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# Role of adaptor protein Toll-Like Interleukin domain containing adaptor inducing interferon β in Toll-Like Receptor 3 & 4 mediated regulation of hepatic drug-metabolizing enzymes and transporter genes.

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**Running title:** Role of TRIF and MAPK in TLR3 & 4-mediated DMET alterations

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**Abbreviations**: NRs, nuclear receptors; PolyI:C, polyinosinic:polycytidylic acid; LPS, lipopolysaccharide; TLRs, Toll-like receptors; DMET, drug metabolizing enzymes and transporters; CYP450, cytochrome P450; TIRAP, Toll/interleukin (IL)-1 receptor (TIR) domain containing adaptor protein; TRIF, TIR domain containing adaptor inducing interferon (IFN)-β

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## Abstract

The expression and activity of hepatic drug metabolizing enzymes and transporters (DMETs) is altered during infection and inflammation. Inflammatory responses in the liver are primarily mediated by Toll-like receptor (TLR)-signaling which involves recruitment of Toll/interleukin (IL)-1 receptor (TIR) domain containing adaptor protein (TIRAP) and TIR domain containing adaptor inducing interferon (IFN)-β (TRIF) that eventually leads to induction of pro-inflammatory cytokines and mitogen-activated protein kinases (MAPKs). Lipopolysaccharide (LPS) activates the gram-negative bacterial receptor, TLR4 and polyinosinic:polycytidylic acid (polyI:C) activates the viral receptor, TLR3. TLR4 signaling involves TIRAP and TRIF, while TRIF is the only adaptor protein involved in the TLR3 pathway. We have shown previously that LPS-mediated down-regulation of DMETs is independent of TIRAP. To determine the role of TRIF, we treated $TRIF^{+/+}$ and $TRIF^{-/-}$ mice with LPS or polyI:C. LPS down-regulated (~40-60%) *Cyp3a11*, *Cyp2a4*, *Ugt1a1*, *Mrp2* mRNA levels, whereas polyI:C down-regulated (~30-60%) *Cyp3a11, Cyp2a4, Cyp1a2, Cyp2b10, Ugt1a1, Mrp2, Mrp3* mRNA levels in $TRIF^{+/+}$ mice. This down-regulation was not attenuated in $TRIF^{-/-}$ mice. Induction of cytokines by LPS was observed in both $TRIF^{+/+}$ and $TRIF^{-/-}$ mice. Cytokine induction was delayed in polyI:C-treated $TRIF^{-/-}$ mice, indicating that multiple mechanisms mediating polyI:C signaling exist. To assess the role of MAPKs, primary hepatocytes were pre-treated with specific inhibitors before treatment with LPS/polyI:C. We found that only the c-jun-N-terminal kinase (JNK) inhibitor attenuated the down-regulation of DMETs. These results show that TRIF-independent pathways can be involved in the down-regulation of DMETs through TLR4 & 3. JNK-dependent mechanisms likely mediate this down-regulation.

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## Introduction

Inflammation and infection have been known to down-regulate expression and activity of drug metabolizing enzymes and transporters (DMETs) and cause changes in clearance of drugs however, the exact mechanism is unclear (Morgan ET, 1997; Renton KW 2001, 2004). Toll like receptors (TLRs) are the major mediators of inflammatory responses in the liver. TLRs promote initial host defense against microorganisms by recognizing distinct microbial/viral components as pathogen-associated molecular patterns (PAMPs) (Rock et al., 1998). Upon recognition of PAMPs, TLR signaling induces potent innate immune responses that signal through adaptor molecules including myeloid differentiation factor 88 (MyD88), Toll/interleukin (IL)-1 receptor (TIR) domain containing adaptor protein (TIRAP), and TIR domain containing adaptor inducing interferon (IFN)-β (TRIF). TLRs promote pro-inflammatory signaling by activating down-stream signaling pathways such as the nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPKs), c-Jun-N-terminal kinase (JNK), p38 and extracellular related protein kinase (ERK) pathways in the liver (Abdulla et al., 2005).

TLRs are present on non-parenchymal cells including Kupffer cells as well as on hepatocytes in the liver (Akira et al., 2001; Liu et al., 2002; Matsumura et al., 2003). We have shown that activation of TLR4 by lipopolysaccharide (LPS; the gram-negative bacterial component) leads to down-regulation of gene expression and activity of DMETs (Ghose et al., 2008, 2009; Shah et al., 2014). LPS-treated rodents are well established models of inflammation, and LPS treatment induces pro-inflammatory cytokines, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF) α. These cytokines act on hepatocytes to reduce the expression of DMET genes. (Renton KW, 2004; Aitken et al., 2006). TLR3 detects viral double-stranded (ds) RNA in the endo-lysosome. TLR3 is involved in the recognition of polyinosinic:polycytidylic acid

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(polyI:C), a synthetic dsRNA analog (Alexopoulou et al., 2001). However, the role of TLR3-mediated regulation of DMETs has not yet been extensively studied. While TRIF is the only adaptor protein involved in mediating TLR3-signaling, TLR4-signaling can be mediated by TRIF or TIRAP. TRIF is important for induction of interferon (IFN)-regulating factor 3, production of IFN-β and also for activation and maturation of dendritic cells (Yamamato et al., 2003). TRIF is involved in mediating NF-κB activation in response to TLR3 ligands and is involved in the MyD88-independent prolonged NF-κB activation in response to TLR4 ligands (Yamamato et al., 2003). While we found no role of TIRAP in TLR4 mediated DMETs down-regulation and cytokine induction in the liver, the plasma cytokine induction by LPS seems to be mediated by TIRAP (Ghose et al., 2011).

