Peroxisome Proliferator-Activated Receptor-α Activation Inhibits Langerhans Cell Function[1]

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Epidermal Langerhans cells (LC) play a pivotal role in initiating and maintaining primary immune responses in the skin. In the present study, we asked whether peroxisome proliferator-activated receptor-α (PPARα) activation modulates LC function. Our results show that PPARα is expressed in immature LC and is down-regulated in mature LC suggesting that an early decrease of PPARα expression in LC may allow them to mature after contact with an Ag. We further show that pharmacologic PPARα activation inhibits LC maturation, migratory capacity, cytokine expression, and the ability to drive T cell proliferation. Moreover, PPARα activation inhibits NF-κB but not stress-activated protein kinase/JNK, p38MAPK, and ERK1/2. In conclusion, PPARα activation by endogenous ligands may provide a molecular signal that allows LC to remain in an immature state within the epidermis for extended periods of time despite minor environmental stimuli. *The Journal of Immunology,* **2007, 178: 4362–4372.**

Peroxisome proliferator-activated receptors (PPAR)[3] are nuclear hormone receptors that are activated by a wide array of endogenous polyunsaturated fatty acids derivatives, oxidized fatty acids, and phospholipids found in oxidized low-density lipoproteins. When activated, they act as transcription factors by entering the nucleus and modulating transcription. In the nucleus, the ligand-bound receptor interacts with response elements of target genes, which involves the recruitment of a complex of regulatory proteins (1, 2). Three subtypes of PPAR have been described in humans and rodents: PPARα, -δ, and -γ. PPARα is expressed in organs with high catabolic rates of fatty acids such as liver and muscles, PPARδ is ubiquitously expressed and PPARγ is expressed predominantly in adipose tissue (3). Furthermore, it has been shown that PPAR are expressed in cells of the immune system, including monocytes/macrophages, B and T lymphocytes, and dendritic cells (DC) (4, 5).

DC are APC that initiate and modulate immune responses. There is increasing evidence that activation of PPAR modulates DC maturation and function. For example, an inverse of expression of CD1a (decreased) and CD1d (increased) and a reduced

capacity to prime Ag-specific cytotoxic lymphocyte responses has been reported after PPARγ activation (6, 7). Furthermore, PPARγ activation inhibits chemokine and chemokine receptor expression (7), resulting in decreased migration of DC and Langerhans cells (LC) to the regional lymph nodes (7, 8), and it was proposed that the MAPK and NF-κB pathways are involved in the regulation of PPARγ-mediated signaling in DC (9). Similarly, PPARα and δ are expressed in DC (6, 10, 11) and diminished DC maturation has been reported after pharmacologic activation of PPARα and δ (11).

Activation of all three PPAR isotypes has been reported to attenuate inflammation. Although activation of PPARα and γ was clearly anti-inflammatory in monocytic cells and in atherosclerotic plaques (5, 12), activation of PPARδ has yielded mixed results (12, 13). Anti-inflammatory effects of PPARα activation were also reported in skin. In a mouse model of allergic contact dermatitis, topical treatment with PPARα agonists was reported to ameliorate ear swelling, which correlated with a reduction in the magnitude of the lymphocytic infiltrate and a decreased expression of proinflammatory cytokines (14). In PPARα knockout animals, it was demonstrated that these effects were receptor mediated (14). However, the mechanisms by which PPARα activation exerts its anti-inflammatory effects in allergic contact dermatitis are not known.

LC, a DC subset resident in the epidermis, are pivotal for the immune response of the skin (15). Located at the interface between the body and its environment, they efficiently capture Ag, which is processed and transferred to the regional lymph nodes to be presented to T cells. The interaction between LC and T cells occurs via MHC molecules, costimulatory molecules (CD80, CD86, CD40), and T cell adhesion molecules (LFA-1, ICAM-1) and determines whether the outcome of Ag presentation is the induction of an immune response or tolerance. Thus, LC are good targets for regulating the adaptive immune response in the skin. A number of regulatory mechanisms modulate LC function. For example, the type of Ag/pathogen or the type of tissue damage influence the maturation and migration of LC to the lymphoid organs (16). Proinflammatory cytokines including IL-1β, IL-6, and TNF-α and chemokines have been implicated in mediating this process. In addition, lipid mediators such as arachidonic acid metabolites are

FIGURE 1. Expression of PPARs in freshly isolated LC (i.e., MHC II$^+$ cells) and keratinocytes (i.e., MHC II$^-$ cells). Cells were fixed, permeabilized, and stained using PPAR or isotype control mAbs. Histograms show fluorescence intensity for PPAR (black histograms) vs isotype-matched control.

present in the epidermis and act as regulatory factors. For example, PGE$_2$ promotes migration and maturation of LC proposing a role of arachidonic acid metabolites in the cutaneous immune response (17, 18). Because certain arachidonic acid metabolites have been reported to exert their function by activating PPAR (19), we here

asked whether PPARα is expressed in LC and whether pharmacologic activation of PPARα modulates LC function.

Materials and Methods

Animals

Mice of the inbred strains C57BL/6 and BALB/c were purchased from Charles River Laboratories and used at 2–3 mo of age. PPAR$\alpha^{-/-}$ mice were bred on a C57BL/6/129S background as described previously (20). Animal experiments were conducted in concordance with to the Institutional Review Board.

LC isolation

Epidermal cells (EC) were isolated by trypsinization. Pieces of mouse ear and trunk skin were incubated in 0.8% trypsin (Merck) for 25–45 min at 37°C. Epidermis was peeled off and incubated for another 30 min at 37°C. Resulting EC suspensions contained 1–3% LC. These LC mature spontaneously in such bulk cultures, i.e., together with keratinocytes. Culture medium used throughout all experiments was Iscove's medium supplemented with 10% heat-inactivated FCS, 50 μg/ml gentamicin (Biochrom and Invitrogen Life Technologies) and 2-ME (Sigma-Aldrich). GM-CSF was obtained from culture supernatants (2% v/v) of the plasmacytoma cell line X63-Ag8 transfected with the mouse *GM-CSF* gene. These cells were a gift of Dr. C. Ruedl (Nanyang Technological University, School of Biological Sciences, Singapore).

Abs and reagents

Anti-mouse PPAR mAbs were obtained from Chemicon International. mAbs used for detecting LC (anti-mouse MHC class II (MHC II)-FITC, clone 2G9) and T cells (anti-mouse CD4-allophycocyanin, clone RM4-5) were purchased from BD Pharmingen. LC were detected in lymph nodes using a rat Ig G$_1$ anti-mouse Langerin mAb binding to an intracellular part of the Langerin molecule (clone 929F3-FITC/CD207; Dendritics) (21). Anti-mouse IL-1β-biotinylated Ab was obtained from R&D Systems. Anti-phospho-p65NF-κB (Ser^{536}), phospho-stress-activated protein kinase (SAPK)/JNK (Thr^{183}/Tyr^{185}), phospho-MEK1/2 (Ser$^{217/221}$), and phospho-p38MAPK (Thr^{180}/Tyr^{182}) were purchased from Cell Signaling Technology, biotinylated anti-mouse or anti-rabbit Abs, and streptavidin-allophycocyanin from Amersham (GE Healthcare). The PPARα agonist WY-14643, oleic acid, and the PPARα antagonist MK866 were purchased from Sigma-Aldrich.

