Peroxisome proliferator-activated receptor γ activators inhibit interleukin-12 production in murine dendritic cells

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Abstract Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. They are divided into three subtypes (α, β or δ, and γ) and are involved in lipid and glucose homeostasis and in the control of inflammation. In this study, we analyzed the expression of PPARs in murine dendritic cells (DCs), the most potent antigen presenting cells. We find that immature as well as mature spleen-derived DCs express PPARγ, but not PPARα, mRNA and protein. We also show that the PPARγ activator rosiglitazone does not interfere with the maturation of DCs in vitro nor modifies their ability to activate naive T lymphocytes in vivo. Finally, we present evidence that PPARγ activators down-modulate the CD40-induced secretion of interleukin-12, a potent Th1-driving factor. These data suggest a possible role for PPARγ in the regulation of immune responses.

Key words: Dendritic cell;
Peroxisome proliferator-activated receptor; Interleukin-12

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family [1]. They are activated by a variety of compounds including hypolipidemic and insulin-sensitizing drugs as well as natural fatty acids and their eicosanoid metabolites [2]. PPARs function as regulators of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation and also appear to control the inflammatory response [3,4]. In addition to regulating gene transcription through the peroxisome proliferator-responsive element, it has recently been demonstrated that PPARs negatively interfere with NF-κB, AP-1 and STAT signaling pathways [5–10]. To date, three subtypes of PPARs, which are characterized by distinct tissue distribution patterns, have been described in humans and rodents: PPARα, β (δ) and γ. PPARα is highly expressed in cells with high catabolic rates of fatty acids [11,12]. PPARβ (δ) is ubiquitously expressed [12,13] whereas PPARγ has a more restricted expression [3]. Along with its expression in adipocyte tissue, colonic mucosa [14] and the vascular wall [15,16], PPARγ has also been shown to be expressed in the spleen white pulp and Peyer's patches [13,17], organs mainly composed of B and T lymphocytes and of dendritic cells (DCs). This tissue-specific location, as well as its expression in monocytes/macrophages [5,6,18–20] and in B [21] and T lymphocytes [22–24] raise the possibility that PPARγ could be involved in innate/adaptive immunity. This speculation is reinforced by the demonstration that PPAR members may act as important immunomodulatory mediators both in vitro and in vivo [5–7,25]. In this report, we investigated the possibility that PPARs may be expressed and may regulate immunostimulatory functions of DCs, for instance by reducing the expression of certain genes involved in T cell sensitization.

DCs are the most potent professional antigen presenting cells (APCs) capable of inducing T cell-mediated immune reactions against a wide range of antigens, including chemical haptens, foreign proteins, infectious agents and tumor-associated antigens [26,27]. This unique property is due to their ability to capture, process and present antigens to naive T lymphocytes in the secondary lymphoid tissues [28]. In response to signals delivered by DCs, T lymphocytes differentiate into type 1 (interleukin (IL)-2, IFNγ) or type 2 (IL-4, IL-5, IL-10) cytokine-producing cells which in turn orientate the immune response towards a cellular or humoral response, respectively. In most tissues, following internalization and processing of exogenous antigens, immature DCs undergo a process of maturation which is a crucial step in their development into fully potent APCs. They increase their expression of MHC class II molecules as well as costimulatory molecules (CD40, CD54, CD80, CD86,) which are necessary to deliver accessory signals required for T cell activation [29,30]. DCs also upregulate their production of T cell-stimulatory cytokines. During antigen-specific interaction between DCs and naive T lymphocytes, CD40/CD40L ligation induces the synthesis of IL-12 by DCs that in turn mediates the differentia-

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Abbreviations: Abs, antibodies; APCs, antigen presenting cells; DCs, dendritic cells; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; KLH, keyhole limpet hemocyanin; LNs, lymph nodes; PPARs, peroxisome proliferator-activated receptors; RSG, rosiglitazone; RT-PCR, reverse transcriptase-polymerase chain reaction; Th, T helper

