Peroxisome Proliferator-Activated Receptor δ Regulates Inflammation via NF- κ B Signaling in Polymicrobial Sepsis

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The nuclear peroxisome proliferator-activated receptor δ (PPAR δ) is an important regulator of lipid metabolism. In contrast to its known effects on energy homeostasis, its biological role on inflammation is not well understood. We investigated the role of PPAR δ in the modulation of the nuclear factor- κ B (NF- κ B)-driven inflammatory response to polymicrobial sepsis *in vivo* and in macrophages *in vitro*. We demonstrated that administration of GW0742, a specific PPAR δ ligand, provided beneficial effects to rats subjected to cecal ligation and puncture, as shown by reduced systemic release of pro-inflammatory cytokines and neutrophil infiltration in lung, liver, and cecum, when compared with vehicle treatment. Molecular analysis revealed that treatment with GW0742 reduced NF- κ B binding to DNA in lung and liver. In parallel experiments, heterozygous PPAR δ -deficient mice suffered exaggerated lethality when subjected to cecal ligation and puncture and exhibited severe lung injury and higher levels of circulating tumor necrosis factor- α (TNF α) and keratinocyte-derived chemokine than wild-type mice. Furthermore, in lipopolysaccharide-stimulated J774.A1 macrophages, GW0742 reduced TNF α production by inhibiting NF- κ B activation. RNA silencing of PPAR δ abrogated the inhibitory effects of GW0742 on TNF α production. Chromatin immunoprecipitation assays revealed that PPAR δ displaced the NF- κ B p65 subunit from the κ B elements of the TNF α promoter, while recruiting the co-repressor BCL6. These data suggest that PPAR δ is a crucial anti-inflammatory regulator, providing a basis for novel

sepsis therapies. (Am J Pathol 2010; 177:1834–1847; DOI: 10.2353/ajpath.2010.091010)

Sepsis is a systemic response to infection characterized by hemodynamic and metabolic derangement that may result in septic shock, multiple organ system failure, and death. Although antibiotic therapy may effectively treat an underlying infection, this treatment is not sufficient to reverse the systemic inflammation and its consequence.¹ The invading microorganisms and their components initiate a cascade of events by stimulating immune competent or parenchymal cells of the host, leading to the overwhelming production of endogenous pro-inflammatory mediators.² This production is regulated at the nuclear level by a rapid activation of transcription factors, including nuclear factor- κ B (NF- κ B), which initiates gene expression of cytokines, adhesion molecules and chemokines, and cytotoxic enzymes, by binding to specific elements in their promoter region.^{3,4}

The peroxisome proliferator activated receptors (PPARs) are a family of nuclear receptors, which require ligand binding and function directly as transcription factors to control gene regulation. Three PPAR isoforms, α , δ (also called β) and γ , have been identified and function as heterodimers with the 9-*cis*-retinoic receptor (RXR) through binding to a specific PPAR-responsive element in the promoter region of certain target genes.⁵ PPAR δ is ubiquitously expressed and particularly abundant in skeletal muscle and liver where it regulates lipid and lipoprotein metabolism.⁶ Its beneficial effects have been described on dyslipidemia in insulin-deficient or obese experimental animals^{7–9} and on thermogenic regulation for genes for fatty acid oxidation and energy dissipation.¹⁰ Other non-metabolic functions of PPAR δ include

Supported by the National Institutes of Health grants R01 GM067202 and R01 AG027990 (B.Z.) and grant R01 GM027673 (J.A.C.).

Accepted for publication June 8, 2010

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cell growth, proliferation, and differentiation and tissue repair in wound healing.¹¹

In general, natural ligands for these transcription factors are represented by fatty acids and prostaglandins. Among a considerable number of synthetic ligands, the class of phenoxyacetic acid derivatives, such as GW0742, has been described as highly selective for PPAR δ .¹² Recent *in vitro* studies in endothelial cells and cardiomyocytes have shown that pharmacological activation of PPAR δ by specific ligands exerts potent anti-inflammatory properties most probably through inhibition of the NF- κ B pathway.^{13–15} In tumor necrosis factor- α (TNF α)-activated endothelial cells, PPAR δ activation seems to reduce the expression of vascular cell adhesion molecule-1 (VCAM-1), E-selectin and secretion of monocyte chemoattractant protein-1 (MCP-1).^{13,15} Similarly, the synthetic ligand GW0742 inhibits production of TNF α in cultured cardiomyocytes subjected to lipopolysaccharide (LPS) challenge.¹⁴

Considering these *in vitro* anti-inflammatory properties of PPAR δ , we investigated the biological effects of PPAR δ modulation on the inflammatory response of sepsis. Specifically, in gain-of-function studies, *in vivo* in septic rats and *in vitro* in J774.A1 macrophages, we demonstrated that treatment with the PPAR δ ligand GW0742 ameliorated the extent of the inflammatory response secondary to bacterial challenge. On the contrary, in loss-of-function studies, *in vivo* in septic mice, we demonstrated that PPAR δ gene deletion was associated with increased susceptibility to bacterial-induced inflammation, tissue injury and high mortality. In J774.A1 macrophages, PPAR δ directly bound to TNF α promoter and inhibited transactivation of NF- κ B also by recruiting BCL6, a transcriptional repressor that is a critical regulator of immune and inflammatory function in immune competent cells.¹⁶ Thus, this study provides evidence that PPAR δ is a novel regulator of the innate immune response by protecting against dysregulated inflammation in sepsis.

Materials and Methods

Rat Model of Cecal Ligation and Puncture

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by U.S. National Institutes of Health (NIH Publication No. 85-23 revised 1996) and commenced with the approval of the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River laboratories, Wilmington MA) weighing 175 to 250 g were anesthetized with thiopentone sodium (70 mg/kg i.p.). Cecal ligation and puncture (CLP) was performed as previously described.¹⁷ After opening the abdomen, the cecum was exteriorized and ligated by a 3.0 silk ligature at its base without obstructing intestinal continuity. The cecum was punctured twice with an 18-gauge needle and returned into the peritoneal cavity. The abdominal incision was closed with 3-silk running suture. Three groups of rats were used in the experiment. The first group ($n = 14$) received an equal volume (1 ml/kg) of dimethyl sulfoxide (100%) instead of

the PPAR δ ligand (CLP + vehicle group). The second group ($n = 14$ for each group) received GW0742 (0.5 mg/kg, CLP + GW0742 group) by i.p. injection. In a third group of rats, surgery was performed as in the CLP group, but the cecum was not ligated nor punctured (Sham group, $n = 12$). To ensure bioavailability of the drug during both early and late time points of the inflammatory response, GW0742 or vehicle was administered at 0, 6, and 12 hours after CLP and every 12 hours thereafter until the end of the observation period. Saline solution (0.9%, 5 ml) was given subcutaneously to replace the fluid and blood loss during operation. Groups of animals ($n = 3$ to 14) were sacrificed at different time points after CLP (1, 3, 6, 18 hours). Plasma samples, peritoneal macrophages, lung, liver, and cecum were collected for histological and biochemical studies described below. In separate survival studies, two groups of rats (CLP + vehicle group and CLP + GW0742 group, $n = 30$) underwent a more severe model of injury by a variation of cecal puncture using a 16-gauge needle and triple puncture (CL3P). After the surgical procedure, rats were awakened and allowed free access to water and food, and survival was monitored for 72 hours.