Flow cytometry analysis

The phenotype of LC was examined using three-color FACS analysis. Cells were resuspended in cold PBS/1% BSA and serially incubated with FITC- and PE-conjugated mAbs directed against various mouse Ags for 20 min on ice. Specificity of staining was confirmed using isotype-matched control mAbs. Fluorescence was measured using a FACSCalibur flow cytometer and data were analyzed with CellQuest software (both from BD Biosciences). Nonviable cells were excluded by 7-aminoactinomycin D
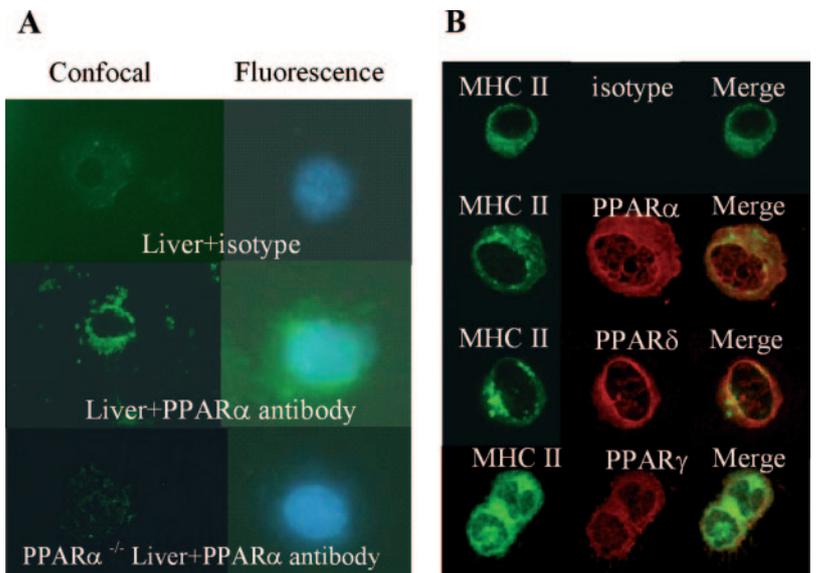
FIGURE 2. Expression of PPARs in freshly isolated LC. Confocal microscopy showing the expression of PPAR in liver cells (*A*) and freshly isolated LC (i.e., MHC II$^+$ EC) (*B*). Cells were fixed in acetone and stained using PPAR or isotype control mAbs. As a positive control, staining was conducted in liver cells from wild-type mice, as a negative control in liver cells from PPAR$\alpha^{-/-}$ mice.

FIGURE 3. PPAR expression during LC maturation. *A,* PPAR expression in LC upon in vitro maturation assessed by flow cytometry: EC suspensions were cultured in the presence of GM-CSF. At T0, T6, T24, and T48 h, cells were fixed, permeabilized, and stained using isotype and PPAR mAbs as described in *Materials and Methods.* LC were gated as MHC II$^+$ cells. *B,* Immunostaining showing PPAR expression after 24 and 48 h of culture. *C,* PPAR expression in LC upon in vivo maturation: mouse ears were treated with 1% TNCB at T0. Three days after sensitization, cells from epidermis and draining lymph nodes were prepared, fixed, permeabilized, and stained using isotype and PPAR mAbs as described in *Materials and Methods.* LC were gated as MHC II$^+$ cells in epidermis and as Langerin$^+$ cells in lymph nodes. Results are presented as mean $\pm$ SEM ($n = 3–4$). Data were analyzed using a one-way ANOVA followed by a Newman-Keuls test. *, $p < 0.05$ compared with T0; &, $p < 0.05$ compared with T6 h; #, $p < 0.05$ compared with T24 h.
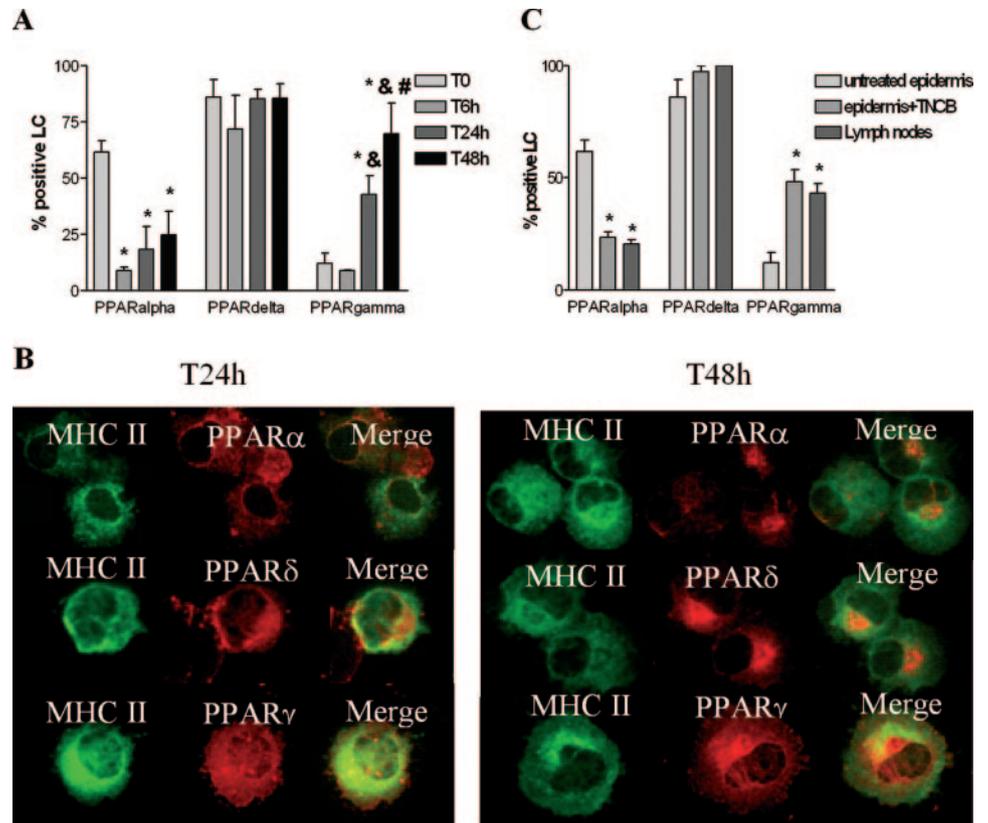
(7-AAD) uptake (Sigma-Aldrich). The expression of PPARs, phosphoproteins, and IL-1β in LC was assessed using two-color FACS analysis. Cells were first fixed and permeabilized with a cell permeabilization kit (Fix & Perm; An der Grub Bio-Research) for 15 min at room temperature, washed, and then incubated with the first-step Ab (1 h, at room temperature), washed, and stained with either streptavidin-allophycocyanin or with biotinylated Abs followed by streptavidin-allophycocyanin for 30 min at room temperature. Unenriched EC suspensions, containing LC, were used. Therefore, LC were identified by counterstaining with an anti-mouse MHC II-FITC mAb. Emigrated LC located in the skin draining lymph nodes were identified with an anti-mouse Langerin-FITC mAb.

Immunocytochemical analysis

Cytospins of epidermal, liver, and adipose cells were fixed in acetone for 5 min. Immunostaining was performed with PPAR or isotype control mAbs

for 1 h at 37°C. Ab binding was visualized using biotinylated donkey anti-mouse Ig followed by a streptavidin-Texas Red conjugate (Vector Laboratories). LC were counterstained with anti-mouse MHC II-FITC mAb. Immunolabeled specimens were mounted in 4',6'-diamidino-2-phenylindole (DAPI)-containing Vectashield (Vector Laboratories). Confocal microscopy was performed with a microlens-enhanced Nipkow disk-based confocal system UltraVIEW RS (PerkinElmer) mounted on an Olympus IX-70 inverse microscope. Images were acquired using the UltraVIEW RS software.

