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Title: Pregnane X Receptor Modulates the Inflammatory Response in Primary Cultures of Hepatocytes

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Running Title: Pregnane X Receptor Inhibits the Acute Phase Response

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Abstract

Bacterial sepsis is characterized by a rapid increase in the expression of inflammatory mediators to initiate the acute phase response (APR) in liver. Inflammatory mediator release is counterbalanced by the coordinated expression of anti-inflammatory molecules such as interleukin 1 receptor antagonist (IL1-Ra) through time. The goal of this study was to determine whether activation of pregnane x receptor (PXR, NR1I2) alters the lipopolysaccharide (LPS)-inducible gene expression program in primary cultures of hepatocytes (PCHs). Pre-activation of PXR for twenty-four hours (hr) in PCHs isolated from wild type mice suppressed subsequent LPS-inducible expression of key inflammatory mediators interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα); but not in PCHs isolated from *Pxr*-null (PXR-KO) mice. Basal expression of key inflammatory cytokines was elevated in PCHs from PXR-KO mice. Stimulation of PCHs from PXR-KO mice with LPS alone produced enhanced levels of IL-1β when compared with wild type. Experiments performed using PCHs from both humanized-*PXR* transgenic mice ($hPXR_{tg}$) as well as human donors indicate that prolonged activation of PXR produces increased secretion of IL1-Ra from cells through time. Our data reveal a working model that describes a pivotal role for PXR in both inhibiting as well as in resolving the inflammatory response in hepatocytes. Understanding the molecular details of how PXR is converted from a positive regulator of drug metabolizing enzymes into a transcriptional suppressor of inflammation in liver will provide new pharmacological strategies for modulating inflammatory-related diseases in liver and intestine.

Introduction

Pregnane X Receptor (PXR, NR1I2) is a ligand-activated nuclear receptor (NR) superfamily member expressed at high levels within the entero-hepatic system of mammals. The biological function of PXR is mediated together with its obligate partner retinoid X receptor alpha (Kliewer et al., 1998; Lehmann et al., 1998). To date, ligands identified for PXR are numerous and are as structurally diverse as naturally occurring steroids (Kliewer et al., 1998), antibiotics (Lehmann et al., 1998), bile acids (Goodwin et al., 2003; Staudinger et al., 2001a; Xie et al., 2001), anti-cancer agents (Desai et al., 2002; Nallani et al., 2004), as well as the active ingredients in several herbal remedies (Brobst et al., 2004; Ding and Staudinger, 2005; Moore et al., 2000). Ligand-activated PXR positively regulates the drug-inducible expression of genes encoding key drug transporters and drug metabolizing enzymes that function coordinately to increase the uptake, metabolism, excretion and efflux of xenobiotics from the body. In this way, PXR activation is associated with increased metabolism and clearance of a myriad of potentially toxic compounds, and is classically thought of as a protective response.

Clinical treatment with PXR activators can also lead to the repression or attenuation of other biochemical pathways in liver and intestine including both energy metabolism and the inflammatory response (Moreau et al., 2008). For example, it was demonstrated nearly 45 years ago that treatment with Rifampicin (Rif), a prototypical ligand of human PXR, leads to a compromised ability to mount an effective immune response in cell-based assays (Paunescu, 1970). *In vivo* studies in rodents suggest that PXR activation suppresses inflammation and the acute phase response (APR) by attenuating the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling (Shah et al., 2007). More recent studies using rodents indicate that PXR activation regulates intestinal barrier function through an interaction with toll-like receptor 4 (TLR4), the molecular target of lipopolysaccharide (LPS) stimulation (Venkatesh et al., 2014). It is now widely accepted that the activation of PXR is associated with general suppression of the immune response, particularly in

intestine (Cheng et al., 2012; Dou et al., 2012; Dou et al., 2014). Currently, little is known regarding the molecular mechanism of this phenomenon in liver or in hepatocytes.

Recent investigations indicate that ligand-mediated activation of liver-enriched nuclear receptors (NRs) liver receptor homologue-1 (LRH-1) and liver x receptor beta (LXRβ) initiate anti-inflammatory mechanisms and pathways that suppress the hepatic acute phase response (APR) (Venteclef et al., 2010). These investigations reveal that post-translational modification of these two NRs by small ubiquitin related modifier (SUMO) is required for suppression of the hepatic expression of the acute phase protein and marker of the APR, haptoglobin. A theme emerges in which metabolic NRs are modified by SUMO proteins to suppress the inflammatory response, particularly in hepatocyte and in macrophage cell types [reviewed in (Treuter and Venteclef, 2011)]. Previous work from our laboratory has revealed that PXR is also the target of SUMO proteins to suppress the tumor necrosis factor alpha (TNFα)-mediated production of interleukin 1 beta (IL-1β) in primary cultured hepatocytes (PCHs) (Hu et al., 2010). While much is known regarding LPS administration as an experimental model of gram negative bacterial sepsis *in vivo* in rodents, very little is known regarding the PXR-mediated modulation of the LPS-inducible gene expression program in PCHs across species. We therefore wanted to further characterize the ability of ligand-mediated activation of PXR to suppress a broad array of LPS-inducible hepatic inflammatory response genes using both mouse and human PCHs as model systems.

Administration of LPS, a glycolipid constituent of the outer membrane of gram-negative bacteria, to live animals or cultured cells initiates a signaling cascade in cells through TLR4 receptor multi-protein complexes that include CD14, Myd88, and MD-2 as co-receptor proteins (Buer and Balling, 2003). Here we perform concentration- and time-response analysis of the LPS-inducible gene expression program in both mouse and human PCHs. Our data reveal that PCHs respond to stimulation with LPS to rapidly induce the expression of key inflammatory mediators including IL-1β and interleukin 6 (IL-6). Using a commercial gene array platform, we show that 24 hr pre-treatment of mouse PCHs with a strong rodent

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PXR activator, pregnenolone 16α carbonitrile (PCN), suppresses subsequent LPS-inducible inflammatory responses in PCHs. Follow-up experiments using PCHs isolated from *Pxr*-null (PXR-KO) mice demonstrate that the diminution of LPS-inducible gene expression by PCN requires functional PXR in hepatocytes. Finally, using PCHs derived from both humanized-*PXR* transgenic mice ($hPXR_{tg}$) and human donors indicate that activation of PXR enhances the secretion of interleukin 1 receptor antagonist (IL1-Ra), a key negative regulator of IL1 signaling, from hepatocytes. Taken together, these data shed new light on the molecular mechanisms that comprise the interface between PXR activation and resolution of the APR in liver in mammals.

Materials and Methods

Isolation and Culturing of Primary Hepatocytes. PXR knockout (PXR-KO) mice were generated as previously described (Staudinger et al., 2001b). The humanized *PXR*-transgenic mice ($hPXR_{tg}$) were previously described (Lichti-Kaiser and Staudinger, 2008). Hepatocytes were isolated from male congenic (C57BL6) wild-type and PXR-KO mice aged 6-10 weeks using a standard collagenase perfusion method as described previously (Staudinger et al., 2003). The primary cultures of human hepatocytes used in this study were derived from samples collected and provided by the KUMC Department of Pharmacology, Toxicology and Therapeutics Hepatocyte Core lab and the KU Liver Center which is sponsored by the Department of Pharmacology, Toxicology and Therapeutics Biospecimen Core lab and the Liver Center at the University of Kansas Medical Center. Fresh isolated human hepatocytes were plated at a cell density of 0.5×10^6 cells/well in 12-well plates previously coated with 0.2 mg/ml type I collagen. The isolated hepatocytes (>80% viability) were maintained in Dulbecco's Modified eagle's Medium supplemented with 100 nM dexamethasone, 100 nM insulin, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% bovine calf serum and kept at 37°C in a humidified incubator with 95% air/5%

CO2. The hepatocytes were allowed to attach to the plate for 4 hr and the medium was then replaced with serum-free Williams E medium as described previously (Staudinger et al., 2003).

Total RNA Isolation, Reverse Transcription, and Real-Time Quantitative-Polymerase Chain Reaction Analysis. Real time quantitative Polymerase Chain Reaction (Q-PCR) was performed as described (Ding and Staudinger, 2005).