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tion of naive T helper (Th) cells to IFNγ-producing cells [31–34]. The demonstration that PPAR activators may regulate the synthesis of cytokines and/or surface molecules by various cell types [5–7] raised the possibility that members of the PPAR family could also modulate the expression of costimulatory molecules and/or cytokines by maturing DC and further influence their function. In this report, we investigated the expression of PPARs in murine splenic DCs by reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot and immunocytochemistry. Our results indicate that PPARγ is expressed in DCs whereas PPARα appears to be absent. We also determined the effects of PPARγ activators on maturation and accessory function of DCs. We show that PPARγ activation does not affect the maturation of DCs nor their ability to sensitize naive T lymphocytes in vivo. Interestingly, we found that PPARγ agonists significantly reduce the CD40-induced synthesis of IL-12 by DCs, suggesting a role for PPARγ in the orientation of immune responses.

2. Materials and methods

2.1. Reagents and antibodies (Abs)

All reagents were purchased from Sigma (St. Quentin-Fallavier, France) unless otherwise indicated. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ_2) was from Cayman Chemical (Ann Arbor, MI, USA). Rosiglitazone (RSG) was kindly provided by Dr. A. Bril (SmithKline Beecham, Rennes, France). Affinity-purified rabbit polyclonal Abs specific for PPARγ have already been described [19]. Affinity-purified rabbit Abs directed against an irrelevant parasite antigen were prepared in the laboratory and were used as a negative control. FITC-conjugated anti-CD80 (clone 16-10A1, hamster IgG), -CD86 (clone GL1, rat IgG2a), -CD40 (3/23, rat IgG2a), biotin-conjugated anti-CD11c (HL3, hamster IgG) and -CD54 (3E2, hamster IgG) Abs were purchased from Becton Dickinson (Le Pont de Claix, France). The anti-MHC class II hybridoma (M5/114, rat IgG2b) was kindly provided by Dr. Ann Ager (NIMR, Mill Hill, UK). FITC-conjugated goat anti-rat IgG (Beckman Coulter, Villepinte, France), FITC-conjugated donkey F(ab′)$_2$ anti-rabbit Igs and FITC-streptavidin (FITC-SA) (Clinisciences, Montrouge, France) were used as second stage reagents. Anti-CD11c-conjugated microbeads (Miltenyi Biotech, Paris, France) were used for MACS purification of DCs. Mouse recombinant GM-CSF was purchased from Biosource (Nivelles, Belgium). Human recombinant Flt-3 ligand was from Immunex Corp. (Seattle, WA, USA).

2.2. Preparation and treatment of DCs

DCs were isolated from spleens obtained from Flt-3 ligand injected C57BL/6 mice as already described [35]. Briefly, spleens were digested with collagenase for 30 min at 37°C and then dissociated in RPMI containing 10 mM EDTA. The cell suspension was separated into low and high density fractions on a Nycodenz gradient (Nycomed Pharma, Oslo, Norway) (1700×*g* for 15 min). Splenic DCs were collected at the interface, washed twice with RPMI and purified by positive selection over two successive MACS columns by using anti-CD11c-conjugated microbeads. After purification, the cells were plated in 100 mm Petri dishes for 2 h at 37°C in RPMI supplemented with 2% ultroser (Life Technologies, Cergy Pontoise, France), a serum free medium supplement. Non-adherent cells were removed by vigorous pipetting and the adherent cells (immature DCs) were allowed to mature for 18 h in RPMI supplemented with 2% ultroser and 10 ng/ml of GM-CSF in the presence or in the absence of 10 μM RSG. In general, non-adherent mature DCs represented at least 99% of the total cell population as assessed by FACS analysis with anti-CD11c monoclonal Ab. For pulse experiments, 50 μg/ml keyhole limpet hemocyanin (KLH) was added (pulsed DCs) or not (unpulsed DCs) to immature DCs for 18 h as reported [35] in the presence or not of RSG.

