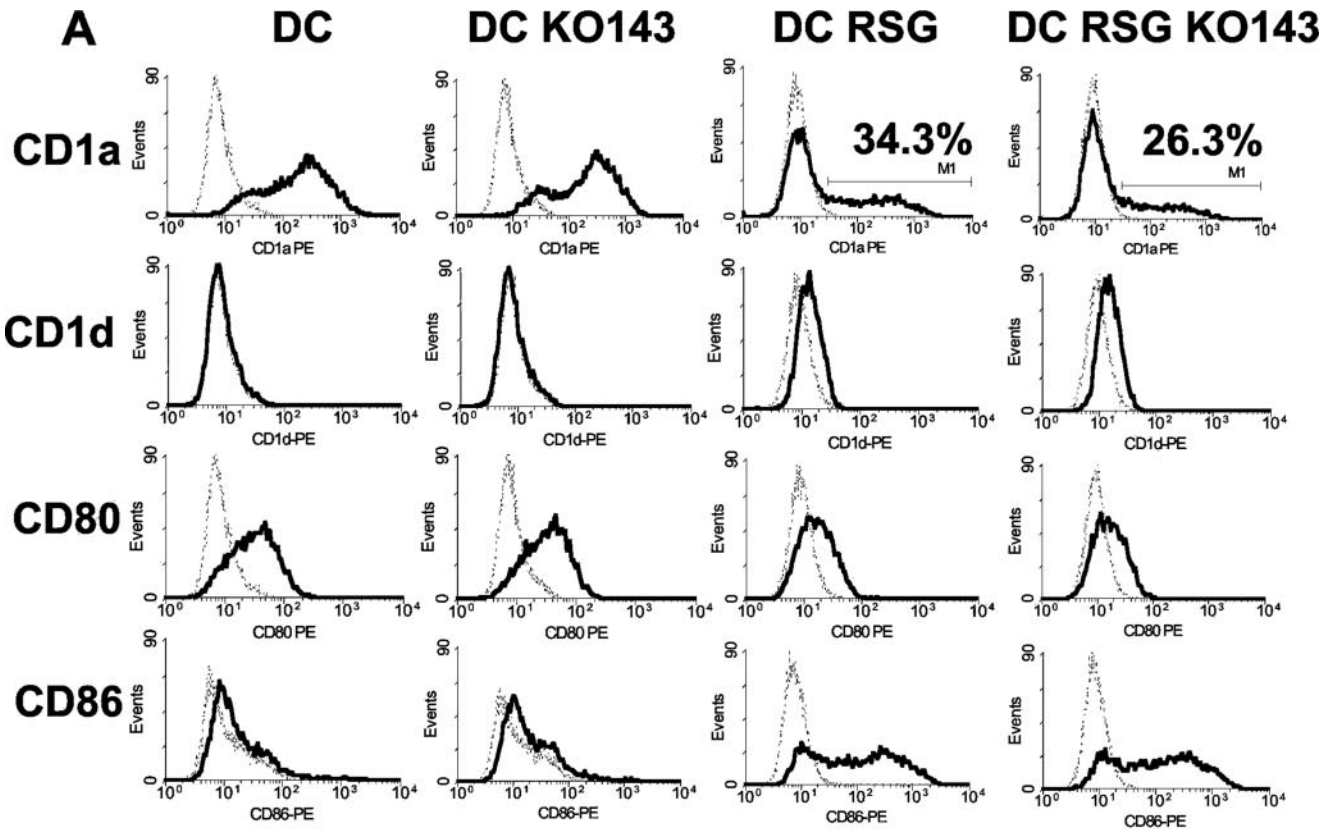

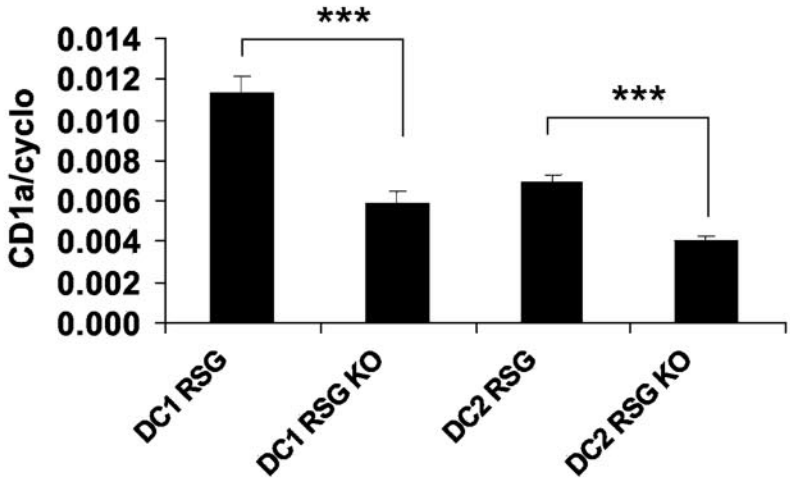

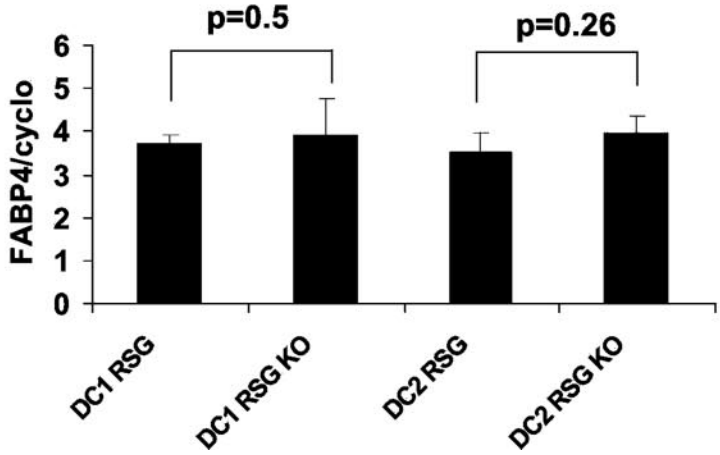

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activated cells in the presence of ABCG2 inhibitor (KO143) and compared the phenotype of the inhibitor-treated and control cells. We and others (19, 24, 25) have recently characterized the phenotype of the PPARγ-instructed DCs. Consistent with the previous findings, PPARγ-activated DCs expressed more CD86 and CD1d, but the expression of CD80 and CD1a were decreased (Fig. 8*A*). The administration of the selective ABCG2 inhibitor (KO143) did not change the cell surface expression of CD80, CD86, or CD1d but the expression of CD1a was slightly decreased on the PPARγ activated cells (Fig. 8*A*). We also assessed the mRNA expression of CD1a and FABP4 by RT-Q-PCR. The transcript level of FABP4 did not change but CD1a expression was reduced consistent with the changes detected on the cell surface expression. Together our data indicate that blocking the ABCG2 transporter in PPARγ-instructed DCs only mildly modify the phenotype of DCs and it is unlikely that it contributes significantly to PPARγ-dependent changes in DC phenotype. However, a subset of PPARγ-regulated genes (*i.e.* CD1a) was slightly modified when the pump was inhibited. This raises the issues that ABCG2 might have some regulatory roles in DCs besides its established cytoprotective function, which is likely to be part of an adaptive response.

DISCUSSION

The functional and adaptive specification of human DCs is a well established concept (26, 27). However the regulatory processes and the molecular underpinnings of functional plasticity or subtype specifications are largely unknown. Previously we and others (19, 24, 25) have provided evidence that nuclear hormone receptors are likely to contribute to these processes.

