

# Effects of Two Anti-TNF- $\alpha$ Compounds: Etanercept and 5-Ethyl-1-phenyl-2-(1H)-pyridone on Secreted and Cell-Associated TNF- $\alpha$ *in Vitro*

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## ABSTRACT

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a potent inflammatory cytokine and its exaggerated production has been implicated in acute, chronic and autoimmune inflammatory diseases. Proteinaceous and non-proteinaceous anti-TNF- $\alpha$  agents have been developed to reduce its circulating levels either by neutralizing, binding or inhibiting the *de novo* synthesis with the aim of achieving desirable therapeutic effects. In the present study, we compared the effects of a protein-based anti-TNF- $\alpha$  drug, etanercept, and a non-protein-based anti-TNF- $\alpha$  small molecule, 5-ethyl-1-phenyl-2-(1H) pyridone (5-EPP), on the LPS-stimulated secretion of TNF- $\alpha$  in the medium and TNF- $\alpha$  associated with the THP-1 cells *in vitro*. Both drugs had marked concentration-dependent inhibitory effects on the LPS-stimulated secretion of TNF- $\alpha$ . However, their effects on the LPS-stimulated cell-associated TNF- $\alpha$  were diametrically opposed to each other. For instance, etanercept further increased the level by up to 12-fold, whereas 5-EPP inhibited the level in a dose dependent manner. In addition, 5-EPP caused a significant reduction in the elevated level of cell associated TNF- $\alpha$  caused by LPS + etanercept. The differences in the levels of cell-associated TNF- $\alpha$  as reported in the present study may partly explain the adverse effects of some protein-based anti-TNF- $\alpha$  drugs including etanercept as opposed to a non-protein-based anti-TNF- $\alpha$  drug such as pirfenidone, a structural analogue of 5-EPP, for treatment of some TNF- $\alpha$  mediated diseases. It was concluded from the findings of this study that drugs which elevate the levels of cell associated-TNF- $\alpha$  will potentially have more adverse events even after reducing the secreted levels of TNF- $\alpha$  than the drugs which reduce both the secreted and cell-associated TNF- $\alpha$  levels.

**Keywords:** Tumor Necrosis Factor-Alpha, 5-Ethyl-1-phenyl-2-(1H)-pyridone, Etanercept, Cytokines

## 1. Introduction

The cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a key regulator of systemic inflammation and the acute phase response. A precursor form of this cytokine is expressed as a 26 kD type II polypeptide transmembrane protein (mTNF) on the surface of activated macrophages, lymphocytes and other cell types (endothelium). The soluble form of TNF- $\alpha$ , a 17 kD polypeptide, is released when mTNF is cleaved by the metalloproteinase TNF- $\alpha$  converting enzyme (TACE) [1]. Both mTNF- $\alpha$  and soluble TNF- $\alpha$  readily form biologically-active homotrimeric structures.

TNF- $\alpha$  elicits its pathophysiological responses by interacting with two structurally distinct transmembrane TNF- $\alpha$  receptors: type I (TNFR1) and type II (TNFR2). Although both receptors are glycoproteins, contain mul-

iple cysteine-rich repeats and share considerable structural and functional homology within their extracellular domains, the intracellular domains of TNFR1 and TNFR2 differ considerably. Signal transduction occurs via both overlapping and discrete pathways. Secreted TNF- $\alpha$  binds to both receptors, while mTNF- $\alpha$  binds mainly to TNFR2 [2-5]. Like TNF- $\alpha$ , these receptors exist both in a soluble form and in a membrane-anchored form. The soluble receptors bind and neutralize the biological activities of TNF- $\alpha$ , whereas the membrane-anchored form of the receptors mediates the pleiotropic pathophysiological effects of this cytokine [6-8].

