Peroxisome Proliferator-Activated Receptor \( \gamma \)
Control of Dendritic Cell Function
Contributes to Development of CD4\(^+\) T Cell Anergy


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Peroxisome Proliferator-Activated Receptor γ Control of Dendritic Cell Function Contributes to Development of CD4+ T Cell Anergy


There is increasing evidence that dendritic cell (DC) immunogenicity is not only positively regulated by ligands of pattern recognition receptors, but also negatively by signals that prevent DC activation and full functional maturation. Depending on their activation status, DCs can induce either immunity or tolerance. In this study, we provide molecular evidence that the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is a negative regulator of DC maturation and function. Sustained PPARγ activation in murine DCs reduced maturation-induced expression of costimulatory molecules and IL-12, and profoundly inhibited their capacity to prime naïve CD4+ T cells in vitro. Using PPARγ-deficient DCs, generated by Cre-mediated ablation of the PPARγ gene, agonist-mediated suppression of maturation-induced functional changes were abrogated. Moreover, absence of PPARγ increased DC immunogenicity, suggesting a constitutive regulatory function of PPARγ in DCs. Adoptive transfer of PPARγ-activated Ag-presenting DCs induced CD4+ T cell anergy, characterized by impaired differentiation resulting in absent Th1 and Th2 cytokine production and failure of secondary clonal expansion upon restimulation. Collectively, our data support the notion that PPARγ is an efficient regulator of DC immunogenicity that may be exploited to deliberately target CD4+ T cell-mediated immune responses. The Journal of Immunology, 2007, 178: 2122–2131.

Dendritic cells (DCs) are professional APCs with a unique capacity to stimulate naïve T cells (1). The functional properties of DCs are closely linked to their maturation status, because maturation-induced expression of costimulatory molecules and production of proinflammatory cytokines, in particular IL-12, are essential for DCs to induce strong T cell-mediated immunity (2, 3). Critical to the functional maturation of DCs is their activation through pattern-recognition receptors, e.g., TLRs that recognize conserved microbial structures (4). Induction of T cell-mediated immunity is characterized by massive clonal expansion and differentiation of naive T cells into cells with effector function, i.e., cytotoxicity for CD8+ T cells and helper function for CD4+ T cells. Under steady-state conditions, i.e., in the absence of inflammation, immature DCs take up tissue Ags, migrate to draining lymph nodes, and present Ag in the absence of proper costimulation and IL-12 secretion, thereby inducing specific immune tolerance toward tissue Ags (3, 5). Several mediators contribute to the maintenance of tolerance by negative interference with DC maturation, such as IL-10 or TGF-β derived from regulatory T cells, as well as DC-derived mediators like prostanoids (6, 7). However, the molecular mechanisms responsible for the maintenance of a tolerogenic state in DCs are not fully understood.

The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor that has been shown to mediate anti-inflammatory effects on several immune cell types (8, 9). Upon ligand binding, PPARγ heterodimerizes with the retinoid X receptor and binds to PPAR response elements located in the promoter region of target genes. Additionally, important anti-inflammatory effects of PPARγ are mediated by negative interference with proinflammatory cell signaling, either via competition for coactivators or via transrepression through physical interaction with proinflammatory transcription factors, in particular NF-κB (9, 10). PPARγ agonists include endogenous ligands such as polyunsaturated fatty acids and prostanoids like the PGD2 metabolite 15-deoxy-Δ12,14-prostaglandin J2, as well as several synthetic ligands such as the antidiabetic thiazolidinediones, e.g., pioglitazone (PIO) and rosiglitazone, which are currently being used for the correction of metabolic disturbances in type II diabetes (13). In monocytes and macrophages, PPARγ agonists inhibit the expression of a number of proinflammatory cytokines (8, 9), and attenuate the oxidative burst (11). In T lymphocytes, PPARγ activation inhibits both Ag-specific and nonspecific T cell proliferation and the production of several proinflammatory cytokines (12, 14). Recently, it has been shown that PPARγ agonists prevent expression of IL-12 in murine DCs (15) and affect maturation of human monocyte-derived DCs as evidenced by altered surface expression levels of costimulatory molecules (16).

However, because PPARγ knockout (k.o.) is embryonally lethal, formal evidence for involvement of PPARγ in immune control mechanisms is lacking (17). Moreover, several reports raised...
the question of whether the anti-inflammatory effects of PPARγ agonists are really receptor-mediated; for instance, it has been shown that at least some effects of both natural and synthetic PPARγ agonists are not due to activation of the receptor (18, 19). Using a conditional k.o. strategy, we in this study provide direct molecular evidence that PPARγ controls DC function both in the steady-state situation and under inflammatory conditions. The consequence of PPARγ activation in DCs is the conservation of a functionally immature state, which is associated with failure to promote activation of naive CD4+ T cells and subsequent clonal expansion and differentiation into effector cells.

Materials and Methods

Mice

Female BALB/c mice and C57BL/6 mice (6–10 wk old) were purchased from Charles River Laboratories. MHC-class II-restricted TCR-transgenic mice OT II (H-2b background) and DO11.10 (H-2d background), both specific for OVA peptide (323–339), as well as PPARγfl/fl mice (20) (purchased from The Jackson Laboratory) were bred in our animal facility. All experiments were performed according to guidelines of the animal ethics committee.