Here we further characterize the role of one of the lipid-regulated transcription factors and provide evidence for the induction of the expression of functional ABCG2 transporter by PPARγ receptor in human monocyte-derived DCs. We demonstrate the up-regulation of the transporter both at the mRNA and the protein level, the proper cell surface localization as well as the xenobiotic transport activity of the pump. Moreover, we identify a specific enhancer region containing three PPAREs, directly binding PPARγ, in the upstream region of the *ABCG2* gene.

A key issue in regulatory processes especially involving xenobiotics response is specificity. We found that ABCG2 induction was receptor and subtype specific. Neither LXR ligands nor retinoids (data not shown), regulators of other transporters, induced the expression of this pump. We also obtained poor responses when cells were treated with PPARα or PPARδ ligands (data not shown). Furthermore, ABCG2 induction was abrogated by the addition of a PPARγ specific antagonist and by using siRNA against PPARγ. Importantly, the PPARγ-dependent regulation appears to be direct. Three PPAR response elements (direct repeat 1, DR1) were identified and characterized in the upstream region of the human *ABCG2* gene. Gel-shift and transient transfection analyses as well as chromatin immunoprecipitation confirmed the specific binding of PPARγ·RXR heterodimer to this enhancer region. By the identification of this upstream enhancer we provide novel insights into the promoter/enhancer structure and regulation of ABCG2 as well. Earlier studies identified several Sp1 sites in the −222 bp to −49 bp promoter region of ABCG2, a CCAAT-box is also present in the −274-bp position, and CpG islands are found in its downstream proximity (28). In addition, a novel Estrogen Response Element (ERE) has been identified in the −188-bp to −172-bp segment, which triggers an enhanced transcription of human ABCG2 in ER-positive cells (29). Our data indicate the presence of a relatively distant (−3946/−3796 bp) major regulatory site, responsible for PPARγ·RXR regulation of this gene. It is also possible that this conserved enhancer contains other binding sites, which might contribute to gene regulation. This issue requires further investigation.

