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PPAR-γ/IL-10 axis inhibits MyD88 expression and ameliorates murine polymicrobial sepsis

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Abstract

Polymicrobial sepsis induces organ failure and is accompanied by overwhelming inflammatory response and impairment of microbial killing. Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a nuclear receptor with pleiotropic effects on lipid metabolism, inflammation, and cell proliferation. The insulin-sensitizing drugs thiazolidinediones (TZDs) are specific PPAR-γ agonists. TZDs exert anti-inflammatory actions in different disease models, including polymicrobial sepsis. The TZD pioglitazone, which is an FDA-approved drug, improves sepsis outcome; however, the molecular programs that mediate the effect of pioglitazone have not been determined. In a murine model of sepsis, we now show that pioglitazone treatment acts to improve microbial clearance and to enhance neutrophil recruitment to the site of infection. We also observed reduced pro-inflammatory cytokine production and high IL-10 levels in pioglitazone-treated mice. These effects were associated with a decrease in STAT-1-dependent expression of myeloid differentiation factor-88 (MyD88) *in vivo* and *in vitro*. IL-10R blockage abolished PPAR-γ-mediated inhibition of MyD88 expression. These data demonstrate that the primary mechanism by which pioglitazone protects against polymicrobial sepsis is by impairing MyD88 responses. This appears to represent a novel regulatory program. In this regard, pioglitazone provides advantages as a therapeutic tool, since it improves different aspects of host defense during sepsis, ultimately enhancing survival.

INTRODUCTION

The overwhelming production of systemic inflammatory mediators is a key feature in severe sepsis and is commonly associated with organ dysfunction and death (1). An important role for myeloid differentiation factor (MyD88), an essential adaptor protein in the TLR/IL-1R1 signaling pathway, has been shown in the production of inflammatory mediators during

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sepsis. *Myd88*-deficient mice fail to produce high levels of IL-6, CXCL1, and CXCL2 during cecal ligation and puncture (CLP)-induced sepsis (2). Therefore, it should be possible to reduce systemic inflammation by modulating MyD88 expression or activity. The expression of MyD88 has been shown to be upregulated by lipopolysaccharide (LPS) and inflammatory mediators such as TNF-α and IFN-γ (3) and downregulated by IL-10 (4). Moreover, we have previously demonstrated that leukotriene B_4 also induces MyD88 expression in a STAT-1-dependent manner (5).

Thiazolidinediones (TZD) are potent insulin-sensitizing drugs that function via activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ). Pioglitazone is a TZD approved by the U.S. Food and Drug Administration used for type 2 diabetes treatment. Rosiglitazone and troglitazone also show potent insulin-sensitizing properties, but they were removed from the market due to undesired side effects (6). In addition to the glucose control, the activation of PPAR-γ by TZDs has also been involved in the modulation of atherosclerosis, adipocyte proliferation and inflammation (7). Indeed, natural and synthetic PPAR-γ ligands have been tested in experimental models of sepsis due to their known anti-inflammatory effects. Evidences show that treatment with TZDs and natural ligands for PPAR- γ reduces the release of IL-6, IL-10, TNF-α, CCL2 and also the neutrophil infiltration in lung and liver induced by polymicrobial sepsis or endotoxemia (8, 9). In addition, treatment of endotoxemic mice with PPAR-γ agonists reduces levels of high mobility group box 1 and markers for organ dysfunction. As consequence, survival of mice is significantly increased by those treatments (8, 10–12). However, the mechanism for the anti-inflammatory effects of PPAR-γ agonists is not fully understood. A well accepted hypothesis is that activated PPAR-γ directly interacts with a transcription factor, resulting in its transrepression (13). Indeed, the synthetic PPAR-γ agonist ciglitazone reduces IKK activity and IκB degradation induced by hemorrhagic-shock (9). Along these lines, the natural PPAR-γ agonist 15d-PGJ2 reduces IFN-α and -γ induced STAT-1 phosphorylation in different cell types (14).

The aim of this study was to determine whether PPAR-γ impairs STAT-1 activation thereby reducing STAT-1-dependent MyD88 expression during CLP-induced sepsis. We found that treatment with pioglitazone, a synthetic agonist of PPAR-γ, inhibited STAT-1 phosphorylation in an IL-10R dependent manner, leading to a reduction in MyD88 expression in peritoneal cells. Pioglitazone treatment increased neutrophil migration to the peritoneal cavity, culminating in reduced bacterial load and a reduced systemic inflammatory response during CLP-induced sepsis.

METHODS

Mice

Female C57Bl/6 mice (8-week old, weight 18–23 g) were obtained from Jackson Laboratory (Bar Harbor, USA). Doxycycline-controlled dnPPARγ-transgenic mice, were bred at IUSM (15). Mice were maintained according to NIH guidelines for the use of experimental animals with the approval of the Indiana University Committees for the Use and Care of Animals.

Sepsis induction

Sepsis was induced by CLP as previously described (16), using a 26G or 18G needle to induce moderate or lethal sepsis, respectively. A group of mice was treated with pioglitazone (20 mg/kg, i.p., Cayman Chemical), 18 hours before CLP surgery. Survival was monitored at each 12 hours for 7 days after CLP surgery. At these times, mice showing signs of imminent death (inability to maintain upright position/ataxia/tremor and/or agonal

breathing) were euthanized. For other protocols, mice were euthanized 6 hours after CLP surgery.