Oral treatment with PIO

Seven days before splenic DC isolation, mice were treated with 30 mg/kg body weight per day PIO (Acros; Takeda Pharmaceuticals) suspended in 2% carboxymethylcellulose by daily oral gavage. Control DCs were isolated from mice fed for 3 days. At day 3, Cre transduction with 2% paraformaldehyde, permeabilized with 0.5% triton, and stained with Alexa647-labeled OVA (MWG Biotec). Purity of isolated splenic DCs from OT-II mice and CD11c+ DCs in vitro coculture assays

Splenic CD4+ T cells from OT-II mice and CD11c+ DCs from C57BL/6 mice were isolated by immunomagnetic separation using CD4+ and CD11c+ MACS microbeads (Miltenyi Biotec). Purity of isolated cells was controlled by FACS staining of CD4, and CD11c, respectively. CD4+ T cells were cocultured with either CD11c+ splenic DCs or BMDCs in triplicates for 5 days in RPMI 1640 medium (Invitrogen Life Technologies) containing 8% FCS, 1% glutamine, and antibiotics. After medium change at day 3, cells were trypsinized at day 4, resuspended, and plated at a density of 5 × 10^5 until day 7. A total of 10 μM PIO (Axxorra) was added to the culture during the whole differentiation procedure from day 0 to 7. Percentage of CD11c+ DCs at day 7, routinely >90%, was not altered by treatment with PIO. For maturation, BMDCs were stimulated with 100 ng/ml LPS for 20 h at day 7.

Isolation of splenic T cells and splenic DCs and in vitro coculture assays

Splenic DCs were isolated by immunomagnetic separation using CD4+ and CD11c+ MACS microbeads (Miltenyi Biotec). Purity of isolated cells was controlled by FACS staining of CD4, and CD11c, respectively. CD4+ T cells were cocultured with either CD11c+ splenic DCs or BMDCs in triplicates for 5 days in RPMI 1640 medium (Invitrogen Life Technologies) containing 8% FCS, 1% glutamine, and antibiotics. OVA (Serva) was added at a concentration of 0.5 mg/ml. For restimulation experiments, CD4+ T cells were separated by density centrifugation using lymphoprep (PAA), washed twice, and cultured in triplicates for another 20 h in the presence of anti-CD3 Ab (clone 145-2C11) coated on 96-well plates. For titration assays, fixed amounts of naive OVA-specific CD4+ T cells were cocultured with BMDCs at ratios of 2:1 to 10:1 (T cells:BMDCs) in triplicates on 96-well plates in the presence of Ag. For analysis of T cell proliferation in vitro, naive freshly isolated CD4+ T cells were labeled for 15 min with 1 μM CFSE, washed twice with PBS, and cocultured with BMDCs in 96-well plates for 5 days in the presence of OVA; proliferation was assessed by flow cytometric analysis of CFSE dilution in CD4+ cells.

Conditional k.o. of PPARγ in BMDCs

Conditional k.o. of PPARγ in vitro was achieved by site-specific recombinase of loxP sites integrated into the PPARγ locus of PPARγfl/fl mice using a membrane-permeable Cre recombinase (HTNCre). Recombinant HTNCre protein was purified from Escherichia coli as described previously (21). Bone marrow precursor cells were isolated from PPARγfl/fl mice or C57BL/6 mice as described above, and incubated with 3 μM HTNCre in IMDM containing 1% FCS for 6 h. Afterward, cells were washed twice and cultured in 100-mm diameter petri dishes at a density of 5 × 10^5 in IMDM supplemented with GM-CSF-containing supernatant for 3 days. At day 3, Cre transduction with 3 μM HTNCre was performed again for 6 h, and cells were cultured for another 4 days in IMDM plus GM-CSF as done before. A total of 10 μM PIO was added to the culture during the whole differentiation procedure from day 0 to 7. Treatment of BMDCs with HTNCre did not induce significant cell death as assessed by flow cytometric analysis of 7-aminoactinomycin D (7-AAD) incorporation. Additionally, percentage of CD11c+ DCs at day 7 as well as DC phenotype (baseline levels of CD40, CD80, CD86) were not altered by Cre recombination.