Analysis of Secreted IL1-Ra in PCHs. A 200 μL aliquot of cell media was removed and combined with 200 μL 2X Laemmli Buffer (4% SDS, 20% Glycerol, 120 mM Tris-Cl pH 6.8, 10% 2-mercaptoethanol, 0.004% Bromphenol Blue) with 50 mM dithiothreitol. Following removal of culture media, cells were harvested by scraping into 1X PBS and pelleted briefly in a microfuge. Following removal of PBS, cells were lysed in 1X Laemmli Buffer and equal amounts were resolved using 10% SDS-PAGE. Western blot analysis was performed as described previously (Xu et al., 2009) using a monoclonal antibody that recognizes human and mouse IL1-Ra (Novus Biologicals, NBP1-96673). Western Blot images were quantitated by densitometry scanning of the X-ray films with the UVP Biodoc-It 220 image analysis system and 1D Gel Analysis Software.

Statistical Analysis. Where appropriate the statistical differences among an experimental group were determined using a one-way analysis of variance followed by the Duncan's multiple range post hoc test. Statistical differences between experimental groups were determined using the student's t-test.

Results

LPS-Inducible Concentration- and Time-response Analysis of Key Inflammatory Mediators in Mouse and Human PCHs. The primary goal of the current study is to determine the extent to which pre-activation of PXR alters the subsequent LPS-inducible gene expression program in a PCHs. Therefore, we first examined LPS-inducible IL-1β gene expression in both mouse and human PCHs performing concentration- and time-response analysis using Q-PCR. Treatment of PCHs isolated from wild type

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male mice aged 6-10 weeks with increasing amounts of LPS (0.01, 0.1, 1, 10, and 100 μg/mL of media) for 12 hr produced robust induction of IL-1β messenger RNA expression at all concentrations examined (**Figure 1A**). Based upon these results, and to provide a relatively strong inflammatory stimulus for our subsequent studies of possible PXR-mediated effects, we reasoned that LPS treatment should be performed using a relatively high concentrations (10 μg/mL) for initiation of the inflammatory response. While all of the time points examined (1, 6, 12, 24, and 48 hr) exhibited significant increases in IL-1β gene expression levels in mouse PCHs, the 6 and 12 hr time points had the largest increases showing an approximately 1,000- and 600-fold increase in IL-1β expression, respectively (**Figure 1B**). When PCHs derived from human donors were used in identical analyses, similar concentration- and time-dependent induction of IL-1β was observed (**Figure 2A and 2B**). Examination of the kinetics of LPS-inducible mouse and human IL-6 produced very similar results (***Supplementary data*, Figures S1-S4)**. Taken together, these data indicate that treatment with LPS induces the expression of key inflammatory mediators in both mouse and human PCHs, even at very low concentrations (10 ng/mL). Our data are in agreement with other previously published investigations regarding the production of inflammatory cytokines in PCHs in response to treatment with LPS (Liu et al., 2002; Panesar et al., 1999). A time-response analysis of CYP3A gene expression using PCN (10 μM) as a prototypical rodent PXR activator and Rif (10 μM) as a prototypical human PXR activator indicates that 24 hr treatment produces maximal CYP3A gene expression that is sustained through the 48 hr time point (***Supplementary data*, Figures S5).** Taken together, these data reveal that pre-treatment with PXR activators for 24 hr and subsequent co-treatment for 12 hr together with LPS is expected to be useful treatment regimen in a broad assessment of the effect of pre-activation of PXR on the subsequent LPS-inducible gene expression program in PCHs.

Pre-activation of NR Superfamily Members PXR, FXR, and LXRα Suppresses LPS-inducible IL-1β Gene Expression in Mouse PCHs. Together, IL-6 and IL-1β impart key aspects of the inflammatory response. IL-1β, In particular, is involved in regulating a variety of cellular activities including cell proliferation,

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differentiation, and apoptosis. Ligand-activation of NR superfamily members including PXR (Zhou et al., 2006), farnesoid x receptor (FXR, NR1H4) (Hollman et al., 2012), liver x receptor alpha (LXRα, NR1H3) (Ghisletti et al., 2007), and peroxisome proliferator antigen receptor alpha (PPARα, NR1C1) (Devchand et al., 1996) has been shown to suppress key aspects of the inflammatory response in several different cell types. Because expression of these four NR family members is highly enriched in hepatocytes, we sought to determine whether ligand-mediated activation of these receptors could alter LPS-inducible IL-1β gene expression in our PCHs (**Figure 3**). As expected, treatment of mouse PCHs with LPS alone for 12 hr increased IL-1β expression dramatically. Pre-activation of PXR, FXR, and LXRα for 24 hr with their cognate ligands suppressed subsequent LPS-inducible IL-1β gene expression. In contrast, pre-activation of PPARα had no effect on subsequent LPS-inducible IL-1β gene expression. Treatment of PCHs with each cognate ligand alone for 24 hr produced comparatively small but statistically significant increases in the expression of IL-1β. Treatment with each ligand also induced expression of their prototypical target genes Cyp3a11, bile salt excretory protein, Cyp7a1, and Cyp4a14 for PXR, FXR, LXRα, and PPARα respectively (data not shown). These data indicate that some, but not all, liver-enriched NR superfamily members may exhibit anti-inflammatory properties in PCHs, namely PXR, FXR and LXRα. It is also possible that these three liver-enriched NRs share a common molecular mechanism of repression of LPS-inducible inflammatory responses in PCHs, likely through alterations in post-translational modifications such as SUMOylation (Treuter and Venteclef, 2011). Unexpectedly, treatment with NR ligands alone induced statistically significant but relatively low levels of IL-1β expression, and was observed consistently throughout our studies and those performed by others (Vavassori et al., 2009). While the molecular mechanism of this phenomenon remains elusive, it could include the presence of cryptic or low-affinity NR response elements within the IL-1β promoter.

Pre-Activation of PXR Suppresses Key LPS-inducible NFκB-Target Genes. An important transcriptional mediator of LPS signaling in cells is nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)

(Sen and Baltimore, 1986). Treatment of primary cultures of mouse hepatocytes with LPS for 12 hr induced expression of sixteen well-known NFκB-target genes (**Table 1**). Treatment with PCN alone for 24 hr suppressed the basal expression of numerous NFκB-target genes (**Table 2**). When compared with LPS treatment alone, treatment with PCN for 24 hr and subsequent co-treatment together with LPS for an additional 12 hr produced significantly lower expression levels of several notable LPS-inducible NFκB-target genes to include IL-1β, IL-6, TNFα, and IL1-Ra (**Table 3**). These data suggest that PCN diminishes the APR by inhibiting the LPS-inducible gene expression program of key inflammatory mediators.

To further examine this PCN-mediated effect, we next chose to examine LPS-inducible gene expression of IL-1β, IL-6, TNFα, and IL1-Ra in PCHs derived from wild type and PXR-KO mice using independently designed real-time Q-PCR primer sets (**Table 4**). In wild type PCHs, treatment with LPS alone for 12 hr induced the expression levels of all four genes examined; whereas pre-activation of PXR with PCN for 24 hr and subsequent co-treatment together with LPS for an additional 12 hr produced approximately half that observed with LPS treatment alone (**Table 5**). Remarkably, treatment with PCN alone for 36 hr produced comparatively small but statistically significant increases in the levels of all four genes examined in PCHs isolated from wild type mice, but this effect was completely absent in the PXR-KO PCHs. All four genes examined were expressed at slightly higher levels in the vehicle treated PXR-KO PCHs when compared with vehicle-treated wild type PCHs. These data suggest a repressive role for PXR in regulating basal expression levels of key inflammatory mediators, and are also consistent with previous reports indicating that deletion of PXR in mice produces elevated levels of cytokine expression (Wallace et al., 2010; Zhou et al., 2006). Treatment of PXR-KO PCHs with LPS alone for 12 hr significantly increased the expression levels of all four genes examined. Contrary to the results obtained with wild type hepatocyte PCHs, pre-treatment of PXR-KO PCHs with PCN for 24 hr and subsequent co-treatment together with LPS for an additional 12 hr did not suppress subsequent LPS-inducible expression of any of the genes examined. These data reveal that PXR is required for PCN-mediated decreases in LPS-

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inducible target gene expression. Of note, the fold-induction of IL-6, TNFα, and IL1-Ra following 12 hr LPS treatment was less robust in the PXR-KO cultures when compared with 12 hr LPS-treated wild type hepatocyte cultures. In stark contrast, LPS-inducible IL-1β expression levels were enhanced in PXR-KO PCHs when compared with that observed in wild type PCHs (**Table 5, bold lettering**). These data reveal that the absence of PXR produces a condition in which the expression of inflammatory mediators is heightened, and suggests that PXR is required for the effective resolution of the IL-1β inflammatory response through time. Closer inspection of the biological function of the gene products identified in our gene expression analysis indicate that one gene product in particular, IL1-Ra, is intimately associated with resolution of the IL1-signaling pathway and is expressed at high levels in hepatocytes.