Pharmacological treatments

For *in vivo* experiments, pioglitazone (20 mg/Kg, i.p) was administered 1, 4 and 18 hours before CLP surgery.

For *in vitro* experiments, pioglitazone, rosiglitazone, troglitazone, and GW9662 (10 μM each, Cayman Chemical) were incubated with 2×10^6 cells for 24 h before immunoblotting or PCR assays. Anti-IL-10R (Biolegend, 20 μg/mL) was incubated for 0.5 h before pioglitazone and/or 100 ng/mL LPS (Sigma-Aldrich) stimulation.

Cell harvest

Elicited macrophages were harvested from the peritoneal cavities of mice by lavage with PBS 4 days after the injection of 2 ml 3% thioglycollate as described previously (17).

Bacterial load

Blood was collected from the orbital plexus of mice and peritoneal cavity was washed with PBS. Aliquots of serial log dilutions were plated in Mueller-Hinton agar dishes as previously described (18).

Cell counts

Leukocyte numbers were determined in the peritoneal cavity and bronchoalveolar lavage fluid (BALF) 6 h after CLP or sham using the Hemavet 950FS System.

Flow cytometry

Peritoneal cells were resuspended in PBS containing 2 mM EDTA and 0.5% FCS. Fc receptor-mediated and nonspecific Ab binding was blocked by addition of excess CD16/CD32 (clone 2.4G2, BD Biosciences Pharmingen) for 10 min at 4°C. The cells were stained with mouse anti-GR1 FITC (1:100, BD Biosciences Pharmingen) for 30 min at 4°C, and the expression of this receptor was analyzed by flow cytometry (FACSCalibur). Data were analyzed with WinMDi and FlowJo Version 7.6.4 software.

Cytokines measurement

TNF-α, IL-10, IL-1β, and IL-6 were measured using DuoSet ELISA (R&D Systems,), following the manufacturer's protocol.

PPAR-γ activity assay

PPAR- γ DNA binding activity in nuclear extracts (10 μg of protein) was assayed using a PPAR-γ transcriptionfactor assay kit (Cayman Chem). For *in vivo* PPAR- γ activity, C57BL/6 mice were treated or not with pioglitazone (20 mg/Kg, i.p) for 4 or 24 h and peritoneal cells were isolated. For *in vitro* activity assay, WT macrophages were stimulated for 1, 4 or 24 h with 10 μM pioglitazone.

Histology

Mice were perfused with 10% formalin before lung harvesting. The tissues were fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with H&E as previously described (19). The images were recorded using an Infinity 1 camera attached to Nikon Eclipse Ci microscope. Capillary congestion, alveolar edema and PMN infiltration were determined as previously described (19).

Immunoblotting

Western blots were performed as previously described (5, 17). Protein samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with primary antibodies against MyD88, total or phosphorylated (S727 or Y701) STAT-1, and phosphorylated JAK2 (Tyr1007/1008) (all at 1:1000; Cell Signaling), or β-actin (1:10,000; Sigma-Aldrich). Densitometric analysis was performed as described previously (5, 17).

RNA isolation and semiquantitative real-time RT-PCR

Total RNA from cultured cells was isolated using the Gene mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Real-time RT-PCR was performed as previously described (5, 17). The sequences for the primers *Ppar*-γ, *Ppar*-α, *Ppar*-δ, *Myd88*, *Stat1*, and *Actin* (all from Integrated DNA Technologies) are listed in Table 1. Relative expression was calculated using the comparative threshold cycle (Ct) and expressed relative to control or WT (ΔΔCt method).

Nuclear isolation

Nuclear and cytoplasmic fractions of peritoneal cells from naïve, sham or septic mice treated or not with pioglitazone were isolated as previously described (5) using a subcellular protein fractionation kit according to the manufacturer's instructions (Pierce).

Statistics

Survival curves are expressed as percent survival and were analyzed by a Log-rank (Mantel-Cox) Test. Bacterial load results are expressed as median. Other results are expressed as mean ± SEM and were analyzed by ANOVA followed by Bonferroni analysis. Differences were considered significant for $P < 0.05$.

RESULTS

Prolonged PPAR-γ activation protects mice against severe polymicrobial sepsis

PPAR-γ activation inhibits activation of TLR and NFκB, which are essential components involved in the control of polymicrobial sepsis (20, 21). It has been shown that PPAR-γ activation protects mice against endotoxic shock and polymicrobial sepsis (12, 22–25). Using the cecum ligation and puncture (CLP) model, we investigated whether PPAR-γ activation exerts different effects *in vivo* depending on the severity of polymicrobial sepsis. Treatment of mice with pioglitazone (20 mg/kg) for 1 or 4 h before CLP did not have any effect in animal survival in neither moderate nor severe sepsis (Supplemental figure 1). When mice were treated with pioglitazone for 18 h before CLP, we observed a significant increase in the survival of severely septic mice, but no effect on the survival of moderately septic mice (Fig. 1A). The same protective effect was also observed in rosiglitazone-treated mice (not shown). We found that pioglitazone treatment substantially decreased bacterial load in severely septic mice in both peritoneal cavity (Fig. 1B) and blood (Fig. 1C). We observed the same pattern when mice were treated with pioglitazone, followed by induction of moderate sepsis (not shown). To determine whether expression of other classes of PPAR was increased following pioglitazone treatment of septic animals, we performed qPCR analysis of peritoneal cells (PCs) harvested 6 h after CLP. PPAR-γ was the most abundantly expressed PPAR during sepsis although PPAR-α was also significantly elevated, albeit to a much lesser extent (Fig. 1D). The increased PPAR-γ expression during CLP was confirmed by immunoblotting (Fig. 1E). Thus, activation of PPAR-γ by pioglitazone treatment results in further expression of PPAR-γ in septic animals. From a translational perspective, these data demonstrate that prolonged PPAR-γ activation enhances survival of severely septic

mice, and survival was associated with reduced bacterial load at the site of infection and in the blood.