In vivo migration assay

Mouse ears were sensitized with 1% 2,4,6-trinitro-1-chlorobenzene (TNCB) (picryl chloride; Kodak Eastman) in acetone:olive oil, 4:1 at time point 0 (T0). A total of 20 μl of WY-14643 (10 mM) in acetone or acetone alone were topically applied at T − 3 h, T + 45 min, and T + 4 h on
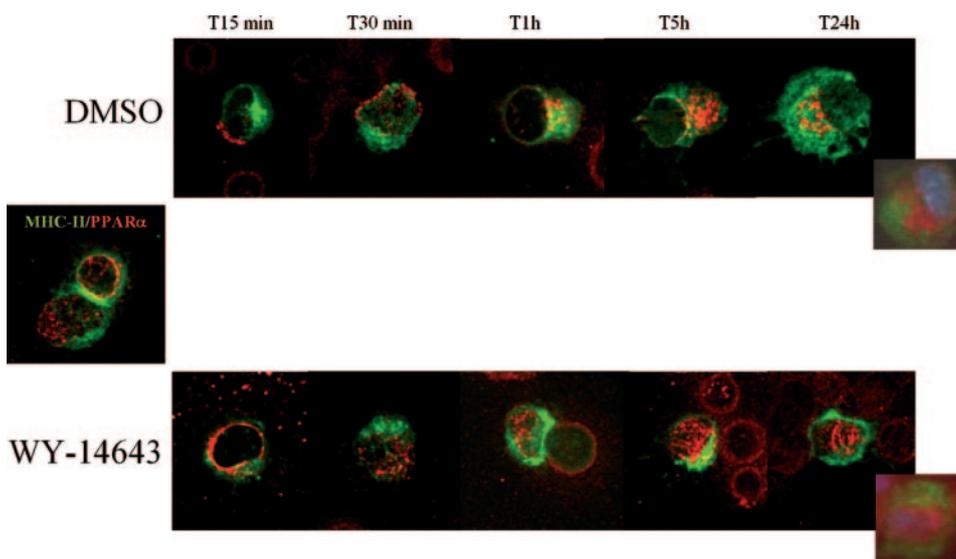
FIGURE 4. Nuclear translocation of PPARα in LC upon WY-14643 treatment. Immunostaining showing PPARα localization in cells after treatment with WY-14643 (0.5 mM) or DMSO for 0, 15, 30, 60 min, and for 5 and 24 h. Cells were fixed, permeabilized, and stained using isotype and PPARα mAbs as described in *Materials and Methods. Insets,* DAPI nuclear stain at T24 h.
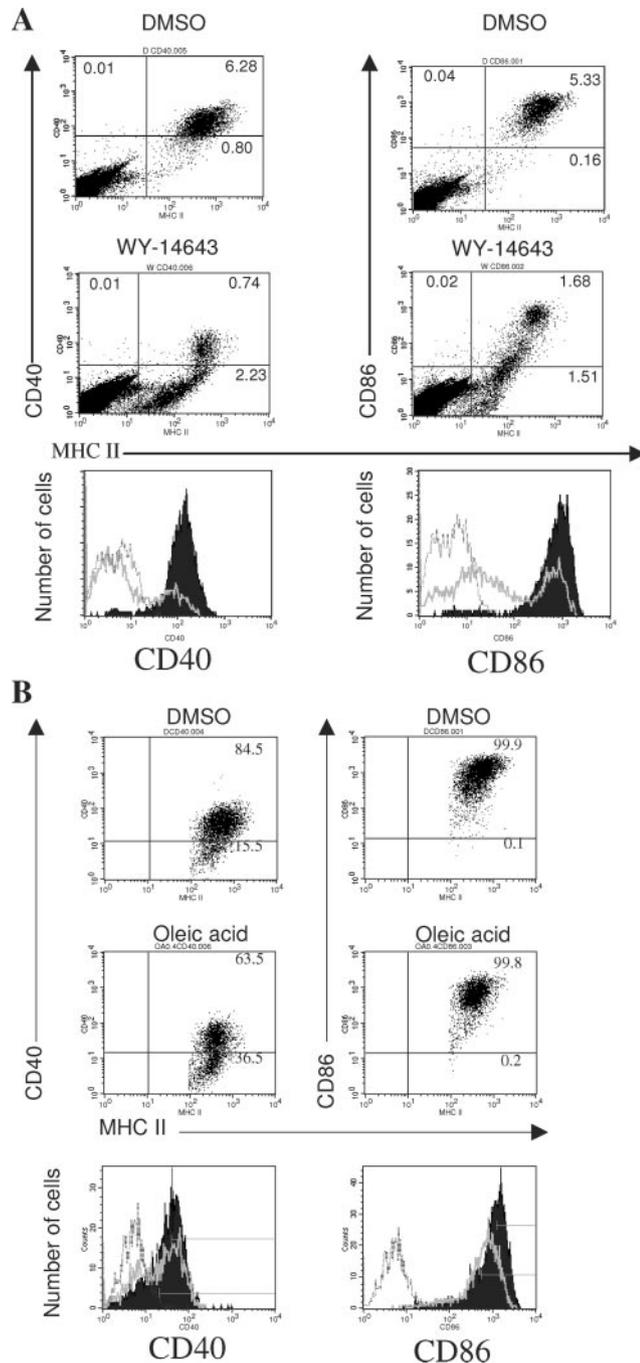
FIGURE 5. PPARα agonists inhibit LC maturation in vitro. EC were incubated for 48 h in culture medium supplemented with GM-CSF in the presence of DMSO or 0.5 mM WY-14643 (*A*) or 0.4 mM oleic acid (*B*). Cells underwent triple staining with MHC II-FITC and CD40-PE or CD86-PE mAbs or rat Ig G2a-PE as isotype control. Note that in the presence of WY-14643 a substantial proportion of LC (i.e., MHC II+ cells) remains negative for CD40 and CD86. The *two bottom panels* demonstrate the overlay of CD40 and CD86 expression on LC, i.e., on cells gated for MHC II expression. The plain black histogram designates DMSO-treated cells, the gray line WY-14643/oleic acid-treated cells, and the dotted black line the isotype control. 7-AAD+, necrotic cells were excluded from these analyses. Representative results from four independent experiments are shown.

the right ear or left ear, respectively. Epidermal sheets were prepared at T24 h by separation with ammonium thiocyanate (Merck), fixed with acetone, and stained with anti-MHC II-FITC mAb. To quantitate emigration of LC from the epidermis, MHC II+ cells in epidermal sheets were counted under an Olympus BX60 epifluorescence microscope using ×40 objective lenses and a calibrated grid (20 fields/sample). Alternatively, cells from auricular lymph nodes were prepared by digestion with 0.2 mg/ml collagenase P (Roche Applied Science) for 30 min and counted in the hemocytometer. Cell suspensions were stained with anti-Langerin-FITC and anti-CD11c-allophycocyanin (clone HL3) mAbs to detect emigrated LC. Absolute numbers of LC per lymph node were calculated on the basis of FACS analyses and hemocytometer cell counts.