In vivo experiments

Naive CD4+ T cells from DO11.10 mice were isolated by immunomagnetic separation, labeled with 1 μM CFSE for 5 min, and injected into the lateral tail vein of BALB/c mice (5 × 10^5 viable cells per animal) at day 1. At day 0, mice received a s.c. immunization with 1 × 10^6 OVA 323–339-loaded CD11c+ splenic DCs from animals treated for 7 days with PIO or vehicle. At day 5, draining lymph nodes and spleens were isolated; proliferation of CD4+ T cells was assessed by flow cytometric analysis of CFSE profiles. For ex vivo restimulation experiments, cells (200,000 cells per 96-well in triplicates) were cultivated in the presence of 5 μM OVA 323–339 (MWG Biotech). Supernatants were analyzed by ELISA after 20 h for the amount of IL-2 and IFN-γ and after 72 h for the amount of IL-4 and IL-10. For recall assays, spleen cells from immunized mice were restimulated for 7 days with OVA (0.5 mg/ml); at day 4, cells were separated by density centrifugation, and cultured for another 3 days in the presence of IL-2 (2 ng/ml). At day 7, equal cell numbers were restimulated with OVA 323–339-loaded CD19+ syngeneic B cells for 20 h; afterward, supernatants were analyzed for cytokine content. Flow cytometric analysis of regulatory T cells was performed directly ex vivo, at day 3 after Ag-specific restimulation, and at day 7 after recall stimulation. For analysis of apoptosis, lymph node cells and spleen cells were cultured for 1 h in the presence of the FLICA reagent FAM-DEVD-FMK, which specifically and irreversibly binds to active caspases 3 and 7 (Axxorra). After extensive washing, cells were stained for CD4 and the Ag-specific TCR KJ1-26; unspecific cell death was assessed by additional staining with 7-AAD.

Cytokine ELISA

Supernatants were collected at the indicated time points. Quantitative ELISA for IL-2, IL-4, IL-10, and IFN-γ were performed using commercially available Abs (BD Biosciences) according to the manufacturer’s recommendations.

Flow cytometry

Single-cell suspensions were stained with fluorescently conjugated Abs to CD11c, CD40, CD80, and CD86 for DCs, and to CD4, CD25, and KJ1-26 for T cells (all obtained from BD Biosciences). Appropriate isotype controls were always included. Cell death was measured by 7-AAD incorporation. Ag uptake was assessed by quantification of Alexa488-labeled OVA in CD11c+ cells after 10-min incubation. For intracellular staining of BMDCs, cells were stained for 5 h with 100 ng/ml LPS in the presence of Golgi Plug and Golgi Stop (BD Biosciences). After surface staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton, and stained with an Ab against FoxP3 (eBioscience). Cells were measured using a FACSCalibur (BD Biosciences) and results were analyzed using FlowJo software (Tree Star).

RNA extraction and RT-PCR

Total RNA was extracted from BMDCs at day 7 after HNTCre treatment and subsequent differentiation by using TRIzol (Invitrogen Life Technologies) according to standard protocols. Reverse transcription was performed with SuperScript (Invitrogen Life Technologies). Sense (5’-GTCAGCGGTTCTGAGAAGGATTG-3’) and antisense (5’-TATCAGGAGATCTCCGGAACAGC-3’) primers were designed to anneal to regions in exons A1 and 4 of PPARγ, respectively, which detect both full-length (700 bp) and recombinant (300 bp) transcripts (20). PCR was performed by 40 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 60 s.

Nuclear protein extraction

Cells were washed with PBS, centrifuged at 2,000 × g for 5 min, washed in 1 ml of buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.1% Nonidet P-40, and 0.5 mM DTT), and pelleted at 2,000 × g for 5 min. Pellets were resuspended in 80 μl of buffer A containing 0.1% Triton.
X-100, incubated at 4°C for 10 min, and centrifuged at 2,000 × g for 5 min. Afterward, the nuclear pellet was resuspended in 60 μl of 20 mM HEPES (pH 7.9), 0.42 M NaCl, 25% (v/v) glycerol, 1.5 mM MgCl₂, and 0.2 mM EDTA, incubated on ice for 30 min, and centrifuged at 15,000 × g for 20 min at 4°C. Extracts were stored at −80°C until use.

**EMSA**

For detection of NF-κB DNA binding, the following sequence was used:

5' - AGT TGA GGG GAC TTT CCC AGG C - 3'

and

3' - TCA ACT CCC CTG AAA GGG TCC G - 5'

Oligonucleotides were annealed in 10 mM Tris (pH 8.0) at 65°C for 1 min and slow cooling at room temperature. Double-stranded oligonucleotides (10 pmol) were 5' - end labeled with [γ-³²P]ATP (5000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs), purified using a G25 quick spin column (Roche Molecular Biochemicals), and diluted to a final volume of 150 μl. Experiments were performed using the Gel Shift Assay System from Promega: nuclear extracts (10 μg per reaction) were preincubated in binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.5 mg/ml poly(dI:dC) for 1 h on ice. For supershift and competition assays, 3 μl of Ab (polyclonal anti-p50-Ab, sc-8414X, and anti-p65-Ab, sc-372X, from Santa Cruz Biochemicals) or an excess of unlabeled double-stranded oligonucleotides were added. After addition of 1 μl of labeled probe to each reaction and 20-min incubation on ice, DNA protein complexes were separated on 8% native polyacrylamide gels at 220 V with 0.5 Tris-buffered EDTA as running buffer. Gels were vacuum-dried and visualized by autoradiography.