2.3. Immunization protocol and in vitro assays

After overnight culture, non-adherent unpulsed or KLH-pulsed DCs were collected, washed and administered at a dose of 3×10^5 cells into the hind footpads of syngeneic mice. Five days later, popliteal lymph node (LN) cells (5×10^5 cells/well in a flat-bottom 96-well plate) were cultured in Click's medium supplemented with 0.5% normal mouse serum and various concentrations of KLH for 4 days at 37°C. During the last 18 h, 0.5 μCi of [^{3}H]thymidine per well was added. At 72 h time point, IFNγ, IL-4, IL-5 and IL-10 production were measured in the culture supernatants from LN cells by enzyme-linked immunosorbent assay (ELISA). IL-4, IL-5 and IL-10 were quantified by two-site ELISA adapted from Pharmingen protocols (Becton Dickinson). The supernatant was assayed for IFNγ using an ELISA kit from R&D Systems (Abingdon, UK).

2.4. RNA extraction and RT-PCR analysis

Total RNA from DCs was obtained using the TRIzol reagent (Life Technologies, Grand Island, NY, USA) and reverse-transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies). Amplification by PCR was performed with the following primers: PPARα, 5′-CCAAGTCACCTTGCTAAAGTAC-3′ and 5′-GGTGTCATCTGGATGGTTGCTC-3′ (fragment size 320 bp); PPARγ, 5′-GTCCATGAGATCATCTACACG-3′ and 5′-GTGCTCTGTGACGATCTGCCT-3′ (fragment size 376 bp). β-Actin-specific primers 5′-TCACCGAGGCCCCCTGAAC-3′ and 5′-GCACGCACTGTAATTCCTC-3′ were used as control (fragment size 324 bp). The numbers of PCR cycles selected were as follows: PPARα (36), PPARγ (36) and β-actin (30).

2.5. Western blot and immunocytochemistry analysis

Total protein was extracted from DCs with sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS–PAGE and transferred to nitrocellulose filters. Proteins were analyzed by Western blot with affinity-purified rabbit anti-PPARγ Abs and horseradish peroxidase-labelled goat anti-rabbit Ig (Sanofi Diagnostics Pasteur, Marnes, France). Specificity of anti-PPARγ Abs was checked by Western blot analysis using in vitro synthesized PPARγ protein [19]. Immunofluorescence staining was performed as previously described [19]. Briefly, immature DCs cultured for 2 h on gelatin-coated coverslips were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 10 min and permeabilized with cold acetone/methanol (1:1) for 5 min. After incubation with anti-PPARγ Abs or the control Abs, proteins were visualized using secondary FITC-conjugated donkey F(ab′)$_2$ anti-rabbit Abs.

2.6. FACS analysis

Phenotypic analysis of DCs was performed with anti-MHC class II hybridoma or biotin-conjugated anti-CD11c, -CD54 or FITC-conjugated anti-CD40, -CD80 and -CD86 Abs. Briefly, 2×10^5 cells/well were plated in 96-well plates, resuspended in 50 μl of culture supernatant or 20 μl of FITC or biotin-conjugated primary Ab diluted in PBS supplemented with 5% fetal calf serum and 0.2% NaN_3 and incubated on ice for 30 min. After washes, FITC-conjugated goat anti-rat IgG or FITC-SA were added when needed for another 20 min, followed by three washes. After the last wash, cells were fixed in 1% paraformaldehyde in PBS and were analyzed on a FACScalibur flow cytometer. Data were analyzed using CellQuest software (Becton Dickinson).

2.7. Stimulation and measurement of IL-12 secretion

L cells transfected with the ligand for CD40 (CD40L) were used to induce CD40 triggering on DCs. Non-transfected L cells were used for control cultures. 10^5 immature DCs were incubated with 10^4 irradiated (10 000 rad) CD40L-transfected L cells in the presence or absence of different doses of RSG. After 24 h, supernatants were collected and IL-12 p40 and IL-12 p70 concentrations were measured by ELISA (R&D Systems).

