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Full length article

Modulation of LPS-induced inflammatory cytokine production by a novel glycogen synthase kinase-3 inhibitor

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ABSTRACT

Sepsis is a serious condition that can lead to long-term organ damage and death. At the molecular level, the hallmark of sepsis is the elevated expression of a multitude of potent cytokines, i.e. a cytokine storm. For sepsis involving gram-negative bacteria, macrophages recognize lipopolysaccharide (LPS) shed from the bacteria, activating Toll-like-receptor 4 (TLR4), and triggering a cytokine storm. Glycogen synthase kinase-3 (GSK-3) is a highly active kinase that has been implicated in LPS-induced cytokine production. Thus, compounds that inhibit GSK-3 could be potential therapeutics for sepsis. Our group has recently described a novel and highly selective inhibitor of GSK-3 termed COB-187. In the present study, using THP-1 macrophages, we evaluated the ability of COB-187 to attenuate LPS-induced cytokine production. We found that COB-187 significantly reduced, at the protein and mRNA levels, cytokines induced by LPS (e.g. IL-6, TNF-α, IL-1β, CXCL10, and IFN-β). Further, the data suggest that the inhibition could be due, at least in part, to COB-187 reducing NF-κB (p65/p50) DNA binding activity as well as reducing IRF-3 phosphorylation at Serine 396. Thus, COB-187 appears to be a potent inhibitor of the cytokine storm induced by LPS.

1. Introduction

Sepsis is caused by a dysregulated host response to infection (Schulte et al., 2013) and septic shock is a leading cause of death (Schulte et al., 2013; Sharma et al., 2017). Thus, there is an ongoing effort to develop therapeutics for this devistating condition (Sharma et al., 2017). Sepsis is entwined with host-response to invasive pathogens during which the innate immune system recognises pathogen-associated molecular patterns (PAMPs) via pathogen recognation receptors including toll-like receptors (TLRs) (McCoy, 2016; Moresco et al., 2011; Schulte et al., 2013; Takeda and Akira, 2001). This recognition induces the production and release of inflammatory mediators (e.g. cytokines including a subset termed chemokines) which help the host organism eliminate the invasion (Dinarello, 2000; Martin et al., 2005; O'Neill and Dinarello, 2000). That said, uncontrolled production of cytokines is thought to be the major driving force in sepsis (Schulte et al., 2013).

In the case of sepsis due to gram-negative bacteria, uncontrolled activation of one member of the TLR family, namely TLR4, via LPS, a component of gram-negative bacteria, leads to production of inflammatory cytokines through the myeloid differentiation factor 88 (Myd88)-dependent and/or -independent pathways (Jope et al., 2016). Sepsis is often characterized by the presence of excessive levels of pro-inflammatory cytokines (e.g. TNF-α and IL-6), as well as the anti-inflammatory cytokine IL-10, in plasma and/or serum (Chaudhry et al., 2013). Nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) plays an important role in cytokine regulation and the level of NF-κB activity is strongly corrolated with the severity of sepsis (Arnalich et al., 2000; Chaudhry et al., 2013; Liu and Malik, 2006).

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Thus, inhibitng LPS-induced cytokine production is a potential therapeutic approach for gram-negative bacteria-induced sepsis.

GSK-3 appears to play an important role in the LPS-induced production of inflammatory cytokines via the TLR4 pathway. Specifically, in the TLR4 Myd88-dependent pathway, GSK-3 is involved in activation of NF-κB as well as the inhibition of activator protein 1 (AP-1) and cAMP response element binding protein (CREB) transcription factors (Jope et al., 2016; Ko and Lee, 2016). In the TLR4 Myd88-independent pathway, GSK-3 may help regulate the production of type I IFNs (e.g. IFN-β) through TANK-binding kinase 1 (TBK1) and interferon regulatory factor-3 (IRF-3) (Jope et al., 2016). Given these observations and the important role of GSK-3 in regulating inflammatory cytokines noted by others (Beurel et al., 2010; Wang et al., 2014, 2011), it is not surprising that GSK-3 inhibitors have been touted as potential therapeutics for sepsis (Martin et al., 2005).

Our group recently described a novel inhibitor of GSK-3 termed COB-187 (Noori et al., 2019). In molecular assays, COB-187 was found to inhibit both GSK-3α and GSK-3β with an IC_{50} in the nM range and to be highly selective for GSK-3 amongst the over 400 kinases screened (Noori et al., 2019). COB-187 appeared to inhibit cellular GSK-3 as evidenced by its ability to inhibit the phosphorylation of canonical GSK-3 cellular substrates (Noori et al., 2019). In the present study we sought to determine if COB-187 could inhibit LPS-induced cytokine storms.

2. Materials and methods

2.1. Cell culture

THP-1 human monocyte cells from American Type Culture Collection (ATCC; Manassas, VA) were cultured and differentiated to the macrophage phenotype via PMA as described previously (Noori et al., 2019). The effects of LPS and COB-187 on the metabolic activity of THP-1 macrophages were evaluated using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay solution (Promega; Madison, WI) according to manufacturer's protocol.

2.2. Compound and LPS treatment

Compound COB-187 [4-hydroxy-4-phenyl-3-(pyridin-4-ylmethyl) thiazolidine-2-thione] was synthesized and purified to >95% purity (Noori et al., 2019). For compound treatments, stock COB-187 (200 mM in 100% DMSO) was diluted in complete culture medium to the final desired concentrations and 0.1% DMSO (v/v) (Sigma-Aldrich). PMA-differentiated THP-1 macrophages (THP-1 macrophages) were pretreated with varying concentrations of COB-187 or 0.1% DMSO (solvent control) for 1 h prior to 4 h stimulation with 10 ng/ml of LPS (Sigma-Aldrich, *E. coli* O111:B4 strain) in medium containing the same concentration of COB-187 or 0.1% DMSO alone.

2.3. ELISA; quantification of cytokine proteins

The presence of inflammatory cytokines in culture supernatants was quantified using the proteome profiler human cytokine array kits (a membrane-based sandwich immunoassay) (ARY005B, R&D Systems; Minneapolis, MN). The procedure was as per the manufacturer's instructions. Blots were scanned and analyzed using the Bio-Rad Chem-iDoc XRS + Molecular Imager (Hercules, CA) and the pixel density of the spots on the blots were measured using Quick Spots software (HLImage^{++}; R&D Systems). The measured density of the negative control spot (background) was then subtracted from all other density measurements. Each density result was then normalized to the density of the reference control spots. Human IL-6, IL-10, IFN-β, and CXCL10/IP-10 Quantikine ELISA kits (R&D Systems) were used according to the manufacturer's protocol.

2.4. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR); quantification of changes in the gene expression

In parallel to collecting the supernatant for cytokine analysis at the protein level, the THP-1 macrophages were harvested, and their mRNA was used to evaluate the expression of IL-6, TNF-α, IL-1β, CXCL10, and IFN-β using RT-qPCR as described previously (Noori et al., 2017). IL-6, TNF-α, IL-1β, CXCL10, IFN-β Taqman® Gene Expression Assays (human, FAM-MGB, Thermo Fisher), Taqman® Gene Expression Master Mix (Applied Biosystems) or SYBR Green Master Mix (Bio-Rad), StepOnePlus™ Real-Time PCR System (Applied Biosystems), and housekeeping genes ACTB and/or GAPDH (human, VIC-MGB, Thermo Fisher) were used to perform the RT-qPCR. ΔΔCT method was utilized for gene expression comparisons relative to the 0.1% DMSO control in combination with LPS.

THP-1 macrophage mRNA transcripts of the TLR signaling pathway were investigated using Human Toll-Like Receptor Signaling Pathway RT^2 Profiler™ PCR Arrays (SABiosciences; Qiagen). In brief, total RNA was isolated as described previously (Noori et al., 2017) and cDNA was synthesized with the RT^2 First Strand Kit (SABiosciences) using an Eppendorf Mastercycler gradient. RT-qPCR was performed using Human Toll-Like Receptor Signaling Pathway RT^2 Profiler™ PCR Arrays (SABiosciences), RT^2 Real-Time™ SYBR Green/ROX PCR Master mix (SABiosciences), and the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Gene expression data was normalized to the reference gene with the most stable expression (*ACTB*). Web-based SABioscience software was used to analyze the data, compare the results from different treatments, and evaluate P-values.

Note that RNA samples yielding a ratio of absorbance 260 nm/absorbance 280 nm between 1.8 and 2, and a ratio of absorbance 260 nm/absorbance 230 nm greater than 1.7 were considered pure. Ribosomal RNA band integrity was also evaluated and samples with RNA Integrity Number (RIN) of 9.5 or higher were used for cDNA synthesis and RT-qPCR.

2.5. TransAm assay; evaluating DNA-binding activity

Nuclear and cytoplasmic protein fractions were extracted using Nuclear Extraction kit (Active Motif; Carlsbad, CA) according to the manufacturer's protocol. The protein content was quantified using the micro BCA protein assay kit (Thermo Fisher) and Nanodrop 2000 Microvolume UV–Vis Spectrophotometer (Thermo Fisher). Five μg of extracted proteins were used in TransAm transcription factor ELISA assays (Active Motif) to measure the DNA binding activity of transcription factors NF-κB and IRF-3. Samples were analyzed using a Synergy HT Multi-Mode Microplate Reader (BioTek).