**FIGURE 1.** PIO impairs Ag-specific CD4⁺ T cell priming by DCs in vitro. BMDCs were generated in the presence (BMDC + Pio) or absence (BMDC) of 10 μM PIO for 7 days (a and c). Alternatively, splenic CD11c⁺ DCs were isolated from animals treated p.o. for 7 days with PIO (splenic DC + Pio) or vehicle (splenic DC) (b and d). Afterward, DCs were cocultured with naïve OVA-specific CD4⁺ T cells in the presence of OVA for 5 days. Coclulture in the absence of Ag served as control. a and b, Cytokine levels (IL-2 and IFN-γ) at day 3 of coculture. c and d, Time course of IL-2 secretion during 5 days of coculture of DCs and CD4⁺ T cells. Cytokine content in supernatants was measured in triplicates by ELISA; *, cytokine secretion compared with DC (*, p ≤ 0.05 and ***, p ≤ 0.01, Student’s t test).

**FIGURE 2.** CD4⁺ T cells primed by PIO-treated DCs exhibit impaired effector function upon restimulation. Cytokine responses upon anti-CD3 restimulation of OVA-specific CD4⁺ T cells that have been cocultured for 5 days either with PIO-treated or untreated BMDCs (a) or with splenic DCs from PIO-treated or mock-treated animals (b). Restimulation of T cells cocultured for 5 days with DCs in the absence of Ag served as control. Cytokine content in supernatants was measured in triplicates by ELISA; *, cytokine secretion compared with T cells primed by DCs (*, p ≤ 0.05 and ***, p ≤ 0.01, Student’s t test).
After appropriate stimulation cells were pelleted, lysed in SDS-sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 0.1% bromphenolblue, 20% glycerol, 200 mM DTT), boiled, and sonicated. Lysates were then subjected to 10% SDS-PAGE followed by Western blotting. Proteins were detected with rabbit anti-phospho p44/42 Ab (Cell Signaling Technology), mouse anti-Hsc70 Ab (Stressgen), and HRP-conjugated anti-rabbit and anti-mouse secondary Abs.

**PAGE and Western blot analysis**

**FIGURE 3.** PPARγ k.o. abrogates PIO-induced changes in BMDC phenotype. BMDCs were generated in the presence or absence of 10 μM PIO. a, At day 7, cells were stained for CD11c and 7-AAD and the percentage of dead cells was determined by flow cytometry (black line, BMDC; gray shadow, BMDC + PIO). b–e, PPARγ-k.o. BMDCs were generated by treatment of bone-marrow precursor cells from PPARγfl/fl mice with a cell-permeant Cre recombinase (HTNCre) and subsequent differentiation in GM-CSF containing medium for 7 days in the presence or absence of 10 μM PIO. As wt controls, either PPARγwt bone marrow was left untreated or C57BL/6 (B6)-derived bone marrow was treated with HTNCre. b, Cre-mediated recombination was determined by RT-PCR of wt (700 bp) and deleted (300 bp) PPARγ transcripts. c, At day 7, BMDCs were incubated with 10 μg/ml Alexa647-labeled OVA for 10 min and Ag-uptake by CD11c+ cells was determined by flow cytometry. d, Surface staining of CD40, CD80, and CD86 on CD11c+ LPS-maturated BMDCs within a 7-AAD-negative life gate. Relative expression levels of CD40, CD80, and CD86 are shown as percentage of expression levels on matured wt BMDCs without PIO. e, IL-12 production of CD11c+ 7-AAD-negative BMDCs was assessed after 5-h LPS stimulation; IL-10 production was assessed after 20-h LPS-stimulation by flow cytometry; percentage of cytokine-producing cells is indicated.
Statistical analysis
Statistical analysis was performed using unpaired Student’s t-test; p values < 0.05 were considered significant. Cell division index, which indicates the average number of cell divisions undergone by the responding T cells, was calculated from CFSE<sup>+</sup> cells using FlowJo software according to the manufacturer’s protocol.

Results
PIO impairs Ag-specific CD4<sup>+</sup> T cell priming by DCs in vitro
We used the PPARγ agonist PIO to investigate the effect of PPARγ on the capacity of DCs to prime naive Ag-specific CD4<sup>+</sup> T cells. BMDCs were generated over 7 days in the presence or absence of PIO with or without PPARγ agonist. We evaluated the IL-12 and IL-10 production of BMDCs by ELISA. CFSE-labeled OVA-specific CD4<sup>+</sup> T cells were cocultured with BMDCs for 5 days in the presence of Ag, and proliferation profiles were analyzed by flow cytometry.
absence of PIO (10 μM), and subsequently cocultured with OVA-specific CD4⁺ T cells from TCR-transgenic animals (OT-II) in the presence or absence of OVA for 5 days (Fig. 1a). Ag-specific activation of naïve CD4⁺ T cells, as determined by measuring T cell release of IL-2 and IFN-γ in culture supernatants, was significantly impaired when T cells encountered their Ag on PIO-treated BMDCs. Direct effects of PIO on CD4⁺ T cells were excluded because only BMDCs were exposed to the PPARγ agonist. To investigate the effects of PPARγ activation on DCs in vivo, splenic DCs were isolated from mice that received PIO p.o. for 7 days at a dosage corresponding to the one used for antidiabetic treatment in humans (22). Similar to results obtained from BMDCs, PIO treatment of splenic DCs lead to strongly diminished OVA-specific T cell priming (Fig. 1b). Importantly, reduced CD4⁺ T cell cytokine production upon priming by PIO-treated DCs was due to delayed T cell responses (Fig. 1, c and d). Thus, PIO treatment of DCs both in vitro and in vivo strongly impairs Ag-specific priming of naïve CD4⁺ T cells.