3. Results

3.1. PPARγ, but not PPARα, is expressed in spleen-derived DCs

To determine the expression of PPARs in DCs, we performed RT-PCR and Western blot analysis on freshly isolated (immature) and on overnight cultured (mature) DCs derived from mouse spleen. RT-PCR analysis demonstrated the pres-

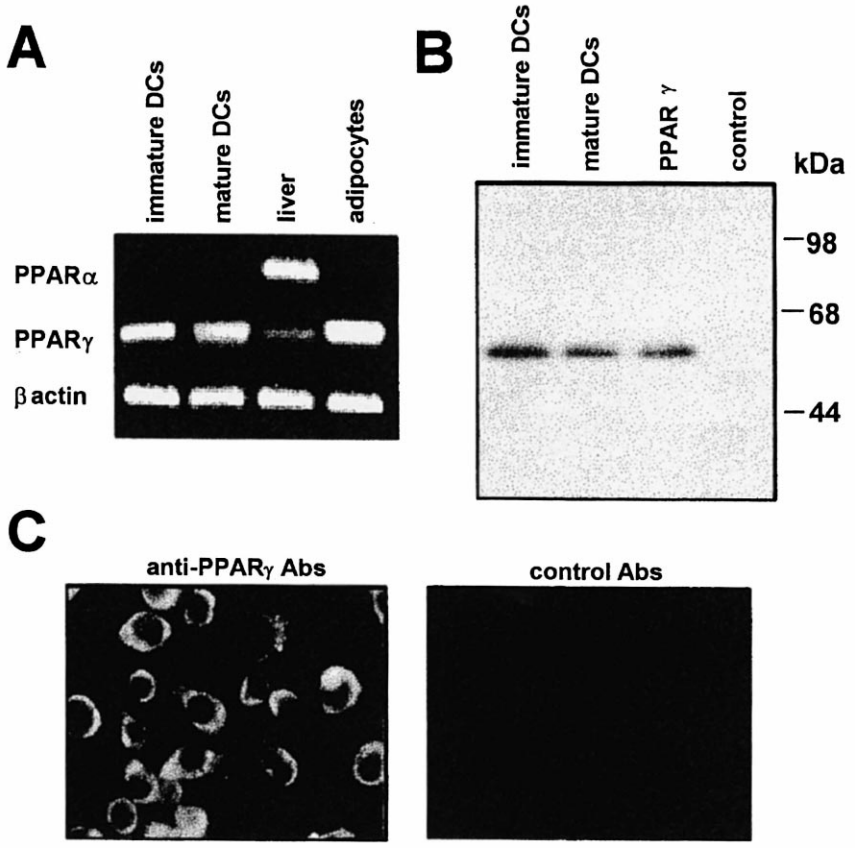

Fig. 1. Expression of PPARγ in murine splenic DCs. A: mRNA expression of PPARα and PPARγ as analyzed by RT-PCR. As positive controls for PPAR mRNA expression, cDNA from mouse adipocyte tissue or from liver was used. B: Western blot analysis of PPARγ protein expression in DCs (5×10^6cells/lane). Western blots were probed with affinity-purified rabbit anti-PPARγ Abs at 2 μg/ml. The identity of the 55 kDa band is confirmed by co-migration with a band seen in in vitro produced PPARγ protein but not in the control in vitro translation products. The affinity-purified rabbit Abs used as a negative control did not reveal any reactivity (not shown). Sizes are indicated in kDa. C: Immunocytochemical analysis of PPARγ expression in DCs. Cultured DCs were stained with 10 μg/ml of the affinity-purified anti-PPARγ Abs or an affinity-purified rabbit Ab specific for an irrelevant parasite antigen (control) (magnification, ×900).

ence of PPARγ, but not PPARα, mRNA in immature as well as in mature DCs (99% pure) (Fig. 1A). Western blot analysis using specific Abs for PPARγ confirmed the presence of PPARγ protein in mouse DCs (Fig. 1B). The identity of the detected 55 kDa band was confirmed by co-migration with a band from in vitro produced PPARγ protein. We noted that PPARγ expression was more pronounced in immature compared to mature DCs. By contrast, consistent with RT-PCR analysis, PPARα protein was undetectable in DCs (not shown). To identify the subcellular location of PPARγ in DCs, indirect immunofluorescence analysis was performed. Staining with anti-PPARγ Abs revealed immunoreactivity in the cytoplasmic compartment at the periphery of the nucleus, but not in the dendrites (Fig. 1C).