The IL1-Ra messenger RNA is detected primarily in the hepatocytes, and its expression is induced by several inflammatory mediators and encodes a secreted antagonist of IL1-signaling (Arend and Gabay, 2000; Arend and Guthridge, 2000). We therefore more closely examined the concentration- and time-dependent LPS-inducible expression of IL1-Ra messenger RNA in wild type PCHs (**Figure 4A**). Treatment with increasing concentrations of LPS (0.01, 0.1, 1, 10, and 100 μg/mL) for 12 hr produced significant increases in the expression level of IL1-Ra. We next treated wild type cultures with high concentration LPS (10 μg/mL) for increasing amounts of time (1, 6, 12, 24, and 48 hr) to examine time-dependent LPS-inducible IL1-Ra expression levels (**Figure 4B**). Significant levels of LPS-inducible IL1-Ra gene expression was observed at the 6, 12, 24, and 48 hr time points. When compared with the time- and concentration-response analysis of IL-1β and IL-6, the detectable induction of IL1-Ra was delayed by several hr, but remained relatively high all the way through the 48 hr time point (**Figure 4B –versus- Figure 1B**). Similar results were obtained using PCHs derived from a human donor to examine the concentration- and time-dependent induction of IL1-Ra messenger RNA (**Figure 5A and 5B**). These data indicate that the kinetics of LPS-inducible IL1-Ra gene expression is distinct from that observed for

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inflammatory mediators IL-1β and IL-6, with expression levels of IL1-Ra increasing at later time points (6-12 hr) and exhibiting a longer period of sustained expression through the 48 hr time point.

There are two major isoforms of the IL1-Ra protein that arise from a single gene called *IL1RN*, and their expression is differentially regulated at the level of transcription by alternative promoters (Butcher et al., 1994). One form of IL1-Ra is a heavily glycosylated and secreted isoform (sIL1-Ra), while the other one is an intracellular isoform (icIL1-Ra). The primary function of sIL1-Ra is to competitively inhibit IL1-binding to cell surface receptors (Arend et al., 1990). The expression and secretion of sIL1-Ra is highly inducible in hepatocytes by inflammatory stimuli, whereas the expression of icIL1-Ra is not inducible and its biological function remains largely unknown (Arend and Guthridge, 2000). The observation that LPS-inducible IL-1β messenger RNA expression was enhanced in the PXR-KO PCHs when compared with LPS-treated wild type PCHs (**Table** 5) prompted us to examine the PXR- and LPS-inducible level of sIL1-Ra and icIL1-Ra proteins in both media and whole cell lysate, respectively (**Figure 6**). When treated with PCN or Rif alone for 36 hr, the level of sIL1-Ra protein increased in the media approximately 2.5- and 3.5-fold in PCHs from wild type and $hPXR_{tg}$ mice, respectively. When treated with LPS for 12 hr, the level of sIL1-Ra protein increased in the media approximately 1.9- and 1.8-fold from wild type and $hPXR_{tg}$, respectively. When treated with PCN or Rif for 24 hr, and then subsequently co-treated with LPS for an additional 12 hr, the level of sIL1-Ra protein increased in the media approximately 1.7- and 3.1-fold from wild type and $hPXR_{tg}$, respectively. When PCHs from PXR-KO mice were used in identical experiments, PCN had no effect on induction of sIL1-Ra protein in media. In contrast, 12 hr LPS treatment produced an enhanced effect in PXR-KO PCHs when compared with PXR-positive cultures. In addition, co-treatment with PCN and LPS together failed to diminish sIL1-Ra levels in media from the PXR-KO PCHs.

Taken together, the studies presented above indicate that PXR activation has both an early negative regulatory role in the LPS-inducible expression of key inflammatory mediators such as IL-1β, as well as a likely positive role in regulating ligand-inducible expression of the secreted form of the IL1-Ra protein at

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later time points. To more closely examine the potential positive role of PXR activation in regulating sIL1-Ra protein levels across species, we chose to perform a longer time-course study using primary cultures of human hepatocytes (**Figure 7**). Treatment of PCHs from a human donor with Rif for 48 hr produced an approximate 3.9-fold increase in sIL1-Ra levels in culture media, whereas 24 hr treatment with LPS increased levels by approximately 1.9-fold. Treatment of human hepatocyte cultures with Rif for 48 hr, followed by co-treatment with Rif and LPS together for an additional 24 hr produced an approximate 8.3-fold increase in sIL1-Ra in the culture media. These data indicate that long-term pre-activation of PXR in both rodent and human PCHs has a strong positive effect upon the secretion of sIL1-Ra from cells, an important and systemic negative regulator of IL1 signaling and key participant in the compensatory anti-inflammatory response in mammals.

Discussion

The liver is a crucial organ that plays a central role in acute phase protein synthesis during bacterial sepsis. The resulting cytokine stimulation rapidly up-regulates expression of acute phase proteins, and simultaneously down-regulates key drug metabolism pathways in liver. While pro-inflammatory cytokines initiate the APR through different cell surface receptors, they share a high level of redundancy with respect to the signal transduction pathways (e.g. Ikappa B kinase, p38, and JNK) by which they exert their influence in the nucleus [reviewed in (Heinrich et al., 2003; Lu et al., 2008; Wajant et al., 2003; Weber et al., 2010)]. Initiation of the acute inflammatory response is mainly achieved through signal-dependent activation of NFkB and AP-1 transcription factors through promoter response elements that regulate expression of genes encoding important pro-inflammatory cytokines.

Treatment of experimental models with LPS stimulates pattern recognition receptors, mainly Toll-like receptor 4 (TLR4), to induce the rapid expression and release of pro-inflammatory cytokines such as TNFα. Secreted TNFα further exerts its inflammatory function through binding to TNF receptor type 1

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(TNFR1) and TNFR2 on various hepatic cell types including hepatocytes. Separate receptor types recognize either IL-6 or IL-1β, respectively, to further initiate and amplify the acute inflammatory response in feed-forward loops. Importantly, treatment of liver with LPS leads to release of pro-inflammatory cytokines IL1β and TNFα from both non-parenchymal cells and hepatocytes. Stimulation with IL-1β induces TNFα secretion from rat hepatocytes, and stimulation of hepatocytes with either IL-1β or TNFα produces IL-6 secretion (Panesar et al., 1999; Yoshigai et al., 2014). Hence, there are multiple levels of interconnection and amplification that occur rapidly between and among inflammatory cytokines following bacterial sepsis.

From a historical perspective, hepatocytes were initially viewed as passive recipients of immune messages from non-parenchymal cells including Kupffer cells (Volpes et al., 1992). However, more recent investigations indicate that this is in fact not the case. Hepatocytes mount a robust response to challenge with either LPS or IL-1β to produce key inflammatory cytokines including IL-6, TNFα, and IL-1β (Liu et al., 2002; Panesar et al., 1999; Spencer et al., 2013; Takano et al., 2012; Yoshigai et al., 2014). It is also now well known that hepatocytes express all necessary machinery to respond to bacterial sepsis including TLR4, CD14, Myd88, and MD-2 (Liu et al., 2002). A recent study indicates that hepatocyte-specific knockout of TLR4 receptor in mice significantly attenuates the systemic serum levels of inflammatory mediators TNFα, IL-6, and IL-1β in response to high fat diet (Jia et al., 2014). Hepatocytes are therefore not merely passive recipients of immune signals from non-parenchymal cells, but instead can be viewed as active participants in mediating an immune response to a variety of signals including sepsis and morbid obesity.