**CD4⁺ T cells primed by PIO-treated DCs show reduced cytokine release upon restimulation**

To investigate the effect of priming by PIO-treated DCs for subsequent function of CD4⁺ T cells, we restimulated the T cells via activation of their TCR. Equal numbers of OVA-specific CD4⁺ T cells that were primed by PIO-treated BMDCs or splenic DCs over 5 days were restimulated with anti-CD3, and cytokine production was determined after 20 h. T cells that were primed by PIO-treated splenic DCs or BMDCs produced significantly less IL-2 and IFN-γ than T cells primed by untreated DCs (Fig. 2, a and b). This result suggests that naïve T cells primed by PIO-treated DCs did not undergo functional differentiation into Th1 cells but rather exhibit impaired effector functions upon restimulation.

**PPARγ is required for PIO-induced changes in DC phenotype**

A direct cytotoxic effect of PIO on BMDCs was excluded because we did not observe 7-AAD incorporation in PIO-treated vs untreated BMDCs by flow cytometric analysis (Fig. 3a). Similar results were obtained for splenic DCs from PIO-treated vs mock-treated animals (data not shown). Because the functional effects of PPARγ agonists on DCs may be caused by receptor-independent mechanisms, we next analyzed whether PIO-induced changes in DC function are indeed mediated by activation of PPARγ. To directly demonstrate involvement of PPARγ in the functional modulation of DCs, we generated PPARγ k.o. BMDCs by means of site-specific recombination using a cell-permeant Cre recombinase in vitro. Ablation of PPARγ was achieved by treatment of BMDCs from PPARγ+/− mice with HTNCre, resulting in deletion of exon 1 and 2 of the PPARγ gene (21). BMDCs from PPARγ+/− mice without HTNCre treatment as well as BMDCs from C57BL/6 mice treated with HTNCre served as wild-type (wt) controls in all experiments. Recombination was detected by RT-PCR of PPARγ transcripts from wt and k.o. BMDCs (700 bp and 300 bp, respectively; Fig. 3b). We next investigated DC functions relevant for Ag-specific T cell activation; i.e., Ag-uptake, costimulation, and cytokine production. Surprisingly, uptake of fluorescently labeled Ag-specific T cell activation; i.e., Ag-uptake, costimulation, and cytokine production. Surprisingly, uptake of fluorescently labeled OVA was significantly increased in PIO-treated BMDCs (Fig. 3c). However, this effect was equally pronounced in PPARγ wt and k.o. BMDCs and therefore does not represent a specific PPARγ-mediated property of PIO. The costimulatory molecules CD40, CD80, and CD86 were equally expressed in LPS-maturated k.o. compared with wt BMDCs in the absence of PIO. Interestingly, PIO treatment of wt BMDCs prevented maturation-induced up-regulation of the costimulatory molecules CD40, CD80, and CD86 (Fig. 3d). Additionally, the LPS-induced production of IL-12 was strongly suppressed by PIO treatment (Fig. 3e). These effects were not present in PPARγ k.o. BMDCs, indicating that PIO-induced phenotypic changes in BMDCs are mediated via activation of PPARγ. An induction in BMDC IL-10 production by PIO was not observed (Fig. 3e).

These results demonstrate that despite increased PIO-induced Ag-uptake, DC activation properties characterized by expression of costimulatory molecules and IL-12 production are negatively regulated by PPARγ activation.