Detection of intracellular cytokines

Mouse ear skin was topically treated with 1% TNCB at T0. WY-14643 (10 mM) or acetone were topically applied as described above. EC suspensions were prepared at T + 5 h, fixed, permeabilized, and stained for IL-1β production. LC were defined as MHC II+ cells. LC and T cells were isolated from lymph nodes by collagenase P digestion. Cells were cultured for 4.5 h with 1 μg/ml brefeldin A (BD Pharmingen) to prevent cytokine secretion. Alternatively, lymph node cells were restimulated for 4.5 h with 100 ng/ml PMA (Sigma-Aldrich). To detect cytokines in LC and T cells, cells were permeabilized and stained with mAbs. LC were identified by double labeling with anti-mouse CD11c-allophycocyanin and anti-Langerin-FITC mAb. All the other DC in skin draining lymph nodes were defined as CD11c+, Langerin− cells. DC/LC were stained with anti-mouse IL-12p40/70-PE (clone C15.6) and anti-mouse TNF-α-PE (MP6-XT22) while CD4+ T cells were stained with anti-mouse IL-10-PE (clone JES5-16E3), anti-mouse IL-4-PE (clone BVD4-24G2), anti-mouse IL-2-FITC (clone JES6-SH4), and anti-mouse IFN-γ-FITC (clone XMG1.2).

Mixed lymphocyte reaction

After incubation of EC with DMSO or WY-14643 (0.5 mM) for 48 h, nonviable cells were largely eliminated using a Nycodenz gradient (22). LC-containing interphases were cocultured with allogeneic CD4+ and CD8+ T cells (3 × 10^5/well) at various ratios for 4 days and proliferation was measured by [³H]thymidine incorporation for the last 16 h (activity 4 μCi/ml = 148 KBq/ml; New England Nuclear/PerkinElmer) in flat-bottom 96-well microtiter plates. Allogenic T cells were isolated from mouse spleens and lymph nodes as described previously (23). Briefly, T cells were isolated from spleens and lymph nodes of BALB/c mice and further purified by negative selection. Cell suspensions were incubated with a lineage mixture consisting of mAb against anti-MHC II (clone M5.114), anti-CD45RA (clone B220), anti-Ly-6C (clone Rb6), anti-heat stable Ag/CD24 (clone M1/69), anti-NK1.1 (clone 4D11) followed by anti-rat/anti-mouse IgG coupled to Biomag beads (Qiagen).

Statistical analysis

Data were analyzed using a paired *t* test or a Student *t* test or using a one-way ANOVA followed by a Newman-Keuls test.

Results

PPARα is expressed in freshly isolated mouse LC

Although previous reports have demonstrated expression of PPARα in macrophages (24) and DC (4), no information was available for LC. To determine the expression of PPAR in LC, we performed flow cytometry analyses and immunohistochemistry followed by confocal microscopy on freshly isolated (immature) LC. We detected marked expression of PPARα and δ and less pronounced expression of PPARγ in immature LC (i.e., MHC II+ EC) and in keratinocytes (i.e., MHC II− EC) (Fig. 1). As a positive control, staining was conducted in wild-type liver cells for PPARα (Fig. 2*A*) and PPARδ and on adipocytes for PPARγ (data not shown). As a negative control, PPARα staining was done on liver cells from PPARα−/− mice (Fig. 2*A*). Immunohistochemistry of cultured LC, revealed cytoplasmic PPARα staining with a perinuclear accentuation (Fig. 2*B*).

Decreased PPARα expression during maturation of LC

We next asked whether PPAR expression was altered during in vitro GM-CSF-induced and in vivo hapten-induced maturation of LC. As shown in Fig. 3*A*, PPARα expression was decreased at 6 h of culture (T6 h) as compared with time 0 (T0), i.e., freshly isolated LC. In contrast, PPARγ expression remained low at T0 and
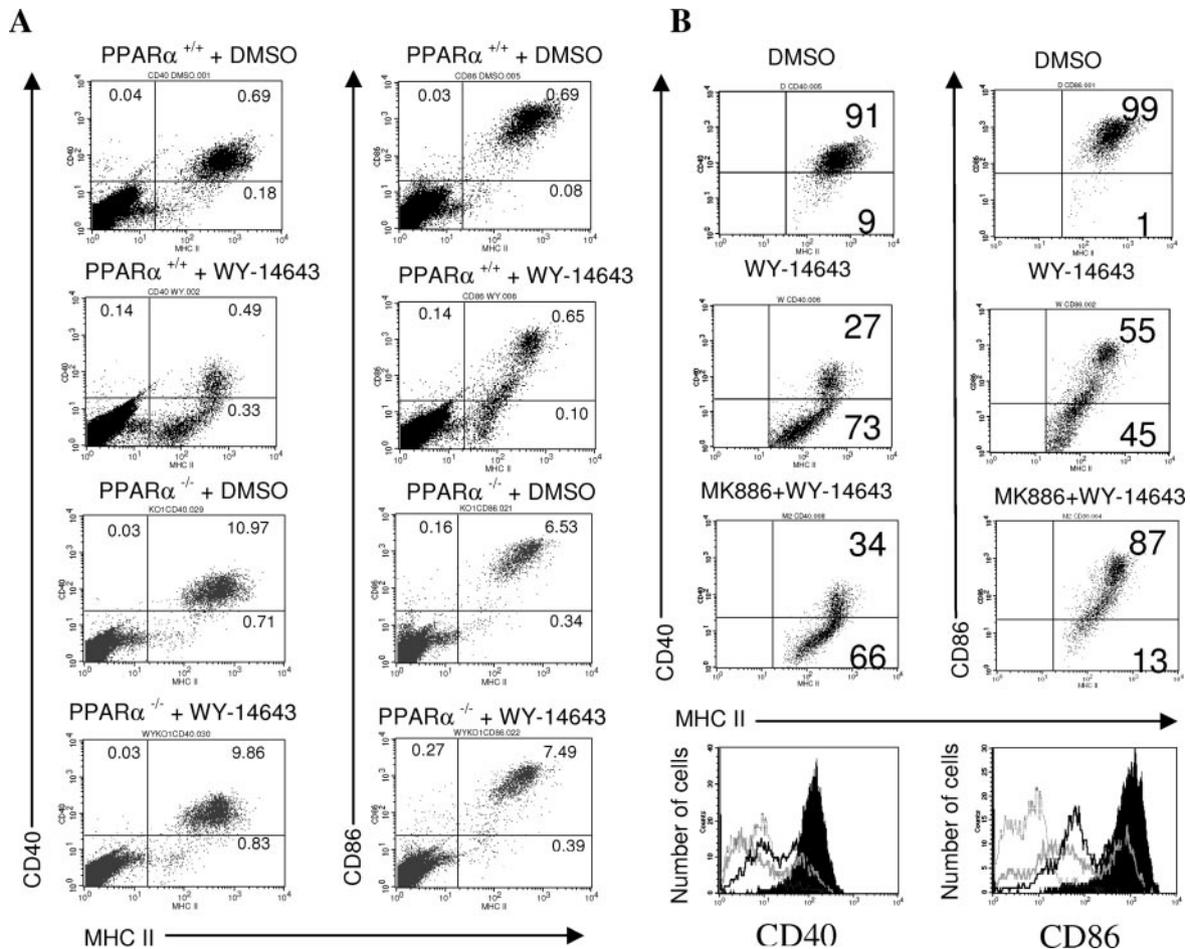
FIGURE 6. Inhibition of LC maturation by PPARα activation requires PPARα. *A*, EC were prepared from PPARα$^{-/-}$ and wild-type mice and incubated for 48 h in presence of 0.5 mM WY-14643 or vehicle (DMSO). Note that the PPARα agonist WY-14643 has no influence on LC phenotype in receptor-deficient mice. *B*, EC suspensions from control mice were incubated for 48 h in presence of 0.5 mM WY-14643 or vehicle or preincubated with 10 μM MK886 for 2 h before 0.5 mM WY-14643 was added for 48 h. The plain black histogram designates DMSO-treated cells, the light gray line WY-14643-treated cells, the dark gray line MK886- and WY-14643-treated cells, and the dotted gray line the isotype control. Cells were analyzed by flow cytometry using a triple staining for MHC II FITC and CD40-PE or CD86-PE or rat Ig G2a-PE as isotype control. 7-AAD$^+$ necrotic cells were excluded from these analyses. Representative results from three independent experiments are shown.