2.6. Western blotting; evaluating the phosphorylation of IRF-3 at serine 396

Phosphorylation of IRF-3 at Serine 396 in 10 μg of extracted nuclear and cytoplasmic proteins from the previous section was evaluated using western blot analysis as described previously (Noori et al., 2019). Rabbit anti-IRF-3 (D83B9) and anti-phospho-IRF-3 (Serine 396, 4D4G) monoclonal antibodies (Cell Signaling Technology; Danvers, MA) were used to detect the level of total IRF-3 and its phosphorylation at Serine 396 [pIRF3 (S^{396})], respectively. IRDye 680LT and 800CW goat anti-rabbit IgG (H + L) polyclonal secondary antibodies (LI-COR Biosciences) and the LI-COR Odyssey Infrared Imaging System were used for visualization and quantification of signals. Odyssey blocking buffer was used to dilute antibodies. The ratio of pIRF3 (S^{396}) to total IRF-3 was used in the statistical analysis.

3. Results

3.1. COB-187 significantly reduces, at the protein level, LPS-induction of chemokines/cytokines involved in sepsis

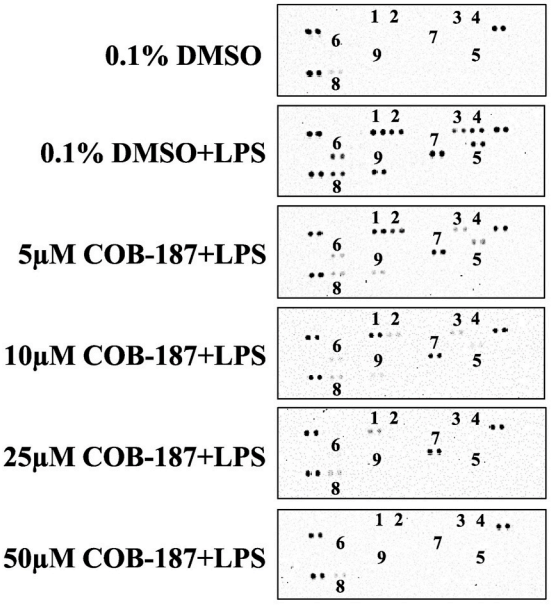
A proteome profiler human cytokine array (protein cytokine array) was used to investigate the effect of COB-187 on LPS induction of cytokines in THP-1 macrophages. Representative blots from this analysis are provided in Fig. 1. As revealed by comparing the top two blots presented in Fig. 1, nine duplicate pairs of dots are distinctly present in the blot prepared from supernatants of LPS treated THP-1 cells (second blot) that are not, or faintly, present in the blot prepared from supernatants of THP-1 cells not treated with LPS (top blot). These dots were the location of capture antibodies for: CCL3/CCL4, CCL5, CXCL1, CXCL10, IL-1β, IL-1ra, IL-8, MIF, and TNF-α. Although the pixel analysis identified additional cytokines that appeared to be elevated, these additional cytokines were all <6% of the internal reference control and were difficult to discern visually. In contrast, the nine proteins highlighted in Fig. 1 were all >45% of the reference standard and were readily discerned visually. Thus, we continued with the analysis of the nine proteins listed in Fig. 1.

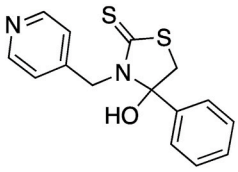
As shown in the four lower blots of Fig. 1, the pixel density of the nine identified dots appeared to decrease, in a dose dependent manner, when the THP-1 cells were treated with COB-187 concurrent with LPS-stimulation. Replicate analysis was performed and the results from the replicates averaged and presented in Fig. 2. As shown in Fig. 2A–D, COB-187 treatment with LPS-stimulation resulted in a significant reduction in the LPS-induced production of the chemokines with the least inhibitory effect observed for CCL3/CCL4 (Fig. 2A), and the most dramatic effect observed for CXCL10 (Fig. 2D). Addition of COB-187 caused a significant decrease in the LPS-induced expression of the pro-inflammatory cytokines TNF-α, IL-1β, MIF, and IL-8, as well as the anti-inflammatory cytokine IL-1ra (Fig. 2). TNF-α and IL-1β were reduced to essentially background levels while the effects on IL-8 and MIF were the less dramatic.

IL-6 and IL-10 are reported to be important in sepsis (Chaudhry et al., 2013; Martin et al., 2005; Schulte et al., 2013) and the protein levels of these cytokines were poorly detected using the cytokine array. Therefore, the Quantikine IL-6 and IL-10 ELISA kits were used to evaluate the expression of IL-6 and IL-10, respectively. As presented in Fig. 3A and B, LPS stimulation significantly increased the secretion of both cytokines. Treatment with COB-187 at concentrations ≥5 μM significantly reduced the LPS-induced IL-6 production in a dose-dependent manner (Fig. 3A). Interestingly, for IL-10 production (Fig. 3B), treatment with the lowest concentration of COB-187 tested (i.e. 2.5 μM) significantly increased the production of anti-inflammatory cytokine IL-10, while COB-187 concentrations higher than 5 μM brought the IL-10 level back to the basal level.

The capture antibody for IFN-β, a cytokine important in sepsis (Mahieu and Libert, 2007), was not included in the proteome profiler human cytokine array. Therefore, the Quantikine IFN-β ELISA kit was used to evaluate the effect of COB-187 on the expression of IFN-β, one of the type I IFNs regulated by the Myd88-independent pathway (Hoshino et al., 2002). As shown in Fig. 3C, LPS stimulation significantly increased the protein production of IFN-β in THP-1 macrophages. Treatment with COB-187 at concentrations ≥2.5 μM resulted in a significant dose-dependent reduction in IFN-β protein levels in the LPS-stimulated THP-1 macrophages (Fig. 3C).

A best fit regression [i.e. third-degree polynomial regression] of the dose response data from Figs. 2 and 3 was performed to determine the IC_{50}s. Note that the IC_{50} value for IL-8 was estimated from the 25 μM and 50 μM COB-187 data points (Fig. 2I) and the IC_{50} for IL-10 was not determined due to the bimodal effect of COB-187 on IL-10 expression (Fig. 3B). Since CXCL10 was reduced to basal levels for all concentrations of COB-187 tested in the protein cytokine array (Fig. 2D), the CXCL10 ELISA kit (data not shown) was used to determine the IC_{50} for this chemokine at concentrations ≤ 5 μM. The IC_{50} values are presented in Table 1 (column labeled Protein). As shown, the IC_{50}s are all in the micromolar range with the lowest value (greatest inhibitory effect) observed for CXCL10 (IC_{50} of 2 μM) and the highest value (least inhibitory effect) observed for MIF (IC_{50} of 48 μM). The average IC_{50} for the cytokines presented in Table 1 was 15 μM with a standard deviation of 16 μM.





COB-187

Coordinate	Target
1	CCL3/CCL4
2	CCL5/RANTES
3	CXCL1/GROα
4	CXCL10/IP-10
5	IL-1β/IL-1F2
6	IL-1ra/IL-1F3
7	IL-8
8	MIF
9	TNF-α

Fig. 1. Blots from the proteome profiler human cytokine protein array reveal that nine chemokines/cytokines are markedly induced by LPS and their expression is reduced by COB-187.
THP-1 macrophages were stimulated with 10 ng/ml of LPS in combination with varying concentrations of COB-187 or 0.1% DMSO (solvent control). LPS treatment was for 4 h and COB-187 was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. Separate proteome profiler blots were exposed to supernatants harvested from the various treatment groups (the treatment groups are labeled to the left of the blot). The four dots in the left most region of each blot and the two dots in the right most region of each blot are the internal reference controls and are used to normalize the data obtained from each blot. Note that each dot is paired, i.e. they are replicates. The numbers demarcate the proteins that appear in response to LPS treatment. These proteins are listed in the table to the right. Inset shows the structure of COB-187 (Noori et al., 2019).