Peritoneal Macrophage Harvest

At 6 hours after CLP, resident peritoneal macrophages were harvested through lavage with ice-cold Dulbecco's modified Eagle's medium (Gibco Technologies, Grand Island, NY), spun down and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The cells were plated at 1×10^6 cells/well into 25 cm² flasks and incubated for 2 hours at 37°C and 5% CO₂. After incubation, the supernatant was collected for TNF α quantification and nonadherent cells were removed by rinsing the plates with cold PBS. Adherent cells were then collected for nuclear protein extraction.

Hemodynamic Measurements

Mean arterial blood pressure was measured in other groups of rats ($n = 4$ to 10 for each group). In a first experiment, rats were anesthetized with thiopentone sodium (70 mg/kg i.p.). The trachea was cannulated to facilitate respiration and the carotid artery was cannulated to measure mean arterial blood pressure by a pressure transducer connected to a MacLab A/D converter (AD instruments, Milford, MA). In these anesthetized rats, mean arterial blood pressure was then monitored for 6 hours after induction of CLP. In a second experiment, animals underwent CLP and were allowed to recover. Fourteen hours later, the animals were anesthetized and the trachea and carotid artery cannulated. In these anesthetized rats, mean arterial blood pressure was then measured for 4 hours (ie, up to 18 hours after CLP). Animals that died before the end of the experiment were excluded from the study.

Murine Model of CLP

Male heterozygous *PPAR* δ -deficient (*PPAR* $\delta^{+/-}$) mice and their wild-type control on a C57BL/6 genetic background¹⁸ weighing 20 to 25 g were anesthetized with thiopentone sodium (400 μ g/10 g i.p.). Similarly to the rat model, after an abdominal incision the cecum was ligated at the colon juncture with a 6–0 silk ligature, punctured twice with a 22-gauge needle and returned into the peritoneal cavity. The incision was closed in layers with a 6–0 silk ligature suture. Sham-operated mice were subjected to the same surgical procedure, ie, laparotomy and cecal isolation, but the cecum was neither ligated nor punctured. After the procedure, mice were fluid resuscitated with 0.6 ml saline solution (0.9%) injected subcutaneously. Mice were sacrificed at 18 hours after CLP and lung, liver, and blood samples were harvested for the histological and biochemical studies. In a separate study, mice were awakened and allowed free access to water and food, and survival was monitored for 5 days.

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation in lung, colon, and liver, as previously described.¹⁷ Tissues were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7.0) and centrifuged for 30 minutes at 20,000 $\times g$ at 4°C. An aliquot of the supernatant was allowed to react with a solution of tetra-methyl-benzidine (1.6 mmol/L) and 0.1 mmol/L H₂O₂. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μ mol of hydrogen peroxide/min at 37°C and expressed in units per 100 mg weight of tissue.

Histopathological Analysis

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with H&E and histopathologically analyzed for alveolar congestion, hemorrhage, infiltration, or aggregation of neutrophils in airspace or vessel wall, thickness of alveolar wall and hyaline membrane formation.

Cell Culture and Transfection of Small Interfering RNA

Murine J774.A1 macrophages (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (100 μ g/ml) under standard incubation conditions at 37°C and 5% CO₂. Cells (at 80% confluence) were then stimulated with LPS of *Escherichia coli* (Serotype 0111:B4, 100 ng/ml) up to 24 hours in the presence or absence of the *PPAR* δ ligand, GW0742 (10 to 1000 nmol/L). The ligand was added as a pretreatment 30 minutes before LPS stimu-

lation. In parallel experiments, small interfering (si)RNA technique was used to silence *PPAR* δ gene. Specifically, nonsilencing RNA (scramble siRNA) or siRNA targeted for *PPAR* δ receptor (*PPAR* δ -siRNA) (1.5 μ g each in 100 μ l) was transfected into J774.A1 macrophages using HiPerfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cells were cultured for 48 hours following siRNA transfection.

Luciferase Assay

Cells were transiently transfected with 1 μ g of pGL3-NF- κ B-luciferase reporter construct using a 3 $\times$ NF- κ B construct as previously described¹⁹ and SuperFect transfection reagent (Qiagen). For normalization of transfection efficiency, 0.1 μ g of the Renilla luciferase pGL4.74[hRLuc/TK] plasmid was co-transfected. At 48 hours after transfection, the cells were treated with LPS (100 ng/ml) for 4 hours before cell harvest. The *PPAR* δ ligand, GW0742 (10 to 1000 nmol/L) was added to the culture media 30 minutes before LPS stimulation. Relative luciferase activity from the firefly NF- κ B-luciferase reporter gene was determined and normalized to Renilla luciferase activity using the Dual Luciferase Reporter Assay System kit according to the manufacturer's instructions (Promega, Madison, WI).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the instructions of the manufacturer. Briefly, at 1 hour after LPS treatment J774.A1 cells were cross-linked with 1% formaldehyde and quenched before harvest and sonication. The sheared chromatin was pre-cleaned with protein G Agarose beads, then immunoprecipitated with antibodies raised against *PPAR* δ , p65 subunit of NF- κ B or BCL6. The negative control was chromatin immunoprecipitated with control IgG; the positive control (Input) was chromatin recovered without immunoprecipitation. The immune complexes were recovered with protein G agarose beads. The eluted immunoprecipitates were digested with RNase A and proteinase K, and DNA was extracted and underwent PCR with primers specific for the murine TNF α promoter region spanning the most proximal NF- κ B binding site: (forward) 5'-CAGCCTTCCCCACTAAAATAACC-3' and (reverse) 5'-ACCCACAAAAACCCTGCTCTG-3'. Densitometric analysis of blots was performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Levels of Cytokines and Chemokines

Plasma levels of interleukin (IL)-1 β , IL-6, IL-10, leptin, keratinocyte-derived chemokine and MCP-1 were determined by a multiplex array system (Linco-Research, St. Charles, MO). Cell supernatant levels of TNF α were evaluated by commercially available solid-phase sandwich enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN), using the protocol recommended by the manufacturer.