Much attention has recently been given to the notion that PXR activation by Rif and its analogues may be beneficial in treatment of inflammatory liver and bowel diseases (Cheng et al., 2012; Jonker et al., 2012; Kakizaki et al., 2011; Wallace et al., 2010). Additional research indicates a key role for PXR in maintaining the barrier function of the gut (Dou et al., 2012; Dou et al., 2014; Venkatesh et al., 2014).

Thus, detailed knowledge of the molecular mechanisms governing PXR-mediated suppression of the APR in these tissues is vital but is currently lacking. It is well known that SUMOylation modifies the transactivation capacity of a myriad of transcription factors, and in most cases correlates with transcriptional suppression [Reviewed in (Gill, 2005)]. For example, the SUMO-modification of liver-enriched NR family members is implicated in suppression of NR-function and in modulation of the APR (Balasubramaniyan et al., 2013; Ghisletti et al., 2007; Treuter and Venteclef, 2011; Venteclef et al., 2010; Zhou et al., 2012). While several liver-enriched NRs are the molecular target of the SUMO signaling pathway, not all of them are. For example, constitutive androstane receptor, a close relative of PXR, is not SUMOylated (unpublished observation). We have previously shown that PXR is SUMOylated in response to TNFα treatment in both human and mouse PCHs (Hu et al., 2010). These observations suggest that shared molecular mechanism(s) exists to govern the conversion of the primary metabolic function of liver during the non-septic or non-inflamed state to one that is involved in resolving the APR during sepsis or injury that likely involve SUMOylation.

Here we present a working model that defines a novel pathway for the feedback inhibition and resolution of the inflammatory response in hepatocytes through time (**Figure 8**). Following injury or infection, we propose that low stoichiometric amounts of SUMO-modified PXR function to directly suppress pro-inflammatory mediators IL-1β, IL-6, and TNFα at the level of transcription, while the remainder of PXR protein is likely ubiquitinated and subsequently degraded by the 26S proteasome in a signal dependent manner through time. Indeed, significantly lower levels of PXR protein are detected in endotoxin-treated mice (Teng and Piquette-Miller, 2005). As the APR ensues through time, newly synthesized PXR protein becomes available for up-regulating ligand-dependent expression of novel or alternative PXR-target genes to include the negative regulator of IL1 signaling, sIL1-Ra, possibly through cryptic or low-affinity PXR-response elements. Of note, a recent investigation indicates that peroxisome proliferator-activated receptor gamma coactivator 1-alpha, a strong PXR-coactivator protein, controls

expression of IL1-Ra in liver (Buler et al., 2012). In this way, PXR activation gains a novel repressive anti-inflammatory function and plays an active role in the resolution of the inflammatory response through time. Future efforts should seek to determine whether this mechanism by which PXR is converted from a positive regulator of drug metabolizing enzymes into a transcriptional suppressor of inflammation in liver will provide new pharmacological strategies for modulating inflammatory-related diseases in liver and intestine.

In the current study, LPS was used to initiate the acute inflammatory response in an effort to determine the extent to which PXR-mediated suppression of the inflammatory response was dependent upon cell surface receptor type, e.g. TLR4 receptor versus TNFR. Data presented here indicate that PXR activation negatively regulates the LPS-inducible gene expression program in hepatocytes similar to that observed with TNFα or phorbol ester stimulation (Hu et al., 2010; Zhou et al., 2006). Taken together, these data indicate that the negative regulatory role for PXR in inflammation is not specific to cell surface receptor type, and likely operates at the level of the promoters for key inflammatory cytokines TNFα, IL1β, and IL-6. In a symmetrical manner, stimulation of the inflammatory response is well known to suppress drug metabolism pathways in liver through the rapid and selective down-regulation of specific CYP enzymes. Several possible mechanisms have been proposed for this phenomenon including the reduction of PXR mRNA levels during sepsis (Beigneux et al., 2002). Another line of thought postulated the disruption the association between PXR-RXRα heterodimer complex by a signal-dependent interaction of NFkB with RXRα, thereby sequestering the active form of PXR that regulates drug-inducible gene expression (Gu et al., 2006). While plausible and not mutually exclusive, the precise signals and molecular mechanisms for inflammation-inducible repression of drug metabolism deserve further scrutiny.

Authorship Contributions

Participated in research design: Sun, Cui, Woody, Staudinger

Conducted experiments: Sun, Cui, Woody

Performed data analysis: Sun, Cui, Woody, Staudinger

Wrote or contributed to the writing of the manuscript: Sun, Woody, Staudinger

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Footnotes:

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Figure Legends

Figure 1. Concentration- and Time-dependent Analysis of the Expression of IL-1β in Mouse PCHs. (**A**) PCHs isolated from wild-type (C57Bl6) mice were treated with either vehicle (0.1% saline in media) or increasing concentrations of LPS (0.01, 0.1, 1.0, 10, or 100 μg/ml) for 12 hr. Total RNA was isolated and the relative expression level of IL-1β was determined using Q-PCR. (**B**) PCHs isolated from wild-type (C57Bl6) mice were treated with LPS (10 μg/ml) for increasing times (1, 6, 12, 24, and 48 hr). Total RNA was isolated and the relative expression level of IL-1β was determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

Figure 2. Concentration- and Time-dependent Analysis of the Expression of IL-1β in Human PCHs. (**A**) PCHs isolated from a human donor were treated with either vehicle (0.1% saline in media) or increasing concentrations of LPS (0.01, 0.1, 1.0, 10, or 100 μg/ml) for 12 hr. Total RNA was isolated and the relative expression level of IL-1β was determined. (**B**)**.** PCHs isolated from a human donor were treated with LPS (10 μg/ml) for increasing times (1, 6, 12, 24, and 48 hr). Total RNA was isolated and the relative expression level of IL-1β was determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

Figure 3. LPS Induces the Expression of the Compensatory anti-inflammatory Response Gene IL1-Ra in Primary Cultures of Mouse Hepatocytes. Primary hepatocytes isolated from wild-type (C57Bl6) mice were treated with either vehicle (0.1% DMSO), PCN (10 μM), GW4064 (1 μM), GW3965 (1 μM), or GW7647 (1 μM) for 24 hr. Media was removed and replenished with media containing the treatments for an additional 12 hr as indicated, respectively. Total RNA was isolated and the relative expression level of IL-1β was determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

Dashed lines indicate a statistical difference between treatment with LPS alone for 12 hr when compared with pre-treatment with cognate ligand for 24 hr and subsequent co-treatment together with LPS for an additional 12 hr.

Figure 4. LPS Induces the Expression of the Compensatory anti-inflammatory Response Gene IL1-Ra in Mouse PCHs. (**A**). PCHs isolated from wild-type (C57Bl6) mice were treated with either vehicle (0.1% saline in media) or increasing concentrations of LPS (0.01, 0.1, 1.0, 10, or 100 μg/ml) for 12 hr. Total RNA was isolated and the relative expression level of IL1-Ra was determined. (**B**). PCHs isolated from wild-type (C57Bl6) mice were treated with LPS (10 μg/ml) for increasing times (1, 6, 12, 24, and 48 hr). Total RNA was isolated and the relative expression level of IL1-Ra was determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

Figure 5. LPS Induces the Expression of the Compensatory anti-inflammatory Response Gene IL1-Ra in Human PCHs. (**A**). PCHs isolated from a human donor was treated with either vehicle (0.1% saline in media) or increasing concentrations of LPS (0.01, 0.1, 1.0, 10, or 100 μg/ml) for 12 hr. Total RNA was isolated and the relative expression level of IL1-Ra was determined. (**B**). PCHs isolated from a human donor was treated with LPS (10 μg/ml) for increasing times (1, 6, 12, 24, and 48 hr). Total RNA was isolated and the relative expression level of IL1-Ra was determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

Figure 6. Analysis of the Secreted Form of IL1-Ra Protein in Culture Media from PCHs Isolated from Wild Type, hPXR$_{tg}$, and PXR-KO Mice. PCHs were isolated from the indicated genotype and were treated with vehicle (0.1% DMSO) or 10 μM PCN for 24 hr. Cell cultures were then divided into four experimental groups and were treated for an additional 12 hr with either vehicle, PCN alone, 10 μg/mL LPS alone, or PCN and LPS together. Western blot analysis of the secreted form (sIL1-Ra) and

intracellular form (icIL1-Ra) of IL1-Ra was performed. Western blot analysis of the secreted form (sIL1-Ra) and intracellular form (icIL1-Ra) of IL1-Ra was performed. Western Blot images were quantitated by densitometric scanning of the X-ray films with the UVP Biodoc-It 220 image analysis system and 1D Gel Analysis Software, and numbers represent densitometric image intensity of sIL1-Ra divided by image intensity of icIL1-Ra.