**k.o. of PPARγ increases priming capacity of DCs**

We next addressed the question of whether PPARγ also regulates APC function in DCs. We therefore compared PPARγ-activated wt vs k.o. BMDCs in their ability to prime naïve CD4⁺ T cells (Fig. 4a). Interestingly, the priming capacity of PPARγ k.o. BMDCs was significantly increased compared with wt BMDCs, indicating that PPARγ is involved in the attenuation of DC-priming capacity (Fig. 4a). Moreover, PIO treatment of PPARγ-k.o. BMDCs did not significantly decrease their enhanced priming capacity, demonstrating that the functional effects of PIO on DCs are mediated via activation of PPARγ and not via receptor-independent effects. The respective T cell activation status obtained by priming with PIO-treated wt and k.o. BMDCs was conserved after
FIGURE 6. PPARγ activation in DCs prevents the induction of a sustained CD4+ T cell response in vivo. At day −1, 5 × 10^6 CFSE-labeled (a) or unlabeled (b–f) DO11.10 CD4+ T cells were injected i.v. into BALB/c mice. At day 0, these mice were immunized s.c. with 1 × 10^6 OVA-loaded splenic DC from mice treated with PIO or vehicle for 7 days; unimmunized mice served as controls. a. After 5 days, spleen cells were isolated and proliferation profile and cell division index (CDI) of CFSE+ KJ1-26+ T cells was determined by flow cytometry. b. Five days after DC immunization, spleen cells were isolated and equal cell numbers were restimulated with 10 μM OVA323–339 peptide. IL-2 and IFN-γ content as well as IL-4 and IL-10 content in culture supernatants were determined by ELISA after 20 and 72 h, respectively. c. Spleen cells from immunized mice were cultured in the presence of 0.5 mg/ml OVA for 7 days. Afterward, CD4+ T cells were harvested and restimulated with splenic B cells loaded with OVA323–339 peptide (recall assay). After 20 h, IL-2 and IFN-γ contents in culture supernatants were determined by ELISA. d. After 7 days restimulation of splenic T cells in vitro, numbers of OVA-specific T cells were assessed by flow cytometric quantification of KJ1-26-positive T cells in total CD4+ T cells; graph shows relative increase in Ag-specific T cells compared with nonimmunized control animals. a–d. Values are depicted as mean ± SEM; significant differences compared with DC without PIO are indicated (*, p < 0.05; **, p < 0.01, Student’s t test). e. For analysis of regulatory T cells, spleen cells isolated 5 days after immunization were either stained ex vivo or first cultured in the presence of OVA for 3 days and afterward stained for CD4, KJ1-26, CD25, and intracellular FoxP3. Dot plots show CD25+, FoxP3+ cells in the CD4+ T cell population. The table depicts the percentage of CD25+FoxP3+ cells in total KJ1-26+ cells ex vivo, 3 days after culture with OVA (restim) and 2 days after secondary restimulation with splenic B cells loaded with OVA323–339 peptide (recall). f. For determination of apoptosis, spleen cells were labeled for active caspase 3 + 7 (FAM) for 1 h, and afterward stained for CD4, KJ1-26, and 7-AAD. Dot plots depict the percentage of early apoptotic (FAM single positive) and late apoptotic (FAM and 7-AAD double positive) cells in total CD4+ T cells 1 day after OVA-specific restimulation. The table depicts the percentage of FAM-positive cells in total KJ1-26+ cells ex vivo, 1 day after culture with OVA (restim) and 1 day after secondary restimulation with splenic B cells loaded with OVA323–339 peptide (recall).

PPARγ activation inhibits NF-κB DNA binding and MAPK signaling in DCs

NF-κB is known to play an important role in DC maturation by different stimuli; for example, it induces expression of costimulatory molecules and IL-12. We therefore investigated whether PPARγ activation in DCs impairs NF-κB DNA-binding activity as a putative molecular mechanism of PPARγ-mediated interference with DC immunogenicity. As depicted in Fig. 5a, untreated wt BMDCs showed a strong induction in NF-κB DNA-binding activity upon LPS stimulation, whereas PIO-treated DCs failed to up-regulate NF-κB DNA binding upon stimulation with LPS. Importantly, NF-κB DNA-binding activity in LPS-treated PPARγ-k.o. DCs was even more pronounced compared with wt BMDCs, and this increase in NF-κB DNA-binding activity was not abrogated by treatment of ko BMDCs with PIO, thus indicating that PIO-induced impairment of NF-κB DNA binding in DCs is dependent on PPARγ.
Another pathway involved in the regulation of DC immunogenicity is the MAPK pathway. We therefore analyzed the impact of PPARγ activation on MAPK signaling by determination of LPS-induced ERK1 phosphorylation (pp42) in PIO-treated PPARγ wt and k.o. BMDCs. As shown in Fig. 5b, LPS stimulation for 15 min led to a strong increase in phosphorylated ERK1 that was not observed in BMDCs pretreated with PIO. However, in PPARγ-k.o. BMDCs, no significant decrease in ERK1 phosphorylation state by PIO was observed, demonstrating that PIO reduces MAPK signaling in a PPARγ-dependent fashion. In conclusion, repression of MAPK signaling and DNA binding of NF-κB both represent molecular mechanisms of PPARγ-mediated impairment of DC maturation.

**PPARγ activation prevents the induction of a sustained CD4+ T cell response by DCs in vivo**

The data presented so far demonstrate a profound influence of PPARγ on the capacity of DCs to promote functional differentiation of naive CD4+ T cells in vitro. To investigate the role of PPARγ on DC function in vivo, we analyzed OVA-specific T cell priming in vivo as well as OVA-specific recall response ex vivo. Mice received CFSE-labeled, naive OVA-specific CD4+ T cells and were subsequently immunized either with OVA-peptide-loaded splenic DCs isolated from PIO-treated or mock-treated animals or left untreated. Proliferation of naive CD4+ T cells after 5 days was not altered in animals immunized with PIO-treated DCs or with mock-treated DCs, as indicated by identical division profiles as well as equal division indices of CFSE-labeled T cells (Fig. 6a). As expected, naive OVA-specific CD4+ T cells from nonimmunized mice did not proliferate. Correspondingly, the number of OVA-specific CD4+ T cells obtained from draining lymph nodes and spleens from mice 5 days after immunization with DCs from PIO-treated or mock-treated animals did not differ between both groups (data not shown). This indicates that PPARγ-activated DCs do not have a migration defect in vivo, as was previously suggested (23). The observation that identical CCR7 expression levels and similar migration profiles toward CCL21 were observed for PIO- and mock-treated DCs in vitro further strengthens this notion (data not shown). CD4+ T cells isolated from spleens (Fig. 6b) and lymph nodes (data not shown) of animals immunized with PIO-treated DCs, however, showed a profound decrease in IL-2 and IFN-γ secretion after OVA-specific restimulation ex vivo as compared with animals immunized with mock-treated DCs. Moreover, secretion of the Th2 cytokines IL-4 and IL-10 was significantly decreased upon restimulation of CD4+ T cells that had been primed by PIO-activated DCs in vivo (Fig. 6b).