Subcellular Fractionation and Nuclear Protein Extraction

Lung and liver samples were homogenized, or primary rat peritoneal and J774.A1 macrophages were rinsed with a buffer containing 0.32 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EGTA, 2 mmol/L EDTA, 5 mmol/L Na₂S₂O₈, 10 mmol/L β -mercaptoethanol, 20 μ mol/L leupeptin, 0.15 μ mol/L pepstatin A, 0.2 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 0.4 nmol/L microcystin. The cell suspension or homogenates were centrifuged ($1000 \times g$, 10 minutes) and the supernatant (cytosol + membrane extract) was collected for evaluation of I κ B α expression. The pellets were solubilized in Triton buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EGTA, 1 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 20 μ mol/L leupeptin A, 0.2 mmol/L phenylmethylsulfonyl fluoride). The lysates were centrifuged ($15,000 \times g$, 30 minutes, 4°C), and the supernatant (nuclear extract) was collected for evaluation of expression of PPAR δ , PPAR α , PPAR γ , RXR α , and DNA binding of PPAR δ and NF- κ B.

Western Blot Analysis

Nuclear content of PPAR δ , PPAR α , PPAR γ , RXR α and cytosolic degradation of I κ B α in liver and lung homogenates or primary rat peritoneal and J774.A1 macrophage lysates were determined by immunoblot analyses using primary antibodies against PPAR δ , PPAR α , PPAR γ , RXR α , or I κ B α and secondary peroxidase-conjugated antibody. Immunoreactive β -actin was also evaluated as a loading control. Immunoreaction was visualized by chemiluminescence. Densitometric analysis of blots was performed using ImageQuant.

Electrophoretic Mobility Shift Assay

This assay was performed in nuclear extracts of livers or lungs as previously described.¹⁷ Oligonucleotide probes corresponding to NF- κ B consensus sequence (5'-AGT-TGAGGGGACTTCCCAGGC-3') or PPARs consensus sequence (5'-GAAACTAGGTCAAAGGTCA-3') were labeled with γ -[³²P]ATP using T4 polynucleotide kinase and purified in Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Ten μ g of nuclear protein were pre-incubated with electrophoretic mobility shift assay (EMSA) buffer (12 mmol/L HEPES pH 7.9, 4 mmol/L Tris-HCl pH 7.9, 25 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 50 ng/ml poly [d(I-C)], 12% glycerol v/v, and 0.2 mmol/L phenylmethylsulfonyl fluoride) on ice for 10 minutes before addition of the radio-labeled oligonucleotide for an additional 10 minutes. The specificity of the binding reactions was determined by co-incubating duplicate nuclear extract samples with 100-fold molar excess of respective unlabeled oligonucleotides (competitor assays). Supershift assays, to further determine the specificity of binding of PPAR δ , was performed by co-incubating samples with antibodies cor-

responding to PPAR α , PPAR δ , or PPAR γ . Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:bisacrylamide) and run in 0.5 $\times$ TBE (45 mmol/L Tris-HCl, 45 mmol/L boric acid, 1 mmol/L EDTA) for 1 hour at constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under a vacuum at 80°C for 1 hour, and exposed to photographic film at -70°C with an intensifying screen. Densitometric analysis was performed using ImageQuant.

Materials

The primary antibodies directed at I κ B α , PPAR δ , RXR α , p65/NF- κ B subunit, BCL6, secondary antibodies, oligonucleotides for NF- κ B and PPARs, and the nonsilencing RNA and PPAR δ -siRNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The primary antibodies directed at PPAR α and PPAR γ were obtained from Thermo Scientific (Rockford, IL). The primary antibody directed at β -actin was obtained from Abcam (Cambridge, MA). When not specified otherwise, all other chemicals and the PPAR δ ligand GW0742 were from Sigma/Aldrich (St. Louis, MO).

Data Analysis

All values in the figures and text are expressed as mean $\pm$ SEM of n observations ($n = 3$ to 17 animals for each group for the *in vivo* experiments; $n = 3$ independent experiments in duplicate for the *in vitro* experiments). The results were examined by analysis of variance followed by the Bonferroni's correction post hoc *t*-test. The Kaplan-Meier log-rank and Gehan-Breslow analyses were used to compare differences in survival rates. A *P* value less than 0.05 was considered significant.

Results

Improvement of Early Hypotension and Survival Rate in Septic Rats Treated with the PPAR δ Ligand

To imitate the clinical scenario of polymicrobial sepsis, rats were subjected to CLP, a well-characterized procedure that induces peritonitis.²⁰ As expected, at 4 hours after CLP vehicle-treated rats exhibited a significant hypotension, which further worsened up to 6 hours after CLP. Treatment with the synthetic PPAR δ ligand GW0742 blunted this initial hypotension: although GW0742-treated animals experienced a transitory hypotension at 4 hours after CLP, mean arterial blood pressure was maintained at normal values at all time points (Figure 1A).

In a second study, we evaluated survival to a more severe model of injury, as obtained by cecal ligation and triple puncture with a 16-gauge needle (CL3P). Under this severe condition, GW0742-treated rats had a higher survival rate (66%) than vehicle-treated rats (33%) at 72 hours after CL3P (Figure 1B).

Figure 1. **A:** Effect of *in vivo* treatment with GW0742 on mean arterial blood pressure in rats subjected to cecal ligation and puncture (CLP). Data represent the mean $\pm$ SEM of 3 to 17 animals for each time point. * $P < 0.05$ versus time 0 in the same treatment group subjected to CLP. **B:** Effect of *in vivo* treatment with GW0742 on survival in rats ($n = 15$ for each group) subjected to cecal ligation and triple puncture (CL3P). * $P < 0.05$ versus vehicle-treated group at Gehan-Breslow analysis. GW0742 (0.5 mg/kg i.p.) was administered at the time of CLP or CL3P procedure and at 6 and 12 hours thereafter.