The goal of this investigation was to determine the role of TRIF in TLR4 and TLR3-mediated down-regulation of DMETs (Schematic 1). We observed that LPS administration led to significant down-regulation (~40-60%) of gene expression of key DMEs including *Cyp3a11, Cyp2a4*, *Ugt1a1* and *Mrp2* in $TRIF^{+/+}$ as well as $TRIF^{-/-}$ mice. Induction of pro-inflammatory cytokines, *IL-1β*, *IL-6* & *TNFα* was not attenuated in LPS-treated $TRIF^{-/-}$ mice. Upon treatment of $TRIF^{+/+}$ mice with polyI:C, we observed significant down-regulation (~30-60%) of gene expression of *Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sult1a1*, *Mrp2* and *Mrp3*. Surprisingly, the down-regulation of DMETs by polyI:C was not attenuated in $TRIF^{-/-}$ mice. PolyI:C administration also leads to induction of gene expression of pro-inflammatory cytokines *IL-1β*, *IL-6* & *TNFα* at 1 and 4h, however, this induction was not observed at 1h, but only at 4h in $TRIF^{-/-}$ mice. This delayed induction indicates that alternate pathways may be involved in recognizing polyI:C-mediated responses.

In order to study the role of MAPKs in regulating gene expression of DMETs, primary hepatocytes were treated with specific inhibitors of JNK, p38 and ERK before treatment with LPS or polyI:C. We found that inhibition of JNK led to complete attenuation of LPS or polyI:C-mediated down-regulation of gene expression of DMETs. Our results indicate that the down-regulation of DMETs during TLR4 and TLR3-induced inflammation can occur through TRIF-independent pathways and in all likelihood involves JNK-dependent signaling pathways.

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## Materials and methods:

### Materials

Highly purified LPS (*Escherichia coli*) and polyI:C (high molecular weight) were purchased from InvivoGen (San Diego, CA) and freshly diluted to the required concentration in 0.9% saline. The sequences of the primers and probes were obtained from the literature as reported previously (Shah et al., 2014). All oligonucleotides were purchased from Sigma Genosys (Houston, TX) and all real-time PCR reagents were purchased from Applied Biosystems, (Foster city, CA). Anti-JNK (#9252) and anti-phospho-JNK (#9251) were purchased from Cell-Signaling (Beverly, MA). Anti-IκB-α antibody (sc-371) was purchased from Santa Cruz biotechnology (San Diego, CA). JNK inhibitor (SP600125), ERK inhibitor (PD98059), and p38 inhibitor (SB203580) were purchased from Calbiochem (Gibbstown, NJ). Cell culture media and media supplements were purchased from Gibco BRL (Gaithersburg, MD). Unless specified, all other materials were purchased from Sigma-Aldrich (St Louis, MO).

### Animals

Adult male C57BL/6 mice, (~6-8 weeks) weighing 20–25 g were obtained from Harlan Laboratories (Houston, TX). Adult male $TRIF^{+/+}$ and $TRIF^{-/-}$ mice (~6-8 weeks) were obtained from Jackson laboratories (Bar Harbor, ME). We did not notice any overt phenotype in our experiments with $TRIF^{-/-}$ mice. The animals were maintained in a temperature and humidity-controlled environment and were provided with water and rodent chow ad libitum. All animal experimental and surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

**Treatments**

The mice were intraperitoneally (i.p.)-injected with saline, polyI:C (5 mg/kg body wt.) or LPS (2 mg/kg body wt.) for 1, 4, 8 & 16 h. Livers were harvested, cryopreserved in liquid nitrogen and stored at -80°C for further studies. All experiments were performed in triplicate and repeated three times. The *in vivo* doses for polyI:C and LPS were found to be non-toxic as evidenced by unchanged liver toxicity markers; aspartate transaminase (AST) and alanine transaminase (ALT).

**Real-time PCR**

Total RNA was isolated from mouse liver using TRIzol reagent (Sigma-Aldrich; St Louis, MO) according to the manufacturer's protocol. cDNA was synthesized using the High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems; Foster City, CA) as described previously (Shah et al., 2014). In short, each 25 µl reaction mixture contained 50-100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer, 200 nM fluorogenic probe, and 15 µl of TaqMan Universal PCR Master Mix. We extrapolated the quantitative expression values from standard curves and these values were normalized to cyclophilin.

**Primary hepatocyte isolation and treatment**

Primary mouse hepatocytes were isolated from C57BL/6 mice using the two step collagenase perfusion technique as described previously (Li et al., 2002; Ghose et al., 2011; Shah et al., 2014). In short, after digestion, the liver was excised and the hepatocytes were purified using a percoll gradient (33%), washed and screened for viability using trypan blue exclusion technique. Only isolations with viability greater than 90% were used for these studies. Cells were plated at a

density of 500,000 cells per well in six-well Primaria plates (BD biosciences; San Diego, CA) and allowed to attach for 4 h. Cells were maintained for 48 h with daily change of medium.

The cells were incubated with serum-free Williams medium E two hours prior to treatment with 50 μg/ml polyI:C (8 h) or 1 μg/ml LPS (16 h). In order to identify the role of MAPKs, the cells were pre-treated with specific inhibitors 1 h prior to treatment with polyI:C or LPS. RNA was then isolated for real-time PCR analysis as described above. The *in vitro* doses for polyI:C and LPS were found to be non-toxic as evidenced by unchanged liver toxicity markers; aspartate transaminase (AST) and alanine transaminase (ALT) , and MTT assays in hepatocytes.

**Immunoblotting**

Whole cell extracts were prepared as described previously (Ghose et al., 2004) and the protein concentration was determined using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Pierce, Rockford, IL, U.S.A). Equal amounts of protein (30 μg) were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were probed with anti-JNK (1:500), anti-P-JNK (1:500) or IκB-α (1:1000) antibody followed by probing with a goat anti-rabbit IgG-AP secondary antibody (1:2000). The membranes were then washed and incubated with Tropix® CDP star® nitroblock II TM ECL reagent as per the manufacturers' instructions (Applied Biosystems). The membranes were analyzed using FlourChem FC imaging system (Alpha Innotech).

**Statistical analysis**

Treatment groups were compared using two-way analysis of variance, followed by a post hoc test (Bonferroni's post hoc test) with $P < 0.05$. The results were presented as mean ± S.D

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## Results

### Role of TRIF in down-regulation of gene expression of DME/transporters by LPS

LPS-administration leads to down-regulation of DME genes and this down-regulation is independent of TIRAP (Ghose et al., 2008). In order to investigate the role of TRIF in LPS-mediated down-regulation of DMEs, we treated $TRIF^{+/+}$ and $TRIF^{-/-}$ mice with LPS (Fig. 1). We found that mRNA levels of *Cyp3a11* (~80%), *Cyp2a4* (~35%), *Ugt1a1* (~45%) and Mrp2 (~45%) were significantly down-regulated in LPS-treated $TRIF^{+/+}$ mice. However, this down-regulation was not attenuated in $TRIF^{-/-}$ mice indicating redundancy in TLR4 signaling pathways.