To assess the influence of PIO-activated DCs on T cell recall responses, spleen cells isolated from immunized mice and control mice were cultured in the presence of OVA for 7 days. Afterward, cytokine production was investigated upon restimulation of equal cell numbers with OVA-peptide-loaded splenic B cells. CD4+ T cells obtained from mice immunized with PIO-treated DCs almost completely lost the ability to produce cytokines after TCR-mediated stimulation (Fig. 6c). The lack in cytokine production was accompanied by a lack in further clonal expansion of CD4+ T cells: whereas CD4+ T cells from animals immunized with mock-treated DCs expanded nearly 70-fold compared with control T cells from nonimmunized mice, OVA-specific T cells from mice immunized with PIO-treated DCs expanded only 5-fold after two rounds of restimulation (Fig. 6d). To assess whether the PIO-induced changes in the CD4+ T cell-priming capacity of DCs in vivo are mediated indirectly via induction of regulatory T cells, we assessed the amount of FoxP3+CD25+ OVA-specific CD4+ T cells after immunization with PIO-treated or untreated DCs (Fig. 6e). However, we did not observe an induction of Ag-specific regulatory T cells by PPARγ-activated DCs either directly ex vivo or after Ag-specific restimulation of lymph node cells or spleen cells in vitro. Another possible explanation for the observed impairment of sustained CD4+ T cell activation by PPARγ-activated DCs may be an increase in T cell apoptosis. Therefore, we assessed the amount of apoptotic Ag-specific T cells both directly after isolation of spleen cells and lymph node cells at several time points after Ag-specific restimulation in vitro using a specific marker for active caspase 3 and caspase 7 (Fig. 6f). However, an induction of apoptosis of OVA-specific T cells that have been primed by PIO-treated DCs was not observed.

These data suggest that PPARγ activation in DCs does not influence initial Ag-specific proliferation of naive CD4+ T cells in vivo but rather prevents T cell differentiation into Th1 and Th2 cells as well as secondary clonal expansion.

**Discussion**

The functional status of DCs determines the outcome of T cell-mediated Ag-specific immune responses. Although knowledge is accumulating rapidly on the mechanisms that lead to full DC maturation, e.g., by stimulation through membrane-bound or intracellular pattern recognition receptors, little is known on the molecular mechanisms that control such activation. In this study, we provide molecular evidence that PPARγ controls immune function of DCs by preventing full maturation in response to stimulation by the TLR4 ligand LPS. As a consequence of PPARγ-constrained DC function, we observed a reduced ability to both prime naive CD4+ T cells and support Th cell differentiation. CD4+ T cells primed by PPARγ-activated DCs failed to express Th1 and Th2 cytokines and did not respond to further TCR-mediated stimulation with secondary clonal expansion, which is characteristic of anergic CD4+ T cells.

PPARγ belongs to a family of nuclear receptors known to interfere with inflammatory cell signaling (24). PPARγ is expressed by different immune cells, such as macrophages, DCs, and T cells (9, 25). It is accepted that PPARγ is a negative regulator of macrophage activation (26, 27) and inhibits production of monocyte inflammatory cytokinones (8). PPARγ also controls helper T cell responses (28). Several studies investigated the effect of PPARγ agonists on the function of DCs. PPARγ agonists prevented LPS and CD40L-induced maturation of murine and human DCs (15, 16), and reduced DC priming of CD8+ T cells (29). However, both synthetic and natural PPARγ agonists do not exclusively act on PPARγ, but have additional PPARγ-independent effects, like direct inhibition of IκB kinase (18). So far, clear molecular evidence was missing for a direct involvement of PPARγ in control of DC function. In this study, we used a conditional k.o. technique using cell-permeable Cre recombinase to abolish PPARγ expression in DCs derived from transgenic animals that contain loxP sites integrated into the PPARγ gene locus. Our experiments unequivocally demonstrate that PPARγ inhibits functional maturation of BMDCs in response to LPS, and we could clearly link the failure of PIO-treated BMDCs to up-regulate costimulatory molecules and produce IL-12 to the activation of PPARγ. Moreover, the APC function of DCs is also controlled by PPARγ, because PPARγ activation strongly reduces the functional CD4+ T cell-priming capacity of DCs. The effects of PIO on DC priming were not observed in PPARγ k.o. DCs and therefore represent a specific, receptor-mediated property of this substance. Importantly, k.o. of PPARγ itself increased the ability of DCs to prime naive CD4+ T cells, which indicates that PPARγ also acts to control DC function under steady-state conditions. At present, it is unclear whether this regulatory role of PPARγ is due to an intrinsic “baseline” receptor.
activity that is ligand-independent, or is mediated by yet unknown endogenous PPARγ-ligands derived either from the DC itself or from interacting T cells. Interestingly, the increase in CD4+ T cell-priming capacity after k.o. of PPARγ was not accompanied by an increase in steady-state expression levels of the costimulatory molecules CD40, CD80, and CD86, or by enhanced IL-12 production, suggesting that PPARγ may additionally regulate DC expression of other costimulatory or perhaps co-inhibitory molecules. Ag uptake was strongly increased by PIO, as has already been described by others (30). However, this increase was not dependent on PPARγ, because PPARγ wt and k.o. BMDCs exhibit equal Ag uptake both in the presence or absence of PIO.