Reduction of Neutrophil Infiltration in Major Organs in Septic Rats Treated with the PPAR δ Ligand

Since a serious consequence of sepsis is the occurrence of multiple organ failure, which is preceded by accumulation of neutrophils in major vital organs, we next evaluated neutrophil infiltration in the lung, liver, and cecum by measurement of the activity of MPO, an enzyme specific to neutrophil lysosomes. MPO activity was significantly elevated at 18 hours after CLP in vehicle-treated rats. Treatment with GW0742 reduced MPO activity approximately by 25% in the lung (Figure 2A), 78% in the liver (Figure 2B), and 39% in the cecum (Figure 2C), thus suggesting a reduction in inflammatory cell influx in these organs.

Figure 2. Effect of *in vivo* treatment with GW0742 (0.5 mg/kg i.p.) on myeloperoxidase (MPO) activity in the lung (**A**), liver (**B**), and cecum (**C**) in rats at 18 hours after cecal ligation and puncture (CLP). Each data point represents the mean $\pm$ SEM of 4 to 10 animals for each group. * $P < 0.05$ versus sham rats. ** $P < 0.05$ versus vehicle-treated rats. Treated rats received GW0742 at the time of CLP procedure and at 6 and 12 hours thereafter.

Reduction of the Inflammatory Response in Septic Rats Treated with the PPAR δ Ligand

To also investigate the effect of GW0742 on systemic inflammatory response, we evaluated plasma levels of the cytokines TNF α , IL-1 β , IL-6, the adipokine leptin, and the chemokine MCP-1. A substantial increase in all of these inflammatory mediators was found in vehicle-treated rats after CLP. Treatment with GW0742 reduced the early production of TNF α at 3 hours after CLP (Figure 3A). This inhibition was then followed by a later significant decline of IL-6 at 6 hours (Figure 3B) and IL-1 β , leptin and MCP-1 at 18 hours after CLP (Figure 3, C–E).

Figure 3. Effect of *in vivo* treatment with GW0742 (0.5 mg/kg i.p.) on plasma levels of TNF α (A), IL-6 (B), IL-1 β (C), leptin (D), and MCP-1 (E) in rats at 18 hours after cecal ligation and puncture (CLP). Each data point represents the mean $\pm$ SEM of 3 to 10 animals for each group. **P* < 0.05 versus sham rats. ***P* < 0.05 versus vehicle-treated rats. Treated rats received GW0742 at the time of CLP procedure and at 6 and 12 hours thereafter.

Because macrophages are an important source of cytokines during sepsis,²¹ in *ex vivo* studies we harvested resident peritoneal macrophages from vehicle- or GW0742-treated rats at 6 hours after CLP and we evaluated their ability to release TNF α and their nuclear expression of PPAR δ . We observed that under basal conditions peritoneal macrophages of sham rats did not express PPAR δ in the nucleus. However, PPAR δ appeared in the nucleus after CLP and its expression was further enhanced by *in vivo* treatment with GW0742 (Figure 4A). *In vivo* treatment with the PPAR δ ligand also inhibited *ex vivo* TNF α production (Figure 4B), and this reduction was associated with further up-regulation of nuclear expression of PPAR δ (Figure 4A). Thus, these data suggest that activation of PPAR δ may improve outcome of septic animals, probably by tempering the inflammatory cytokine response to bacterial stimuli.

Down-Regulation of PPAR δ Expression and Up-Regulation of NF- κ B Activation in the Liver Following Polymicrobial Sepsis

Since PPAR δ is highly abundant in the liver, where it functions as a modulator for lipid homeostasis,⁶ we next investigated whether induction of polymicrobial sepsis was associated with changes in PPAR δ expression. To determine the inflammatory signaling pathways affected by GW0742 treatment, we also investigated the kinetics of NF- κ B activation. By Western blot analysis, we observed that the nuclear content of PPAR δ decreased in a

time-dependent manner in livers of vehicle-treated rats subjected to CLP (Figure 5A). This event was associated with cytosolic degradation of I κ B α (Figure 5B) and increased DNA binding of NF- κ B (Figure 5, C and E). Treatment with GW0742 reversed the reduction of PPAR δ expression, whereas it decreased NF- κ B activation. However, treatment with GW0742 did not affect degradation of I κ B α (Figure 5B). Thus, it appears that activation of PPAR δ may regulate the activation of the pro-inflammatory NF- κ B at the nuclear level in the liver. Similarly, treatment with GW0742 significantly reduced DNA binding of NF- κ B in septic lungs (Figure 5, D and F), thus confirming the beneficial effects of PPAR δ on organ injury.

Increased Mortality, Cytokine Production, and Lung Injury in PPAR δ -Deficient Mice Following Polymicrobial Sepsis

To confirm the physiological role of PPAR δ in sepsis, we performed additional studies in genetically altered mice. Because PPAR δ gene knockout leads to high embryonic lethality or growth retardation in surviving mice,^{18,22} heterozygous PPAR δ ^{+/-} mice (ie, with partial PPAR δ deficiency) and wild-type control PPAR δ ^{+/+} mice were used in a model of CLP. In a survival study, heterozygous PPAR δ ^{+/-} mice were more susceptible to the lethal effect of CLP when compared to wild-type mice; 92% of PPAR δ ^{+/-} mice and 33% of wild-type mice were dead within 5 days after CLP (Figure 6). In the lung of wild-type

Figure 4. Effect of *in vivo* treatment with GW0742 (0.5 mg/kg i.p.) on PPARδ expression and production of TNFα in *ex vivo* rat peritoneal macrophages. **A:** Western blot analysis of PPARδ and β-actin (used as loading control protein) in nuclear extracts. Blot is representative of three independent experiments. **B:** Supernatant levels of TNFα production. Data are mean ± SEM of three independent experiments. **P* < 0.05 versus sham rats. ***P* < 0.05 versus vehicle-treated rats. Peritoneal macrophages were harvested at 6 hours after cecal ligation and puncture (CLP). Treated rats received GW0742 at the time of CLP procedure.

mice, histological examination revealed extravasation of red cells and accumulation of inflammatory cells into the air spaces at 18 hours after CLP (Figure 7A, C, E, and G). In *PPARδ*^{+/-} mice, lung injury was more severe and consisted of reduced alveolar air spaces, large hemorrhagic areas, infiltration of inflammatory cells, and obstructed bronchial airways (Figure 7, B, D, F, and H). The larger influx of neutrophils in *PPARδ*^{+/-} mice was further confirmed by a marked increase of tissue MPO activity when compared to values of wild-type mice (Figure 8A). Increased mortality of heterozygous *PPARδ*^{+/-} mice was also associated with higher plasma levels of the pro-inflammatory keratinocyte-derived chemokine and TNFα (Figure 8, B and C). Thus, a partial deficiency of *PPARδ* seems to negatively impact the outcome of septic animals, probably by enhancing the inflammatory cytokine response and lung neutrophil infiltration.