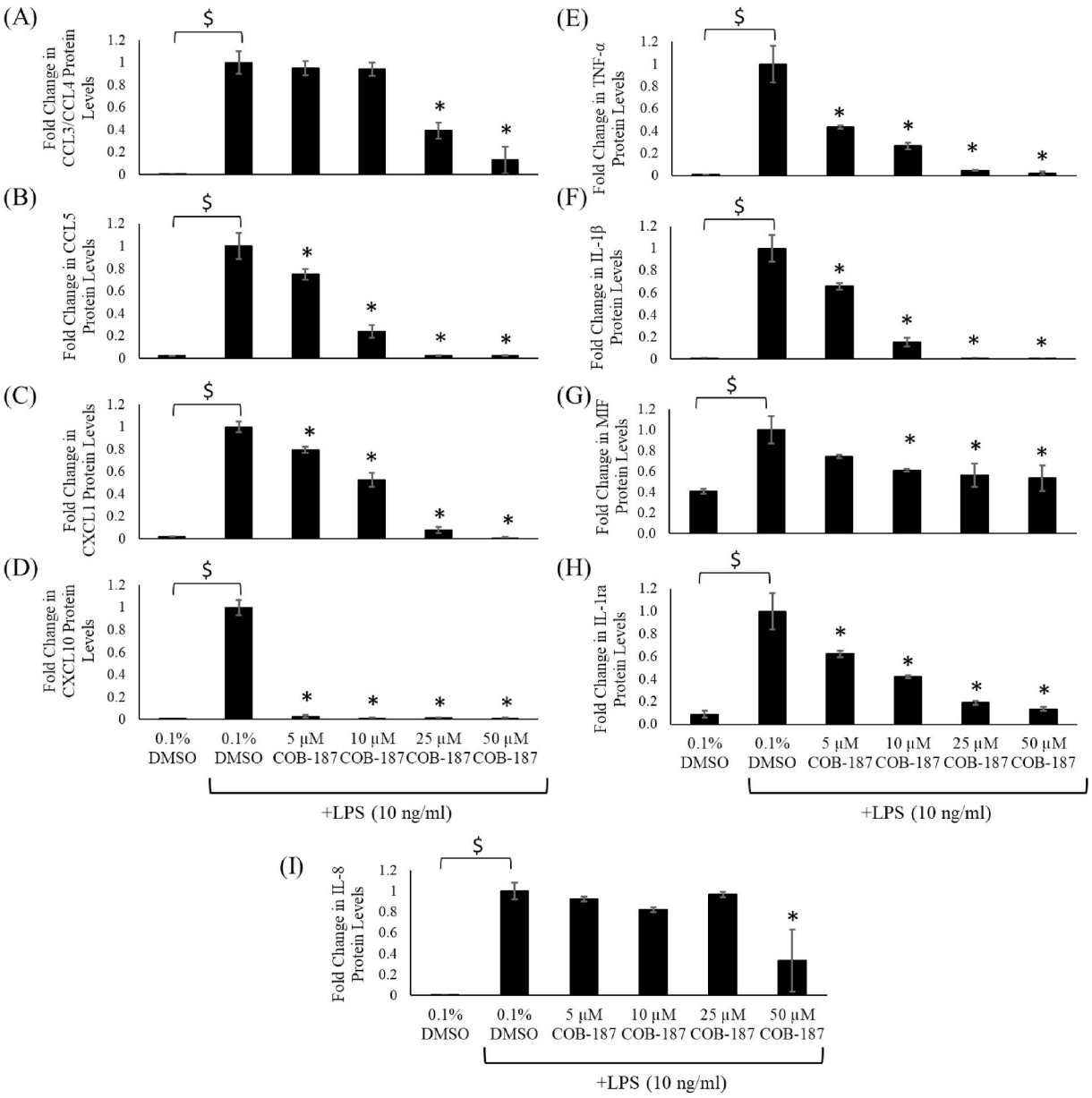


Fig. 2. COB-187 significantly reduces LPS-induced protein levels of a variety of chemokines/cytokines in THP-1 macrophages. THP-1 macrophages were stimulated with 10 ng/ml of LPS in combination with varying concentrations of COB-187 or 0.1% DMSO (solvent control). LPS treatment was for 4 h and COB-187 was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. The level of secreted (A) CCL3/CCL4, (B) CCL5, (C) CXCL1, (D) CXCL10, (E) TNF-α, (F) IL-1β, (G) MIF, (H) IL-1ra, and (I) IL-8 was measured by the proteome profiler human cytokine array. The COB-187 dose response was determined in three independent experiments. Pixel density data was processed as described in the methods, the results normalized to the 0.1% DMSO control in the presence of LPS and the average of the three replicates (+/-S.E.M.) were plotted vs the concentration of COB-187. A one-way ANOVA coupled with post hoc Dunnett's test was used for the statistical analysis. $^{\$}$Stimulation with LPS significantly increased the secretion relative to the 0.1% DMSO control ($P < 0.05$). *Significantly different ($P < 0.05$) relative to the 0.1% DMSO control in the presence of LPS.

3.2. COB-187 significantly reduces, at the mRNA level, the LPS-Induced expression of TLR pathway molecules

To gain further insight into the effect of COB-187 on LPS-induced cytokine/chemokine expression via TLR4 signaling, the effects of 4-h LPS stimulation alone and in combination with COB-187 treatment was evaluated at the mRNA level using the Human Toll-Like Receptor Signaling Pathway RT^2 Profiler™ PCR Array (TLR PCR array). Note that this array allows for the detection of a multitude of cytokines/chemokine transcripts as well as a host of other TLR-related transcripts. As expected, from the protein cytokine array and ELISA results (Figs. 1–3), 4-h stimulation with 10 ng/ml of LPS drastically induced the expression of cytokines and chemokines in the TLR signaling pathway (Table 2), with the exception of IFN-α1, IFN-γ and IL-2 (Supp. Table 1). The mRNA expression of all the inflammatory cytokines and chemokines that were significantly induced by LPS, were significantly inhibited by treatment with 25 μM COB-187 (Table 2); the exception being lymphotoxin alpha which was slightly induced by LPS and not inhibited by COB-187 (Supp. Table 1). These findings are in line with results from the protein cytokine array and ELISA kits (Figs. 1–3) where treatment with 25 μM COB-187 resulted in a significant inhibition in LPS-induced protein levels of CXCL10, IFN-β, IL-10, IL-1β, IL-6, and TNF-α. Note that the other proteins evaluated in Figs. 1–3, namely CCL3/CCL4, CCL5, CXCL1, IL-1ra, and MIF, were not in the TLR array. Overall, COB-187 has a dramatic inhibitory effect on a host of cytokine and chemokine mRNA transcripts induced by LPS.