The central role of TNF- $\alpha$  in the pathogenesis of chronic inflammatory diseases has led to the development and widespread clinical uses of protein-based anti-TNF- $\alpha$  agents for treatment of rheumatoid arthritis [9,10],

Crohn's disease [11], psoriasis [12], ankylosing spondylitis [13,14], and Behcet's disease [15]. The FDA has approved five protein-based anti-TNF- $\alpha$  drugs and all of them act, in essence, as neutralizing antibodies of secreted TNF- $\alpha$ , thus preventing its interactions with cell surface receptors. However, none of these protein-based anti-TNF- $\alpha$  agents have any reported effect on the synthesis of TNF- $\alpha$ . Lastly, these drugs are only effective when administered via intravenous, intramuscular or subcutaneous injection—a significant therapeutic consideration as compared with a non-protein-based anti-TNF- $\alpha$ .

While the role of soluble TNF- $\alpha$  in many diseases has been investigated for over three decades, the contributions of mTNF to TNF- $\alpha$ -associated pathophysiology have only been appreciated relatively recently. Early studies found that mTNF mediates various cytotoxic and inflammatory functions of leucocytes via direct cell-cell contact and binding to TNFRs on target cells [16-18]. More recently, the binding of mTNF to TNFRs was shown to initiate reverse signaling (*i.e.*, receptor-mediated ligand signal transduction) which altered the physiology of the mTNF-expressing cells. Binding of anti-TNF- $\alpha$  antibodies or soluble TNFRs to mTNF phosphorylates an intracellular signaling domain that triggers changes in the level of intracellular calcium [18,19], initiates synthesis and release of various cytokines [18,20,21], increases expression of the E-selectin adhesion molecule [22], and alters the cellular response to inflammatory stimuli [23].

Pirfenidone, a low molecular weight pyridone, has been shown to inhibit TNF- $\alpha$  release *in vitro*, block endotoxin-induced and *staphylococcus aureus* enterotoxin B-induced endotoxic shock in animal models and inhibit TNF- $\alpha$  synthesis at the translational level [24-27]. The pirfenidone analogs, fluorofenidone and 5-ethyl-1-phenyl-2-(1H) pyridone (5-EPP), also offer a similar degree of protection against endotoxin, lipopolysaccharide/D-galactosamine (LPS/D-GalN) and cecal ligation and puncture (CLP) models of septic shock [28,29].

The ability of orally-effective pirfenidone to reduce the production and secretion of TNF- $\alpha$  in animal models has led to clinical investigations in diseases where this cytokine has been implicated in the underlying pathophysiology such as secondary progressive multiple sclerosis (SPMS) and idiopathic pulmonary fibrosis (IPF). For instance, three clinical trials have shown efficacy of pirfenidone treatment for SPMS [30-32], a crippling disease which may be a TNF- $\alpha$ -driven process [33-35] and IPF [36,37]. Recently, Noble *et al.* published the results of two randomized clinical trials of the CAPACITY study and suggested that pirfenidone offers an appropri-

ate treatment option for patients with IPF [38]. Based in part on these clinical results, pirfenidone has been approved for the treatment of IPF in both Japan and the European Union.

Protein-based anti-TNF- $\alpha$  drugs and pyridone compounds such as pirfenidone have one thing in common—both significantly reduce the elevated levels of biologically active soluble TNF- $\alpha$ , albeit by different mechanisms. Protein-based anti-TNF- $\alpha$  drugs bind to TNF- $\alpha$  and thereby block further biological interactions, whereas pyridone-based compounds inhibit the enhanced synthesis of TNF- $\alpha$  intrinsic to inflammatory events. This difference in mechanism of action may explain why pirfenidone appeared to arrest the progression of SPMS and stabilized the condition in clinical studies [31], while infliximab exacerbated the progression of SPMS and forced the discontinuation of the trials [39].

In order to better delineate these differences in mechanism of action, we compared the effects of the protein-based anti-TNF- $\alpha$  drug, etanercept, with that of the novel pyridone, 5-EPP, on the regulation of soluble and cell-associated TNF- $\alpha$  in LPS-stimulated THP-1 cells *in vitro*. While little is presently known about 5-EPP, its chemical structure would imply that it likely possesses pharmacological and toxicological properties similar to those of pirfenidone and fluorofenidone.