Increased Lung NF-κB Activation in Septic *PPARδ*-Deficient Mice

Having established that sepsis is more severe in heterozygous *PPARδ*^{+/-} mice, we next determined whether the different pathophysiologic response to bacterial stimuli *in vivo* correlates to different function of DNA binding of PPARδ and NF-κB in the lung. Thus, we assessed the DNA binding of PPARs by EMSA using an oligonucleotide with binding site for all three PPAR receptor types α, γ, and δ.²³ We found that in sham animals of both genotypes there was a constitutive binding of PPARs, which

was markedly down-regulated at 18 hours after CLP (Figure 9, A, first panel, and 9C). The presence of PPARδ binding sites within the complex was confirmed by the addition of specific anti-PPARδ antibody, which resulted in the appearance of a supershifted complex in wild-type *PPARδ*^{+/+} mice. As expected, supershift analysis demonstrated that heterozygous *PPARδ*^{+/-} mice exhibited a modest constitutive binding of PPARδ to DNA in the lung at basal conditions, which was significantly blunted after CLP when compared with wild-type animals (Figure 9B). Supershift analysis also revealed the presence of PPARα and PPARγ binding: nuclear extracts treated with specific anti-PPARα antibody produced a prominent supershift band; whereas a weaker and lower band was detected using anti-PPARγ antibody. Induction of sepsis appeared also to down-regulate binding activity of PPARα and nearly eliminated PPARγ band in both genotypes (Figure 9B). On the contrary, *PPARδ* deficiency was associated with higher DNA binding activity of NF-κB at 18 hours after CLP when compared with wild-type animals (Figure 9, A, second panel, and D).

Increased Liver NF-κB Activation in Septic *PPARδ*-Deficient Mice

Similarly to the changes observed in the lung, we also found that constitutive DNA binding of PPARs was reduced under basal conditions in the liver of heterozygous *PPARδ*^{+/-} mice when compared to wild-type *PPARδ*^{+/+} mice. Binding of PPARs was further down-regulated in both genotypes after CLP. However, DNA binding of NF-κB was significantly higher in heterozygous *PPARδ*^{+/-} mice when compared to wild-type *PPARδ*^{+/+} mice after CLP (Figure 10, A–B). Furthermore, to rule out off-target effects of the *PPARδ* gene deletion on expression of other receptors, we evaluated the nuclear expression of PPARδ, PPARγ, PPARα, and RXRα by Western blot analysis (Figure 10, C–F). In sham heterozygous *PPARδ*^{+/-} mice, PPARδ expression was significantly reduced at basal conditions; whereas there was no difference in the basal expression of the other receptors. After sepsis, PPARδ along with PPARγ, PPARα, and RXRα was down-regulated in both genotypes of mice; however, the degree of PPARδ and PPARα decrease was significantly worse in heterozygous *PPARδ*^{+/-} mice when compared with wild-type mice. Thus, our data suggest that partial loss of PPARδ function further enhances the inflammatory response, which in turn may further affect PPARα expression.

PPARδ Regulation of TNFα Production in J774.A1 Macrophages

Based on these *in vivo* observations, we investigated the molecular anti-inflammatory mechanisms of PPARδ activation in LPS-stimulated J774.A1 macrophages. Unstimulated macrophages did not express PPARδ in the nucleus. However, on stimulation with LPS (100 ng/ml) PPARδ nuclear expression increased and was further enhanced in the presence of GW0742 (Figure 11A). Stim-

Figure 5. Sepsis induces down-regulation of PPAR δ expression and up-regulation of NF- κ B activity. **A:** Western blot analysis of PPAR δ and β -actin (used as loading control protein) in nuclear extracts of livers. **B:** Image analysis of $\text{I}\kappa\text{B}\alpha$ expression relative to β -actin expression in cytosolic extracts of livers determined by densitometry of Western blots ($n = 3$ time-course experiments). **C** and **D:** Representative autoradiographs of EMSA for NF- κ B DNA binding in liver (**C**) and lung (**D**); DNA binding complexes are indicated with a left bracket. **E** and **F:** Image analyses determined by densitometry of three independent EMSAs for DNA binding of NF- κ B in liver (**E**) and lung (**F**). Fold increase was calculated versus respective sham value (time 0) set to 1.0. Tissue samples were obtained from vehicle-treated and GW0742-treated rats at 0, 1, 3, 6, and 18 hours after cecal ligation and puncture (CLP). Treatment with vehicle or GW0742 (0.5 mg/kg i.p.) was given at the time of CLP procedure and at 6 and 12 hours thereafter. * $P < 0.05$ versus vehicle-treated group.

ulation of macrophages with LPS for 24 hours induced a marked production of TNF α , which was inhibited by pretreatment with the PPAR δ ligand GW0742 in a dose-dependent manner (Figure 11B). The possible involvement of PPAR δ in TNF α production was further examined using a knockdown strategy by siRNA specific to PPAR δ . As shown in Figure 11C, treatment of PPAR δ -siRNA transfected cells with the ligand GW0742 did not inhibit LPS-induced increase TNF α production, thus confirming that inhibition of the cytokine release by GW0742 is PPAR δ -dependent.

Figure 6. Mortality was significantly higher in heterozygous PPAR $\delta^{+/-}$ than wild-type PPAR $\delta^{+/+}$ mice subjected to polymicrobial sepsis ($P = 0.007$ at log-rank analysis). Wild-type PPAR $\delta^{+/+}$ ($n = 12$) and heterozygous PPAR $\delta^{+/-}$ ($n = 12$) mice underwent cecal ligation and puncture (CLP), and were monitored for survival for five days.

PPAR δ Recruitment of BCL6 and Direct Transrepression of TNF α Expression at the NF- κ B Promoter Region

Since the *de novo* synthesis of TNF α in macrophages is regulated by NF- κ B transcription,²⁴ we also determined the activation of the NF- κ B signaling pathway. In a time-course study, we observed that the cytosol content of $\text{I}\kappa\text{B}\alpha$ was degraded as early as 15 minutes after LPS stimulation, and was replenished thereafter (Figure 12, A). In cells pretreated with the PPAR δ ligand GW0742 (100 nmol/L), $\text{I}\kappa\text{B}\alpha$ was similarly degraded at 15 minutes after LPS stimulation. In a subsequent experiment, macrophages were transfected with a 3xNF- κ B promoter-luciferase reporter plasmid. On expression of the reporter gene, luciferase activity was increased at 4 hours after LPS stimulation in comparison to control cells, thus suggesting an increase of NF- κ B activation. Pretreatment of the cells with the PPAR δ ligand significantly reduced NF- κ B activation in a dose-dependent manner (Figure 12B). Thus, our data demonstrated that PPAR δ activation inhibits NF- κ B at the nuclear level without affecting the upstream cytosolic events.