PPAR-γ activation increases neutrophil recruitment and decreases cytokine generation and lung inflammation

We and others have shown that neutrophils are key cells involved in control of polymicrobial sepsis (26–28). We also showed that high mortality in severe sepsis is a consequence of impaired neutrophil migration to the site of infection, although neutrophils and cytokines accumulate in other target organs (27, 29). We speculated that PPAR-γ activation could prevent neutrophil migration failure to the peritoneal cavity during severe sepsis, leading to enhanced microbial clearance. Our results showed a decrease in the neutrophil numbers in the peritoneal cavity 6 h after severe sepsis and PPAR-γ activation prevented neutrophil migration failure to the infectious site (Fig. 2A and B). The same pattern was also observed in monocyte recruitment (not shown).

An overwhelming inflammatory response due to cytokine storm is a major cause of organ dysfunction and death in septic patients (30, 31). We determined whether pioglitazone treatment would inhibit the production of proinflammatory cytokines TNF-α, IL-6, and IL-1β in septic animals. Levels of TNF-α and IL-1β in both peritoneal cavity and blood were reduced in pioglitazone-treated septic mice when compared to untreated septic mice (Fig. 2C and D). IL-6 levels were decreased in the blood but not in the peritoneal cavity (Fig. 2C and D). In contrast, levels of the anti-inflammatory cytokine ıL-10 were greatly enhanced in both peritoneal cavity and blood in pioglitazone-treated septic mice (Fig. 2C and D). These data suggest that pioglitazone improves mouse survival by enhancing bacterial clearance, increasing neutrophil recruitment, and by favoring an anti-inflammatory milieu at the site of infection.

Prolonged PPAR-γ activation decreases lung inflammation in septic mice

One of the major consequences of sepsis is the development of lung injury (27). Therefore, one possible mechanism by which PPAR-γ improves animal survival is by decreasing the inflammatory response in the lung. Indeed, we observed that pioglitazone treatment inhibited accumulation of neutrophils in lungs of septic mice (Fig. 3A). We did not observe significant changes in monocyte or lymphocyte numbers (Fig. 3A). The effect of pioglitazone-mediated protection in the lung pathology was also confirmed by examination of lung sections. Pioglitazone treatment decreased leukocyte recruitment to the lung, and decreased alveolar edema and capillary congestion (Fig. 3B and C). We also detected the presence of pro- and anti-inflammatory cytokines in the bronchoalveolar lavage fluid of mice treated with pioglitazone (Fig 3D). The data show that pioglitazone treatment resulted in decreased levels of TNF-α and IL-1β but dramatically increased IL-10 levels. These data show that in addition to improving features involved in the control of microbial burden at the site of infection, PPAR-γ activation also ameliorates lung injury, an important event associated with mortality in sepsis (32–34).

PPAR-γ activation inhibits MyD88 expression *in vivo* and *in vitro*

We hypothesized that prolonged PPAR-γ activation inhibits inflammatory responses in sepsis by inhibiting the expression of TLRs or the TIR adaptor MyD88, which is essential for TLR and IL-1β receptor responsiveness (35, 36). Our data show that MyD88 expression was decreased in peritoneal macrophages treated with different PPAR-γ agonists (Fig. 4A). Pioglitazone and rosiglitazone were more potent than troglitazone in inhibiting MyD88 expression in macrophages. Endogenous PPAR-γ did not inhibit MyD88 expression in macrophages from PPAR-γ DN and from WT macrophages treated with GW9662 (data not shown). Pioglitazone treatment decreased MyD88, but not TLR4 expression in monocyte-

derived human macrophages (Fig. 4B). Next, we investigated whether the time of PPAR-γ activation correlates with the decrease in MyD88 expression *in vivo* and *in vitro*. Our results show that pioglitazone enhances PPAR-γ activation 4 h after treatment *in vitro* and peaks after 24 h both *in vivo* and *in vitro* (Fig. 4 C and D). We only observed a decrease in MyD88 levels 24 h after pioglitazone treatment *in vivo* and *in vitro* (Fig. 4C and D). These data further suggest that pioglitazone-induced PPAR-activation leads to decreased MyD88 levels. We also confirmed that pioglitazone effects are restricted to PPAR-γ activation. Macrophages were pretreated with the PPAR-γ receptor antagonist GW9662, followed by challenge with pioglitazone for 24 h. We did not see any changes in MyD88 expression when macrophages were treated with GW9662 only and prevented pioglitazone-mediated MyD88 inhibition (supplemental figure 2). We then investigated whether pioglitazone inhibited MyD88 expression during sepsis *in vivo*. MyD88 expression was increased 6 h after CLP at both the mRNA level (Fig. 4E) and protein level (Fig. 4F and G), and treatment with pioglitazone significantly decreased MyD88 levels in these mice (Fig. 4E–G). Activation of PPAR-γ inhibited MyD88 specifically but did not affect TLR4 expression (Fig. 4F) or the TLR adaptor TRIF (not shown). These data indicate that PPAR-γ activation provides an endogenous brake involved in both homeostatic and inducible MyD88 expression both *in vitro and in vivo*.