In this study, we report that both etanercept (ET) and 5-EPP limited the bioavailable TNF- $\alpha$  in the medium following LPS-stimulation. In marked contrast, however, the levels of cell associated TNF- $\alpha$  in etanercept-treated cells increased several-fold, while these levels were significantly reduced in 5-EPP-treated cells. These results may have important ramifications with respect to adverse events between the use of some protein-based anti-TNF- $\alpha$  drugs and drugs from the pyridone family in the management of TNF- $\alpha$ -driven diseases.

## 2. Materials and Methods

### 2.1. Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Etanercept (Enbrel™) was purchased from a pharmaceutical supplier in 1 cc syringes at 50 mg/ml and was used in both native carrier and dialyzed (in PBS) forms with no differences in efficacy or toxicity between the two. Heating (95°C  $\times$  10 minutes) of etanercept stock solution completely inactivated all TNF- $\alpha$ -neutralizing activity as assessed by TNF- $\alpha$  bioassay (data not shown).

Five-EPP was synthesized at the Solanan Research Laboratory according to the procedure described earlier [40]. Briefly, the starting compound 5-ethyl-2-pyridone

(J & W Pharmed LLC, Levittown, PA) was reacted with bromobenzene in the presence of a Cu-Zn catalyst under a blanket of argon to produce 5-EPP. After extraction, purification and re-crystallization, the material was found to contain >99.8% 5-EPP as determined by reverse phase HPLC and NMR analysis with a sharp melting point of 58.5°C. All drugs and reagents used in our experiments were diluted in RPMI-1640 with 2% FBS and supplemented as described below.

## 2.2. *In Vitro* Cell Treatment

THP-1 cells (ATCC, Manassas, VA), a mononuclear cell line which can be differentiated into macrophage-like cells [41], were collected from culture media by centrifugation at  $250 \times g$  and resuspended at  $1 \times 10^6$  cells/ml in RPMI-1640 supplemented with 10% FBS (Hyclone, Logan, UT), 50 mg/ml gentamicin, 25 mM HEPES, 1.25 g/L sodium bicarbonate, 100  $\mu$ M 2-mercaptoethanol and 50 ng/ml phorbol myristate acetate (PMA; to facilitate differentiation and cell adherence to the culture plates). Five hundred  $\mu$ l of cell suspension were added to each well of a 24-well Costar plate (Thomas Scientific, Swedesboro, NJ) and allowed to incubate for 18 hours at 37°C under 5% CO<sub>2</sub>. After incubation, cells were washed 2X and fresh media (not containing PMA) were added. Cells were allowed to incubate for an additional 24 hours to abate the effects of PMA on cell activation. Media were then removed and cells treated with LPS, 5-EPP, or etanercept (ET) dissolved in RPMI for the time described for each experiment. Following incubation, culture media and cell lysates (to be discussed later) were collected and analyzed for TNF- $\alpha$  by bioassay or ELISA, respectively.

## 2.3. TNF- $\alpha$ Bioassay

TNF- $\alpha$  was measured in culture media using a modification of the TNF- $\alpha$  bioassay utilizing Wehi-164 var13 cells (ATCC) as described previously [42]. The modification of the protocol involved replacing neutral red staining with the more sensitive and reproducible soluble formazan dye-based viability assay to determine TNF- $\alpha$  mediated cytotoxicity. Briefly, Wehi-164 cells were plated at  $1.5 \times 10^4$  per well in Falcon Primaria 96-well plates (Thomas Scientific) and allowed to adhere. After 6 hours, culture media from drug-treated THP-1 cells (as described previously) were centrifuged to remove cell debris and added to the Wehi-164 cells. Serial 1:2 dilutions were performed in-well for all samples. Serial dilutions of recombinant human TNF- $\alpha$  standard (3.9 - 500 pg/ml; BD Biosciences, San Jose, CA) were also included to determine the quantity of TNF- $\alpha$  in the experimental samples. Finally, 2  $\mu$ g/ml actinomycin-D were added to each well and the cells were incubated over-