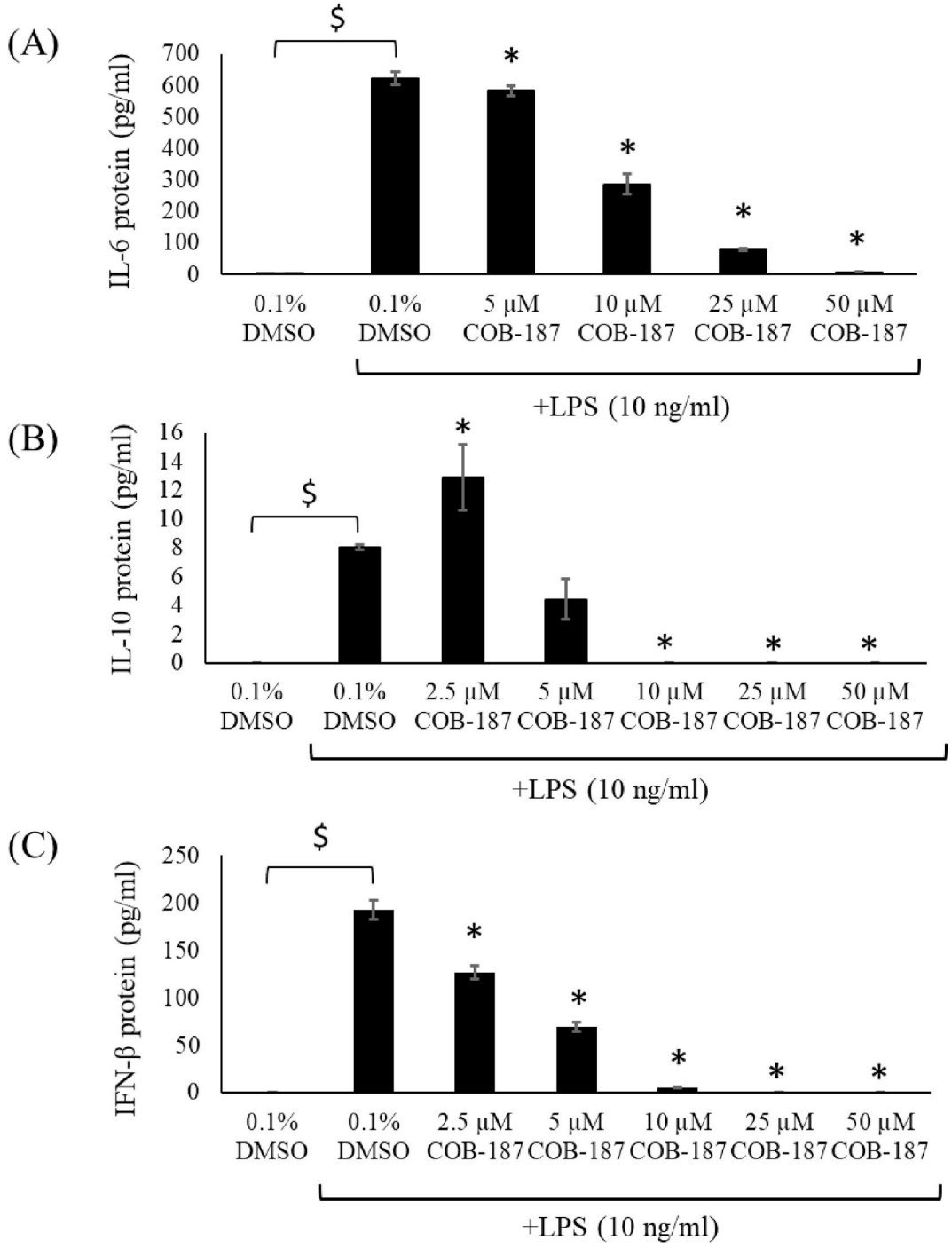


Fig. 3. Concurrent treatment of LPS-stimulated THP-1 macrophages with COB-187 results in a dose-dependent reduction in IL-6 and IFN-β protein expression, while concurrent treatment first increases the protein expression of anti-inflammatory cytokine IL-10, then significantly reduces it to the unstimulated level.
THP-1 macrophages were stimulated with 10 ng/ml of LPS in combination with varying concentrations of COB-187 or 0.1% DMSO (solvent control). LPS treatment was for 4 h and COB-187 was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. The levels of secreted (A) IL-6, (B) IL-10, and (C) IFN-β were measured by ELISA in at least two independent experiments performed in duplicate. The average of the independent values (+/-S.E.M.) were plotted vs the concentration of COB-187. A one-way ANOVA coupled with post hoc Dunnett's test was used for the statistical analysis. $^{\$}$Stimulation with LPS significantly increased the secretion of IL-6, IL-10, and IFN-β relative to the 0.1% DMSO control ($P < 0.05$). *Significantly different ($P < 0.05$) relative to the 0.1% DMSO control in the presence of LPS.

CXCL10, IL-6, IL-1β, TNF-α, and IFN-β are among the most important and well-studied chemokines/cytokines involved in sepsis (Bakshi et al., 2017; Chaudhry et al., 2013; Herzig et al., 2014; Jope et al., 2016; Mahieu and Libert, 2007; Schulte et al., 2013). Therefore, the dose-response of COB-187 inhibition of these cytokines was further investigated using RT-qPCR (Fig. 4). [Note that IL-10 was not further investigated due to its bimodal protein response (Fig. 3B)]. A best fit regression [i.e. third-degree polynomial] of the data presented in Fig. 4 was performed to determine the IC_{50}. The IC_{50} for all of the genes was ≤10 µM (Table 1, column labeled mRNA). These results are in line with the IC_{50} values determined at the protein level (Table 1, column labeled Protein) and the PCR array data presented in Table 2. Note that 50 µM of

Table 1
IC_{50} values (i.e. the concentration of COB-187 where the LPS-induced protein secretion or mRNA expression is reduced by 50%) for chemokines and cytokines in LPS-stimulated THP-1 macrophages. ELISA and RT-qPCR were used to determine the protein and mRNA expression of a variety of chemokines and cytokines in response to 4-h LPS stimulation in the presence of varying concentrations of COB-187. The COB-187 dose response was determined in at least two independent experiments performed in duplicate. Dose-dependent data from these replicate experiments were used to determine the IC_{50} [NP: Not Performed].

	IC_{50} (μM)	
Cytokines/Chemokines	Protein	mRNA
CCL3/CCL4	22	NP
CCL5	7	NP
CXCL1	11	NP
CXCL10	2	3
TNF-α	5	4
IL-1β	6	10
MIF	48	NP
IL-ra	8	NP
IL-8	43	NP
IL-6	12	3
IFN-β	4	10

Table 2
COB-187 significantly reduces LPS-induced mRNA expression of cytokines in the TLR pathway.
The effect of LPS and concurrent treatment with COB-187 on the mRNA expression levels of a host of cytokines and chemokines in THP-1 macrophages were evaluated using Human Toll-Like Receptor Signaling Pathway RT^2 Profiler™ PCR Array in three independent experiments. Web-based SABioscience software was used to analyze the data and determine P-values. LPS treatment was for 4 h and COB-187 or 0.1% DMSO (carrier control) was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. Results of LPS stimulation in combination with 0.1% DMSO were compared with results of the 0.1% DMSO control, and the corresponding fold changes and P-values for the cytokine genes in the TLR pathway are presented in the second and third columns. Molecules with fold change $\geq$1.0 and P-value $<$ 0.05 are considered to be significantly induced by LPS and are presented in this table. Results of the LPS stimulation concurrent with 25 μM COB-187 were compared to: (i) the results of LPS stimulation in combination with 0.1% DMSO (fourth and fifth columns), and (ii) the 0.1% DMSO control (sixth and seventh columns). For the fourth and fifth columns, molecules with fold change $\leq$1.0 and P-value $\leq$ 0.05 are considered to be significantly inhibited by COB-187. *Addition of 25 μM of COB-187 to the LPS treatment resulted in a cytokine level statistically identical to treatment with 0.1% DMSO alone.

	LPS + DMSO		LPS +25 μM COB-187			
	Relative to DMSO		Relative to LPS + DMSO		Relative to DMSO	
Gene symbol	Fold change	*P*-value	Fold change	*P*-value	Fold change	*P*-value
CCL2	338.35	0.00	0.00	0.00	1.04*	0.72
CSF2	623.17	0.00	0.00	0.00	2.65	0.00
CSF3	909.43	0.00	0.01	0.00	4.70	0.02
CXCL8 (IL-8)	341.39	0.00	0.16	0.00	54.73	0.01
CXCL10	21673.35	0.00	0.00	0.00	2.15*	0.18
IFN-β1	105.65	0.00	0.19	0.00	19.59	0.01
IL-10	183.58	0.00	0.01	0.00	2.55	0.04
IL-12A	6.74	0.00	0.38	0.05	2.56*	0.22
IL-1α	45.42	0.00	0.05	0.00	2.35	0.01
IL-1β	1094.51	0.00	0.05	0.00	51.92*	0.06
IL-6	1596.92	0.00	0.00	0.00	2.41*	0.09
TNF-α	107.15	0.00	0.22	0.00	24.07	0.01

COB-187 effectively returned the LPS-induced expression level of all of the cytokines to basal levels (Fig. 4).

In addition to the cytokines and chemokines presented in Table 2, the TLR PCR array revealed that COB-187 significantly inhibited LPS induction of a variety of TLR pathway molecules, including co-stimulatory molecules (e.g. *CD80* and *CD86*), kinases (e.g. *IRAK2*, *MAP2K3*, *MAP4K4, RIPK2, and TBK1*), adapter molecules (e.g. *MYD88*), and transcription factors (e.g. *IRF1*, *REL*, *RELA*, *NFKB1*, *NFKB2*, *NFKBIA*) (Table 3). Overall, 55 of the 84 genes in the TLR PCR array were induced by LPS and of these 38 (~69%) were significantly reduced by COB-187; 14 (~25%) to background levels (Table 3 and Supp. Table 1). The level of gene induction by LPS is broad ranging from a 1.27 to a 21,673-fold increase. Interestingly, the most remarkable effect of LPS-induced gene expression occurs with cytokines/chemokines where inductions between two and three orders of magnitude are common (Table 2). In contrast, for the other genes, with the exception of *CD80* and *IRAK2*, the induction never reaches two orders of magnitude (Table 3).

The expression of five genes (*HSPA1A, HSPD1*, *JUN*, *MAPK8IP3*, and *TLR9*) was significantly increased upon concurrent treatment with 25 μM COB-187 and LPS relative to LPS treatment alone but these increases were quite modest (ranging from 1.4 to 2.7 fold increase) with the exception of *HSPA1A* which was increased 32.9 fold with addition of COB-187 (Table 3). We did observe 9 genes (*ECSIT*, *FOS*, *HRAS, IRAK1, LY96*, *NFKBIL1*, *SIGIRR*, *TLR10* and *TOLLIP*) that were not induced by LPS but that did exhibit an increase in expression upon concurrent treatment with COB-187 and LPS relative to DMSO with LPS or DMSO alone suggesting that COB-187 may induce these genes (Supp. Table 2). Again, these effects were relatively small; averaging less than an order of magnitude (Supp. Table 2).

3.3. COB-187 reduces the activity of NF-κB (p65/650) in LPS-stimulated THP-1 macrophages

GSK-3 has been implicated in the production of pro-inflammatory cytokines, chemokines, and IFN-β in the TLR4 pathway through direct activation of NF-κB (Jope et al., 2016; Ko and Lee, 2016) and/or indirect activation of IRF-3 (Jope et al., 2016), respectively. GSK-3 has also been implicated in the production of anti-inflammatory cytokines through inhibition of transcription factors AP-1 and CREB (Jope et al., 2016; Ko and Lee, 2016). As shown in Figs. 1–4 and Tables 1 and 2, COB-187 significantly reduced the expression of pro-inflammatory cytokines and chemokines in the LPS-stimulated THP-1 macrophages. Since the amount of secreted IL-10, an anti-inflammatory cytokine, was relatively low (<20 pg/ml, Fig. 3B) compared to the other cytokines (e.g. IL-6 was ~600 pg/ml, Fig. 3A), we focused on evaluating the effect of COB-187 on the activity of NF-κB and IRF-3, which are involved in the production of pro-inflammatory cytokines and IFN-β, rather than CREB and AP-1, which are mainly involved in the production of anti-inflammatory cytokines like IL-10. Note that NF-κB and IRF-3 are in the Myd88-dependent and Myd88-independent TLR4 signaling pathways, respectively.