Figure 7. Analysis of the Secreted Form of IL1-Ra Protein in Culture Media from Human PCHs. PCHs from a human donor were treated with vehicle (0.1% DMSO) or 10 μM Rif for 24 hr. The cultures were then divided into four experimental groups and were treated for an additional 12 hr with either vehicle, Rif alone, 10 μg/mL LPS alone, or Rif and LPS together. Western Blot images were quantitated by densitometric scanning of the X-ray films with the UVP Biodoc-It 220 image analysis system and 1D Gel Analysis Software, and numbers represent densitometric image intensity of sIL1-Ra divided by image intensity of icIL1-Ra.

Figure 8. Model of the Mechanism of PXR-mediated Interaction with the Inflammatory Response in Hepatocytes.

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Table 1. Genes Increased by LPS. PCHs isolated from wild type mice were treated for 12 hours with either vehicle (0.9% saline) or LPS (10 μg/mL) (n=4). Total RNA was isolated and Q-PCR was performed per manufacturer's instructions (SA Biosciences). Data are expressed as fold induction ± standard deviation (S.D.) where $p \leq 0.05$.

Gene Name	Fold Induction	S.D.
Cxcl3	393.9	80.8
IL-1β	314.4	77.4
IL-6	128.9	43.2
Csf3	63.4	20.4
IL12β	52.5	12.4
Ccl5	50.5	11.5
Ptgs2	50.5	10.6
Ltb	50.0	10.6
IL1-Ra	49.7	9.2
IL-1α	31.0	7.8
Cxcl1	10.2	2.3
TNFα	10.0	4.6
Sele	6.3	2.9
Cd74	6.3	2.1
Vcam1	5.0	1.1
Bcl2a1a	3.9	1.3

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Table 2. Genes Suppressed by PCN. PCHs isolated from wild type mice were treated with either vehicle (0.1% DMSO) or PCN (10 μM) for 24 hours (n=3). Total RNA was isolated and Q-PCR was performed per manufacturer's instructions (SA Biosciences). Data are expressed as fold suppression ± standard deviation (S.D.) in the PCN treated group when compared with vehicle treated cells ($p \leq 0.05$).

Gene Name	Fold Suppression	S.D.
Selp	5.1	2.4
C3	4.2	2.0
Csf2	4.1	0.3
TNFsf10	4.1	2.0
Agt	4.1	1.9
Myd88	3.3	1.4
Aldh3a2	3.3	1.6
Csf2rb	3.2	1.5
F8	3.2	1.5
Ifnb1	2.6	1.0
Cfb	2.6	1.0
IL1-Ra	2.5	0.8
Ifn□	2.2	0.8
Trp53	2.1	0.8
Akt1	2.1	0.8
Ccl22	2.1	0.8
Nqo1	2.1	0.8
Cxcl3	2.1	0.6
Mitf	2.0	0.8
Fas	2.0	0.8
Stat3	2.0	0.8
TNFrsf1b	2.0	0.8
Rel	2.0	0.8
Stat5b	2.0	0.8
Xiap	2.0	0.8
Irf1	2.0	0.8
Fasl	2.0	0.7

Table 3. Expression Profiling of Primary Cultures of Mouse Hepatocytes Co-treated with LPS and PCN when compared with LPS Alone. PCHs from wild type mice were pre-treated with PCN or vehicle (0.1% DMSO) for 24 hours (n=6). Cultures were divided into two experimental groups and were treated with LPS alone or were co-treated with PCN and LPS together for an additional 12 hr (n=3). Total RNA was isolated and Q-PCR was performed per manufacturer's instructions (SA Biosciences). Data are expressed as fold suppression $\pm$ standard deviation (S.D.) in the co-treated (PCN + LPS) group when compared with LPS alone ($p \leq 0.05$).

Gene Name	Fold Suppression	S.D.
Ptgs2	2.5	0.6
Mmp9	2.5	0.8
Cd83	2.5	0.8
Cd74	2.5	0.6
IL-6	2.0	0.4
IL1-Ra	2.0	0.5
IL-1β	1.6	0.2
TNFα	1.6	0.3

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Table 4. Primer Sequences used for Independent Analysis of Gene Expression. Primer sequences were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank/).

Gene	Primer Sequences	
mIL-1β	Forward	5' gAA ATg CCA CCT TTT gAC AgT g 3'
	Reverse	5' CTg gAT gCT CTC ATC Agg ACA 3'
mIL-6	Forward	5' CTg CAA gAg ACT TCC ATC Cag 3'
	Reverse	5' AgT ggT ATA gAC Agg TCT gTT gg 3'
mIL1-Ra	Forward	5' TAg ACA Tgg TgC CTA TTg ACC T 3'
	Reverse	5' TCg TgA CTA TAA ggg gCT CTT C 3'
mTNFα	Forward	5' CAg gCg gTg CCT ATg TCT C 3'
	Reverse	5' CgA TCA CCC CgA AgT TCA gAT g 3'
hIL-1β	Forward	5' ATg ATg gCT TAT TAC AgT ggC AA 3'
	Reverse	5' gTC ggA gAT TCg TAg CTg gA 3'
hIL-6	Forward	5' ACT CAC CTC TTC AgA ACg AAT Tg 3'
	Reverse	5' CCA TCT TTg gAA ggT TCA ggT Tg 3'
hIL-1Ra	Forward	5' CAT TgA gCC TCA TgC TCT gTT 3'
	Reverse	5' CgC TgT CTg AgC ggA TgA A 3'
m/h β-actin	Forward	5' CAA gAT CAT TgC TCC TCC Tg 3'
	Reverse	5' TAA CAg TCC gCC TAg AAg CA 3'

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Table 5. Expression Level of IL-1β, IL-6, TNFα, and IL1-Ra in Wild Type and PXR-KO PCHs. PCHs isolated from wild type (n=6) or PXR-KO mice (n=6) were pre-treated for 24 hr with either vehicle (0.1% DMSO) or PCN (10 μM). Cultures were split into two experimental groups (n=3) and then were treated with vehicle, PCN, LPS (10 μg/mL), or were co-treated with PCN and LPS together for an additional 12 hr. Total RNA was isolated and real time Q-PCR was performed using custom designed primer pairs (**Table S4**). Data are expressed as fold induction ± standard deviation (S.D.) and are considered statistically significant (^) when compared with the vehicle treated wild type experimental group ($p \leq 0.05$). The asterisks (*) in the wild type [PCN + LPS] experimental group indicate a statistical difference when compared with LPS-treatment alone. The **bold numbers** in the PXR-KO experimental group indicate that IL-1β expression was enhanced in both of the LPS alone and the [LPS + PCN] experimental groups, when compared with IL-1β expression levels in wild type hepatocyte cultures.