PPAR-γ activation inhibits serine phosphorylation and nuclear translocation of the MyD88 transcription factor STAT-1

We have previously shown that STAT-1 is the major transcription factor responsible for MyD88 expression (37). We have also shown that the suppressor of cytokine signaling-1 (SOCS-1) inhibits MyD88 levels by preventing STAT-1 activation (37). We hypothesize that PPAR-γ inhibits MyD88 expression by preventing STAT-1 activation. Initially, we investigated whether PPAR-γ activation alters STAT-1 expression. No changes in STAT-1 mRNA expression were observed when macrophages were treated with pioglitazone, rosiglitazone, troglitazone (Fig. 5A) or the PPAR-γ antagonist GW9662 (data not shown). Furthermore, we did not observe any changes in STAT-1 mRNA levels in macrophages from PPAR-γ receptor DN mice (data not shown). *In vivo* experiments showed that while STAT-1 expression was increased after sepsis, pioglitazone did not affect STAT-1 levels *in vivo* (Fig. 5B). Since no changes in STAT-1 expression were observed, we speculated that PPAR-γ might alter phosphorylation of STAT-1 at the residue important for DNA binding activity (Y701) or at the site required for maximal transcriptional activity (S727) (38–40). We found that phosphorylated Y701 was not detected under any conditions tested (not shown), but we found that peritoneal cells from septic mice exhibited higher STAT-1 S727 phosphorylation than peritoneal cells from sham mice (Fig. 5C). Pioglitazone treatment inhibited STAT-1 S727 phosphorylation compared to septic mice (Fig. 5C). Since SOCS-1 inhibits JAK/STAT-1 activation, we sought to determine the levels of SOCS-1 in pioglitazone-treated mice during sepsis. No differences in SOCS-1 expression were detected in any group tested (not shown). Furthermore, the decrease in STAT-1 phosphorylation was not dependent on JAK-2 phosphorylation (Fig. 5D). Even though sepsis increased JAK-2 phosphorylation, pioglitazone treatment did not decrease JAK-2 phosphorylation (Fig. 5D). To further investigate the molecular programs involved in PPAR-γ-mediated STAT-1 action, we investigated whether PPAR-γ activation influenced STAT-1 nuclear translocation *in vivo*. Peritoneal cells from septic mice exhibited enhanced STAT-1 nuclear translocation, an event prevented by PPAR-γ activation (Fig. 5E). These data suggest that PPAR-γ activation decreases MyD88 expression by preventing two important steps involved in STAT-1 activation— serine phosphorylation and nuclear translocation.

PPAR-γ-induced IL-10 accounts for the inhibition of MyD88 expression in macrophages

Since we previously observed that pioglitazone treatment greatly enhances IL-10 levels *in vitro* and *in vivo* (41–44) and coupled with the fact that IL-10 decreases MyD88 expression (4, 45), we speculated that IL-10 mediates PPAR-γ effects on MyD88 levels. We determined whether pioglitazone enhanced IL-10 production and whether it further enhanced LPS-induced IL-10 levels in thioglycollate-elicited macrophages. We found that pioglitazone significantly enhanced IL-10 production in elicited macrophages and amplified LPS-induced IL-10 levels (Fig. 6A). To investigate whether enhanced IL-10 in pioglitazone-treated cells accounted for inhibition of MyD88, macrophages were pretreated with anti-IL-10 receptor antibody, followed by pioglitazone and LPS treatment for 24 h to further investigate the role of PPAR-γ and IL-10 in inducible MyD88 expression. Pioglitazone inhibited LPS actions (Fig. 6B). However, when cells were pretreated with anti-IL10R, we observed an enhancement in MyD88 levels, which occurred even in the presence of pioglitazone (Fig. 6B). We further investigated whether blocking IL-10 actions would also prevent PPAR-γ-inhibition of STAT-1 activation. As we observed during CLP, macrophage treatment with pioglitazone decreased LPS-induced STAT-1 S727 phosphorylation but not STAT-1 Y701 phosphorylation, an event mediated by endogenous IL-10 actions (Fig. 6B). These results are consistent with the existence of a novel regulatory program in which PPAR-γ activation enhances IL-10 levels to decrease STAT-1-dependent MyD88 expression, which further inhibits TIR-dependent macrophage activation and improves animal survival during sepsis (Fig. 6C).

DISCUSSION

This study demonstrates the therapeutic potential of the PPAR-γ ligand pioglitazone in the most widely used models of sepsis and septic shock, which resemble human sepsis in many parameters (31, 46, 47). Specifically, we demonstrate that prolonged PPAR-γ activation protects mice against severe CLP-induced sepsis by ameliorating important steps in the pathogenesis of sepsis which leads to increased survival, including: 1) restoring neutrophil recruitment to the site of infection, 2) increasing bacterial clearance at the site of infection and in blood, 3) decreasing cytokine storm, and 4) inhibiting MyD88 expression and, therefore, the actions of TIR (Toll-IL-1 receptor) domains, such as members of the Interleukin-1 Receptor and Toll-Like Receptor Superfamily. Importantly, these results suggest a novel PPAR-γ /IL-10 network that inhibits MyD88 levels in macrophages that dampens systemic inflammatory response.