The NF-κB transcription factor family consists of five subunits namely p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel (Tergaonkar, 2006). Note that high activity of the p65/p50 NF-κB dimer is strongly corrolated with the severity and mortality of sepsis (Arnalich et al., 2000; Chaudhry et al., 2013; Liu and Malik, 2006). As shown in Fig. 5 and 4-h stimulation with 10 ng/ml of LPS increased the DNA binding activity of p50 and p65 subunits of NF-κB more than 70% in THP-1 macrophages relative to control. Concurrent treatment with COB-187 at concentrations $\geq$25 μM significantly reduced the DNA binding activity of p50 (Fig. 5A) and p65 (Fig. 5B) NF-κB subunits relative to vehicle control (0.1% DMSO) in LPS-stimulated THP-1 macrophages, suggesting that COB-187 inhibits LPS-induced NF-κB activity.

3.4. COB-187 reduces IRF-3 phosphorylation at serine 396 in LPS-stimulated THP-1 macrophages

Given that COB-187 inhibits LPS induction of IFN-β (Figs. 3C and 4E

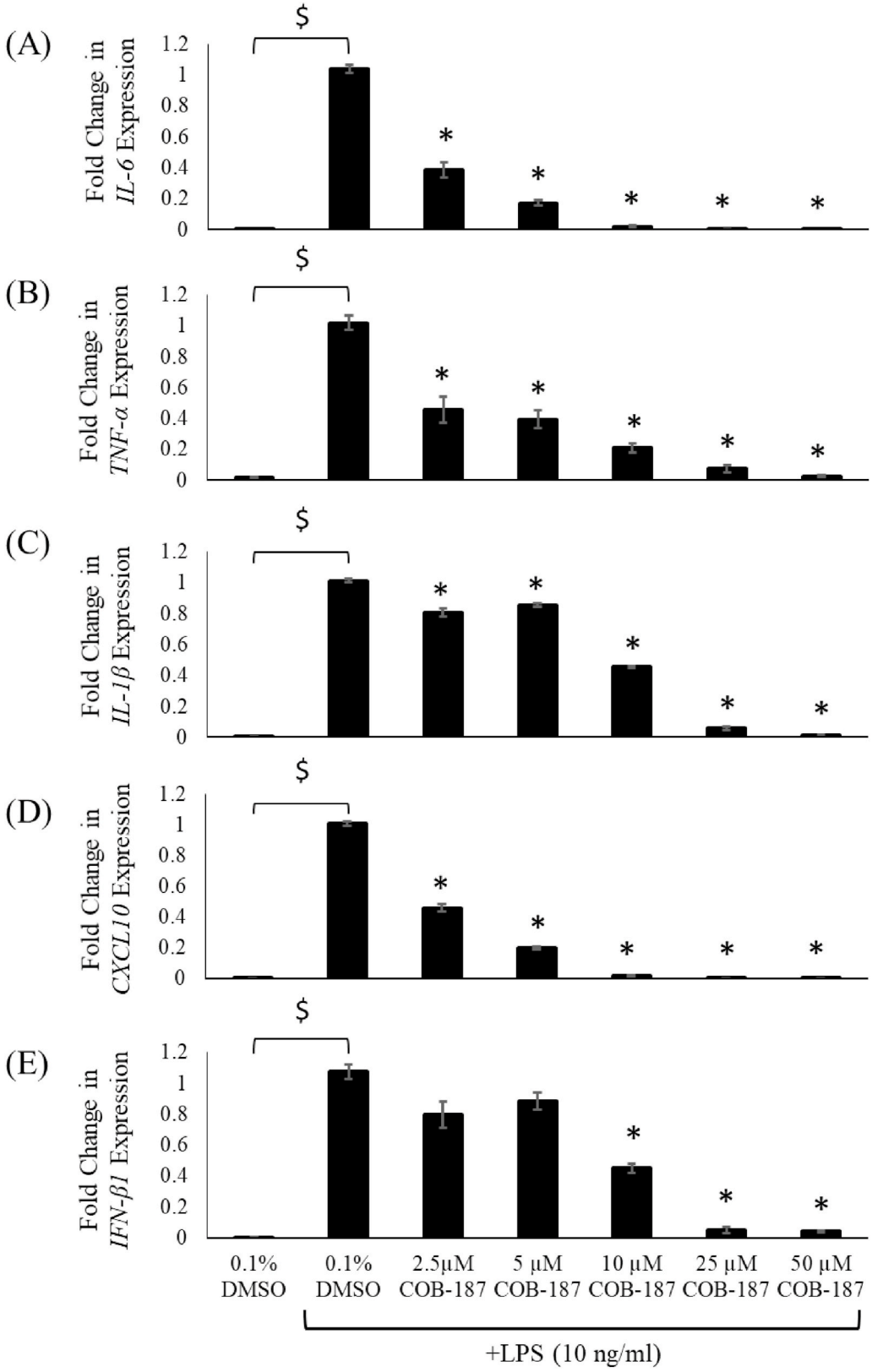


Fig. 4. Concurrent treatment of LPS-stimulated THP-1 macrophages with COB-187 results in a dose-dependent reduction in mRNA expression levels of *IL-6*, *TNF-α*, *IL-1β*, *CXCL10*, and *IFN-β1*, which are cytokines well-known to be involved in sepsis.

THP-1 macrophages were stimulated with 10 ng/ml of LPS in combination with varying concentrations of COB-187 or 0.1% DMSO (solvent control). LPS treatment was for 4 h and COB-187 was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. Subsequently, the mRNA levels of (A) *IL-6*, (B) *TNF-α*, (C) *IL-1β*, (D) *CXCL10*, and (E) *IFN-β1* were determined via RT-qPCR. The results at each dose were averaged for the three independent experiments performed in triplicate. These averages (+/-S.E.M.) were plotted vs the concentration of COB-187. *ACTB* was used as a house-keeping gene. A one-way ANOVA coupled with post hoc Dunnett's test was used for the statistical analysis. $^{\$}$Stimulation with LPS significantly increased the mRNA level of cytokines relative to the 0.1% DMSO control ($P < 0.05$). *Significantly different ($P < 0.05$) relative to the 0.1% DMSO control in the presence of LPS.

and Tables 1 and 2) which is a down-stream cytokine in the Myd88-independent pathway, the phosphorylation state of IRF-3 at Serine 396 [pIRF-3 (S^{396})], a hallmark of active IRF-3 (Bakshi et al., 2017), was assessed using western blot analysis to gain insights into the effect of COB-187 on IRF-3 activity. As shown in Fig. 6, pIRF-3 (S^{396}) is not present, or present at an undetectable level, in the nucleus or the cytoplasm of unstimulated THP-1 macrophages. Upon stimulation with LPS, IRF-3 is reported to get phosphorylated at Serine 396 and translocate into the nucleus (Bakshi et al., 2017). Thus pIRF-3 (S^{396}) should be detected in the nucleus and limited, if any, pIRF-3 (S^{396}) should be

Table 3
COB-187 significantly inhibits the LPS-induced expression of a variety of TLR-related transcripts in THP-1 macrophages.
The effect of LPS and concurrent treatment with COB-187 on the mRNA expression levels of a variety of TLR-related genes in THP-1 macrophages were evaluated using Human Toll-Like Receptor Signaling Pathway RT^2 Profiler™ PCR Array in three independent experiments. Web-based SABioscience software was used to analyze the data and determine P-values. LPS treatment was for 4 h and COB-187 or 0.1% DMSO (carrier control) was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. Results of LPS stimulation in combination with 0.1% DMSO were compared with results of the 0.1% DMSO control and the corresponding fold changes and P-values for the TLR-related genes are presented in the second and third columns. Molecules with fold change ≥1.0 and P-value < 0.05 are considered to be significantly induced by LPS and are presented in this table. Results of the LPS stimulation concurrent with 25 μM COB-187 were compared to: (i) the results of LPS stimulation in combination with 0.1% DMSO (fourth and fifth columns), and (ii) the 0.1% DMSO control (sixth and seventh columns). Bold text demarcates genes that were induced by LPS and significantly reduced by COB-187, i.e. fold change ≤1.0 and P-value ≤ 0.05 in the fourth and fifth columns. *Addition of 25 μM of COB-187 to the LPS treatment resulted in an expression level statistically identical to treatment with DMSO alone.