Since our data suggested that PPAR δ may modulate inflammatory mediator production by regulating nuclear events, we next evaluated whether PPAR δ may directly interfere with the NF- κ B binding to the specific κ B elements situated in the promoter region of TNF α by chro-

Figure 7. Representative histology photomicrographs of lung sections of heterozygous *PPARδ*^{+/-} and wild-type *PPARδ*^{+/+} mice subjected to polymicrobial sepsis. Lungs were harvested 18 hours after CLP and were stained with H&E. **A** and **B**: Normal lung architecture in a sham *PPARδ*^{+/+} mouse (**A**) and a sham *PPARδ*^{+/-} mouse (**B**) at magnification $\times 100$; **C** and **D**: Normal lung architecture in a sham *PPARδ*^{+/+} mouse (**C**) and a sham *PPARδ*^{+/-} mouse (**D**) at magnification $\times 400$. **E** and **F**: Lung derangement in a *PPARδ*^{+/+} septic mouse (**E**) and a *PPARδ*^{+/-} septic mouse (**F**) at magnification $\times 100$. **G** and **H**: Lung derangement in a *PPARδ*^{+/+} septic mouse (**G**) and a *PPARδ*^{+/-} septic mouse (**H**) at magnification $\times 400$. A similar pattern was seen in $n = 3$ to 6 different tissue sections in each experimental group.

matin immunoprecipitation analysis. The recruitment of the transcription repressor BCL6 was also evaluated. J774.A1 macrophages were pretreated with GW0742 for 30 minutes and stimulated with LPS (100 ng/ml) for 1 hour afterward or remained as controls. Under control conditions and on LPS stimulation, *PPARδ* as well as the co-repressor BCL6 weakly associated with the κ B elements of the *TNFα* promoter. Pretreatment with GW0742 further increased *PPARδ* and BCL6 association with the *TNFα* promoter. On the contrary, the p65 subunit of NF- κ B strongly associated with the *TNFα* promoter in response to LPS. Pretreatment with GW0742 prevented the p65 subunit of NF- κ B from binding the promoter region in a dose-dependent manner (Figure 12, C–D). Thus, our data demonstrate that *PPARδ* inhibits *TNFα* production by direct inhibition of NF- κ B transactivation.

Figure 8. Inflammatory response is enhanced in heterozygous *PPARδ*^{+/-} mice after polymicrobial sepsis. **A**: Myeloperoxidase (MPO) activity in the lung, **(B)** plasma levels of keratinocyte-derived chemokine (KC), and **(C)** *TNFα* in heterozygous *PPARδ*^{+/-} and wild-type *PPARδ*^{+/+} mice 18 hours after cecal ligation and puncture (CLP). Each data point represents the mean $\pm$ SEM of 3 to 6 animals for each group. * $P < 0.05$ versus sham mice of the same genotype. ** $P < 0.05$ versus wild-type mice.

Discussion

The present study demonstrates the anti-inflammatory role of *PPARδ* and the therapeutic potential of its activation in a clinically relevant model of sepsis. Specifically, we demonstrate that during sepsis *PPARδ* is down-regulated in the liver and lung. Genetic *PPARδ* deficiency enhances lung injury, systemic release of pro-inflammatory cytokines, and mortality in septic mice. On the contrary, treatment with the *PPARδ* ligand GW0742 blunts early hypotension, reduces neutrophil infiltration in lung, liver, and cecum, and the plasma elevation of cytokines in septic rats. The *in vivo* protective effects of *PPARδ* activation are probably due to the fact that the initial sequence of inflammatory events, mainly production of pro-inflammatory mediators and recruitment of neutrophils in major organs, is interrupted through a negative

Figure 9. PPAR δ deficiency is associated with increased activity of NF- κ B in the lung after polymicrobial sepsis. **A:** Representative autoradiographs of EMSA for PPARs (first panel) and NF- κ B (second panel) DNA binding in the lung of heterozygous PPAR $\delta^{+/-}$ and wild-type PPAR $\delta^{+/+}$ mice 18 hours after cecal ligation and puncture (CLP). **B:** Specificity of DNA binding was confirmed by incubation with cold oligonucleotide for PPARs. Supershift assay was also performed in samples incubated with antibodies against PPAR α , PPAR δ , or PPAR γ . **C and D:** Image analyses of DNA binding of PPAR (**C**) and NF- κ B (**D**) in the lung determined by densitometry. Fold increase was calculated versus respective sham value set to 1.0. Data represent the mean $\pm$ SEM of 3 to 5 animals for each group. * P < 0.05 versus sham mice of the same genotype. ** P < 0.05 versus wild-type mice.

modulation of NF- κ B-mediated transcription. Our hypothesis is supported by the observation that PPAR δ directly interacts to the TNF α promoter and prevents the p65 subunit of NF- κ B from binding to its κ B response elements region in LPS-stimulated macrophages.

There is emerging evidence that the acute phase response, which is induced by infection, results in marked down-regulation of nuclear hormone receptors involved in the lipid and glucose metabolism.^{17,25–27} We have previously demonstrated that the onset of inflammation and cardiovascular derangement of septic shock is preceded by down-regulation of PPAR γ expression in bronchial epithelial cells, smooth muscle, and endothelial cells of thoracic aortas.¹⁷ Experimental *in vitro* studies indicate that pro-inflammatory cytokines can directly induce changes in the levels of RXR α , PPAR α , PPAR γ , and

liver X receptor- α in Hep3B human hepatoma cells.²⁵ Similarly, hearts from endotoxin-treated mice exhibited reduced expression of PPAR γ , RXR α , and thyroid receptor.²⁶ As with these nuclear receptors, TNF α has also been shown to down-regulate PPAR δ expression and function in oligodendrocytes.²⁷ In our study, we observed that during polymicrobial sepsis expression and DNA binding function of PPAR δ are altered in the liver and lung. Consistent with the previous studies demonstrating that several nuclear receptors are down-regulated during an inflammatory response,^{17,25–27} we also observed that the other receptors PPAR α , PPAR γ , and RXR α were significantly decreased after CLP compared with basal values of sham normal animals. Interestingly, the expression of PPAR δ decreased in a time-dependent fashion during sepsis in rats. This temporal decrease well correlated with the occurrence of hypotension and the severity of the inflammatory response. Of more importance, using a genetic loss-of-function approach, our data also reveal for the first time that PPAR δ has a crucial role in the regulation of the inflammatory process after bacterial infection *in vivo*, as PPAR δ -deficient mice were considerably more susceptible to sepsis than wild-type mice.