T6 h, but increased at T24 h while PPARδ was constitutively expressed throughout LC maturation in vitro (Fig. 3*A*). Confocal microscopy indicated that PPAR expression polarizes in mature LC in areas of high lysosomal activity (Fig. 3*B*). Similar results were obtained from LC isolated from epidermis and draining lymph nodes 3 days after hapten-mediated (TNCB) sensitization (Fig. 3*C*). These results demonstrate an inverse regulation of PPARα and PPARγ expression during LC maturation.

PPARα translocates to the nucleus of LC upon WY-14643 treatment

To further delineate the intracellular localization of PPARα, we cultured EC in the presence of WY-14643 (0.5 mM), a high-affinity PPARα agonist, or DMSO for 0, 15, 30, 60, 300 min, and 24 h. As shown in Fig. 4, PPARα was primarily found in perinuclear localization between T0 to T5 h of cell culture in vehicle-treated cells (before its expression decreased at 6 h as described above). In contrast, after addition of WY-14643 to the culture medium, PPARα translocated to the nucleus after 30 min and remained in this location for at least 24 h (Fig. 4).

PPARα agonists inhibit LC maturation in vitro

Next, we asked whether activation of PPARα would alter LC maturation. We treated EC suspensions with WY-14643 (0.5

mM), a synthetic, and oleic acid (0.4 mM), a natural PPARα agonist (25, 26) or vehicle for 48 h and analyzed the phenotype of LC after exclusion of necrotic 7-AAD$^+$ cells. WY-14643 (Fig. 5*A*) and oleic acid (Fig. 5*B*) treatment inhibited LC maturation by preventing the full up-regulation of MHC II, CD40, and CD86 expression, demonstrating that PPARα agonists inhibit LC maturation in vitro. The percentage of LC recovery after 48 h of cell culture was comparable between vehicle and WY-14643/oleic acid-treated cell cultures (DMSO: 79.5 ± 2.5, n = 10; WY-14643: 73.3 ± 1.6, n = 10; oleic acid: 83.1 ± 4.0, n = 4; p > 0.05). To determine whether the inhibition of LC maturation results from PPARα-induced peroxisome proliferation, we treated cells with 4 phenylbutyric acid (4PB), which is a known peroxisome proliferator that acts independently from PPARα (27). Treatment of EC with 4PB at doses known to induce peroxisome proliferation (2.5 and 5 mM) for 48 h increased the expression of MHC II in a dose-dependent manner (mean fluorescence intensity: DMSO: 532; 2.5 mM 4PB: 914; 5 mM 4PB: 1129), but did not modify the expression of CD40 or CD86 on LC, if compared with vehicle-treated cells (data not shown). These findings demonstrate that pharmacologic activation of PPARα inhibits LC maturation and that peroxisome proliferation alone does not account for this effect.
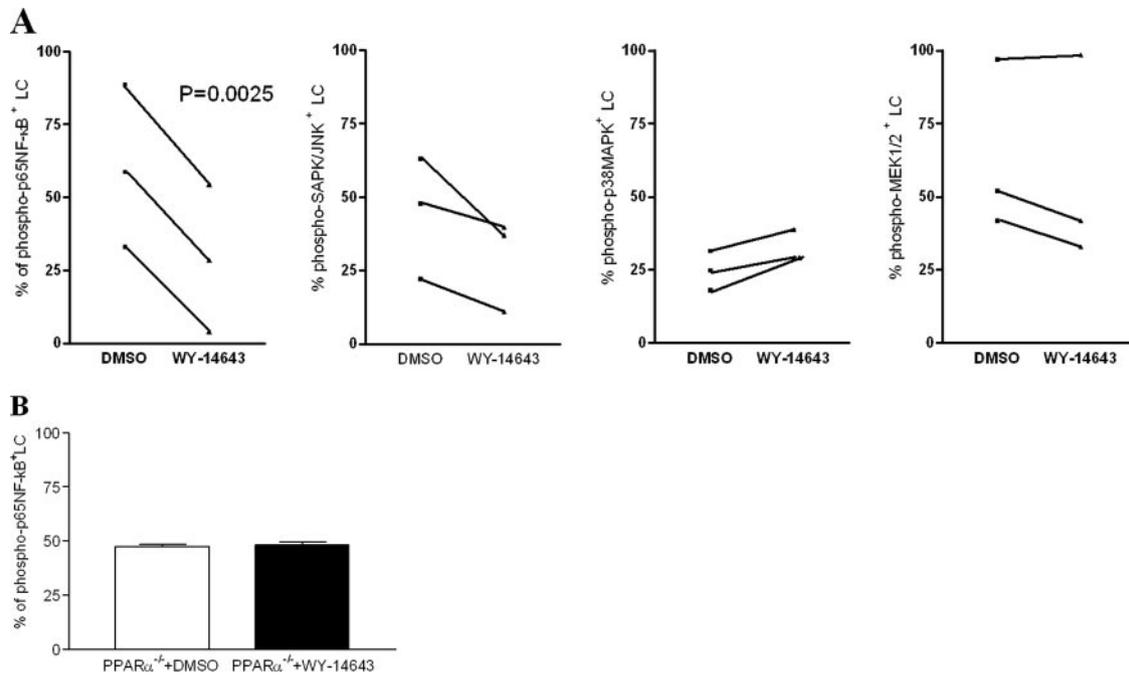
FIGURE 7. PPARα activation decreases phospho-p65NF-KB in LC. EC suspensions were incubated with DMSO or WY-14643 (0.5 mM). After 24 h, cells were fixed, permeabilized, and doubled-stained with MHC II-FITC/phospho-p65NF-κB or phospho-SAPK/JNK or phospho-p38MAPK or phospho-MEK1/2 Abs. Staining with fluorochrome-matched isotype controls was conducted in parallel. Data were analyzed using a paired *t* test. *A*, Results are shown from three independent experiments using wild-type control cells. *B*, EC from one PPARα$^{-/-}$ mouse was divided into six plates, three were incubated with DMSO, and three with WY-14643 (0.5 mM).

WY-14643 mediated inhibition of LC maturation requires PPARα

To determine whether PPARα is required for the inhibitory effects of WY-14643 on LC maturation, EC were isolated from PPARα$^{-/-}$ mice and treated with WY-14643 or vehicle. As shown in Fig. 6*A*, no difference was observed in the maturation of PPARα$^{-/-}$ cells and wild-type controls, but WY-14643 failed to inhibit maturation of PPARα$^{-/-}$ LC, indicating that PPARα is required. To further test, whether transcriptional effects are involved, we preincubated cells with MK886, a noncompetitive inhibitor of PPARα that has been shown to prevent the conformational changes that allow binding of PPARα to DNA (28). As shown in Fig. 6*B*, pretreatment of LC with MK886 partially reversed the effect of WY-14643. The effects were more pronounced for CD86 and MHC II than for CD40. Together, these results indicate that the inhibitory effects of WY-14643 on LC maturation require the presence of the receptor and its DNA-binding activity.