3.2. PPARγ activation has no effect on the maturation of DCs in vitro

We then assessed the effect of PPARγ activation on the expression of cell surface molecules involved in APC function on DCs undergoing maturation. To this end, immature DCs were cultured for 18 h (the period necessary for spontaneous in vitro DC maturation) in the presence of 10 μM of the highly potent synthetic PPARγ activator RSG. First, we checked that RSG did not induce cell death as already described in the literature for numerous other cell types [19,21,24,36,37]. Enumeration of cells excluding trypan blue revealed that 90–95% of DCs were alive after a 18 h culture in the presence or absence of RSG. DC staining with FITC-Annexin V and propidium iodide confirmed that DCs did not undergo apoptosis after RSG treatment (data not shown). Afterwards, the expression of cell surface molecules was monitored. As shown in Fig. 2, the overnight culture of immature DCs induced their maturation characterized by an increased expression of MHC class II molecules as well as the costimulatory molecules CD80, CD86, CD40 and CD54. Treatment of immature DCs with RSG (10 μM) for 18 h did not modify the phenotypic features of mature DCs. This suggests that activation of PPARγ does not interfere with the maturation of DCs in vitro nor alter the synthesis of surface molecules known to participate in T cell activation and differentiation.

3.3. PPARγ activation does not affect the accessory function of DCs in vivo

We then evaluated the effect of RSG on the ability of DCs pulsed in vitro with antigens to sensitize T cells in vivo. To this end, DCs were pulsed with KLH during overnight culture as previously reported [35] in the presence or absence of RSG. After washing, DCs were injected into the hind footpads of syngeneic mice and the popliteal LNs were harvested 5 days later and restimulated in vitro with KLH. The data in Fig. 3