### Role of TRIF in regulation of hepatic cytokine mRNA levels by LPS

Since cytokines are involved in down-regulation of hepatic DMET genes during inflammation, we determined the changes in gene expression of pro-inflammatory cytokines (Fig. 2). As expected, there was significant induction of *IL-1β*, *IL-6* and *TNFα* mRNA levels in the livers of LPS-treated $TRIF^{+/+}$ mice; however, no attenuation of this induction was observed in LPS-treated $TRIF^{-/-}$ mice. We have shown previously that TLR4-mediated induction of cytokines in the liver is independent of the adaptor protein TIRAP (Ghose et al., 2008). Our results indicate that TLR4 signaling can proceed through TIRAP or TRIF, suggesting redundancy in the signaling pathway.

### Regulation of gene expression of DMETs in polyI:C-treated C57BL/6 mice

Our next goal was to determine the role of the TLR3 ligand, polyI:C in regulating gene expression of DMETs. This would provide the basis for further investigation into the role of

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TRIF. Therefore, we treated C57BL/6 mice with 5 mg/kg of polyI:C for 8 & 16 h (Fig. 3). We show for the first time that activation of TLR3 by polyI:C leads to down-regulation of several DMET genes. mRNA levels of the key phase I enzyme, *Cyp3a11* were significantly down-regulated at 8 h (~35%) and 16 h (~60%) in C57BL/6 mice. mRNA levels of *Cyp1a2* and *Cyp2a4* (~30-40%) were significantly down-regulated at 8 and 16 h. *Cyp2b10* mRNA levels were down-regulated (~35%) at 8 h. The mRNA levels of key phase II genes, *Ugt1a1* and *Sult1a1* were down-regulated significantly (~40% and ~60% respectively) at 8 h. mRNA levels of key transporters, *Mrp2* and *Mrp3* were significantly (~30-35%) down-regulated at 8 and 16 h respectively.

**Activation of cytokines, nuclear receptors (NRs) and cell-signaling pathways by polyI:C**

We have previously shown that activation of TLR2 and TLR4 leads to the induction of pro-inflammatory cytokines *IL-1β, IL-6* and *TNFα* (Ghose et al., 2008; 2009). JNK and NF-κB are known to be critical components of the TLR signaling pathway (Kawai et al., 2005) and the alterations of hepatic DMETs in LPS-induced inflammation involves cross-talk between cell-signaling components and nuclear receptors (NRs). We have also shown that activation of TLR2 and TLR4 leads to the activation of JNK, NF-κB and alterations in expression of NRs (Ghose et al., 2008; 2009). However, it is not clear if activation of polyI:C will lead to activation of these signaling components and whether they play a role in mediating the effects on DMETs. We show significant induction of mRNA levels of pro-inflammatory cytokines, *IL-1β* (~120-folds), *IL-6* (~25-folds) and *TNFα* (~80-folds) in the livers of polyI:C-treated C57BL/6 mice (Fig 4 a). There was down-regulation of the gene expression of *CAR* (~60%) and *RXRα* (~25%) at 8 and 16 h respectively where as a slight up-regulation of gene expression of *PXR* at 16 h was seen (Fig 4 b). We also observed activation of phosphorylated JNK (P-JNK) in whole cell extracts (Fig 4 c).

Activation of NF-κB was determined by degradation of the inhibitory subunit, IκBα after polyI:C treatment (Fig 4 c). We see activation of NF-κB at 1 h indicating that these signaling components are involved in mediating TLR3-signaling pathways.

**Role of TRIF in regulation of gene expression of DMETs by polyI:C.**

Since we have shown that the TLR3 ligand, polyI:C down-regulates DMET genes, our next objective was to determine the role of TRIF in polyI:C-mediated regulation of DMET genes. Maximal down-regulation of DMETs was observed at 8 h on treatment with polyI:C; hence, we chose the 8 h time-point for this study (Fig. 5). We found that the RNA levels of *Cyp3a11* (~40%), *Cyp2a4* (~40%), *Cyp2b10* (~45%), *Ugt1a1* (~50%), *Sult1a1* (~50%) and *Mrp2* (~30%) were significantly down-regulated in $TRIF^{+/+}$ as well as $TRIF^{-/-}$ mice at 8 h and the extent of down-regulation was similar. These results suggest that the down-regulation of DMETs through TLR3 was independent of TRIF.

**Role of TRIF in regulation of hepatic cytokine mRNA levels by polyI:C.**

Induction of pro-inflammatory cytokines *IL-1β, IL-6* and *TNFα* in $TRIF^{+/+}$ mice was completely suppressed in polyI:C-treated $TRIF^{-/-}$ mice at 1 h (Fig 6A). Since cytokines have been implicated in altering the gene expression of DMETs, this finding was surprising. On further investigation, we found significant induction of cytokines at 4 h (*IL-1β*; ~60 fold, *IL-6*; ~35-fold and *TNFα*; ~40-fold) in polyI:C-treated $TRIF^{-/-}$ mice (Fig 6B). There is a delayed response in the induction of cytokines in $TRIF^{-/-}$ mice suggesting that polyI:C can potentially mediate viral responses through multiple mechanisms.

**Roles of MAPKs in TLR4- or TLR3-mediated down-regulation of DMETs**

We studied the role of NF-κB in regulating DMETs during TLR3 & 4 activation by using specific activation inhibitor NAI (6-amino-4-(4-phenoxyphenylethylamino)quinazoline) in primary mouse hepatocytes. We found that inhibiting activation of NF-κB did not attenuate down-regulation of DMET genes which is why we chose to focus on the MAPKs. MAPK have been implicated in altering the gene expression of DMETs during inflammation due to their cross-talk with NRs (Ghose et al., 2004; Li et al., 2002). In order to determine their roles in TLR4 and TLR3-mediated down-regulation of DMETs, we isolated primary hepatocytes from C57BL/6 mice and treated them with chemical inhibitors of JNK (SP600125; 10 μM), ERK (PD89059; 20 μM) and p38 (SB203580; 25 μM), 1 h before treatments with LPS (1 μg/mL; 16 h) or polyI:C (50 μg/mL; 8 h). We observed that treatment of primary hepatocytes with LPS led to down-regulation of gene expression of *Cyp3a11* and *Ugt1a1* (Fig 7a). PolyI:C treatment leads to down-regulation of gene expression of *Cyp3a11*, *Cyp2a4* and *Sultn* (Fig 7b). This down-regulation of DMEs was completely attenuated on treatment of specific JNK inhibitor where as ERK and p38 inhibitor had no effect in attenuating the LPS or polyI:C mediated down-regulation of DMEs.