night. After 20 hours, media were removed and cell viability was determined by MTS assay (Promega, Madison, WI) using manufacturer's instructions. Absorbance at 492 nm was measured with a Thermo Multiskan EX (Thermo Fisher Scientific, Inc, Waltham, MA). Levels of TNF- $\alpha$  in the samples were calculated based on the formula derived from the standard curve for the recombinant TNF- $\alpha$  standard.

## 2.4. Cell Lysate Analysis for TNF- $\alpha$ by ELISA

After media were removed from THP-1 cells as described in the previous section, the adherent cells were washed 3X in PBS and then incubated at 4°C for 15 minutes with lysate buffer (10 mM HEPES, 1mM EDTA, 60 mM KCl, 0.5% NP-40, 1 mM DTT, 1 mM PMSF) containing protease inhibitor cocktail. After 2 freeze-thaws, lysate mixtures were collected and centrifuged at  $10,000 \times g$  at 4°C and supernatants collected and stored at -80°C until analyzed for TNF- $\alpha$  by ELISA. Immediately prior to TNF- $\alpha$  quantification, supernatants from each sample group were normalized for protein content as determined by BCA kit (Promega). The TNF- $\alpha$  OptEIA ELISA kit was purchased from BD BioSciences and the protocol used in this study was per manufacturer's instructions. TMB conversion was measured at 450 nm and 540 nm on a Thermo Multiskan EX plate reader.

It was necessary to measure secreted TNF- $\alpha$  and cell-associated TNF- $\alpha$  by different assays. The secreted TNF- $\alpha$  in the present study was measured using bioassay because etanercept adsorbs TNF- $\alpha$  and does not remove it from the assay tubes. The presence of the etanercept-bound TNF- $\alpha$  in the tubes could still bind to the antibodies utilized in the ELISA and this would have resulted in aberrantly high values. This is consistent with reports by Scallon, *et al.* [43] on the binding properties of TNF- $\alpha$  antagonists. The bioassay measures biologically active available TNF- $\alpha$  only and is better suited for measuring cytokines in the presence of neutralizing agents. Although attempts were made to measure TNF- $\alpha$  in the cell lysate by bioassay, it was not possible to separate the resulting cytotoxicity caused by TNF- $\alpha$  from that of the residual lysis buffer present in the assay tubes. The dialysis of lysis buffer against isotonic saline introduced more variables that further confounded the results.

## 2.5. Apoptosis Assay

THP-1 cells were incubated in 6-well plates with RPMI supplemented with PMA at  $1 \times 10^6$  cells/ml for 12 h. Fresh media not containing PMA were then added and the cells incubated for 24 hours. Next, cells were incubated for 18 h with control media; 1 ng/ml LPS; 1 ng/ml LPS + 200  $\mu$ g/ml 5-EPP; 1 ng/ml LPS + 0.1  $\mu$ g/ml eta-

nercept; 200  $\mu\text{g/ml}$  5-EPP alone; 0.1  $\mu\text{g/ml}$  etanercept alone; or anti-FAS mAb as a positive control. Cell lysates were then collected and analyzed for caspase-3 activity using CaspACE Colorimetric Assay kit (Promega) per manufacturer's instructions. The absorbance was measured at 405 nm on a Thermo Multiskan EX plate reader.

## 2.6. Statistical Analysis

Data are expressed as the mean  $\pm$  SEM for at least three replicates. Statistical differences between LPS treatment alone and various other treatment groups were analyzed using one-way ANOVA with Tukey's multiple comparison post-test and a value of  $P < 0.05$  was considered to be the minimum level of statistical significance. For figure 4, the same statistical comparison was also performed between LPS + ET and LPS + ET + 5-EPP. The statistical software utilized in this study was Graph Pad's Prism for Apple Macintosh, version 4.0c.