	LPS + DMSO		LPS +25 μM COB-187			
	Relative to DMSO		Relative to LPS + DMSO		Relative to DMSO	
Gene symbol	Fold change	*P*-value	Fold change	*P*-value	Fold change	*P*-value
CASP8	1.36	0.02	0.83	0.12	1.13*	0.20
CD80	291.07	0.00	0.01	0.00	1.75	0.01
CD86	6.33	0.00	0.06	0.00	0.37	0.00
CHUK	1.44	0.01	0.91	0.17	1.32	0.02
CLEC4E	17.61	0.03	0.04	0.03	0.77*	0.97
EIF2AK2	10.48	0.00	0.11	0.00	1.14*	0.12
ELK1	1.62	0.03	0.92	0.47	1.49	0.00
HSPA1A	1.43	0.01	32.93	0.00	47.03	0.00
HSPD1	1.40	0.03	2.11	0.01	2.95	0.00
IKBKB	1.36	0.00	0.79	0.03	1.07*	0.49
IRAK2	192.62	0.00	0.11	0.00	20.60	0.00
IRF1	9.50	0.00	0.05	0.00	0.46	0.00
JUN	3.07	0.00	1.43	0.03	4.37	0.00
LTA	3.01	0.00	1.02	0.88	3.09	0.01
LY86	1.33	0.05	0.76	0.05	1.01*	0.90
MAP2K3	13.72	0.00	0.39	0.00	5.40	0.00
MAP2K4	1.66	0.00	0.84	0.01	1.39	0.00
MAP4K4	14.90	0.00	0.27	0.00	4.02	0.00
MAPK8	1.68	0.00	0.92	0.38	1.54	0.01
MAPK8IP3	1.27	0.03	2.21	0.00	2.81	0.00
MYD88	9.60	0.00	0.11	0.00	1.01*	0.92
NFKB1	52.81	0.00	0.06	0.00	3.04	0.00
NFKB2	41.92	0.00	0.08	0.00	3.39	0.01
NFKBIA	43.65	0.00	0.08	0.00	3.41	0.01
NR2C2	1.33	0.02	0.80	0.12	1.07*	0.55
PELI1	37.15	0.00	0.11	0.00	4.12	0.00
PPARA	1.69	0.00	0.86	0.43	1.45*	0.11
PRKRA	1.40	0.01	0.91	0.51	1.28*	0.13
PTGS2	64.69	0.00	0.27	0.00	17.65	0.00
REL	27.57	0.00	0.10	0.00	2.80	0.00
RELA	2.72	0.00	0.47	0.00	1.29 *	0.05
RIPK2	6.88	0.00	0.40	0.02	2.74	0.01
TBK1	2.76	0.00	0.58	0.02	1.60*	0.08
TICAM1	4.20	0.00	0.91	0.50	3.83	0.00
TICAM2	2.39	0.00	0.22	0.00	0.52	0.01
TLR2	1.74	0.00	1.61	0.11	2.79	0.03
TLR3	1.36	0.01	0.56	0.02	0.76*	0.17
TLR8	1.27	0.02	0.33	0.00	0.41	0.00
TLR9	2.38	0.03	2.72	0.04	6.48	0.01
TNFRSF1A	1.74	0.00	0.58	0.00	1*	0.95
TRAF6	1.35	0.02	1.16	0.33	1.56	0.04
UBE2N	2.10	0.00	0.72	0.01	1.51	0.02

detected in the cytoplasm of LPS-stimulated THP-1 macrophages. Consistent with these expectations, pIRF-3 (S^{396}) was detected, but only in the nuclear fraction of 4-h LPS-stimulated THP-1 macrophages (Fig. 6). Concurrent treatment with 50 μM of COB-187 significantly reduced the level of pIRF-3 (S^{396}) found in the nucleus of LPS-stimulated THP-1 macrophages (Fig. 6). Somewhat surprisingly given this observation, no significant effect of COB-187 on IRF-3 DNA binding activity was detected in LPS-stimulated THP-1 macrophages using the IRF-3 TransAm assay (Supp. Fig. 1). This latter observation could be due to insensitivity of the TransAm assay [note that treatment with LPS resulted in only a ~50% increase in the DNA binding activity of IRF-3 relative to control (Supp. Fig. 1)]. Setting that caveat aside, the results presented in Fig. 6 suggest that COB-187 inhibits LPS-induced IRF-3 activation.

3.5. *COB-187 treatment post-LPS induction significantly reduces CXCL10 transcripts*

In all of the above studies, we treated the THP-1 macrophages with COB-187 1 h prior to LPS challenge. This raises the question as to what effect COB-187 would have on THP-1 macrophages that have been stimulated with LPS prior to treatment with COB-187. To gain insight to this issue, we treated THP-1 cells with COB-187: 1 h prior to, simultaneous with, 1 h after, and 2 h after, initiation of a 4-h LPS challenge. We subsequently harvested the mRNA and performed RT-qPCR to determine the relative level of *CXCL10* mRNA present for each condition. As revealed in Fig. 7, COB-187 significantly inhibited *CXCL10* mRNA levels at all treatment conditions including 1 and 2 h treatments post initiation of the LPS challenge.

4. Discussion

Several studies have demonstrated the efficacy of GSK-3 inhibitors in models of endotoxic shock (Dugo et al., 2017; Martin et al., 2005; Noh et al., 2012). Further, in aggregate, numerous reports indicate that GSK-3 inhibitors abate sepsis through attenuation of LPS-induced cytokine/chemokine production (Gao et al., 2018; Huang et al., 2009; Jing et al., 2004; Kontzias et al., 2012; Martin et al., 2005; Steinbrecher et al., 2005). The present study revealed that COB-187, a novel GSK-3 inhibitor (Noori et al., 2019), significantly attenuatets the expression of a plethora of LPS-inducible genes. Specifically, LPS induced 55 of the 84 genes in a TLR array of which 69% of those induced were significantly inhibited by COB-187, and 25% were reduced to basal levels. Cytokines/chemokines known to be induced by LPS and important in sepsis (Bakshi et al., 2017; Chaudhry et al., 2013; Huang et al., 2009; Jope et al., 2016; Martin et al., 2005; Schulte et al., 2013; Tay et al., 2012), were inhibited by COB-187 with relatively high potency (Table 1). While in the majority of our studies COB-187 was added pre-LPS challenge, treatment with COB-187 post-LPS stimulation did cause a significant reduction in induced *CXCL10* mRNA (Fig. 7).

These results are in line with the previous studies utilizing other GSK-3 inhibitors [e.g. (Gao et al., 2018; Huang et al., 2009; Jing et al., 2004; Kontzias et al., 2012; Martin et al., 2005; Rehani et al., 2009; Steinbrecher et al., 2005)] and demonstrate the ability of COB-187 to attenuate the cytokine storm induced by LPS. The inhibitory effect of COB-187 does not appear to be due to a non-specific toxic effect since COB-187 had no statistical effect on THP-1 macrophage metabolic activity (Supp. Fig. 2). In regards to the anti-inflammatory cytokine IL-10, previous studies found that GSK-3 inhibitors increased the production of IL-10 (Huang et al., 2009; Martin et al., 2005; Noh et al., 2012; Tay et al., 2012). A similar result was observed in the present study for LPS-stimulated THP-1 macrophages treated with 2.5 μM of COB-187 (Fig. 3B). However, at ≥ 10 μM of COB-187, the IL-10 protein level was reduced to basal level and treatment with 25 μM COB-187 significantly reduced LPS-induced *IL-10* mRNA (Table 2). GSK-3 may regulate IL-10 production, in part, through CREB via the Myd88-dependent