PPARα activation decreases the amount of phospho-p65NF-κB

To further explore the mechanism by which PPARα activation inhibits LC maturation, we assessed WY-14643-mediated changes in phospho-p65NF-κB, phospho-SAPK/JNK, phospho-MEK1/2, and phospho-p38MAPK, which are critically involved in signaling of DC maturation, differentiation, and apoptosis (29–31). Based on reports from the literature (32, 9) and preliminary experiments assessing phosphoprotein expression in LC, we chose a 24-h time point for these experiments. Whereas PPARα activation decreased the amount of phospho-p65NF-κB compared with vehicle-treated LC, the amount of phospho-SAPK/JNK, phospho-p38MAPK, and phospho-MEK1/2 remained essentially unchanged (Fig. 7*A*). To verify the requirement of PPARα, we treated EC suspensions isolated from PPARα$^{-/-}$ mice with WY-14643 or vehicle. Lack of PPARα abrogated the effects of WY-14643 on phospho-

p65NFκB, indicating that PPARα is indispensable for these effects (Fig. 7*B*). Furthermore, MK886 pretreatment partially reversed the decrease in phospho-p65NF-κB (DMSO: 60% of LC; WY-14643: 29%; MK886+WY-14643: 38%, $n = 3$) after WY-14643 treatment, again indicating a genomic effect.

PPARα activation decreases migratory capacity of LC

We next evaluated the effect of PPARα activation on the migratory capacity of LC. The contact allergen TNCB was applied to mouse ears and the ears were concurrently treated with WY-14643 or vehicle as described in *Materials and Methods*; 10 mM or 0.32% WY-14643 lies within the published range of concentrations used in vivo: Sheu et al. (14) used 1 mM (or 0.032%) in mice and Kippenberger et al. (33) (5%) 150 mM WY-14643 solution in humans. After 24 h, we quantified the number of LC remaining in the epidermis and those emigrated to the draining lymph nodes. Results shown in Fig. 8, *A* and *B*, demonstrate that PPARα activation prevents the departure of LC from the epidermis. The number of LC in the epidermis was significantly higher in WY-14643-treated mice whereas in draining lymph nodes the number and the percentage of LC was significantly lower (~2- to 3-fold) compared with vehicle-treated mice. At the same time, the percentages and absolute numbers of non-LC DC (CD11c$^+$, MHC II$^+$, Langerin$^-$) in draining lymph nodes were not significantly different between WY-14643-treated mice and controls (acetone: percent DC: 2.71 ± 0.76; 51032 ± 31290 DC/lymph node; WY-14643: percent DC: 2.25 ± 0.73; 31294 ± 9433 DC/lymph node, $n = 5$–10, $p > 0.1$). Furthermore, the data in Fig. 8, *C* and *D*, show that the departure of LC from the epidermis was not significantly altered in the absence of PPARα. These results demonstrate that PPARα activation impairs LC emigration from the epidermis to the draining lymph nodes.
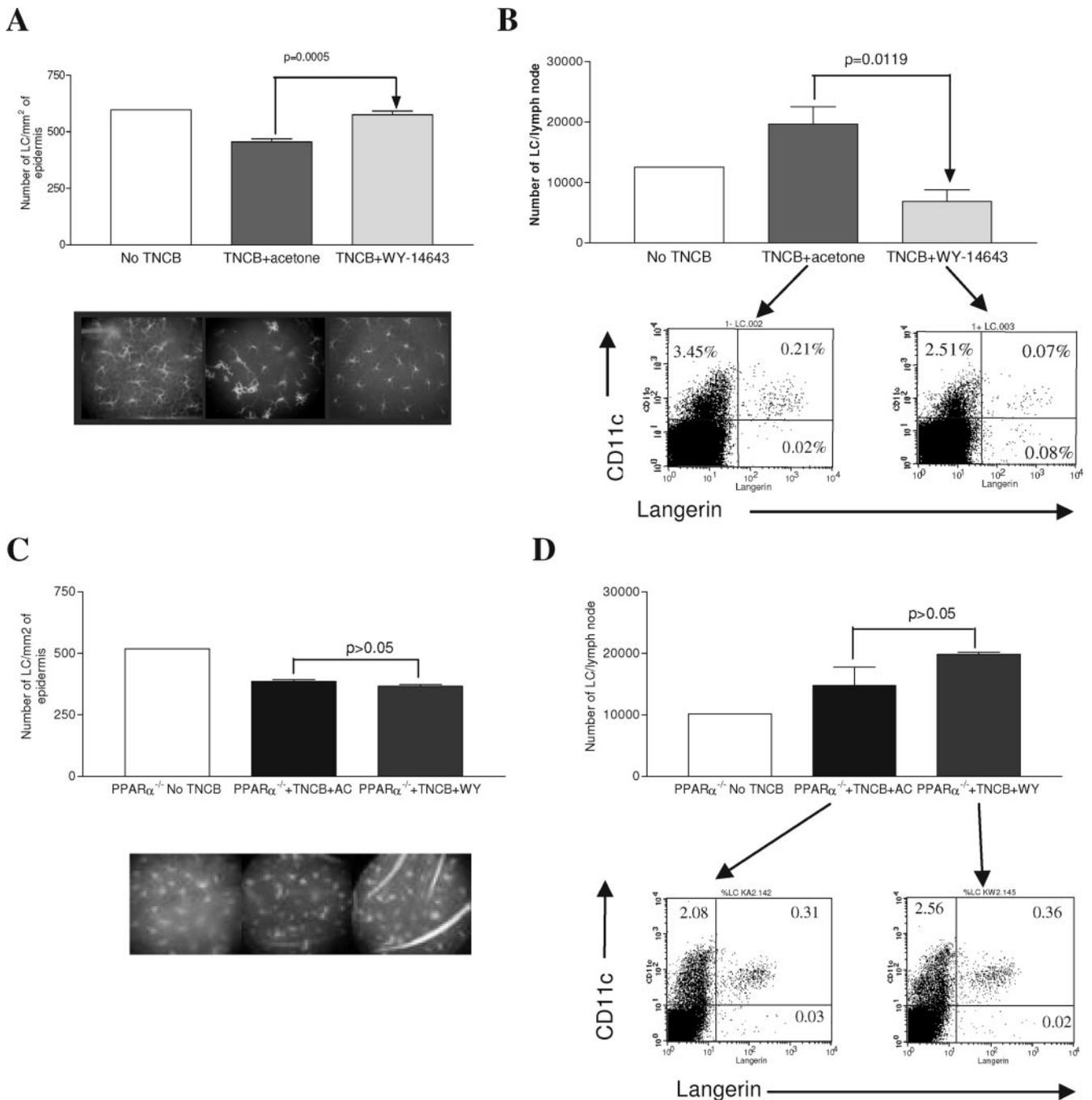
FIGURE 8. PPARα activation impairs the migratory capacity of LC. Ears from PPARα$^{-/-}$ and wild-type mice were treated with 1% TNCB at T0. WY-14643 (10 mM) or acetone were topically applied at T − 3 h, T + 45 min, and T + 4 h. Epidermal sheets and cell suspensions from draining lymph nodes were prepared at T24 h. Epidermal sheets were fixed and stained with an MHC II-FITC mAb. To quantitate emigration of LC from the epidermis, labeled cells in epidermal sheets were counted (20 fields for each sample). *A*, Results obtained from wild-type mice; *C*, results from PPARα$^{-/-}$ mice. Lymph node cells were fixed, permeabilized, and double stained with Langerin-FITC and CD11c-allophycocyanin mAbs. Fluorochrome-matched isotype controls were done in parallel. *B*, Results obtained from wild-type mice; *D*, results from PPARα$^{-/-}$ mice. Bars represent the mean numbers of LC ± SEM from 3 to 10 mice per group. Data were analyzed using a Student *t* test. Results from one untreated control and PPARα$^{-/-}$ mouse are shown as a reference.