## 3. Results

### 3.1. Effects of Etanercept and 5-EPP on Cellular Cytotoxicity *in Vitro*

It was important first to ensure that the working concentrations of the two drugs examined in this study were not cytotoxic. The highest concentration of 5-EPP or etanercept that did not affect cellular viability was determined by incubating THP-1 cells with or without 1 ng/ml LPS combined with 1 - 500  $\mu\text{g/ml}$  5-EPP or 0.001 - 1  $\mu\text{g/ml}$  etanercept in 96-well plates for 3, 6, 9, or 24 h. Cellular viability was determined by MTS assay (Figure 1) and trypan blue exclusion (data not shown). Since incubation periods for all subsequent experiments was within the

time frame of 6 hours, the results of the cytotoxicity studies for this period are shown in Figure 1. However, the results for the shorter and longer incubation time-points showed similar results (data not shown). Five-EPP had no effect on THP-1 cell viability at concentrations below 300  $\mu\text{g/ml}$  and etanercept had no effect on the cell viability at concentrations of 0.250  $\mu\text{g/ml}$  or lower for any length of incubation period used in this study.

The cell viability assays were confirmed by analyzing caspase-3 activity, an indicator of apoptosis. There were no significant ( $P > 0.05$ ) differences in caspase-3 activity between LPS-treated cells and drug-treated groups using the highest non-toxic concentrations of either 5-EPP or etanercept as determined in the previous cell viability experiment (data not shown).

### 3.2. Effects of 5-EPP and Etanercept on Secreted and Cell-Associated TNF- $\alpha$ *in Vitro*

The effects of various concentrations of 5-EPP and etanercept on LPS-stimulated TNF- $\alpha$  secreted in the medium and cell-associated TNF- $\alpha$  in the cell lysate are summarized in Figures 2(a) and 2(b), respectively. Five-EPP had a dose dependent inhibitory effect on both LPS-stimulated TNF- $\alpha$  in the medium and TNF- $\alpha$  associated with the cell lysate. Both were maximally inhibited at 200  $\mu\text{g/ml}$ , a concentration previously determined not to compromise cell viability. Although etanercept caused a dose-dependent reduction in bioavailable TNF- $\alpha$  secreted from LPS-treated THP-1 cells (maximal effect at 0.01  $\mu\text{g/ml}$ ) (Figure 2(a)), it additionally elevated the LPS-stimulated cell associated TNF- $\alpha$  levels as compared to LPS alone. This elevation was by 8-fold at etanercept concentration of 0.1  $\mu\text{g/ml}$  (Figure 2(b)).