## Discussion

Infection and inflammation can alter the expression and activities of several DMETs (Morgan ET, 1997). This can have potentially harmful consequences in patients and therefore, it is extremely important to understand the mechanisms behind alterations of DMET during inflammation. We have shown that expression and activities of hepatic DMETs are altered in a gram-negative and gram-positive bacterial-inflammatory model (Ghose et al., 2009; Gandhi et al., 2012); however, the role of viral infections in altering DMETs has not been studied. We show for the first time that treatment of mice with the TLR3 ligand polyI:C leads to down-regulation of gene expression of DMET, induction of pro-inflammatory cytokines, alterations in nuclear receptor levels and activation of cell signaling components. We also show that TRIF-independent signaling pathways may be involved in down-regulation of DMETs by TLR3 or TLR4. However, *in vitro* results show that this down-regulation is likely mediated by the MAPK, JNK.

Activation of TLR4 can recruit the downstream adaptor proteins, TIRAP or TRIF. Initial studies with TIRAP$^{-/-}$ mice had shown that TIRAP is important for the induction of cytokines by LPS in dendritic cells and peritoneally derived macrophages (Horng et al., 2002; Yamamoto et al., 2002). However, our studies with TIRAP$^{-/-}$ mice indicated that TIRAP is not essential for down-regulation of hepatic DMETs through TLR4 signaling (Ghose et al., 2008). In this study, we show that LPS-mediated induction of pro-inflammatory cytokines and down-regulation of DMETs occurs in TRIF$^{-/-}$ mice, indicating redundancy in TIRAP or TRIF-dependent signaling pathways. Experiments in TIRAP/TRIF double-knockout mice will most likely block the effects of LPS on DMET genes.

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PolyI:C administration led to down-regulation of several DMET genes such as *Cyp3a11, Cyp2a4, Cyp2b10, Cyp1a2, Ugt1a1 Sult1a1, Mrp2* and *Mrp3* in $TRIF^{+/+}$ mice at 8 and 16 h. We show for the first time that TLR3 activation by viral components can down-regulate hepatic DMET genes. We also show that activation of TLR3 by polyI:C leads to (a) induction of pro-inflammatory cytokines, *IL-1β*, *IL-6* and *TNFα*, (b) alterations of nuclear receptor levels of *CAR*, *PXR* and *RXRα* and (c) activation of signaling components like JNK and NF-κB. Since TRIF is the only adaptor protein involved in TLR3 signaling, we expected attenuation of down-regulation of DMET genes in $TRIF^{-/-}$ mice. Surprisingly, the down-regulation of DMETs on polyI:C treatment was also observed in $TRIF^{-/-}$ mice. Since cytokines have been implicated in altering gene expression of DMETs during inflammation, we expected no changes in the induction pattern of pro-inflammatory cytokines in polyI:C-treated $TRIF^{-/-}$ mice. Surprisingly, the induction of hepatic *IL-1β, IL-6* and *TNFα* mRNA levels was completely abolished in polyI:C-treated $TRIF^{-/-}$ mice at 1 h. Further investigation showed that this induction is delayed, and there is significant induction of these cytokines in livers of $TRIF^{-/-}$ mice after 4 h of polyI:C treatment. This indicates that in the absence of TRIF, polyI:C might be activating other signaling pathways to induce cytokine expression in the liver.

Retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) contain caspase-recruiting domains (CARDs) and can detect intracellular viral products such as genomic RNA to signal IRF3, IRF7 & NF-κB (Onoguchi et al., 2007; Yoneyama et al., 2004). These receptors are important in sensing dsRNA because dendritic cells or fibroblast cells lacking in TLR3 secrete type I IFNs on exposure to dsRNA (Hemmi et al., 2004). RIG-I and MDA5 have been implicated in mediating antiviral responses via the IFN-β promoter stimulator (IPS-1 or CARDIF) adaptor protein. Previous studies have shown that induction of interferons

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was attenuated and delayed in TRIF$^{-/-}$ mice treated with polyI:C (Kumar et al., 2008). Cytokine production was totally inhibited only in IPS-1 and TRIF-double knockout mice indicating that both TRIF and IPS-1 play important roles in mediating anti-viral responses. This evidence indicates that in our studies, the redundancy of down-stream pathways (IPS-1 and TRIF) is likely to be responsible for DMET down-regulation by polyI:C in TRIF$^{-/-}$ mice. Studies in polyI:C-treated IPS-1/TRIF double knockout mice will likely attenuate the down-regulation of DMETs during viral infections.

Inflammation and infection lead to alterations in the activity of a variety of target genes and transcription factors through several signal transduction cascades, including the three main MAPKs; JNK, ERK and p38. In order to investigate the roles of JNK, ERK and p38 in regulating DMET genes during gram-negative and viral infections, we treated primary hepatocytes with specific inhibitors 1 h prior to treatment with LPS or polyI:C. We found that the TLR4 and TLR3-mediated down-regulation of DMETs was completely attenuated by the JNK inhibitor, where as ERK and p38 inhibitors had no effects on attenuating the down-regulation of DMETs. JNK is found in 3 isoforms and the JNK inhibitor we used inhibited all 3 isoforms (Bennett et al., 2001). JNK1 and JNK2 are present in most tissues and JNK3 is found almost exclusively in the brain. JNK1/JNK2 double knockout mice are embryonically lethal and JNK1 knockout and JNK2 knockout mice show redundancy in mediating the effects of LPS in the liver (Kosters et al., 2004). Experiments with polyI:C in JNK1 and JNK2 knockout mice will help identify which isoform plays an important role in regulating DMET genes during viral infections.