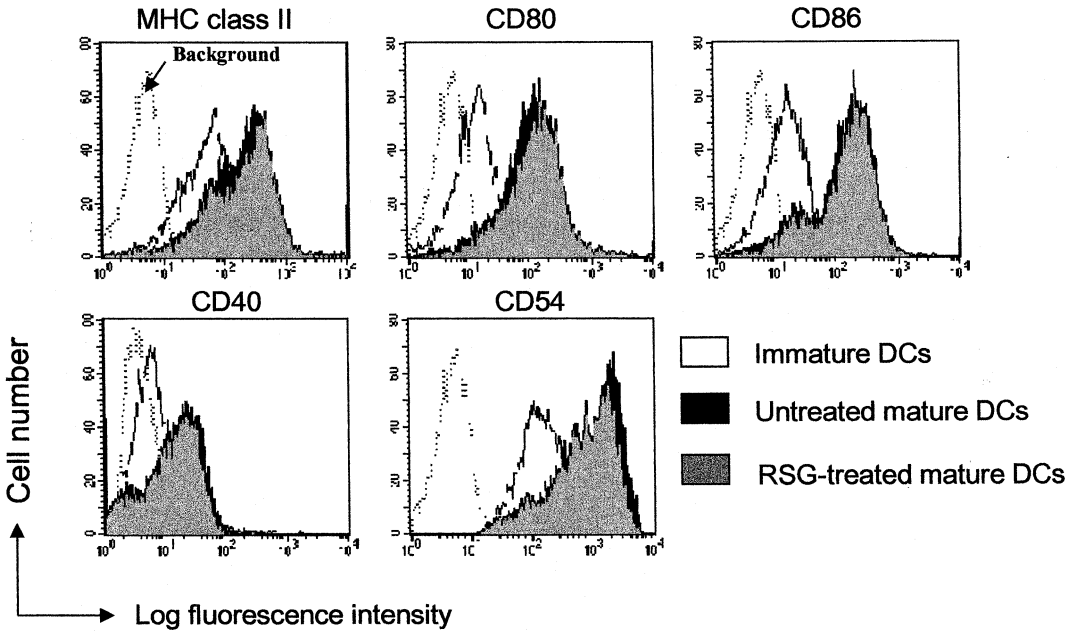

Fig. 2. Effects of the PPARγ activator RSG on the surface expression of MHC class II, CD80, CD86, CD40 and CD54 on DCs undergoing maturation. Immature DCs were cultured in the presence or absence of RSG. After 18 h, untreated mature DCs and RSG-treated mature DCs were stained with the indicated Abs and analyzed by flow cytometry. The background represents DCs stained with the secondary Ab alone (MHC class II) or FITC-SA (CD54) or unstained DCs (CD80, CD86 and CD40). One representative experiment out of seven is shown.

indicate that administration of DCs pulsed ex vivo with KLH resulted in T cell priming, as assessed by KLH-dependent proliferation in culture. The proliferative response of LN cells from mice injected with RSG-treated KLH-pulsed DCs was identical suggesting that the T cell response was not altered. We next analyzed the cytokines released by LN cells primed with the different DC preparations in vivo. KLH-pulsed DCs induced the activation of cells secreting IFNγ, IL-4, IL-5 and IL-10. Similarly, RSG-treated KLH-pulsed DCs sensitized cells produced equal quantities of IFNγ, IL-4, IL-5 and IL-10. This suggests that PPARγ activation during the pulse step does not affect the APC function of DCs.

3.4. PPARγ activators reduce the CD40-induced secretion of the Th1-promoting factor IL-12 in DCs

To further study the role of PPARγ in DC function, we

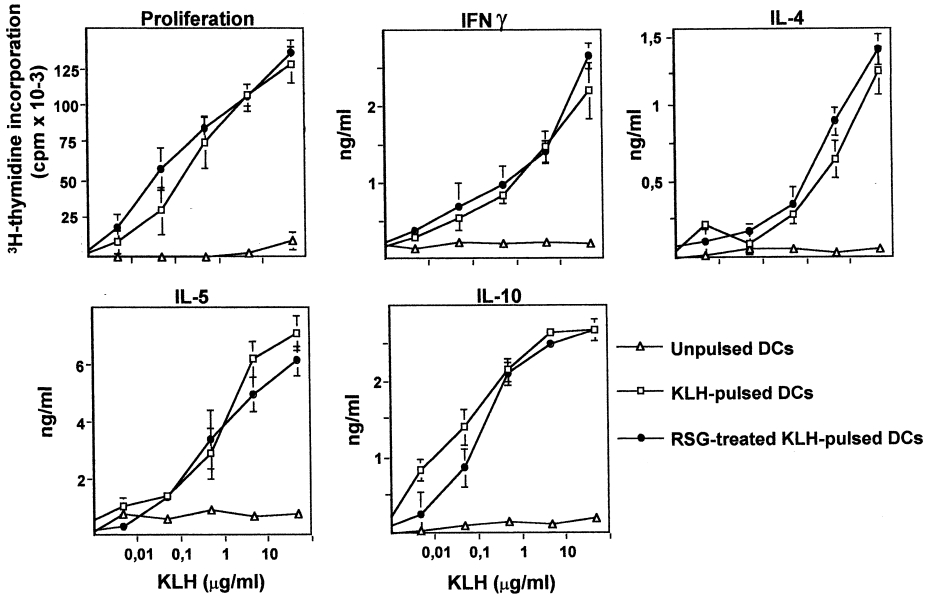

Fig. 3. Effect of RSG on the ability of KLH-pulsed DCs to prime T lymphocytes in vivo. Immature DCs were pulsed or not (unpulsed DCs) extracorporally with 50 μg/ml of KLH in the absence (KLH-pulsed DCs) or the presence (RSG-treated KLH-pulsed DCs) of RSG for 18 h. Then, DCs were injected into the hind footpads of naive mice and 5 days later, draining LNs were harvested and cultured with various doses of KLH. Cytokine production and proliferation were measured after 3 and 4 days of culture, respectively. Results represent the mean of triplicate cultures ± S.D. One representative experiment out of five is shown.