Contrary to the down-regulation observed in liver and lung *in vivo* after polymicrobial sepsis, we observed that bacterial challenge *in vivo* or LPS stimulation *in vitro* induced nuclear expression of PPAR δ in macrophages. Previous studies have reported that PPAR δ is an important regulator of lipid uptake and efflux in macrophages and participates to the control of the chronic atherogenic inflammation in mice.^{28–30} In our study, induction of PPAR δ by LPS correlated with a pro-inflammatory phenotype of the macrophage. Interestingly, treatment with the PPAR δ ligand further increased PPAR δ expression and suppressed TNF α production in a dose-dependent manner, thus indicating a direct involvement of PPAR δ in the control of the bacterial-induced inflammatory response. Additional *in vitro* studies confirm that PPAR δ ligands are cytoprotective, suppressing the expression of LPS-induced pro-inflammatory genes, including inducible nitric oxide synthase, cyclooxygenase-2, and cytokines.^{28,30,31} Taken together with our data, these studies suggest that changes of PPAR δ expression and function, which are induced by microbial pathogens, may also depend on the cell immunological function and specificity.

In our study, the biological relevance of PPAR δ during sepsis is also underlined by the fact that the activation of the receptor by the exogenous ligand GW0742 results in potent anti-inflammatory effects both *in vivo* in polymicrobial sepsis and *in vitro* in LPS-stimulated macrophages. The mechanism by which the PPAR δ ligand reduces the sepsis-induced systemic inflammatory response appears to involve regulation of the NF- κ B-mediated transcription. In support of this hypothesis, in our study we found that septic PPAR δ -deficient mice had increased binding of NF- κ B in the lung when compared to wild-type mice. *In vitro* reports have suggested that the NF- κ B pathway control several inflammatory genes, such as the gene for cytokines and chemokines.³ Numerous experimental studies have proven that activation of NF- κ B is implicated in sepsis.^{4,17,32} With particular clinical relevance, NF- κ B

Figure 10. PPAR δ deficiency is associated with increased activity of NF- κ B in the liver after polymicrobial sepsis. **A** and **B**: Image analyses of DNA binding of PPAR (**A**) and NF- κ B (**B**) in the liver determined by densitometry. Fold increase was calculated versus respective sham value set to 1.0. Data represent the mean $\pm$ SEM of 3 to 5 animals for each group. * P < 0.05 versus sham mice of the same genotype. ** P < 0.05 versus wild-type mice. **C–F**: Image analysis of PPAR δ (**C**), PPAR γ (**D**), PPAR α (**E**), and RXR α (**F**) expression relative to β -actin expression in nuclear extracts of livers determined by densitometry of Western blots (n = 3 to 5 animals for each group).

binding activity has been found to be increased in patients with acute inflammation and sepsis and to be correlated with clinical severity and mortality.³³ NF- κ B activation increased markedly before death in mononuclear leukocytes of patients with systemic inflammatory response syndrome.³⁴ In our study, we observed a temporal correlation between NF- κ B activation and the inflammatory response as NF- κ B was significantly increased already at 1 hour after CLP and appeared to precede or coincide with the increase in plasma cytokines in the rat. The PPAR δ ligand was able to blunt the systemic inflammatory response and to reduce neutrophil infiltration in major vital organs, thus suggesting a negative modulation of gene expression of cytokines and chemokines. We found that *in vivo* treatment of rats with GW0742 reduced significantly lung and liver activation of NF- κ B at late time points only, ie, at 6 and 18 hours after CLP. This inhibition temporally correlated with a significant suppression of the systemic release of IL-6, IL-1 β , MCP-1, and leptin. However, consistent with the role of TNF α as important early mediator of the innate host inflammatory response in stimulating and/or enhancing the excessive production of other pro-inflammatory cytokines and adipokines,³⁵ we also found that that treatment with GW0742 significantly inhibited the release of circulating levels of this cytokine in the early phase of sepsis (ie, at 3 hours after CLP). Thus, taken together, these data suggest that in addition to lung and liver tissues the anti-inflammatory effects of

GW0742 may target other immune or parenchymal cells, including TNF α -producing cells, especially at earlier time points of the inflammatory response.

The present study is in agreement with other reports that ascribe to PPAR δ a regulatory role on transcription and inflammatory mediator production. Other PPAR δ ligands have been shown to inhibit cytokine-induced expression of VCAM-1 in endothelial cells¹³ and to suppress expression of MCP-1 and IL-1 β in bone marrow-derived macrophages.²⁸ In murine cardiomyocytes the PPAR δ ligand GW0742 suppressed LPS-induced TNF α through inhibition of NF- κ B. Similarly, overexpression of PPAR δ substantially decreased TNF α , whereas overexpression of a mutant PPAR δ abrogated the inhibitory effect.¹⁴ Under basal conditions NF- κ B is retained in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits, by association with its inhibitory protein I κ B α . On stimulation with a variety of pro-inflammatory signals such as LPS, TNF α , interleukins, oxidants, bacteria, and viruses, I κ B α is phosphorylated and degraded. These events allow NF- κ B to translocate into the nucleus where it binds to κ B elements and initiate transcription.^{3,4} We found that GW0742, while inhibiting the inflammatory action of LPS, did not prevent the LPS-induced cytosolic degradation of I κ B α in macrophages, thus indicating that PPAR δ acts as an inhibitory modulator of NF- κ B at the nuclear level. In support of a nuclear function of PPAR δ , our results showed that the PPAR δ

Figure 11. PPAR δ activation reduced TNF α production in LPS-stimulated J774.A1 macrophages. **A:** Western blot analysis of PPAR δ and β -actin (used as loading control protein) in nuclear extracts of macrophages stimulated with LPS (100 ng/ml) up to 24 hours. Blot is representative of three independent experiments. **B:** Effect of the PPAR δ ligand GW0742 (10–1000 nmol/L) on TNF α production at 24 hours after LPS stimulation. **C:** Effect of GW0742 (100 nmol/L) on TNF α production at 24 hours after LPS stimulation in macrophages transfected with scramble siRNA or PPAR δ -siRNA. Data are mean $\pm$ SEM of three independent experiments performed in quadruplicate. * P < 0.05 versus control cells with media alone. ** P < 0.05 versus vehicle treatment.

ligand also affected the protein expression of its own receptor. Treatment with GW0742 was, in fact, able to revert down-regulation of PPAR δ , which returned to normal constitutive expression in the liver of septic rats *in vivo*, and to increase nuclear content in LPS-stimulated macrophages *in vitro*. On the contrary, gene silencing of endogenous PPAR δ abrogated the anti-inflammatory effects of GW0742 in macrophages. Thus, these results prove that the cytoprotective properties of GW0742 are due to the maintenance and/or recruitment of PPAR δ at the nuclear site in target cells.