Most PPAR- γ agonists belong to the TZD family (6, 48). These compounds are currently used in the treatment of type 2 diabetes and some cardiovascular complications. Since pioglitazone is currently FDA-approved for treatment of type-2 diabetes, we decided to use only pioglitazone in our study. Rosiglitazone and pioglitazone have been beneficial in treating various kidney injuries including nephropathy resulting from diabetes, hypertension, and cyclosporine-induced renal injury (6); however the consequences of chronic TZD intake on host defense systems remain to be determined.

That PPAR-γ activation decreases inflammatory responses in different disease models is well established (6, 13, 25). In the CLP model, PPAR-γ activation improves animal survival, and the mechanisms underlying animal protection are diverse (25). Zingarelli, et. al., showed that 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and ciglitazone protects rats against polymicrobial sepsis by inhibiting activation of two inflammatory transcription factors, NFκB and AP-1, and improving hemodynamic performance (8). Furthermore, 15d-PGJ_2 treatments also improve renal, liver, and pancreatic function in LPS-challenged mice (10). PPAR-γ activation also inhibits the expression/activation of different inflammatory mediators involved in the pathogenesis of sepsis, including reactive oxygen and nitrogen intermediates,

gelatinase B, and cyclooxygenase 2 (25, 49). Here, we also observed that pioglitazone treatment decreases the generation of pro-inflammatory cytokines responsible for the cytokine storm such as IL-1β, TNF-α and IL-6. These cytokines are involved in the infiltration of neutrophils, leading to tissue necrosis and favoring overwhelming inflammatory response (27, 29). We did not observe an effect of endogenous PPAR-γ activation in the control of sepsis, since treatment of mice with the PPAR-γ antagonist before severe CLP did not alter animal survival or bacterial load (not shown). Other laboratories have reported that PPAR-γ antagonist or PPAR-γ deficient mice are protected from CLP, which is somewhat contradictory to our findings (24, 49, 50). The reasons for such discrepancy include severity of the CLP, time of treatment, mouse strain, and species differences (rat *vs*. mice).

An important finding in our study is that in addition to improving local inflammatory responses, we also observed that pioglitazone prevented acute lung injury in septic mice. This amplified response, also called cytokine storm, results in systemic inflammation that affects several organs, including lungs (51). The lung is particularly affected, and acute lung injury secondary to sepsis is characterized by edema, inflammatory cell infiltration, and impaired gas exchange that can lead to multi-organ failure (30, 52). PPAR-γ activation inhibited CLP-induced edema formation and neutrophil recruitment to the lung, and inhibited cytokine production favoring leukocyte activation. Our findings are in agreement with laboratories that have reported that PPAR-γ activation decreases neutrophils sequestration in lung and production of proinflammatory cytokines (9, 53).

We have previously demonstrated the involvement of TLRs in the inflammatory cytokines production and systemic activation of neutrophils in sepsis. Mice lacking TLR2, TLR4 or TLR9 show attenuated systemic inflammatory response and reduced mortality rates under polymicrobial sepsis (24, 26, 28, 54). These effects were due to enhanced bacterial load control in the knockout mice as consequence of the reduced systemic activation of neutrophils and increased recruitment of these cells to the infection site (24, 26, 28, 54). Based on that, we speculate that the overwhelming inflammatory response triggered by the recognition of diverse ligands to specific TLRs in polymicrobial sepsis can be modulated by the partial abrogation of TLRs signaling. Here we demonstrate that pioglitazone and other TZDs specifically decrease mRNA and protein levels of MyD88, the main adaptor protein of TLRs, in both naïve macrophages and cells from septic mice. Therefore, it can be suggested that pioglitazone-induced decrease of MyD88 expression dampens the excessive TLR signaling, improving the outcome to polymicrobial sepsis.

MyD88 expression is controlled by different transcription factors including NFκB (55), IL-6 responsive elements (56), and STAT-1 (37). We speculated that PPAR-γ activation would decrease STAT-1 activation leading to inhibition of MyD88 levels. PPAR-γ inhibition of STAT-1 DNA binding activity in IFN-α and β activated cells was previously reported (57, 58). However, PPAR-γ inhibition of STAT-1 activity *in vivo* and in a sepsis model or in LPS-stimulated macrophages remains to be determined. Our data show that PPAR-γ activation impairs both upstream effects of STAT-1, such as nuclear translocation, and phosphorylation of STAT-1 on the S727 residue, which is required for optimal transcriptional activity (59). The intracellular molecular programs involved in PPAR-γ regulation of different steps involved in STAT-1 activation remain to be investigated.

PPAR-γ activation elicits numerous anti-inflammatory programs by impairing activation of different transcription factors such as NFκB, AP-1, and STAT-1 through the sequestration of essential, shared co-activators (13). A well-known STAT-1 inhibitor is the suppressor of cytokine signaling-1 (SOCS1) (60, 61). We did not detect any changes in SOCS1 levels when mice or cells were treated with pioglitazone (data not shown). Park et al., have shown