Our study is the first of its kind to show that activation of TLR3 will lead to down-regulation of gene expression of several DMETs. The down-regulation of DMETs and transporters during inflammation and infection is a complex process, and the molecular mechanisms are not fully

understood. We show that TLR4 and TLR3-mediated down-regulation of DMET genes can occur through TRIF-independent pathways, indicating redundancy in the signaling mediated by downstream adaptor proteins. However, the MAPK JNK is a major player in regulating DMET genes during infections caused by gram-negative and viral components. Although a safe and potent human JNK inhibitor is yet to be discovered, our *in vitro* studies indicate that JNK-dependent phosphorylation processes could be targeted to reverse or attenuate inflammation mediated effects on drug metabolism.

## Authorship contributions

*Participated in research design:* Ghose and Shah

*Conducted experiments:* Shah and Omoluabi

*Contributed new reagents and analytic tools:* Ghose, Moorthy

*Performed data analysis:* Shah and Ghose

*Wrote or contributed to the writing of the manuscript:* Shah, Ghose and Moorthy

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**Footnotes:**

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**Figure Legends:**

Schematic 1: Inflammation-associated cell-signaling pathways in the liver. Activation of TLR4 and TLR3 recruits the adaptor proteins (TIRAP and TRIF) to induce downstream signaling which eventually leads to alterations in gene expression of DMETs.

Fig: 1: Regulation of DME mRNA levels in $TRIF^{+/+}$ and $TRIF^{-/-}$ mice following LPS administration. $TRIF^{+/+}$ and $TRIF^{-/-}$ were i.p. injected with saline or LPS (2 mg/kg) and livers were harvested at 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of Cyp3a11 & Ugt1a1 were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated $TRIF^{+/+}$ mice was set to 1, fold change after LPS-treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and LPS groups. The experiments were repeated three times.

Fig 2: Regulation of cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice following LPS administration. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were i.p. injected with saline or LPS (2 mg/kg) and livers were harvested at 1 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of IL-1β, IL-6 and TNFα were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated $TRIF^{+/+}$ mice was set to 1, fold change after LPS-treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and LPS groups. The experiments were repeated three times.

Fig 3: Regulation of DMET mRNA levels in C57BL/6 mice by poly I:C. C57BL/6 mice were i.p. injected with saline or polyI:C (5 mg/kg) and livers were harvested at 8 h (A) & 16 h (B) (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I, phase II and transporter genes were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after polyI:C-treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and poly I:C groups. The experiments were repeated three times.

Fig 4: Regulation of cell-signaling components on activation of TLR3**.** (a) Cytokines: C57BL/6 mice were i.p. injected with saline or polyI:C (5 mg/kg) and livers were harvested at 1 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of IL-1β, IL-6 and TNFα were determined by real-time PCR analysis as described earlier. (b) Nuclear Receptors: C57BL/6 mice were i.p. injected with saline or polyI:C (5 mg/kg) and livers were harvested at 8 and 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of CAR, PXR and RXRα were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline treated mice was set to 1, fold change after polyI:C treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and polyI:C groups. The experiments were repeated thrice. (c) Cell-signaling pathways: Whole cell extracts were prepared from the livers of saline and polyI:C-treated mice at 1 h, and the samples were analyzed by immunoblotting. The phosphorylated forms of JNK (P-JNK) and degradation of IkBα were measured as markers of JNK and NF-κB activation, respectively. The experiments were repeated three times.

Fig 5: Regulation of DMET mRNA levels in $TRIF^{+/+}$ and $TRIF^{-/-}$ mice following polyI:C administration. $TRIF^{+/+}$ and $TRIF^{-/-}$ were i.p. injected with saline or polyI:C (5 mg/kg) and livers were harvested at 8 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I, phase II and transporter genes were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated $TRIF^{+/+}$ mice was set to 1, fold change after polyI:C-treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and polyI:C group in $TRIF^{+/+}$ mice and # indicates significant difference ($p < 0.05$) between saline and polyI:C group in $TRIF^{-/-}$ mice. The experiments were repeated three times.

Fig 6: Regulation of hepatic cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice following poly I:C administration. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice (n = 5 per group) were i.p. injected with saline or polyI:C (5 mg/kg) and livers were harvested at 1 h (A) and 4 h (B). RNA was isolated from the livers and mRNA levels of IL-1β, IL-6 and TNFα were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated $TRIF^{+/+}$ mice was set to 1, fold change after polyI:C-treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and polyI:C groups. # indicates significant difference in polyI:C-treated $TRIF^{+/+}$ and $TRIF^{-/-}$ mice. The experiments were repeated three times.

Fig 7: Regulation of DMEs by MAPKs on LPS or polyI:C treatments. Primary hepatocytes from C57BL/6 mice were treated with DMSO or JNK inhibitor (SP600125, 10 μM), ERK inhibitor (PD98059, 20 μM) or p38 inhibitor (SB203580, 25 μM) for 1 h, before treatment with saline or

(a) LPS (1 μg/mL) for 16 h or (b) polyI:C (50 μg/mL) for 8 h. RNA was isolated, and real-time PCR was performed as described earlier. n = 5-6 per group. All data are presented as ±SD and standardized for cyclophilin mRNA levels. * indicate significant difference ($p < 0.05$) between saline and LPS or polyI:C groups. The experiments were repeated three times.