A control of DC function is important to regulate Ag-specific immune responses. Several molecular mechanisms involving interference with signal transduction pathways have been characterized that prevent full activation of APCs in response to TLR-mediated signals (31, 32). We demonstrate in this study that PPARγ interferes with LPS-induced NF-κB DNA-binding activity in DCs, which is known to be important for full functional maturation (33, 34). Additionally, we show that PPARγ activation decreased LPS-induced phosphorylation of ERK1, thus interfering with MAPK signaling. This is in line with observations made by Appel et al. (35), who showed that the PPARγ agonist troglitazone interfered with TLR ligand-induced MAPK signaling in human DCs resulting in reduced DC activation. Thus, it appears that the immunostimulatory capacity of DCs is stringently controlled by regulatory mechanisms that involve signaling via PPARγ.

Given the potent activity of PPARγ on DC function in vitro, we investigated whether this is also true for the in vivo situation. Analysis of CD4+ T cell priming in vivo revealed that Ag-specific CD4+ T cell proliferation was induced to the same extent both by PPARγ-activated and untreated DCs. However, CD4+ T cells primed by PPARγ-activated DCs exhibited impaired effector function, and secondary clonal expansion was severely blunted upon restimulation. It has already been shown that initial proliferation of naïve CD8+ T cells can occur in the absence of APC-derived signals that are essential for full T cell activation and differentiation (36). A lack of sufficient IL-2 signaling during the priming of CD8+ T cells is associated with failure to differentiate into effector cells and to undergo secondary clonal expansion (37). Accordingly, proliferation of CD4+ T cells primed by PPARγ-activated DCs was not impaired. However, low IL-2 production by these T cells during priming suggests that the observed lack of differentiation and clonal expansion may be due to insufficient IL-2 signaling. We therefore propose that PPARγ controls DC function in a complex manner that permits survival of Ag-reactive CD4+ T cells, but induces CD4+ T cell anergy instead of immunity upon secondary Ag encounter. The observed lack in both Th1 and Th2 cytokines produced by CD4+ T cells that were primed by PPARγ-activated DCs in vivo is in contrast to a switch toward Th2 responses by PPARγ-activated DCs observed by Gosset et al. (16). However, Gosset et al. (16) used shorter treatment periods with PPARγ agonists, probably resulting in less pronounced control of DC function.

Interestingly, it has been shown that agonists of the vitamin D receptor, another member of the steroid hormone receptor superfamily, also inhibit maturation and Ag-presenting capacity of DCs in a NF-κB-dependent fashion (38, 39). However, this process involves induction of IL-10 secretion by DCs that in turn proved to be essential for induction of regulatory T cells (40). In contrast to these findings, we did not observe an increase in IL-10 production by PPARγ-activated DCs. Correspondingly, we did not find an increase in regulatory T cells upon encounter with PPARγ-activated DCs in vivo. Furthermore, it has been shown that vitamin D receptor-treated DCs are capable of selective induction of T cell apoptosis (41). Increased cell death could also contribute to the observed lack in effector function in CD4+ T cells primed by PPARγ-activated DCs. We did not, however, observe increased T cell apoptosis in vitro (data not shown) or ex vivo, therefore excluding this possibility. These observations demonstrate that despite a close relationship between these receptor families and similar net effects on the immunostimulatory capacity of DCs, the underlying mechanisms differ considerably.

If PPARγ activity dominates DC function, it is difficult to envisage how sufficient immunity to infections arises. However, patients taking PPARγ agonists for metabolic control of type II diabetes are not immune-suppressed. One possible explanation for inflammation-induced reversal of PPARγ-mediated effects on DCs is that proinflammatory mediators like IFN-γ and TNF-α are able to reduce PPARγ protein levels both by increased protosomal degradation and by reduced gene expression (42, 43). It will be important to determine in future experiments, which molecular mechanisms override PPARγ-mediated control of DC function in vivo and thus contribute to the induction of immunity in response to infection.

Collectively, our data provide clear molecular evidence that PPARγ controls DC function in response to proinflammatory stimuli, but also under steady-state conditions. PPARγ activation restrains costimulatory signals in DCs resulting in inhibition of naive CD4+ T cells to undergo differentiation and subsequent clonal expansion. The elucidation of the molecular mechanisms controlling DC function will be crucial for the development of treatment strategies, on the one hand to improve tolerogenic DC function for treatment of autoimmune diseases, and on the other hand for the targeted disinhibition of DC function to improve vaccine strategies.

Disclosures
The authors have no financial conflict of interest.

References