	Wild Type Hepatocyte Cultures			
	Vehicle	**LPS**	**PCN + LPS**	**PCN**
IL-1β	1.0 ± 0.1	^1723.2 ± 21.7	^*884.4 ± 96.2	^96.11 ± 8.3
IL-6	1.0 ± 0.1	^379.9 ± 5.1	^*154.2 ± 17.9	^15.1 ± 2.5
TNFα	1.0 ± 0.2	^54.2 ± 8.7	^*24.5 ± 3.1	^8.2 ± 1.2
IL1-Ra	1.0 ± 0.3	^35.2 ± 4.2	^*15.3 ± 1.2	^5.4 ± 0.5
	PXR-KO Hepatocyte Cultures			
	Vehicle	**LPS**	**PCN + LPS**	**PCN**
IL-1β	^204.4 ± 24.1	**^3351.2 ± 294.4**	**^4094.8 ± 201.2**	^246.11 ± 38.3
IL-6	^25.9 ± 2.9	^176.2 ± 5.1	^199.22 ± 11.9	^25.1 ± 4.6
TNFα	^5.8 ± 0 .8	^34.2 ± 18.6	^38.4 ± 25.3	^8.1 ± 4.2
IL1-Ra	^3.7 ± 0.6	^21.9 ± 4.2	^25.3 ± 3.7	^5.2 ± 0.5

Figure 1A

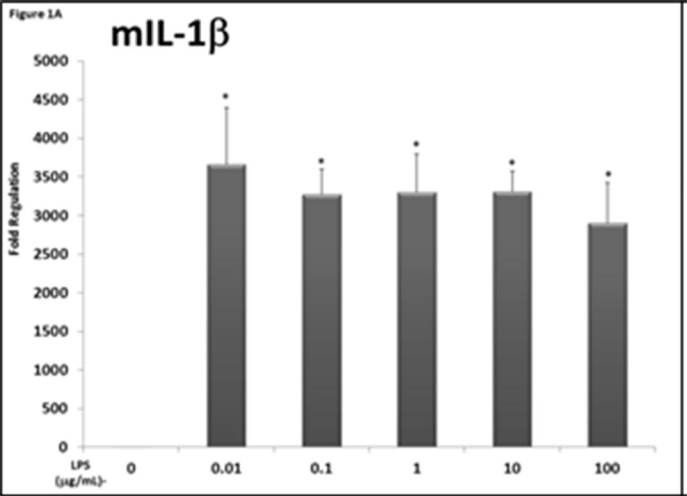


Figure 1B

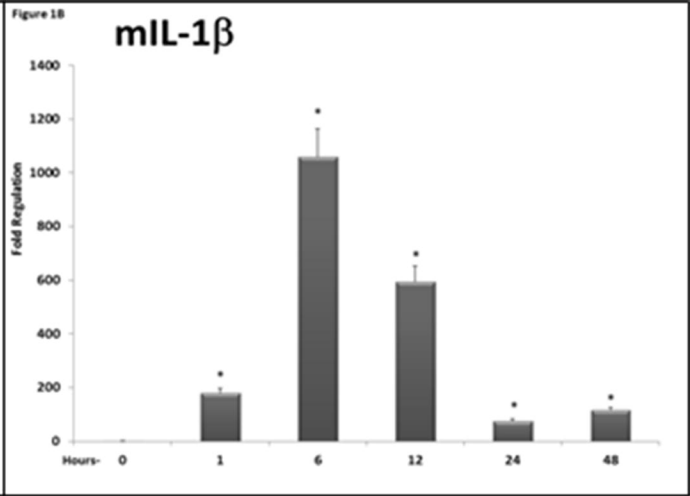


Figure 2A

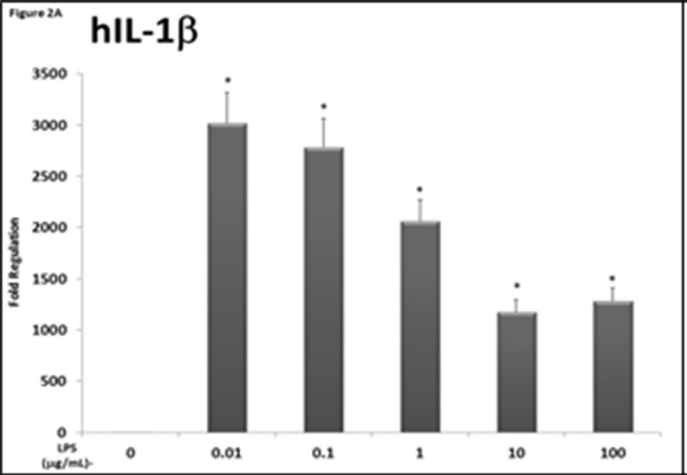


Figure 2B

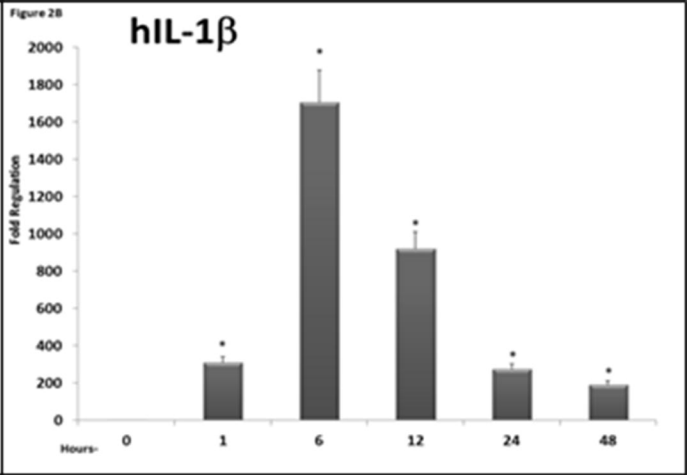


Figure 3

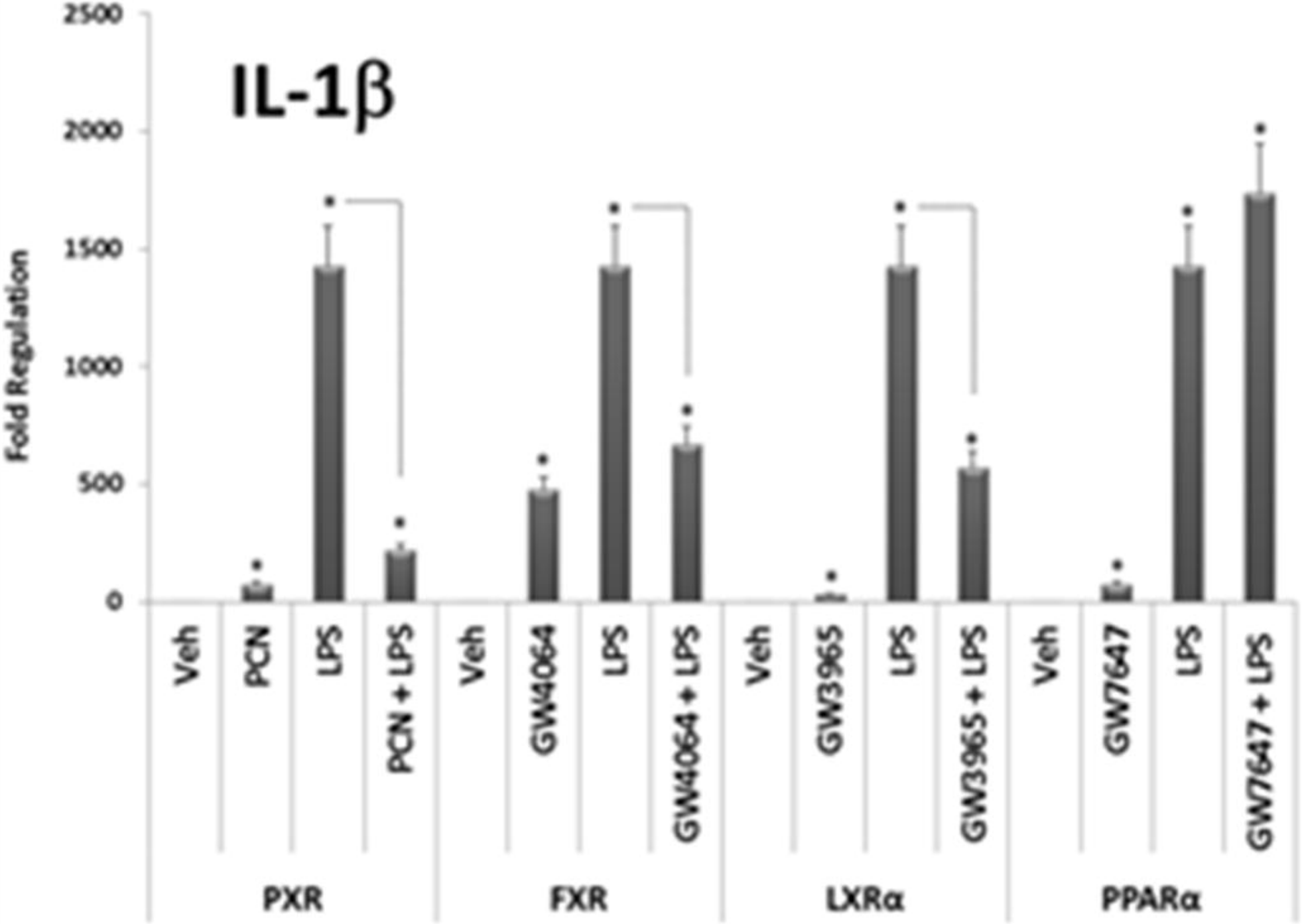

IL-1β
Fold Regulation
2500
2000
1500
1000
500
0
Veh
PCN
LPS
PCN + LPS
Veh
GW4064
LPS
GW4064 + LPS
Veh
GW3965
LPS
GW3965 + LPS
Veh
GW7647
LPS
GW7647 + LPS
PXR
FXR
LXRα
PPARα