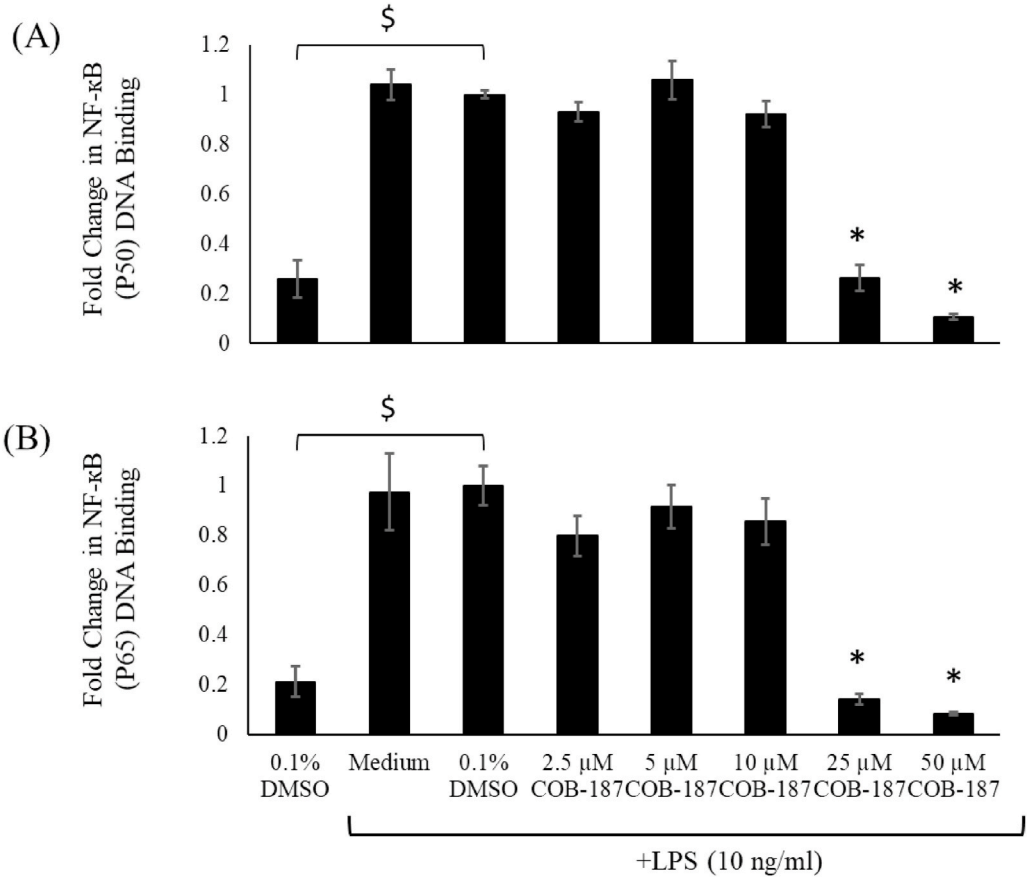


Fig. 5. COB-187 inhibits LPS-induced NF-κB (p50/p65) DNA binding activity in THP-1 macrophages.
THP-1 macrophages were stimulated with 10 ng/ml of LPS in culture medium or with 10 ng/ml LPS in combination with varying concentrations of COB-187 or 0.1% DMSO (solvent control). LPS treatment was for 4 h and COB-187 was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. The nuclear protein fraction was harvested and TransAm DNA binding ELISA assay was used to measure the DNA binding activity of NF-κB. Treatment of THP-1 macrophages with 25 and 50 μM of COB-187 for 5 h caused a significant reduction in LPS-induced DNA binding activity of **(A)** p50 and **(B)** p65 subunits of the NF-κB transcription factor. The results at each dose were averaged for the two independent experiments performed in duplicate. Results were normalized to the 0.1% DMSO control in the presence of LPS and the averages (+/-S.E.M.) were plotted vs the concentration of COB-187. A one-way ANOVA coupled with post hoc Dunnett's test was used for the statistical analysis. $Stimulation with LPS significantly increased the DNA binding activity of p50 and p65 subunits of NF-κB relative to 0.1% DMSO control ($P < 0.05$). *Significantly different ($P < 0.05$) relative to the 0.1% DMSO control in the presence of LPS.

pathway (Ko and Lee, 2016). We have no data on the effect of COB-187 on CREB. Interestingly, the THP-1 macrophages secreted relatively low amounts of IL-10 which could be due to the short incubation time (4 h) of THP-1 macrophages with LPS.

We gained insights into the effect of COB-187 on the activity of the "pro-inflammatory" NF-κB and IRF-3 transcription factors in the TLR4 Myd88-dependent and Myd88-independent pathways, respectively. LPS ligation of TLR4 induces the activation of NF-κB in the Myd88-dependent pathway (Ansaldi et al., 2011; Takada et al., 2004) and the elevated activity of NF-κB after bacterial infection is strongly correlated with the severity of sepsis (Arnalich et al., 2000; Chaudhry et al., 2013; Liu and Malik, 2006). In line with this observation, we found that LPS dramatically induced the DNA binding activity of p50 and p65 subunits of NF-κB (Fig. 5). Takada et al. (2004) reported a complete suppression of LPS-induced NF-κB activity in GSK-3β gene-deleted cells, and demonstrated the pivotal role of GSK-3 in activation of NF-κB (Takada et al., 2004). These obervations suggest that inhibition of GSK-3 could reduce the activation of NF-κB and consequently abate symptoms associated with sepsis (Jope et al., 2016; Klamer et al., 2010; Ko and Lee, 2016); a conjecture that has been bolstered by numerous reports (Ansaldi et al., 2011; Kotliarova et al., 2008; Wilson and Baldwin, 2008). In the present study, we observed that treatment with COB-187 at $\geq$ 25 μM significantly reduced the LPS-induced DNA binding activity of p50 and p65 (Fig. 5). Furthermore, treatment with 25 μM of COB-187 significantly down-regulated the LPS-induced mRNA level of p50 (*NF-κB1*), p65 (*RELA*) and *Myd88* in THP-1 macrophages (Table 3). These findings suggest that COB-187 inhibition of LPS-induced cytokine expression via the Myd88-dependent pathway may be due, in part, to the inhibition of the NF-κB DNA binding activity (Fig. 5) as well as reduction in mRNA expression of the adapter molecules Myd88, and p50 and p65 subunits of NF-κB (Table 3).

LPS stimulation also activates the Myd88-independent pathway which trigers IFN-β production through the sequential events of TRIF recruitment, TBK1 activation, IRF-3 phosphorylation at Serine 396, IRF-3 dimerization and translocation to the nucleus, and subsequent IRF-3 binding to the IFN-β gene promoter (Bakshi et al., 2017; Jope et al., 2016). As shown in Figs. 3C and 4E, COB-187 at $\geq$ 10 μM reduces IFN-β expression, while the phosphorylation of IRF-3 at Serine 396 was only inhibited at 50 μM of COB-187 (Fig. 6). The effect of COB-187 on LPS-induced IRF-3 activation is further clouded by our finding that COB-187 did not alter LPS-induced IRF-3 DNA binding (Supp. Fig. 1). These somewhat paradoxical observations could be explained by the fact that activated NF-κB and c-Jun are required to bind with IRF-3 on the IFN-β gene enhancer/promoter region to induce the expression of *IFN-β1* gene (Fitzgerald et al., 2003; Jin et al., 2014; Kawai and Akira, 2007; Núñez Miguel et al., 2007; Sin et al., 2012). While we did not investigate c-Jun DNA binding activity, we did observe that LPS-induced NF-κB activity was inhibited at COB-187 $\geq$ 25 μM (Fig. 5).

In addition to IFN-β, expression of CCL5 and CXCL10 have been reported to be regulated through the Myd88-independent pathway (Bandow et al., 2012; Yoo et al., 2014). Further, LPS induction of co-stimulatory surface molecules (e.g. *CD80* and *CD86*) is reported to be mediated through IFN-β (Hoebe et al., 2003; Mahieu and Libert, 2007). In the present study we observed that COB-187 significantly reduced (i) the protein levels of CCL5 (Fig. 2B), CXCL10 (Fig. 2D), and IFN-β (Fig. 3C), (ii) the mRNA expression levels of *CXCL10* (Table 2 and Fig. 4D), *TBK1* (Table 3), and *IFN-β1* (Fig. 4E), *CD80* and *CD86* (Table 3), and (iii) the phosphorylation level of IRF-3 at Serine 396 (Fig. 6). Thus, COB-187 may inhibit, at least in part, the TLR4 Myd88-independent pathway.

As mentioned above, numerous studies have reported efficacy of GSK-3 inhibitors in animal models of sepsis raising the potential for a