PPARα activation reduces IL-1β production by LC

IL-1β, a cytokine expressed in many cell types including LC, and secreted during inflammatory processes, is involved in the migration of LC from the epidermis to draining lymph nodes. We therefore assessed the ability of pharmacologic PPARα activation to alter IL-1β expression in EC. We treated TNCB-sensitized mouse ears with WY-14643 or acetone as described above and measured IL-1β expression. As shown in Fig. 9*A*, WY-14643 treatment reduced IL-1β production in LC, which may account for the effects

of PPARα on the migratory capacity of LC. Fig. 9*B* shows that PPARα is required for these effects because IL-1β production by LC was not significantly altered in TNCB-sensitized PPARα$^{-/-}$ mice treated with WY-14643.

Decreased cytokine expression in draining lymph nodes after PPARα activation

Once maturation and migration to the regional lymph nodes have occurred, LC interact with T cells directly via surface molecules.
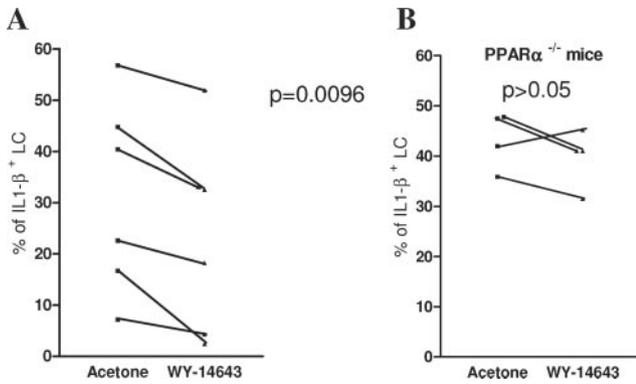
FIGURE 9. PPARα activation diminishes TNCB-induced IL-1β expression. To stimulate IL-1β secretion, mouse ears were treated with 1% TNCB at T0. WY-14643 (10 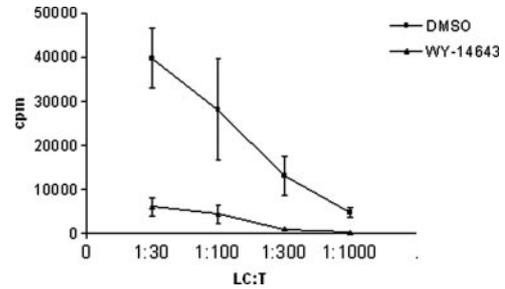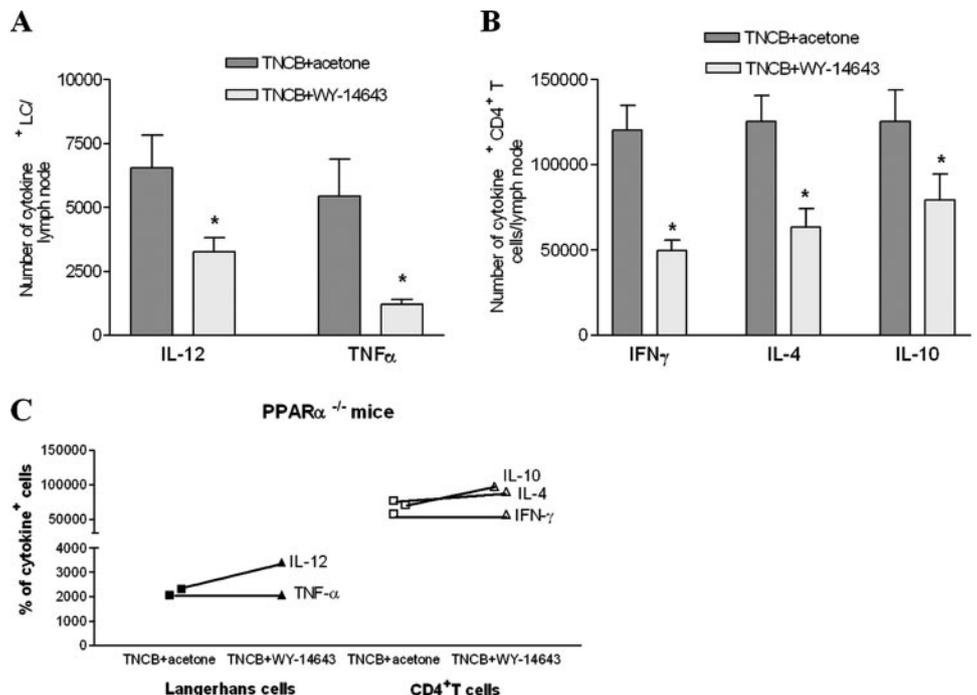mM) or acetone were topically applied at T − 3 h, T + 45 min, and T + 4 h to the right and left ears, respectively. EC suspensions were prepared at T + 5 h, fixed, permeabilized, and stained for IL-1β expression. LC were defined as MHC II⁺ cells. *A*, Results obtained from six independent experiments using cells from wild-type control mice ($n = 3$). *B*, Results obtained from one experiment in PPARα$^{-/-}$ mice ($n = 4$). Data were analyzed using a paired *t* test.

This interaction is modulated by cytokines that can be derived from either cell population. To explore the ability of PPARα to alter the cytokine production in these cells, we sensitized mice with TNCB and treated them with WY-14643 or acetone as described above. PPARα activation decreased the number of IL-12 and TNF-α-positive LC in draining lymph nodes (2- and 4.5-fold, respectively) (Fig. 10*A*). In addition, a decrease in IFN-γ, IL-4, and IL-10 was observed in CD4⁺ T cells before (data not shown) and after in vitro restimulation with PMA (Fig. 10*B*). In contrast, IL-2 production was not significantly altered (data not shown). Fig. 10*C* shows that these effects are PPARα dependent. These results indicate that WY-14643 not only impairs the migratory capacity of LC, but also alters cytokine production in LC and CD4⁺ T cells in the lymph nodes.

FIGURE 11. PPARα activation impairs the ability of LC to drive T cell proliferation. EC were preincubated with WY-14643 (0.5 mM) or DMSO for 48 h. Cells were extensively washed and LC were enriched by using a density gradient and counted. Allogeneic T cells were cocultured with graded doses of LC for 72 h and then pulsed with [³H]thymidine for 16 h. Assays were done in triplicate. Data are represented as the mean of three independent experiments ± SEM.

PPARα activation impairs the ability of LC to drive T cell proliferation

Because PPARα activation altered cytokine production in both LC and T cells in vivo, we tested the ability of WY-14643-treated LC to drive T cell proliferation in vitro. As shown in Fig. 11, PPARα activation strongly compromised LC driven allogenic T cell proliferation. Thus, the immunogenic potential of LC was decreased in MLR when LC were preincubated with the PPARα agonist WY-14643.