Figure 4A

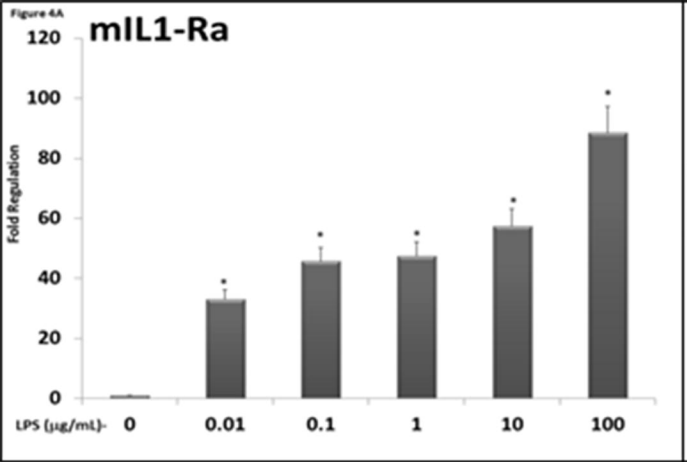


Figure 4B

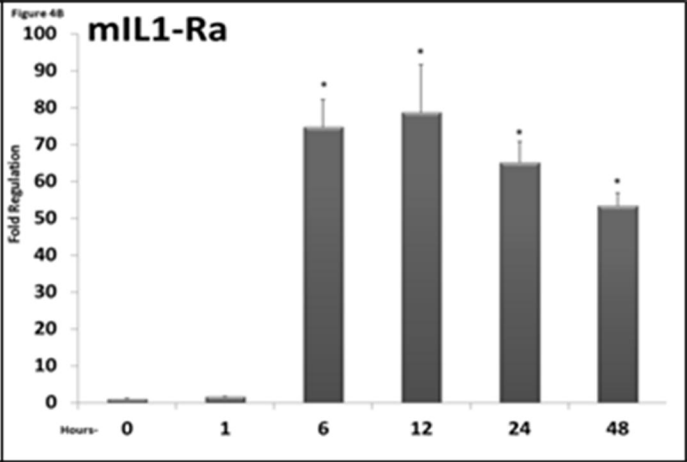


Figure 5A

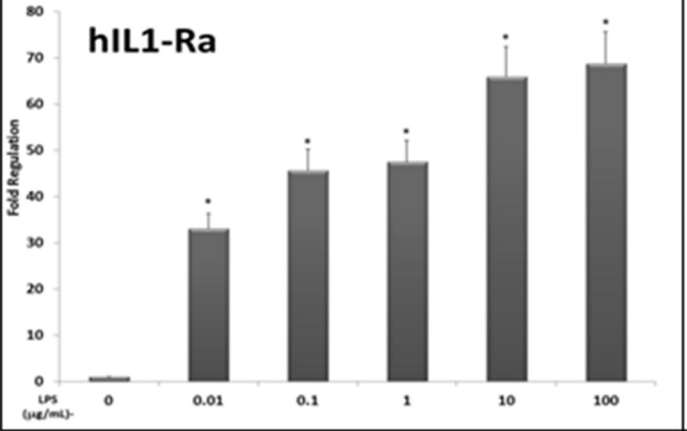


Figure 5B

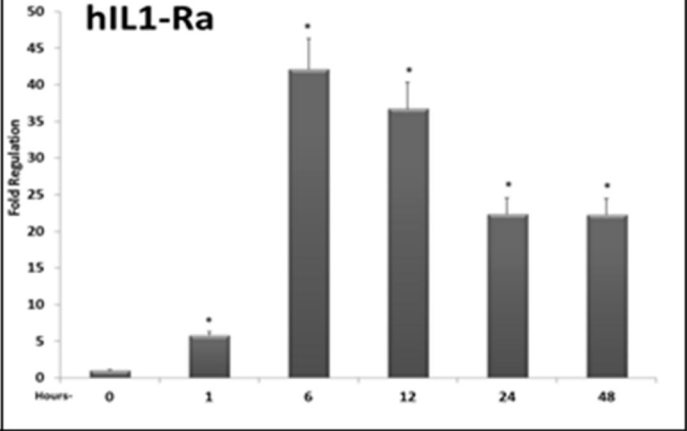


Figure 6

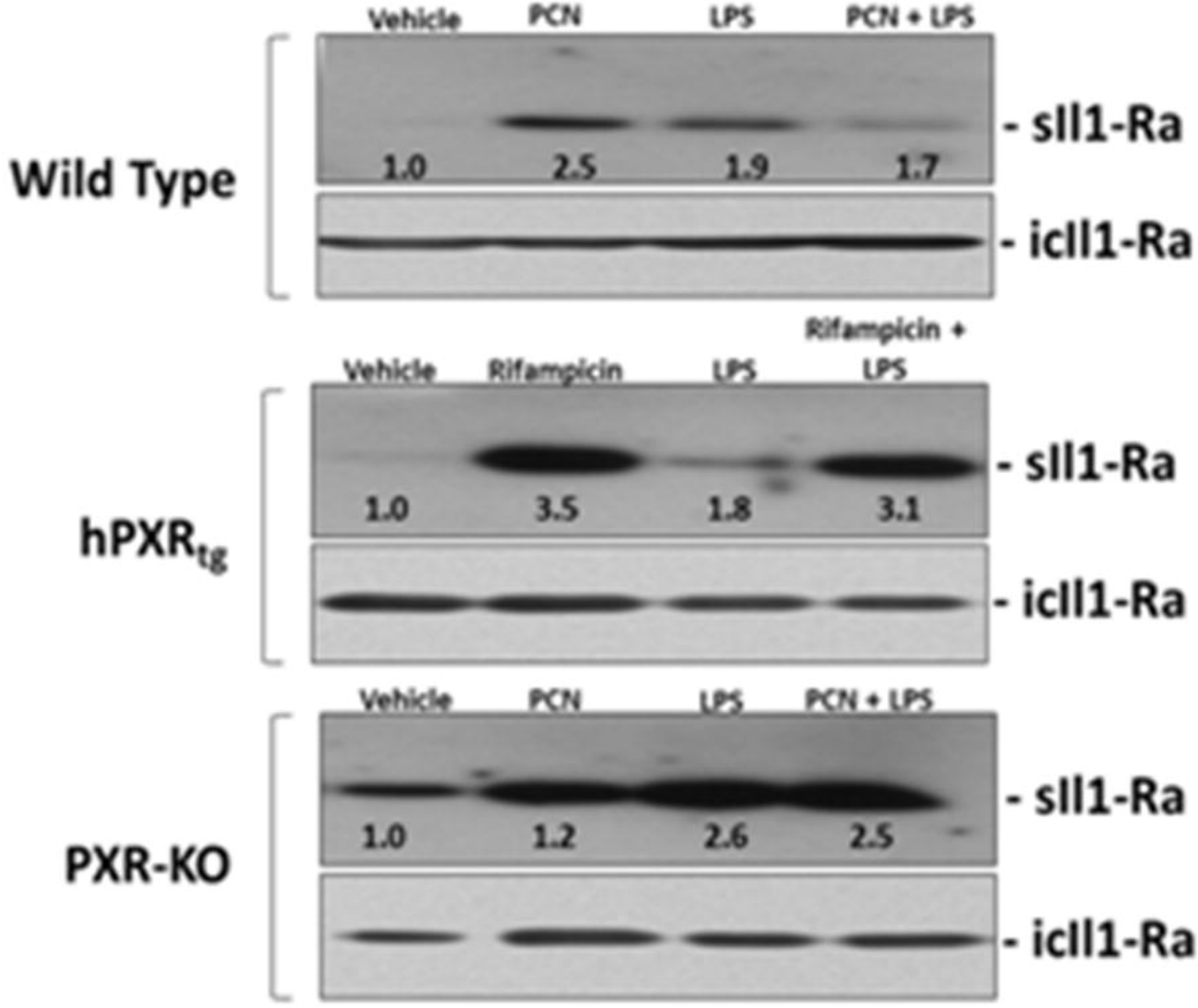

Vehicle
PCN
LPS
PCN + LPS
Wild Type
1.0
2.5
1.9
1.7
- sIl1-Ra
- icIl1-Ra
Rifampicin +
Vehicle
Rifampicin
LPS
LPS
hPXR$_{tg}$
1.0
3.5
1.8
3.1
- sIl1-Ra