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Schematic 1

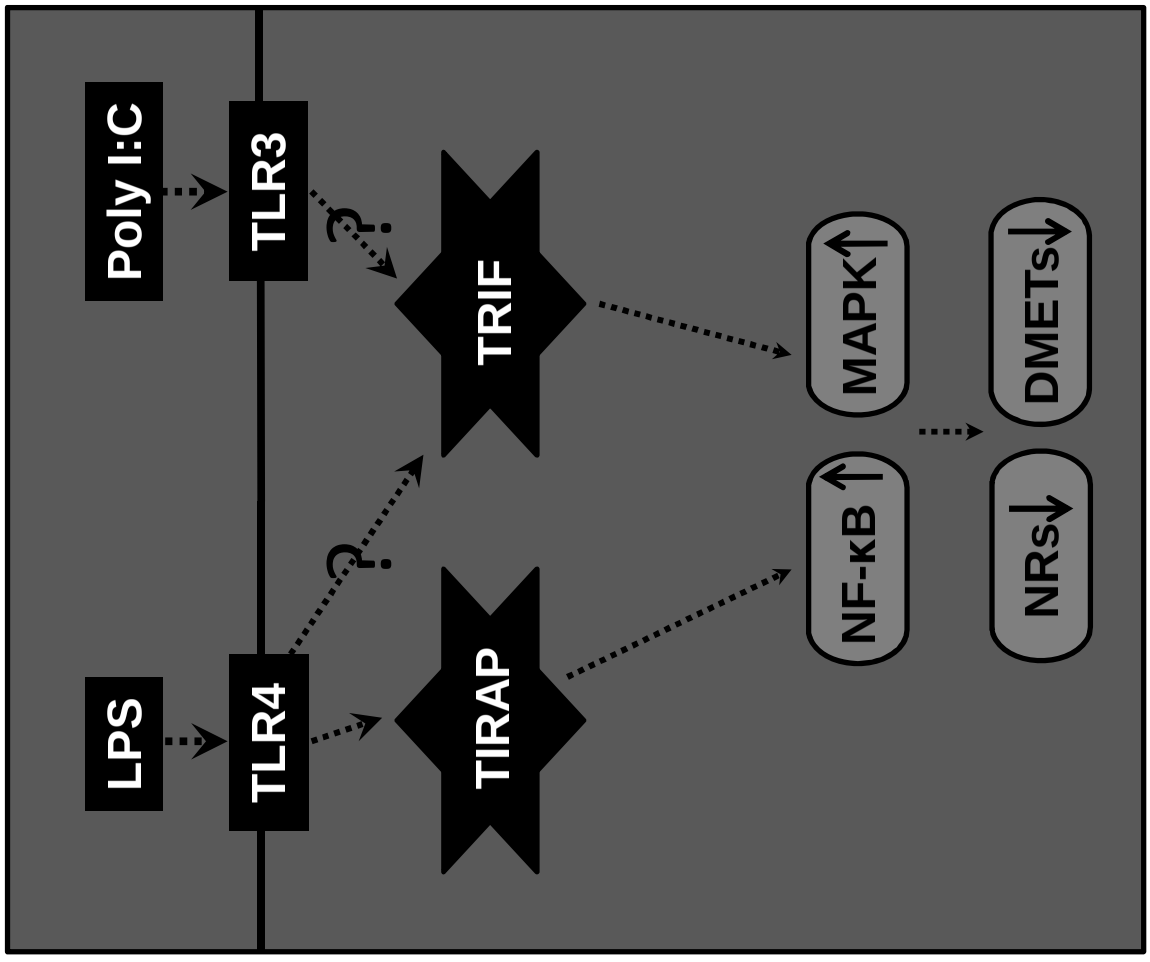

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Fig.1

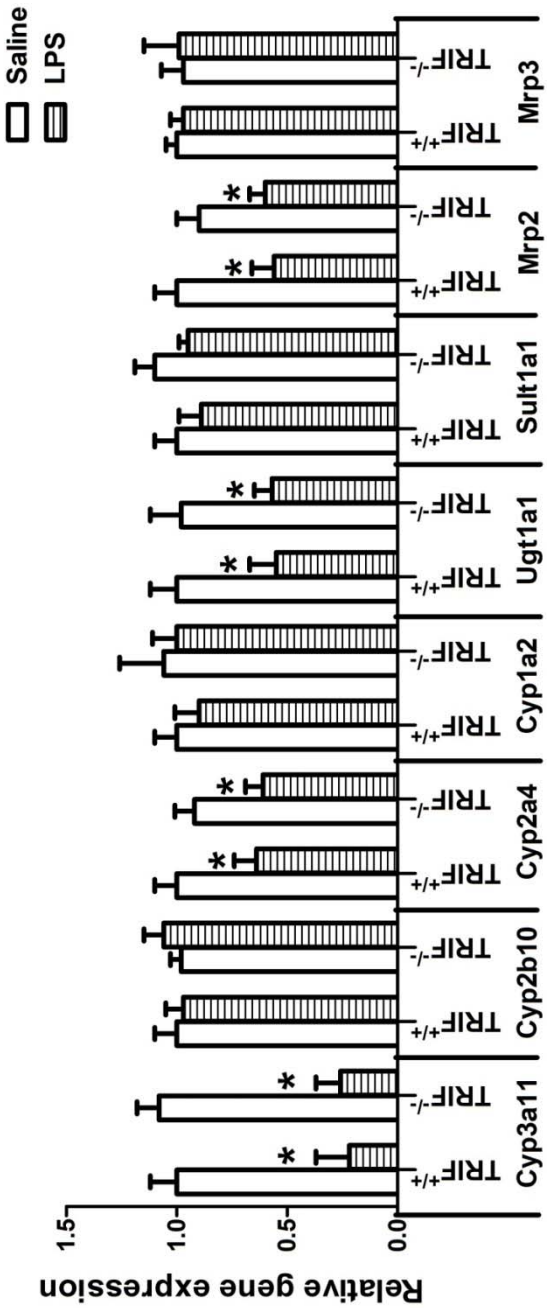