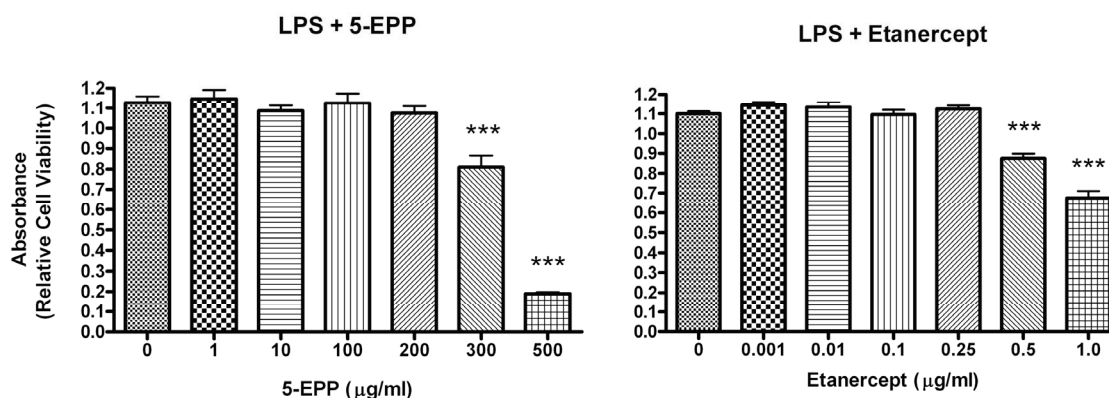
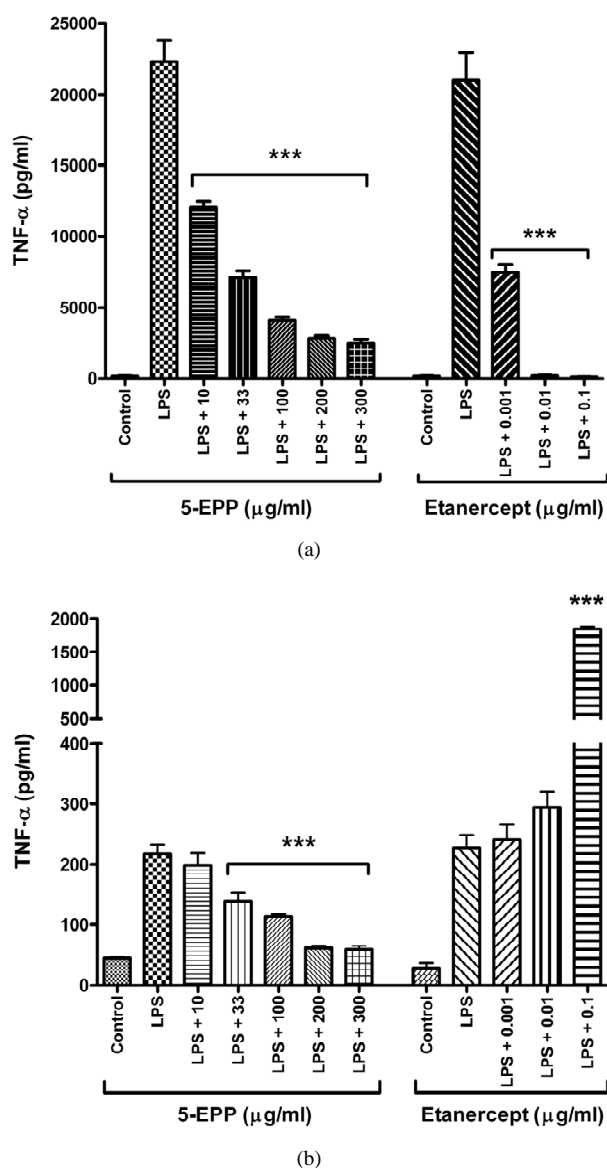


Figure 1. Effects of 5-EPP or etanercept on THP-1 cell viability. PMA-transformed THP-1 cells were incubated on 96-well plates with 1 ng/ml LPS alone, 1 ng/ml LPS plus the indicated concentration of 5-EPP, or 1 ng/ml LPS plus the indicated concentration of etanercept for 6 hours. A MTS assay was used to determine cell viability. Values represent mean  $\pm$  SEM of at least 3 replicates. All treatments groups were simultaneously compared via one-way ANOVA and Tukey multiple comparison test. \*\*\* $P < 0.001$  versus LPS alone.