- icIl1-Ra
Vehicle
PCN
LPS
PCN + LPS
PXR-KO
1.0
1.2
2.6
2.5
- sIl1-Ra
- icIl1-Ra

Figure 7

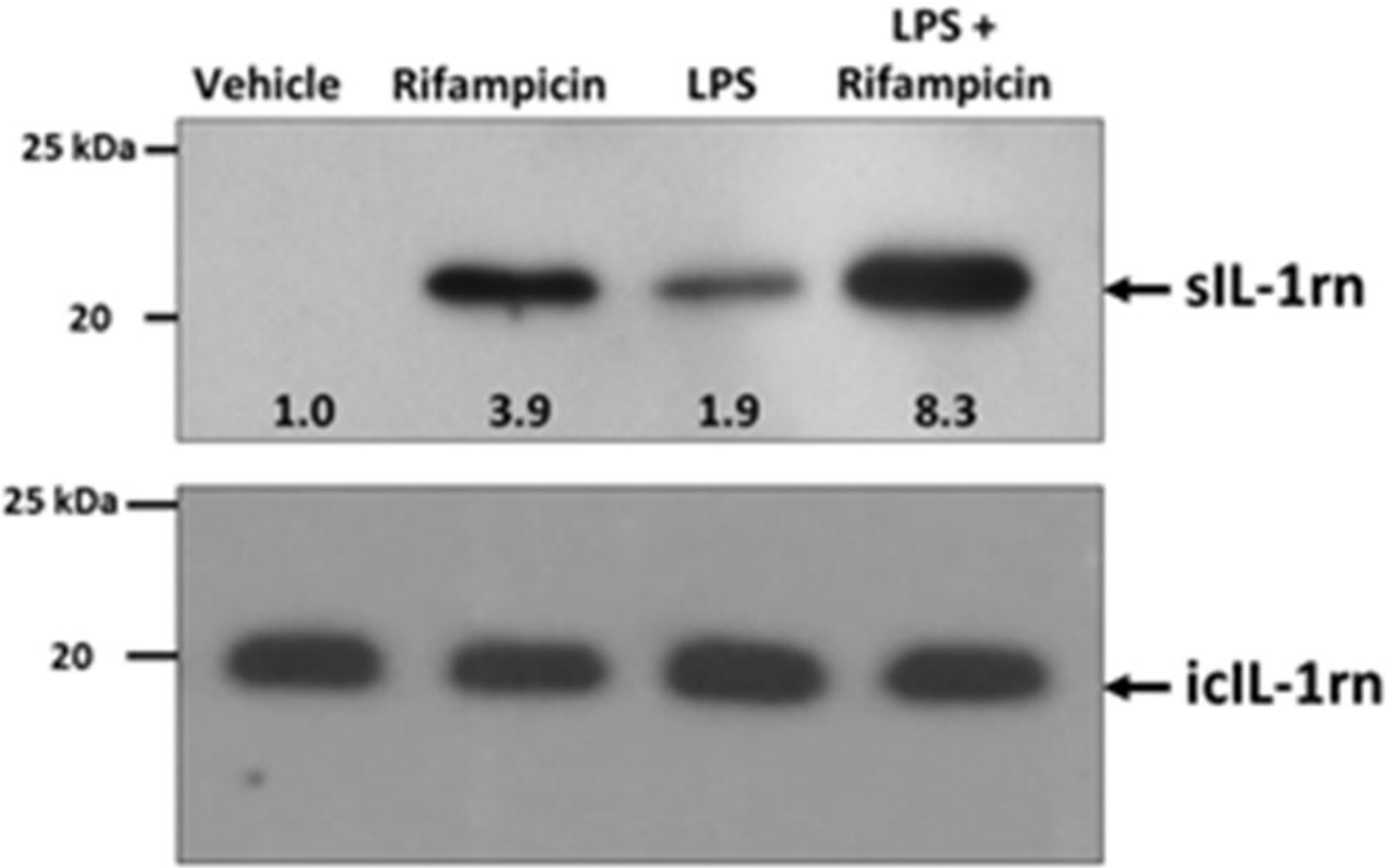
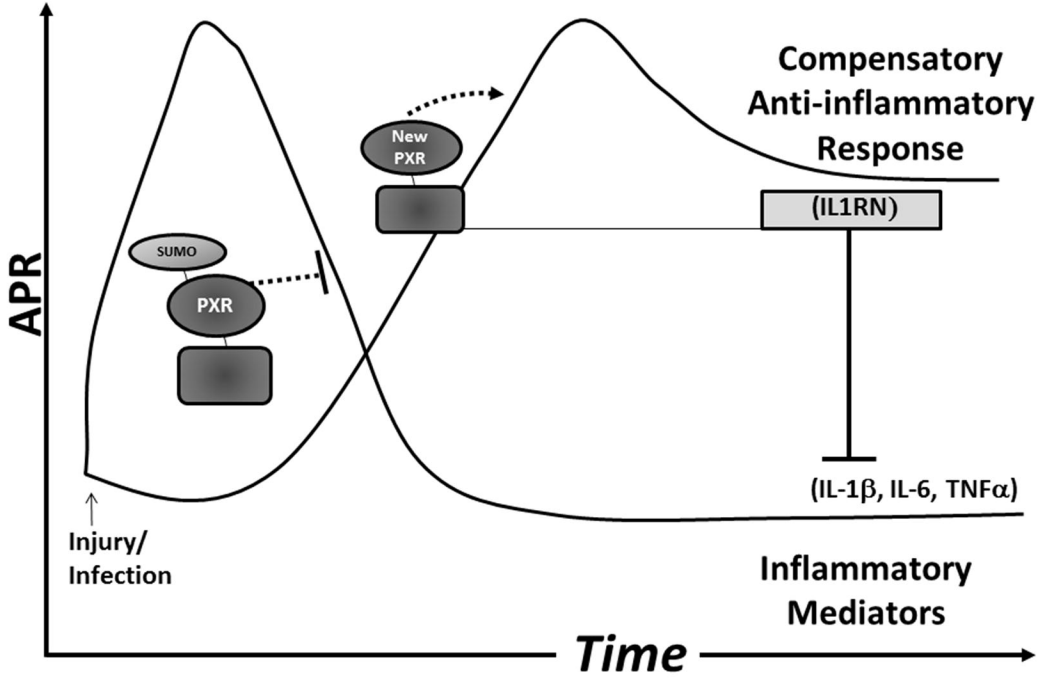

Vehicle
Rifampicin
LPS
LPS + Rifampicin
25 kDa
20
1.0
3.9
1.9
8.3
sIL-1rn
25 kDa
20
icIL-1rn

Figure 8


APR
Time
Compensatory
Anti-inflammatory
Response
New
PXR
(IL1RN)
SUMO
PXR
(IL-1β, IL-6, TNFα)
Injury/
Infection
Inflammatory
Mediators