that rosiglitazone and 15d-PGJ_2 inhibit activation of JAK2 and STAT-1 in glia cells and astrocytes in a manner independent of PPAR-γ activation (57). Furthermore, PPAR-γ activation may enhance the expression of other members of the SOCS family, such as SOCS2 and SOCS3, which could inhibit the JAK/STAT-1 pathway. However, such events remain to be determined. The discrepancy between the work of Park and our current findings could be due to different agonists used, cell types, and inflammatory stimuli. In addition to the direct anti-inflammatory effect of pioglitazone, PPAR- γ activation also enhanced the secretion of anti-inflammatory molecules, such as IL-10 (62, 63). We found that pioglitazone alone enhanced basal and LPS-induced IL-10 in macrophages *in vitro* and in the peritoneal cavity and blood of septic mice. IL-10 is known to decrease TLR responses and, more specifically, MyD88 expression (4) in macrophages. In addition, it has also been shown that IL-10 inhibits IFN-γ and LPS-induced STAT-1 activation in different cell types (64, 65). Therefore, we speculated that IL-10 is a key component in the PPAR-γ inhibition of STAT-1 activation and, consequently, MyD88 expression. Our data show that blocking IL-10R action prevents pioglitazone-mediated inhibition of STAT-1 S727 phosphorylation and MyD88 expression. The fact that IL-10 mediates anti-inflammatory actions of PPAR-γ has been shown in different inflammatory models, such as allergic asthma and ischemia-reperfusion (62, 63). However, the molecular programs involved in IL-10 mediated S727 STAT-1 phosphorylation that further inhibits MyD88 levels remain to be determined. The signaling programs involved in PPAR-γ-induced IL-10 production are currently a topic of intensive research in our laboratory.

Our findings suggest the existence of a novel regulatory program elicited by PPAR-γ activation involving IL-10 production that inhibits STAT-1-dependent MyD88 expression. Importantly, changes in MyD88 levels were critical in preventing cytokine storm, which in turn improved animal survival, reduced bacterial load, and reduced neutrophil recruitment to the lungs, all of which are key components involved in sepsis protection. Thus, in view of the fact that pioglitazone is currently used to treat type 2 diabetes, this drug represents a potential new therapeutic approach to treating overwhelming inflammatory conditions observed in septic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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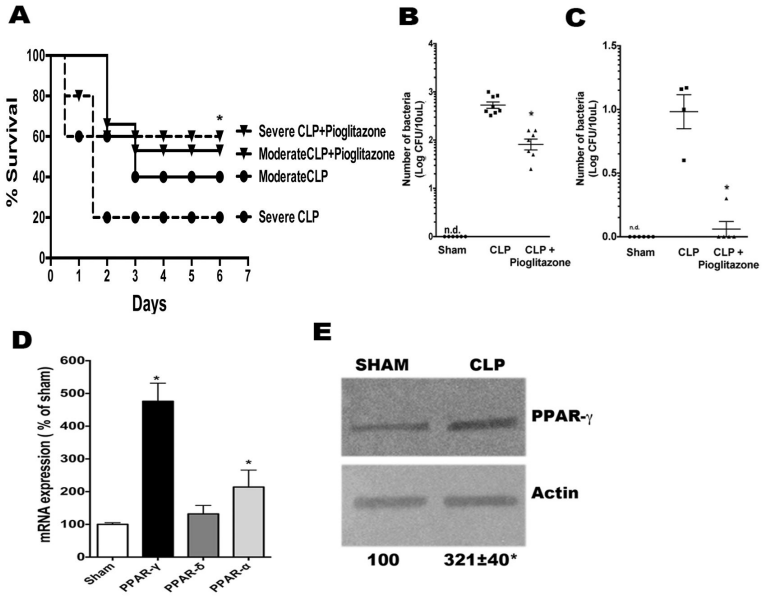

Fig. 1. Pioglitazone treatment enhances animal survival and microbial clearance in polymicrobial sepsis

(A) Survival rates of C57BL/6 mice (n = 10 per group) treated with 20 mg/Kg pioglitazone for 18 h before severe or moderate CLP-induced sepsis. The survival rate was determined at each 12 h up to 7 days after surgery. **P* < 0.05. Colony-forming units (CFU) in peritoneal cavity (B) or blood (C) 6 h after CLP in C57BL/6 mice treated or not with pioglitazone 18 h prior to CLP. (D) Ppar-γ, -δ, -α mRNA expression in the peritoneal cells 6 h after CLP or Sham determined by qPCR as described in Material and Methods. (E) PPAR-γ or actin protein expression detected by immunoblotting of peritoneal cells harvested 6 h after CLP or sham. Numbers under lanes indicate the relative density of PPAR-γ determined from densitometric analysis and expressed as the mean ± SEM from three individual experiments. Values of the WT control group were set as 100%. Data are expressed as mean ± SEM from at least three independent experiments; **P* < 0.05 relative to the sham group or CLP *vs* pioglitazone treatment.

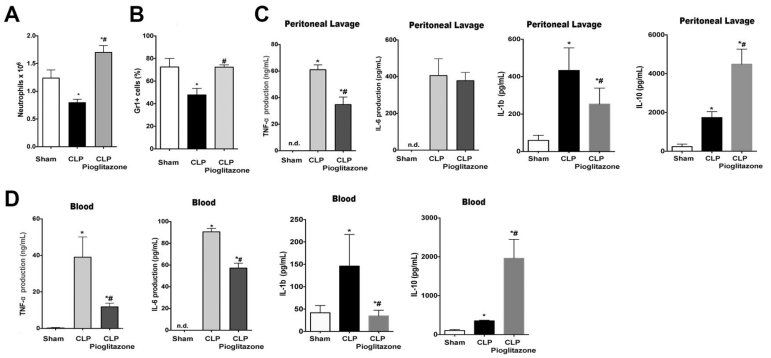

Fig. 2. PPAR-γ enhances neutrophil recruitment to the site of infection and decreases pro-inflammatory cytokine production