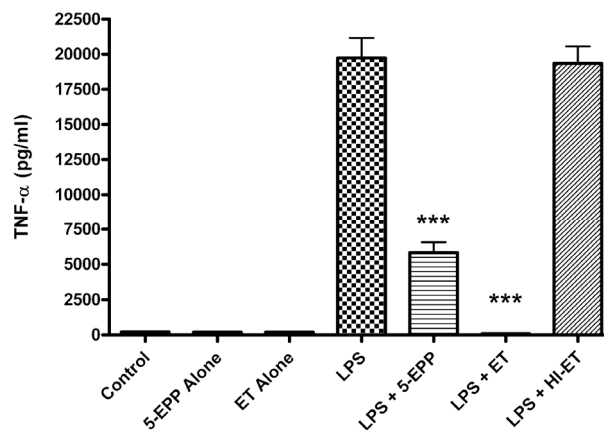


**Figure 2.** Dose response of 5-EPP or etanercept on secreted (a) or cell-associated (b) TNF- $\alpha$  Levels. PMA-transformed THP-1 cells were incubated for 3 h on 24-well plates with control media, 1 ng/ml LPS, or 1 ng/ml LPS plus the indicated concentrations of either 5-EPP or etanercept. Culture media (a) were analyzed for secreted TNF- $\alpha$  via bioassay and lysates (b) were analyzed for cell-associated TNF- $\alpha$  via ELISA. Values represent mean  $\pm$  SEM of at least 3 replicates. All treatments groups were simultaneously compared via one-way ANOVA and Tukey multiple comparison test. \*\*\* $P < 0.001$  versus LPS alone.

In order to compare the effects of 5-EPP with etanercept on the LPS-stimulated secretion of TNF- $\alpha$  in the medium and TNF- $\alpha$  associated with the cell lysate, THP-1 cells were treated in 24-well plates either with media alone, 200  $\mu$ g/ml 5-EPP alone, 0.1  $\mu$ g/ml etaner-

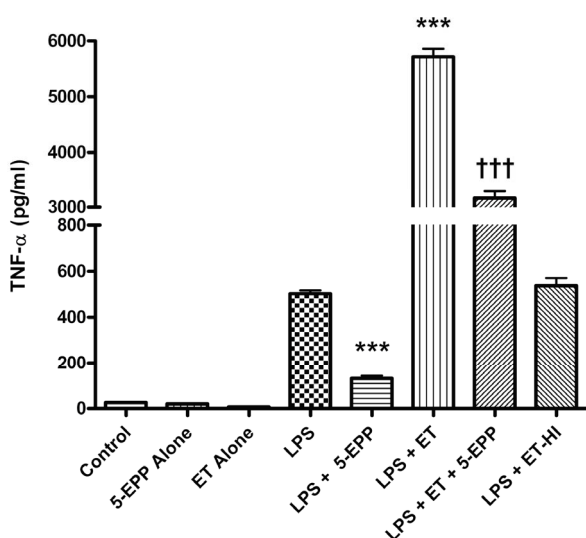
cept alone, 1 ng/ml LPS, 1 ng/ml LPS + 200  $\mu$ g/ml 5-EPP, 1 ng/ml LPS + 0.1  $\mu$ g/ml etanercept, 1 ng/ml LPS + 0.1  $\mu$ g/ml heat-inactivated (HI) etanercept. There was a 70% reduction in the LPS-stimulated secretion of TNF- $\alpha$  by 5-EPP at 200  $\mu$ g/ml and similarly a 100% reduction by etanercept at 0.1  $\mu$ g/ml as compared with the LPS- alone (**Figure 3**). Heat-inactivated etanercept had no effect on the LPS-stimulated secretion of TNF- $\alpha$  indicating the bioavailability of the most of the TNF- $\alpha$  in the medium (**Figure 3**). There was no detectable amount of TNF- $\alpha$  in the medium when cells were treated alone either with 200  $\mu$ g/ml EPP or 0.1  $\mu$ g/ml etanercept.

The effects of 5-EPP at 200  $\mu$ g/ml and etanercept at 0.1  $\mu$ g/ml on 1 ng/ml LPS-stimulated levels of cell-associated TNF- $\alpha$  are summarized in **Figure 4**. Cells exposed to 1 ng/ml LPS + 200  $\mu$ g/ml 5-EPP contained approximately 25% of the TNF- $\alpha$  compared with the cells treated with LPS alone. However, there was an approximate 12-fold increase in the amount of cell-associated TNF- $\alpha$  in the lysate from cells treated with 1 ng/ml LPS + 0.1  $\mu$ g/ml etanercept compared with the lysate from LPS-only treated cells. It is interesting that when cells were treated with 1 ng/ml LPS + 200  $\mu$ g/ml 5-EPP + 0.1  $\mu$ g/ml etanercept, there was a 50% reduction in the cell associated TNF- $\alpha$  as compared to cells treated with 1 ng/ml LPS + 0.1  $\mu$ g/ml etanercept. Heat-inactivation of etanercept abolished its ability to further elevate the LPS-stimulated cell-associated TNF- $\alpha$  and the level returned