The PPARγ-dependent up-regulation of ABCG2 is not restricted to DCs. It was also detected in the monocyte/macrophage cell line, MM6. It is an intriguing question whether this regulation is a DC and/or at least myeloid cell type-specific phenomenon, or ABCG2 can be induced in other PPARγ-positive cell types as well. In our preliminary experiments, by using monocytes cultured with M-CSF, we obtained only a slight FABP4 induction indicating a weak PPARγ response in macrophages and failed to detect any ABCG2 induction (data not shown). Our data still raise the possibility, that in PPARγ receptor positive tumor cells activation of this receptor may cause the appearance of a multidrug resistance phenotype, because of ABCG2 overexpression. There would be obvious therapeutic ramifications if this scenario proved to be true. The expression of ABCG2 is clearly associated with the modulation of xenobiotic absorption, distribution, and toxicity in many different tissues. The wide substrate specificity and tissue distribution of this active transporter thus plays a major role in pharmacotherapy. In hematopoietic stem cells the side population (SP) phenotype, based on Hoechst 33342 efflux, is provided by a high level ABCG2 expression. More recently, pheophorbide A, PPIX and heme have all been shown to be ABCG2 substrates, indicating a role for this protein in the cytoprotection of stem cells and erythrocytes (9, 30). Our experiments fully support this role in dendritic cells by showing that PPARγ-activated DCs accumulated less Hoechst dye and mitoxantrone. This effect was reverted by a specific and selective ABCG2 inhibitor, indicating that the effect is ABCG2-dependent. Consistent with the enhanced mitoxantrone efflux, we also found that PPARγ-treated DCs showed an increased resistance to the anti-neoplastic and immunosuppressive drug, mitoxantrone. Again, this effect was caused by the function of enhanced ABCG2 expression and activity. Therefore this pathway, if activated, arms the DCs with the capacity to withstand xenobiotic and

FIGURE 8. **Inhibition of ABCG2 activity slightly modifies expression of CD1a.** *A*, characterization of CD1a, CD1d, CD80, and CD86 cell surface expression on DCs treated with ligands: 2.5 μM RSG alone or with 1 μM ABCG2 inhibitor (KO143). Data obtained with specific antibodies indicated (*solid line*) *versus* isotype-matched control (*dotted line*). *B*, CD1a and FABP4 transcripts levels were determined from vehicle or PPARγ ligand-treated DCs (2.5 μM RSG) alone or with 1 μM ABCG2 inhibitor (KO143). Gene expression data are expressed as a ratio of CD1a and FABP4 transcripts relative to cyclophilin A (*cyclo*) expression ± S.D. ***, $p < 0.001$.

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toxic noxa. Interestingly, it has been shown that PPARγ ligand treatment induced the expression of an UDP-glucuronosyltransferase (UGT1A9) in human hepatocytes and macrophages (31). UGTs catalyze the glucuronidation reaction, which is a major pathway in the catabolism and elimination of numerous xenobiotics. These results suggest that in PPARγ has the potential to coordinately regulate both the inactivation and elimination of xenobiotics and thus protects these cells. Recently, tumor antigen-loaded DCs have been introduced to elicit an anticancer immune response as part of cell therapy protocols. DC therapy is often combined with chemotherapy. Our results suggest that the application of a PPARγ ligand may protect the antigen presenting cells by selective induction of ABCG2 expression. The findings reported here may prove to be useful in designing more efficient cell therapy protocols. Obviously, this activation should only be utilized in the case of PPARγ negative tumor cells, as PPARγ activators may elicit an ABCG2-dependent multidrug resistance in the tumor cells as well. It remains to be defined how this mechanism contributes to DC function and activity. It is also not clear what is the function of this pathway under normal, non-pathological conditions. In macrophage ABCA1 and ABCG1 have an important role in the efflux of cholesterol (11, 13). It is a debated issue whether ABCG2 can transport any lipids. It was reported that ABCG2 mediated the efflux of cholesterol on bacterial cell membrane (32) but unlike some other members of the ABCG subfamily, ABCG2 seems not to be critically involved in lipid metabolism, because in ABCG2-deficient mice, the plasma levels of cholesterol and phospholipids were normal (30). We failed to detect any enhanced cholesterol efflux on PPARγ activated monocyte-derived DCs (data not shown). Other multidrug transporters have roles in enhanced DC transmigration and leukotriene production (33, 34). These data prompted us to investigate the effect of blocked ABCG2 activity in PPARγ-activated cells. We did not detect major changes in DC phenotype. However, we observed that in the presence of the ABCG2 inhibitor DCs expressed less CD1a. Previous results showed that PPARγ activators negatively regulate CD1a (19, 25), therefore it is formally possible that regulated ABCG2 expression contributes to this by exporting signaling molecules. It is also possible that certain PPARγ ligands might be transported by ABCG2 providing a negative feedback regulation to ligand responsiveness. However we do not have any data to support this scenario yet.

In summary, our study revealed that PPARγ-activated DCs express an increased level of functional ABCG2 transporter, and these cells acquire relative protection against endo- and xenobiotics. This activation may have a significant effect on the differentiation or drug-dependent modulation of DC function and may also have therapeutic ramification.

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