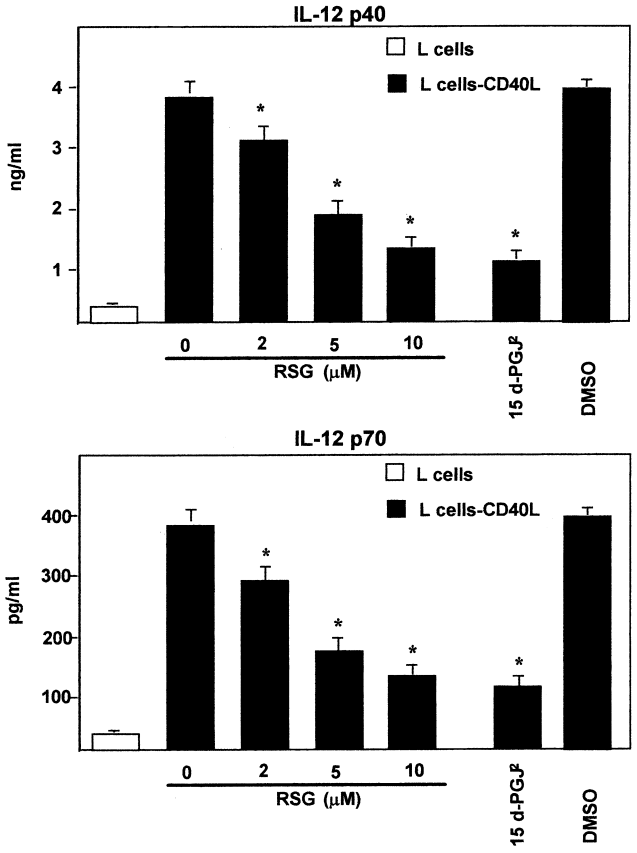

Fig. 4. Effect of PPARγ activators on the secretion of IL-12 p40 and IL-12 p70 by CD40L-stimulated murine DCs. DCs were cultured with L cells expressing or not CD40L for 24 h in the absence or presence of increasing concentrations of RSG or with 15d-PGJ_2 (final concentration: 5 μM) or vehicle (DMSO). Cell viability was not altered by the indicated treatments (data not shown). Results are expressed as means ± S.D. of triplicates and are representative of five independent experiments. Data were statistically analyzed using the Student's *t* test and considered to be significant when $P < 0.05$ (compared to medium or to vehicle). Significant differences are designated by *.

stimulated DCs to produce IL-12 in a CD40L-dependent manner, a situation that occurs during the interactions between DCs and T lymphocytes. To this end, immature DCs were incubated for 24 h with transfected cells expressing CD40L in the presence or not of RSG and the production of IL-12 was measured. Compared to DCs cultured with untransfected L-cells, CD40 ligation dramatically increased the concentration of IL-12 p40 and of the bioactive heterodimer IL-12 p70 in the DC culture supernatants (Fig. 4). Interestingly, this induction was reduced, in a concentration-dependent manner, by RSG. The maximal inhibitory activity was achieved with RSG at 10 μM (72 ± 4% inhibition). In the same manner, the CD40-induced synthesis of IL-12 by DCs was reduced in the presence of the naturally occurring PPARγ activator 15d-PGJ_2 (80 ± 3% at 5 μM). Examination of IL-12 p40 mRNA levels in DCs by RT-PCR indicated that the inhibitory effects of RSG on IL-12 synthesis probably occur at the transcriptional level (not shown). These findings suggest that activation of PPARγ in DCs may negatively regulate expression of genes that are important in immune responses.

4. Discussion

Recent reports suggest that PPARγ may play a role in the control of the inflammatory response, particularly by affecting macrophage function [5,6,17–20]. For instance, PPARγ activation reduces the production of certain inflammatory mediators and cytokines in activated macrophages in response to a variety of exogenous agents. This novel function for PPARγ in modulating inflammation prompted us to investigate the possibility that PPARγ may be expressed and may regulate gene expression in DCs, the most potent professional APCs. Indeed, DCs have the unique capacity to prime and to educate naive T lymphocytes. In these mechanisms, inflammatory mediators and/or cytokines play a key role in initiating and polarizing the immune response.