Discussion

Expression of PPARα in LC

Although previous studies using PPARα-specific ligands demonstrated anti-inflammatory effects of PPARα activation in skin (14), they did not provide insight into potential mechanisms. We here show for the first time that PPARα is expressed in freshly isolated (immature) murine LC, primarily in perinuclear distribution (Fig. 2). This localization is compatible with the formation of chaperone complexes and with phosphorylation by MAPK. Although it is not

FIGURE 10. Decreased cytokine expression in draining lymph nodes of TNCB-sensitized mouse ears after PPARα activation. Ears from PPARα$^{-/-}$ and control mice were treated with 1% TNCB at T0. WY-14643 (10 mM) or acetone were topically applied at T − 3 h, T + 45 min, and T + 4 h on the right ear and left ear, respectively. LC (*A* and *C*) and CD4⁺ T cells (*B* and *C*) were detected with Langerin-FITC/CD11c-allophycocyanin/IL-12-PE or TNF-α-PE mAbs and CD4-allophycocyanin/IFN-γ-FITC or IL-4-PE or IL-2-PE- or IL-10-PE mAbs, respectively. Stainings with fluorochrome-matched isotype controls were conducted in parallel. CD4⁺ T cells were restimulated for 4.5 h with PMA. Bars show the numbers of cytokine⁺ cells ± SEM per auricular lymph node obtained from wild-type control mice ($n = 3$–4) and one PPARα$^{-/-}$ mouse. Data were analyzed using a Student *t* test.

know whether this occurs in EC, in hepatocytes it was shown that PPARα associates with heat shock protein 90 and the cochaperone, hepatitis B virus X-associated protein 2 (XAP2) (34, 35). Moreover, PPAR phosphorylation within the AF1 region of domain A/B via cytosolic MAPK has been reported to occur in response to environmental changes and extracellular signals (36). In addition, the proximity to the nucleus may be of physiological relevance allowing for rapid translocation of ligand-bound PPARα into the nucleus where heterodimerization with RXR and response element binding takes place. Indeed, we demonstrate here that treatment with WY-14643, a selective agonist, induces translocation to the nuclear compartment (Fig. 4). We further show both in vitro and in vivo that PPARα expression is markedly diminished in mature LC (Fig. 3), in concordance with a recent report describing PPARα in immature, but not mature, bone marrow-derived murine DC (10). Moreover, we found that pharmacologic activation of PPARα by WY-14643, a synthetic, and oleic acid, a natural PPARα agonist, inhibits the maturation of LC. These effects require the presence of the receptor in LC, because maturation is not altered in PPARα$^{-/-}$ cells and thus these results indicate an important role of PPARα in the regulation of LC maturation. Even though we ruled out toxic effects of WY-14643 in our specific experimental settings, it should be emphasized that other agonists may have different levels of toxicity and these can be species dependent.

PPARα modulates NF-κB signaling in LC

The NF-κB and MAPK pathways have been shown to regulate both LC maturation and cytokine expression (29–31). Recently, the balance between NF-κB and JNK/AP1 activity was shown to control human LC survival (37). In DC, NF-κB modulates their maturation by controlling the expression of MHC II and costimulatory molecules (29). Furthermore, it has been proposed that the NF-κB pathway plays a role in DC migration (38). We describe here in LC that PPARα activation decreases the amount of phospho-p65NF-κB, but does not significantly affect the level of phospho-SAPK/JNK, phospho-p38MAPK, and phospho-MEK1/2 (Fig. 7). PPARα can regulate NF-κB activity either transcriptionally or by protein-protein interactions (39–41). Notably, in our experiments, when transcriptional activity of PPARα was blocked, the inhibition of LC maturation was partially reversed and phospho-p65NF-κB was partially restored. This indicates that the expression of MHC II, CD40, and CD86 in murine LC is determined by the amount of phospho-p65NF-κB and that PPARα-mediated inhibition of LC maturation in part is due to the ability of PPARα to regulate p65NF-κB transcription (39–41). Further investigations would be required to dissect the relative contributions of the effects of PPARα on gene transcription vs its direct interaction (transrepression) with p65NF-κB in murine LC in vitro. In LC, analyses of PPARα binding to DNA or its interactions with other proteins, as demonstrated in other cellular systems, are currently not feasible due to technical problems in acquiring sufficiently large numbers of highly enriched LC.

Effects of PPARα activation on LC migration and cytokine expression

We report here that PPARα impairs the migration of LC to the skin-draining lymph nodes. The in vivo migration of LC after application of a contact allergen was markedly decreased in WY-14643-treated mice compared with vehicle-treated control mice. In concordance with a recent report, these effects were not observed at low WY-14643 doses (8), but instead occurred in a PPARα-dependent manner when high doses were applied (Fig. 7). Similarly, the presence of PPARα was required for the inhibitory effects of WY-14643 on cytokine expression. Measuring IL-1β in

EC after sensitization with a contact allergen, we observed decreased IL-1β expression in LC after WY-14643 treatment compared with vehicle in a PPARα-dependent manner. The cytokine IL-1β is mainly produced by LC (42). It is the first cytokine to be expressed during contact sensitization (43) and it is thought to direct the migration of LC to the draining lymph nodes (44–47) in multiple ways, i.e., it not only down-regulates E-cadherin, releasing LC-keratinocyte contacts (48), but it also induces the expression of other cytokines (43) and matrix metalloproteinases (49) involved in the emigration of LC from the skin (50, 51). In the early phase of contact sensitivity, mRNA for IL-1β is rapidly up-regulated in LC followed by the production of several other cytokines and chemokines, such as TNF-α, GM-CSF, IFN-γ, IFN-γ-inducible protein 10, and MIP-2, most of them produced by keratinocytes (52). As a complex interplay of cytokines and chemokines is necessary to regulate the migration of LC from the skin to the draining lymph node (53), it is tempting to speculate that the WY-14643-induced IL-1β decrease may be upstream of NF-κB inhibition, accounting for decreased LC emigration. This could be specific to LC because DC migration seems to mainly be controlled by a balance between p38MAPK and MEK1/2 (54).

Furthermore, decreased IL-1β expression and impaired migration of LC to the lymph nodes were reflected by a smaller number of LC-producing TNF-α and IL-12 in the skin draining lymph node (Fig. 10). TNF-α is known to be a regulatory cytokine involved in inflammatory processes enhancing the expression of other proinflammatory cytokines including IL-1, IL-6, IL-8. On the one hand, TNF-α stimulates the release of active forms of NF-κB by acting on IκB kinase (55) and in contrast, PPARα activation was demonstrated to inhibit the inflammatory response by preventing the release of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (56, 57). The decrease in TNF-α$^+$ LC (4.5-fold) reported here is greater than the decrease in IL-12$^+$ LC and the number of emigrated LC to the lymph nodes (2-fold). Thus, one may speculate that PPARα activation inhibits the production of IL-1β in the epidermis, resulting in the decrease of migratory capacity of LC and in their production of TNF-α. Ultimately, this may result in a decreased adaptive immune response (14).

Effects of PPARα on T cell responses in the lymph nodes

Finally, we found that the ability of WY-14643-treated LC to drive T cell proliferation in vitro is markedly impaired on a per cell basis. This is accompanied by decreased cytokine expression in CD4$^+$ T cells (Fig. 10). Interestingly, these experiments did not reveal a preferential inhibition of Th1 or Th2 cytokines, suggesting that PPARα activation does not interfere with the Th1-Th2 balance. Of note, IL-2 levels were not altered after topical application of TNCB/WY-14643 neither in freshly isolated CD4$^+$ T cells nor following in vitro restimulation, suggesting that survival of CD4$^+$ T cells was not involved.

The implications of these findings are severalfold. 1) PPARα activation inhibits the development of the immunostimulatory capacity of LC by maintaining them in an immature stage. Because the endogenous ligands of PPARα are unsaturated fatty acids such as arachidonic acid and its derivatives, which are present in the epidermis in the steady state (17), PPARα activation by naturally occurring ligands may be part of a cue that allows LC to remain in an immature state within the epidermis for extended periods of time despite minor environmental stimuli. Thereby, PPARα could be involved in the counterbalance of ongoing immune responses and thus prevent tissue destruction after activation by arachidonic acids and derivates released from damaged cells. 2) The observed early decrease of PPARα expression in freshly isolated LC may be

required to allow them to mature after contact with an Ag, especially when accompanied by a strong inflammatory stimulus. 3) PPARα activity can be modulated by exogenous compounds. Therefore, PPARα is a promising drug target in inflammatory skin disease.

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Disclosures

The authors have no financial conflict of interest.

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