(A) C57BL/6 were pretreated or not with pioglitazone for 18 h and subjected to severe CLP or sham and after 6 h peritoneal neutrophil numbers were determined using Hemavet as described in the Material and Methods. (B) Mice were treated as in A and the number of $GR1^+$ cells were determined by FACS. (C) C57BL/6 mice were treated as in A followed by determination of the levels of TNF-α, IL-6, IL-1β and IL-10 in the peritoneal lavage fluid by ELISA. (D) Mice were treated as in A and serum was harvested 6 h after CLP for detection of TNF-α, IL-6, IL-1β and IL-10 by ELISA. Data are mean ± SEM; **P* < 0.05 *vs*. sham group, #*P* < 0.05 pioglitazone-treated mice *vs*. untreated.

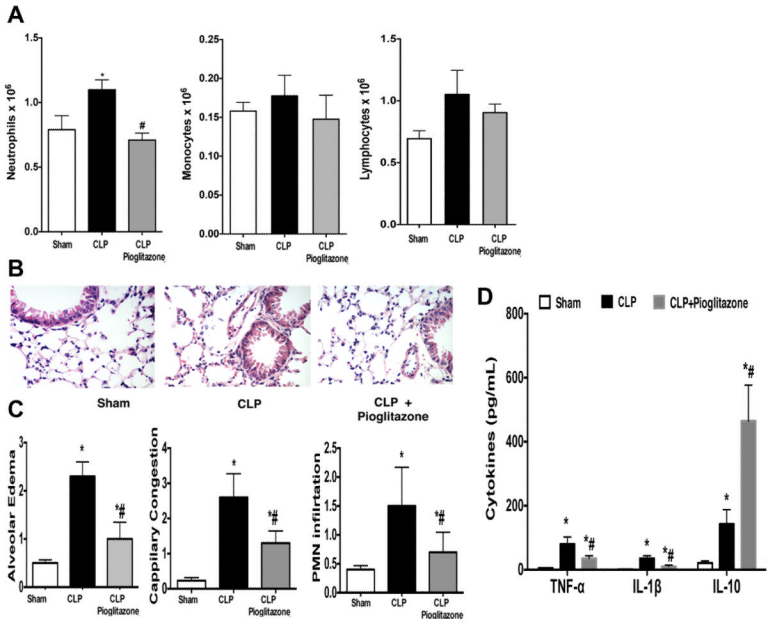

Fig. 3. Pioglitazone treatment decreases inflammatory response in the lung

(A) leukocyte recruitment (neutrophil, monocytes and lymphocytes) in C57BL/6 mice treated or not with pioglitazone for 18 h followed by CLP. Leukocytes present in the BAL: neutrophils, monocytes, and lymphocytes were determined using Hemavet, 6 h after CLP surgery. (B) Histological sections stained with H&E from mice treated as in A. (C) Lung histology scores: alveolar edema, capillary congestion, and polymorphonuclear neutrophil (PMN) infiltration were determined as described in Material and Methods. (D) Cytokine concentrations in BAL: IL-1β, TNF-α IL-6, and IL-10 were determined by ELISA. n = 6–8 in each group. Data are mean ± SEM; **P* < 0.05 *vs*. sham group, #*P* < 0.05 pioglitazone-treated mice *vs*. untreated.