All members of the PPAR family exert a bimodal regulatory mechanism on transcription. PPARs heterodimerize with RXR and recognize direct PPAR response elements on promoter region of target genes and induce transcription. Similar to other nuclear receptors, PPARs are also able to bind to co-repressors or other transcription factors and cause transrepression. Among the PPAR

Figure 12. PPAR δ inhibits TNF α production by direct inhibition of NF- κ B transactivation. **A:** Western blot analysis of I κ B α expression in cytosol extracts of macrophages stimulated with LPS (100 ng/ml) up to 24 hours with or without GW0742 (100 nmol/L). Blot is representative of three independent experiments. Equal loading was confirmed by Ponceau staining after transfer of proteins on nitrocellulose membrane (data not shown). **B:** Effect of GW0742 on NF- κ B promoter activity 4 hours after LPS stimulation. Cells were transfected with a 3xNF- κ B promoter-luciferase plasmid and a Renilla luciferase vector (for normalization). Promoter activity of NF- κ B was evaluated by luciferase assay and was expressed as fold increase of activity of control basal cells set to 1. * P < 0.05 versus cells with media alone (basal). **C:** Representative PCR gels from chromatin immunoprecipitation assays demonstrating binding of p65 subunit of NF- κ B, PPAR δ , and BCL6 to TNF α promoter. Chromatin was immunoprecipitated using an antibody recognizing the p65 subunit of NF- κ B, PPAR δ , or BCL6. Positive control (Input) was chromatin recovered without immunoprecipitation. Cells were stimulated with LPS (100 ng/ml) for 1 hour with or without GW0742 (10 to 100 nmol/L), which was added to the cells 30 minutes prior LPS. PCR was performed with primers specific for the murine TNF α promoter region spanning the most proximal NF- κ B binding site. **D:** Image analysis of promoter binding relative to Input recovery. Data are mean $\pm$ SEM of three independent experiments. * P < 0.05 versus cells with media alone (basal). ** P < 0.05 versus vehicle treatment.

family members, PPAR δ appears to distinguish itself for a more pronounced repression activity. This inhibitory mechanism may also occur in the absence of a specific ligand.³⁶ In our *in vitro* studies, the mechanism by which activation of PPAR δ results in reduced production of TNF α , whose gene is NF- κ B-dependent, seems to involve reduced interaction of NF- κ B with its *cis*-regulatory elements. We observed, in fact, that pretreatment with GW0742 of LPS-treated macrophages caused a dramatic dose-dependent reduction in the binding of the p65 subunit to the κ B site of the TNF α promoter. This reduction was inversely correlated with increased binding of PPAR δ to the promoter, confirming the involvement of this nuclear receptor in the changes of NF- κ B binding observed in the *in vivo* studies. We also found that GW0742 increased the association of the transcriptional repressor BCL6 with the TNF α promoter. Transcriptional regulation of TNF α can involve the activation of distinct sets of transcription factors binding at least two regions of the

promoter, which contains NF- κ B, Egr-1, cAMP-response element, and activator-protein-1 binding sites.^{24,37,38} Therefore, since the 5'-flanking regulatory regions of *TNF α* gene appears to lack PPRE, our results suggest that once the ligand-activated PPAR δ recruits BCL6 to the κ B promoter regions it prevents the binding of the p65 subunit of NF- κ B to its κ B response element and thereby inhibits its ability to induce gene transcription. Our data further confirm previous studies that have suggested that BCL6 and PPAR δ associate in controlling expression of pro-inflammatory cytokines when PPAR δ is in its repressive mode in smooth muscle or immune competent cells.^{15,28} It has been also reported that the lack of BCL6 expression in pancreatic β cells prevented PPAR δ -mediated repression of inflammatory responses.³⁹ In addition to recruitment of co-repressors, it is possible that PPAR δ may interact directly with NF- κ B subunits. For example, PPAR δ physically interacted with p65 in psoriatic lesions, leading to a p65-dependent repression of PPAR δ , but not of PPAR α or PPAR γ .⁴⁰

However, since PPAR δ activation has been shown to induce key genes involved in lipid metabolism, we cannot rule out that other beneficial effect of GW0742 in sepsis may be secondary to energy homeostasis. Nevertheless, it has been recently demonstrated that, even in conditions of dysmetabolism, PPAR δ appears to mediate anti-atherogenic effects mainly through inhibition of vascular inflammation and macrophage recruitment rather than through regulation of macrophage cholesterol homeostasis.^{41,42} Consistent with this notion, our data predict a model where PPAR δ /BCL6 associates with κ B binding sites of various pro-inflammatory mediators, thus interfering with NF- κ B transactivation and the subsequent inflammatory process.

In summary, our results demonstrate the biological relevance of PPAR δ in the innate immune process of sepsis and identify a novel role of PPAR δ as a crucial anti-inflammatory factor. Although it is difficult to extrapolate our findings to the human condition, especially considering that the other side of anti-inflammation is immunoparalysis, which compromises the recovery of the patient, our data clearly show that treatment with a PPAR δ ligand confers a survival advantage in septic animals. Therefore, being ubiquitously present in many organs and also being induced in macrophages by bacterial challenge, PPAR δ can represent an ideal target for the clinical treatment of sepsis. Nevertheless, other studies, investigating whether PPAR δ activation may also help in maintaining immune competence, are certainly required.

Acknowledgments

We are grateful to Dr. Gary Gilkeson (Medical University of South Carolina, Charleston, South Carolina) for providing the heterozygous PPAR δ -deficient (PPAR $\delta^{+/-}$) and the wild-type homozygous PPAR $\delta^{+/+}$ mice.

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