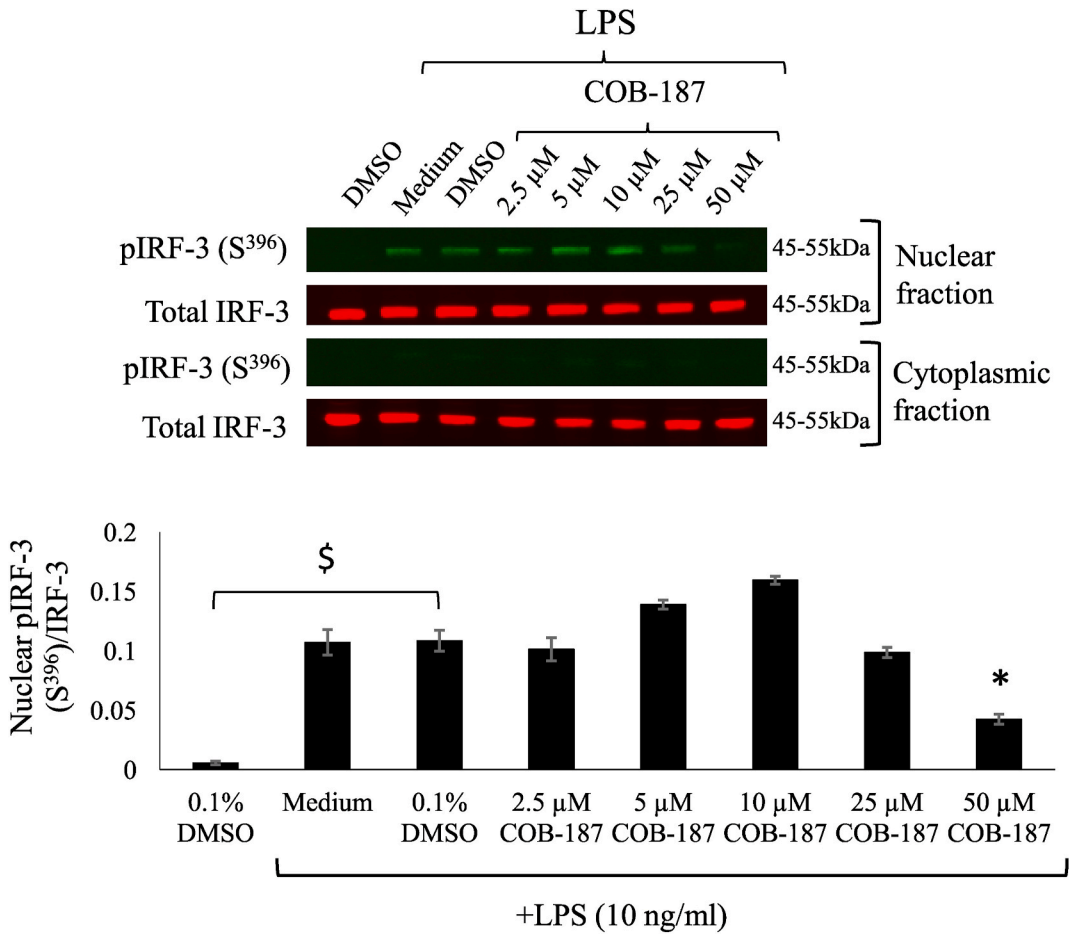


Fig. 6. Concurrent treatment with 50 μM of COB-187 significantly reduces LPS-induced IRF-3 phosphorylation at Serine 396 [pIRF3 (S^{396})] in THP-1 macrophages. THP-1 macrophages were stimulated with 10 ng/ml of LPS in culture medium or with 10 ng/ml LPS in combination with varying concentrations of COB-187 or 0.1% DMSO (solvent control). LPS treatment was for 4 h and COB-187 was added 1 h prior to treatment with LPS and was maintained throughout the LPS treatment. Nuclear and cytoplasmic protein fractions were extracted, and western blot analysis was used to evaluate the levels of pIRF3 (S^{396}) and total IRF-3. (Upper panel: images from western blot analysis; Lower panel: quantification of nuclear pIRF-3 (S^{396}) relative to total nuclear IRF-3). Upper panel images are representative results of three separate experiments. The results from the quantitative analysis of each experiment were averaged (+/-S.E.M.) and plotted vs the concentration of COB-187 in the lower panel graph. A one-way ANOVA coupled with post hoc Dunnett's test was used for the statistical analysis. $^{\$}$Stimulation with LPS significantly increased the phosphorylation of IRF-3 at Serine 396 relative to the 0.1% DMSO control ($P < 0.05$). *Significantly different ($P < 0.05$) relative to the 0.1% DMSO control in the presence of LPS.

clinical use of GSK-3 for sepsis. Several GSK-3 inhibitors are being investigated in clinical trials, but the majority of these focus on neurological disorders or cancer. In considering GSK-3 inhibitors for sepsis, it is important to appreciate that the translation of promising reagents to the clinic for sepsis is froth with disappointment. Indeed, the 2019 Final Report from the NIH Working Group on Sepsis ("NAGMSC Working Group on Sepsis," 2019) emphasized that there are no accepted treatments for sepsis despite decades of work and dozens of clinical trials. We have found that COB-187 does sharply attenuate LPS-induced cytokine production and is quite selective in molecular kinase screens relative to the most clinically advanced GSK-3 inhibitor Tideglusib (Noori et al., 2019). The selectivity of COB-187 may ultimately make COB-187 a better clinical candidate. In this regard, studies that compare the effect of established GSK-3 inhibitors and COB-187 on LPS-induced cytokine expression would be insightful.

Finally, we would like to remark on four unresolved issues raised by the study. First, we *infer* that the inhibition of cytokine induction is due to COB-187 inhibiting GSK-3. This inference is based on (i) our finding that COB-187 is a selective and potent inhibitor of GSK-3 (Noori et al., 2019) and (ii) previous work by others demonstrating GSK-3 inhibitors diminish LPS-induced cytokine expression (Gao et al., 2018; Huang et al., 2009; Jing et al., 2004; Kontzias et al., 2012; Martin et al., 2005; Steinbrecher et al., 2005). Again, comparing multiple GSK-3 inhibitors with COB-187 would be insightful. Second, the studies were done with THP-1 macrophages at a short time point. Complementary studies with freshly isolated cells, that consider short-term and late-phase proteins are clearly needed. Third, given that GSK-3 has a multitude of substrates (Cormier and Woodgett, 2017; Frame and Cohen, 2001), and plays a key role in numerous physiological processes (e.g. glycogen metabolism) (Beurel et al., 2015; Frame and Cohen, 2001; Jope et al., 2007; Saraswati et al., 2018), GSK-3 inhibition could cause numerous side effects. That said, GSK-3 inhibitors have progressed through clinical trials (del Ser et al., 2013; Hoglinger et al., 2012) and lithium, a known GSK-3 inhibitor, is used clinically. Finally, several of the mechanisms of viral-related cytokine storms are similar to bacterial-related mechanisms [e.g. overlap in TLR4 and TLR3 (viral response) signaling pathways (Uematsu and Akira, 2007)]. Given the current COVID-19 epidemic and the fact that cytokine storms are often a complication of coronavirus infections (Channappanavar and Perlman, 2017; Fung et al., 2020), it would be quite timely to probe the effect of COB-187 on viral-related cytokine storms.

In summary, COB-187 inhibits a plethora of LPS-induced, THP-1 macrophage expressed, TLR-related genes, demonstrating that COB-187 is a potent inhibitor of LPS-induced cytokine storms.

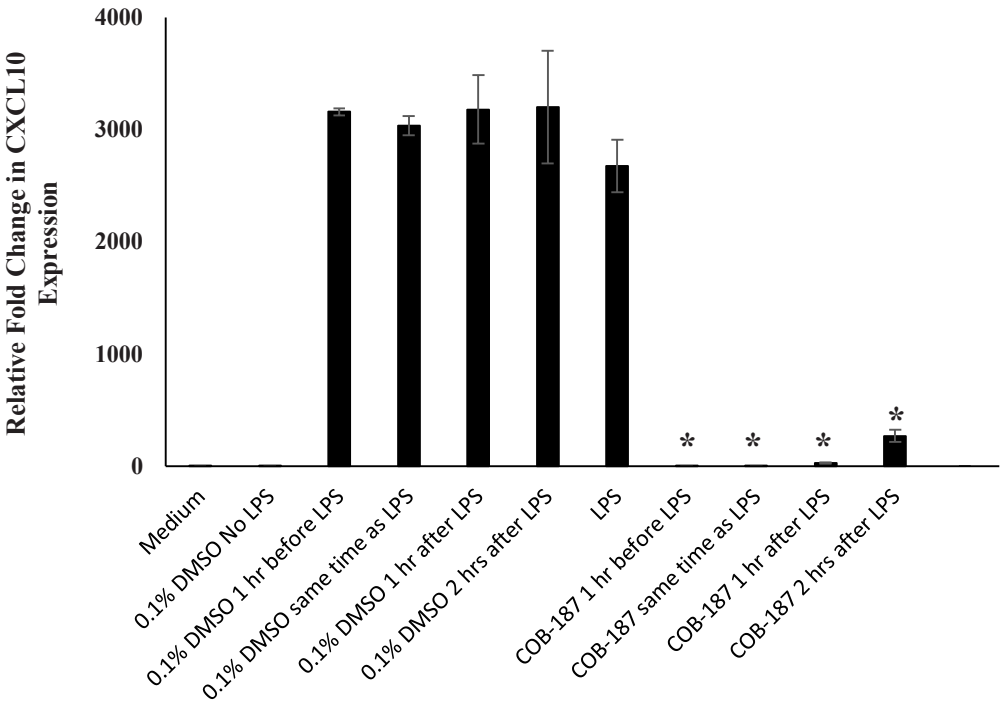


Fig. 7. COB-187 treatment post-LPS challenge results in a significant reduction in mRNA expression levels of *CXCL10*. THP-1 macrophages were treated with 0.1% DMSO or 25 μM COB-187 1 h prior, at the same time, 1 h post or 2 h post challenge with 10 ng/ml of LPS. Four h after LPS challenge, the mRNA levels of *CXCL10* was determined via RT-qPCR for each treatment condition and normalized to the media only control. The results for each condition was averaged for two biological replicates performed in duplicate. These averages (+SD) were plotted vs the treatment condition. A one-way ANOVA coupled with post hoc Tukey's test was used for the statistical analysis. *Significantly different ($P < 0.05$) relative to the time matched 0.1% DMSO control in the presence of LPS.

CRediT authorship contribution statement

Mahboubeh S. Noori: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Maria C. Courreges:** Conceptualization, Validation, Investigation, Data curation. **Stephen C. Bergmeier:** Conceptualization, Validation, Resources, Writing - review & editing, Funding acquisition. **Kelly D. McCall:** Conceptualization, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Douglas J. Goetz:** Conceptualization, Formal analysis, Validation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.173340.

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