In this report, we show for the first time that PPARγ, but not PPARα, is expressed in murine DCs. Indeed, in freshly isolated spleen-derived DCs (immature) as well as in mature DCs, we found relatively high amounts of PPARγ principally located in the cytoplasm around the nucleus. We also confirmed this finding in the bone marrow-derived mouse DC line DC2.4 (not shown) as well as in human monocyte-derived DCs (P. Gosset, in preparation). Interestingly, this preferential cytoplasmic location in DCs differs from that of PPARγ in macrophages since, in these cells, it is located in the nucleus [19,20]. On the other hand, in adipocyte [38] and in endothelial cells [36] PPARγ is located principally in the cytoplasm.

We then assessed the effects of PPARγ activation on DC viability, maturation and function. Treatment of immature DCs with the synthetic pharmacological compound RSG, an anti-diabetic drug with high affinity for PPARγ, for 18–24 h did not affect DC viability, unlike other cell types such as macrophages [19], endothelial cells [36], astrocytes [37] and B and T lymphocytes [21,24] where PPARγ agonists have been shown to induce non-apoptotic or apoptotic cell death. Since maturation is an essential process during which DCs acquire optimal immunostimulatory properties, we examined the influence of PPARγ activators on the expression of a number of cell surface molecules known to be upregulated during maturation. Our data show that DCs that have undergone maturation in vitro in the presence of RSG express the same levels of MHC class II, CD80, CD86, CD40 and CD54 molecules compared to untreated mature DCs. Similarly, we found that RSG does not modify the expression of costimulatory molecules on CD40-activated murine DCs (not shown). We then evaluated the influence of PPARγ activation on the ability of antigen-pulsed DCs to sensitize T cells in vivo. We found that treatment of immature DCs with RSG did not influence the antigen-specific T cell response in terms of proliferation and cytokine production. This suggests that PPARγ activation in DCs undergoing maturation does not interfere with the steps of antigen capture, processing and presentation to naive T lymphocytes. We then studied whether PPARγ activation may interfere with the CD40-mediated IL-12 induction in DCs. This mode of stimulation occurs in the LNs during DC/T cell interactions and the subsequent IL-12 synthesis by DCs is particularly important in the polarization of the acquired immune response [31,32,34]. Our data show that PPARγ activation by RSG or by the endogenous ligand 15d-PGJ_2, a PGD_2 metabolite, decreases the production of IL-12 p40 and p70 in DCs stimulated via CD40/CD40L interaction.

It is therefore tempting to speculate that PPARγ activation in DCs, for instance by endogenous fatty acid derivatives, may play a role in the polarization of immune responses. At this stage, it is important to mention that in our model of immunization using pulsed DCs (Fig. 3), we could not test this hypothesis since the PPARγ agonist was added during the pulse step, but not during the antigen presentation step that occurs during DC/T cell contact. In vivo experiments are therefore warranted to test this assumption.

Although the role of certain prostanoids, particularly the cAMP-elevating factor PGE_2, in the negative control of IL-12 synthesis has already been demonstrated [39,40], our data suggest that other members of the polyunsaturated fatty acid family may also inhibit IL-12 production in DCs via cAMP-independent pathway(s). In this study, we did not explore the mechanism by which PPARγ down-modulates IL-12 synthesis in DCs. However, since the NF-κB is the major signaling pathway triggered by CD40–CD40L interaction [41] and since activation of PPARγ has already been shown to negatively interfere with the expression of a subset of genes by antagonizing the NF-κB pathway [5,6,42,43], it is likely that PPARγ activators inhibit IL-12 production in DCs by interfering with NF-κB.

In conclusion, our data indicate that the nuclear hormone receptor PPARγ is expressed in mouse DCs and that during immune responses, PPARγ activation by endogenous fatty acid-derived ligands may play a role in adaptive immunity by affecting the synthesis of the Th1-driving cytokine IL-12.

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