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Fig.2

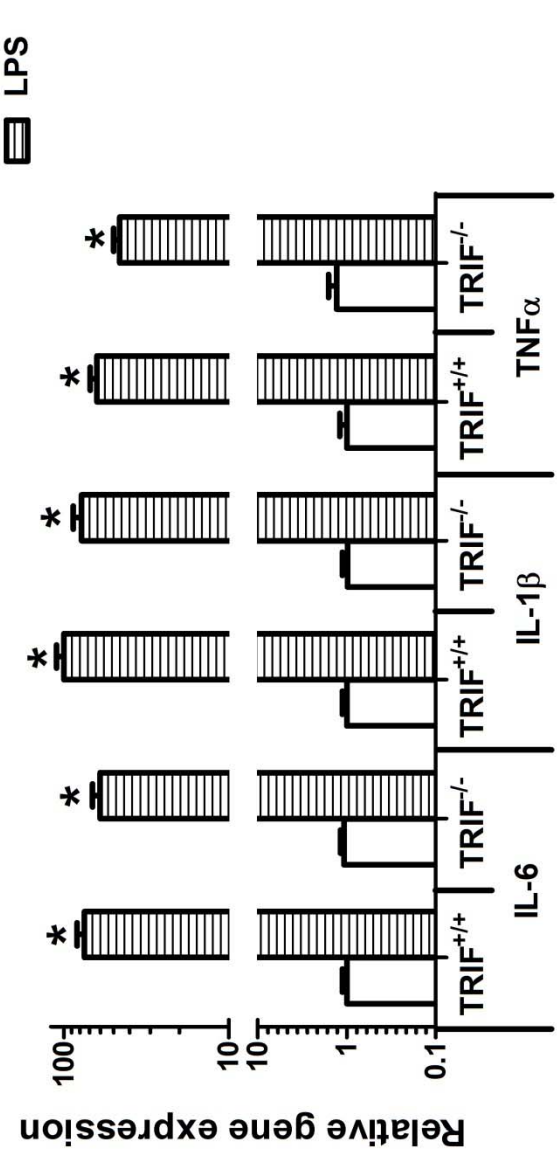

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Fig.3