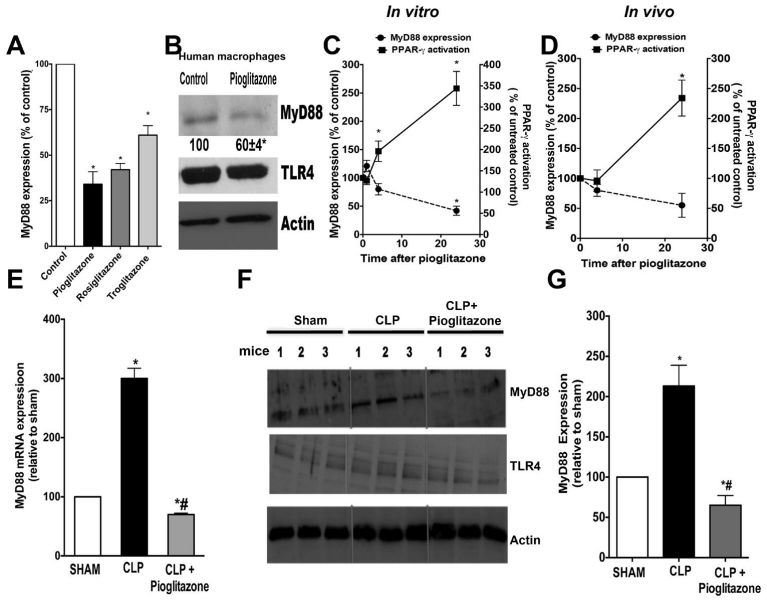

Fig. 4. PPAR-γ inhibits expression of MyD88 *in vitro* and *in vivo*

(A) Peritoneal macrophages were treated with 10 μM of PPAR-γ agonists: rosiglitazone, pioglitazone, troglitazone for 24 h, followed by determination of MyD88 and Actin expression by qPCR as described in Materials and Methods. (B) Monocyte-derived human macrophages were treated with pioglitazone for 24h and subjected to immunoblot for MyD88, TLR4 and Actin. Numbers under lanes indicate the relative density of PPAR-γ, determined from densitometric analysis and expressed as the mean ± SEM from three individual experiments, with the values of the untreated group set as 100% control. (C) Macrophages were treated with pioglitazone for the indicated time points and the expression of MyD88 and Actin mRNA was determined by qPCR and PPAR-γ activity was determined as described in Material and Methods. (D) C57BL/6 mice (n=3 per time point) were treated with 20 mg/Kg pioglitazone via intraperitoneal injection for the indicated time points and the expression of MyD88 and Actin mRNA was determined by qPCR and PPAR-γ activity was determined in the peritoneal cells as described in Material and Methods. (E–G) C57BL/6 mice were treated with pioglitazone for 18 h followed by CLP surgery. Peritoneal cells from 3 different mice were harvested 6 h after induction of sepsis, lysed, and subjected to qPCR (E) immunoblotting (F) for determination of MyD88, TLR4 and Actin. (G) Densitometric analysis of the bands shown in D. Data are mean ± SEM; **P* < 0.01 *vs*. sham group or control group, and #*P* < 0.01 pioglitazone-treated mice *vs*. untreated.

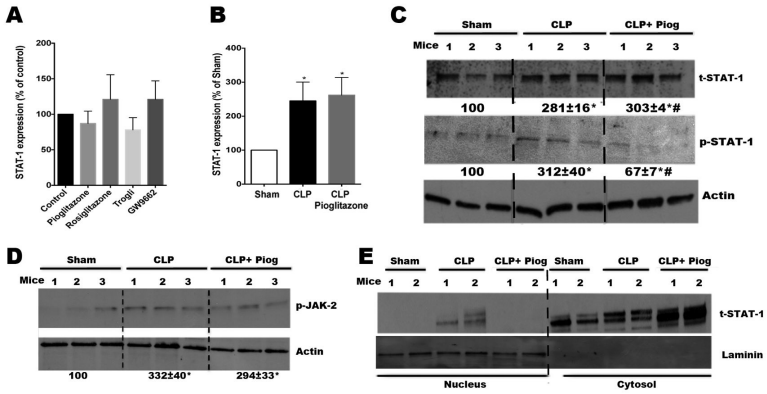

Figure 5. PPAR-γ inhibits STAT-1 nuclear translocation and transcriptional activity

(A) Peritoneal macrophages were treated with 10 μM of PPAR-γ agonists: rosiglitazone, pioglitazone, troglitazone for 24 h, followed by determination of STAT-1 and Actin expression by qPCR as described in Material and Methods. (B–E) C57BL/6 mice were treated or not with pioglitazone for 18 h followed by CLP surgery. Peritoneal cells were harvested 6 h after sepsis induction, lysed, and subjected to qPCR for determination of STAT-1 expression and immunoblotting for determination of total STAT-1, STAT1 pS727 (D), or JAK2, laminin and Actin (E). The numbers underneath the bands represent mean densiometric analysis of the bands shown. Data are mean ± SEM; **P* < 0.01 *vs*. sham group, and #*P* < 0.01 pioglitazone-treated mice *vs*. untreated.

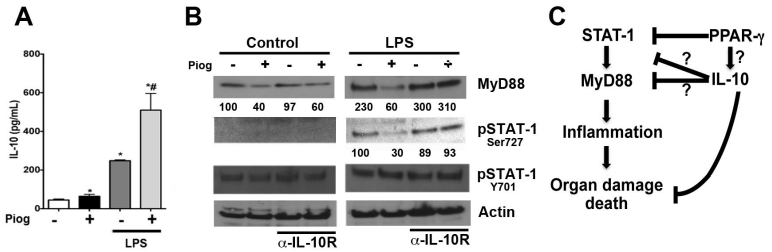

Figure 6. IL-10 mediates PPAR-γ inhibition of STAT-1 dependent MyD88 expression

(A) TG-elicited peritoneal macrophages were treated or not with pioglitazone for 18 h, followed by LPS stimulation for 24 h. IL-10 in the supernatant was measured by ELISA. Data are mean ± SEM; **P* < 0.05 *vs*. untreated cells, #P < 0.05 *vs*. LPS-challenged. (B) Cells were pretreated with anti-IL-10R (20 μg/mL) for 0.5 h before the addition of 10 μM pioglitazone for 24 h and before LPS stimulation for another 24 h. Cell lysates were subjected to immunoblotting for MyD88, STAT-1 pS727, STAT-1 pY701, and Actin. The numbers underneath the bands represent mean densiometric analysis of the bands shown. Data are representative of two independent experiments. (C) Proposed model of PPAR-γ regulation of IL-10 and MyD88 expression and enhancement of animal survival after CLP procedure.

Table 1

Target	Forward	Reverse
ppar α	ACTCCACCTGCAGAGCAACCA	TAGATCTCCTGCAGTAGCGGG
ppar δ	CCCTGGCAAAGCATTTGTAT	AATCCTTGGCCCTCTGAGAT
ppar γ	TTGAGCCCAAGTTCGAGTTTGCTG	ATTCTAGAGCCCGCAGAATGGTGT
myd88	TAGTCGCAGACAGTGATGAAC	CTGCAGAGCAAGGAATGTGA
stat1	GATCACTCTTTGCCACAACCA	TAGAGCATGAAATCAAGAGCCT
β *actin*	CTGCCTGACGGCCAAGTC	CAAGAAGGAAGGTCGGAAAAGAG