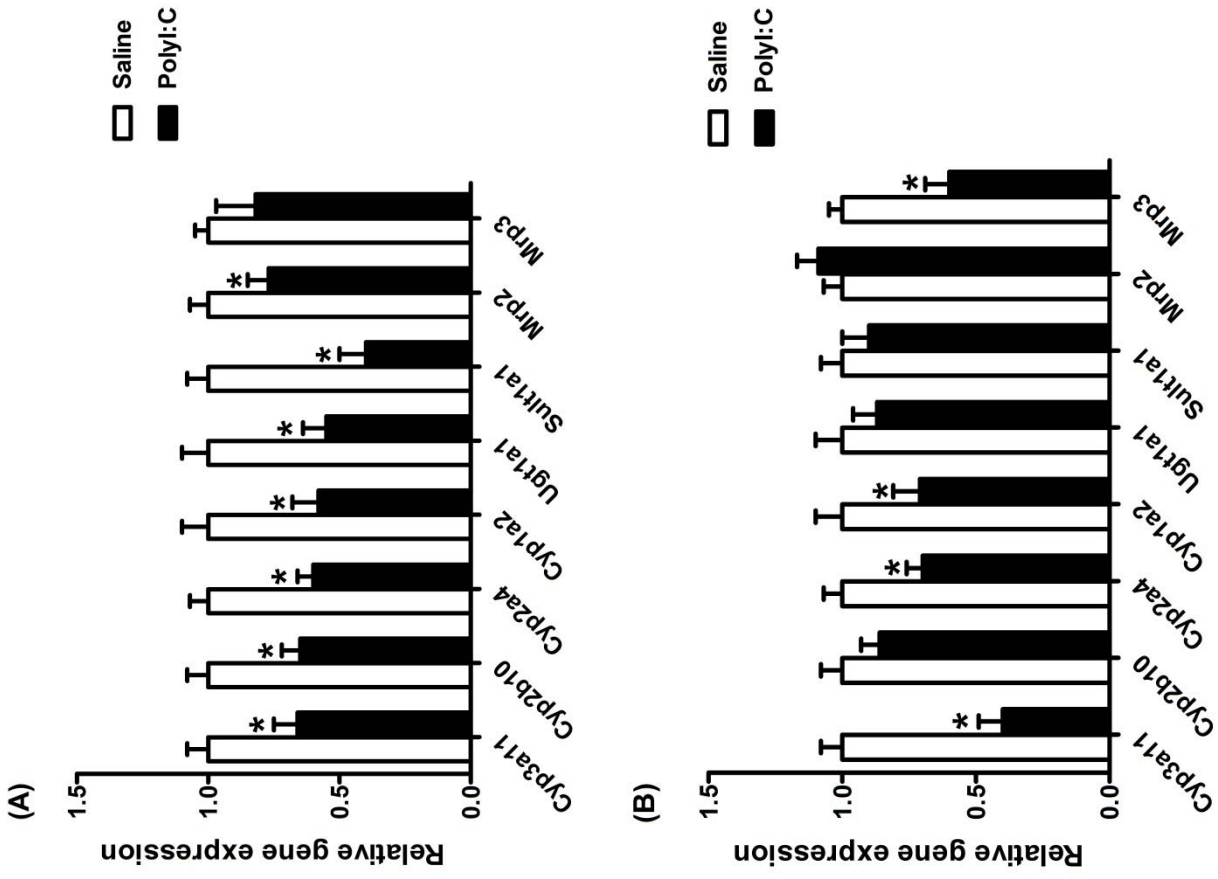

Fig 4

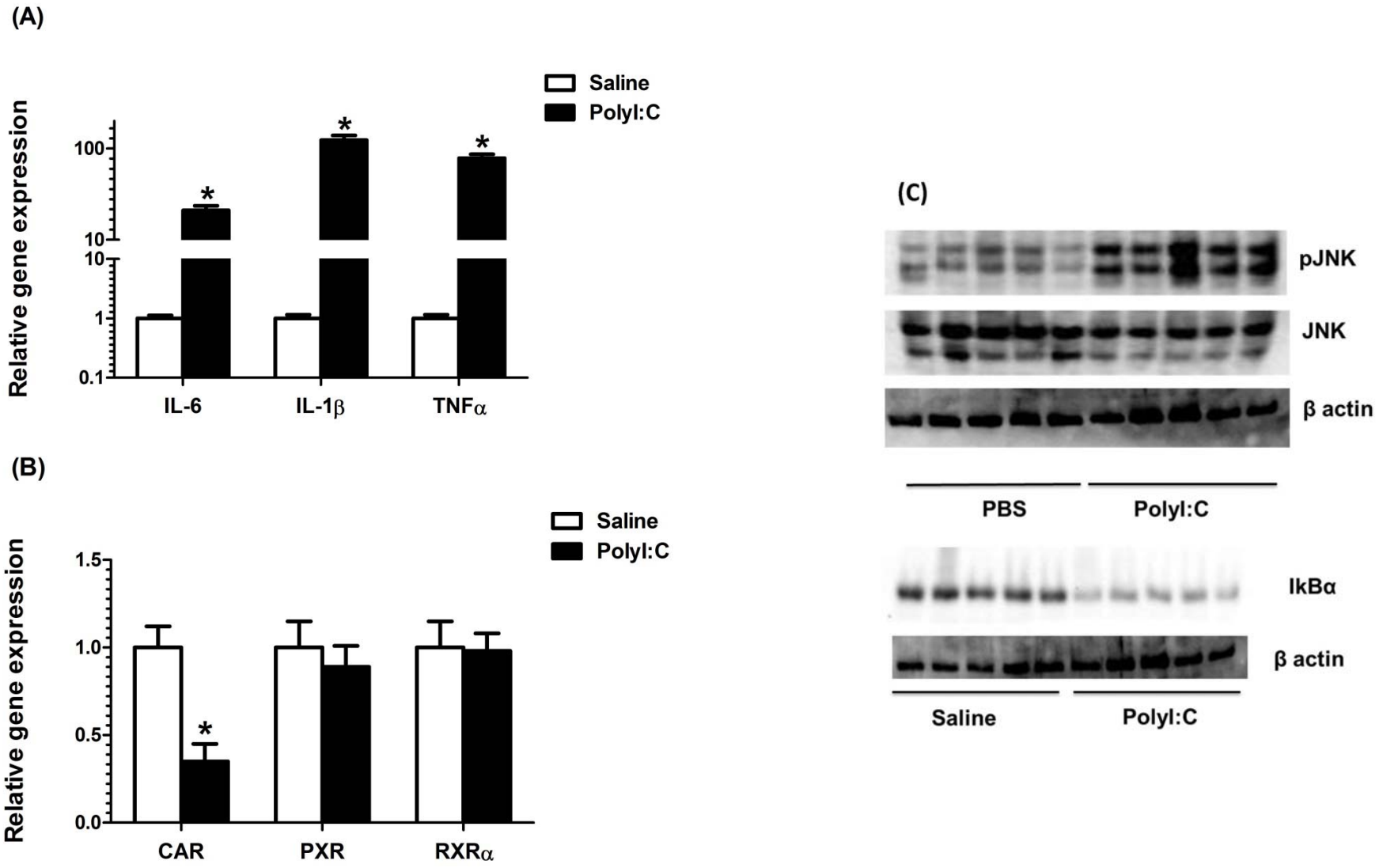

**Fig.5**

Fig.6

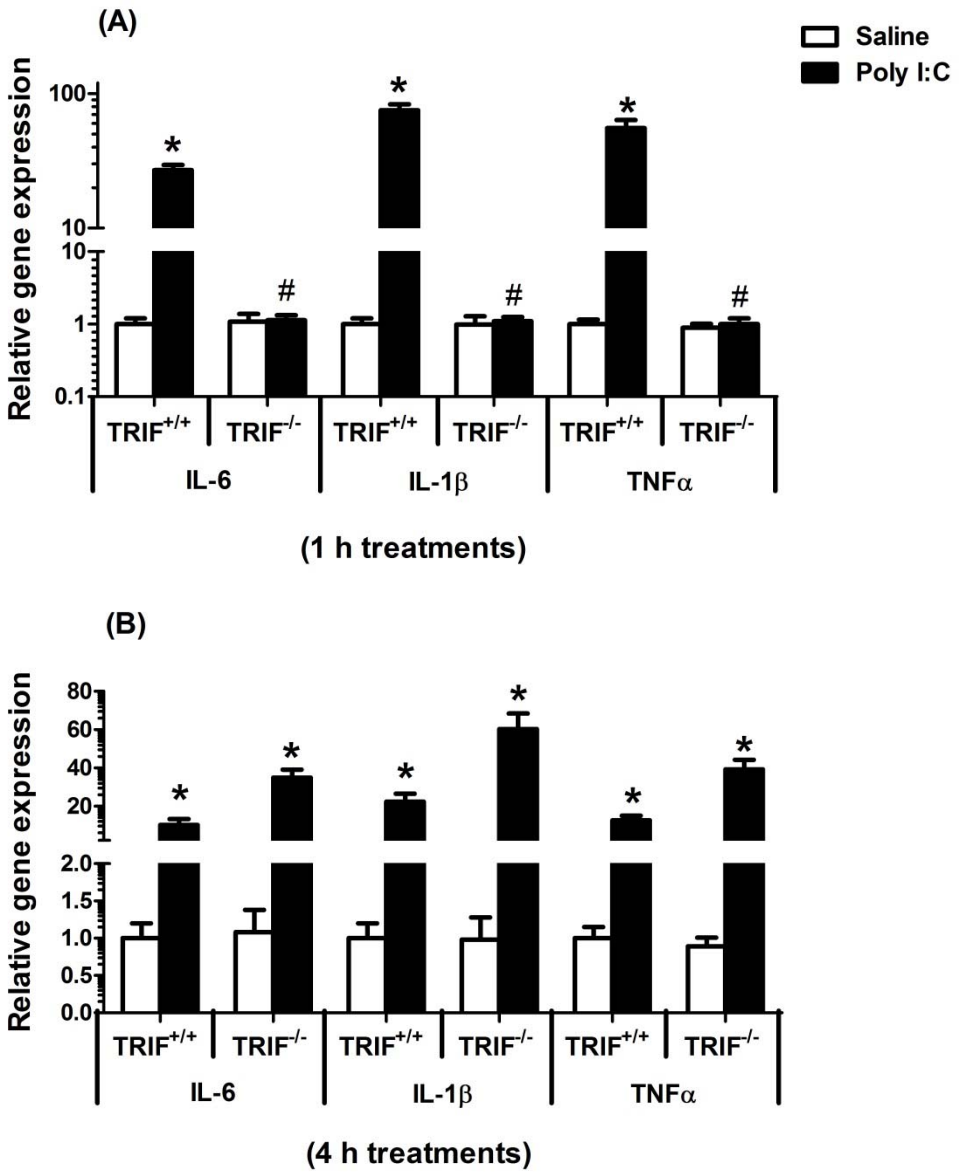

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Fig.7