**Figure 3.** Comparison between the effects of 5-EPP and etanercept on secreted TNF- $\alpha$ . PMA-transformed THP-1 cells were incubated for 3 hours on 24-well plates with culture media, 200  $\mu$ g/ml 5-EPP alone, 0.1  $\mu$ g/ml etanercept (ET) alone, 1 ng/ml LPS, 1 ng/ml LPS + 200  $\mu$ g/ml 5-EPP, 1 ng/ml LPS + 0.1  $\mu$ g/ml ET, or 1 ng/ml LPS + 0.1  $\mu$ g/ml heat-inactivated (HI) ET. Culture media were analyzed for secreted TNF- $\alpha$  via bioassay. Values represent mean  $\pm$  SEM of at least 3 replicates. All treatments groups were simultaneously compared via one-way ANOVA and Tukey multiple comparison test. \*\*\* $P < 0.001$  versus LPS alone.





**Figure 4.** Comparison between the effects of 5-EPP and etanercept on cell-associated TNF- $\alpha$ . PMA-transformed THP-1 cells were incubated for 3 hours on 24-well plates with one of the following treatments: culture media, 200  $\mu$ g/ml 5-EPP alone, 0.1  $\mu$ g/ml etanercept (ET) alone, 1 ng/ml LPS alone, 1 ng/ml LPS + 200  $\mu$ g/ml 5-EPP, 1 ng/ml LPS + 0.1  $\mu$ g/ml ET, 1 ng/ml LPS + 0.1  $\mu$ g/ml ET + 200  $\mu$ g/ml 5-EPP, 1 ng/ml LPS + 0.1  $\mu$ g/ml heat-inactivated (HI) ET. Media were then removed, cells were washed and treated with lysate buffer. Lysates were analyzed for cell-associated TNF- $\alpha$  via ELISA. Values represent mean  $\pm$  SEM of at least 3 replicates. All treatments groups were simultaneously compared via one-way ANOVA and Tukey multiple comparison test. \*\*\* $P < 0.001$  versus LPS alone. ††† $P < 0.001$  between LPS + ET and LPS + ET + 5-EPP.

to that found in the lysates from cells treated with LPS alone.

#### 4. Discussion

Tumor necrosis factor- $\alpha$  is a potent proinflammatory cytokine and key component of the normal immune response. However, exaggerated and prolonged TNF- $\alpha$  production has been implicated in the pathogenesis of a number of acute, chronic inflammatory and autoimmune diseases. Early reports associated elevation of TNF- $\alpha$  with septic shock, meningococcal disease, multiple sclerosis and rheumatoid arthritis [34,44-46]. Recognition of TNF- $\alpha$  as a crucial regulator of the early inflammatory cytokine cascade in rheumatoid arthritis [47] and successful proof-of-concept studies with anti-TNF- $\alpha$  antibody-based therapies in multiple species [48-51], led to the development of five new protein-based anti-TNF- $\alpha$  agents for treatment of several inflammatory diseases.

Despite their proven clinical effectiveness in a majority of patients, protein-based TNF- $\alpha$  antagonist therapy has some limitations. All agents must be injected or de-

livered intravenously every one to four weeks to be effective. The multiple immunomodulatory functions of TNF- $\alpha$  may be of equal or greater importance to its pro-inflammatory role, as its differential effects on T cells, B cells, dendritic cells and apoptosis are critical in controlling the immune response to infectious agents. For example, protein-based anti-TNF- $\alpha$  therapy leads to a greater risk for both opportunistic infections as well as the reactivation of latent tuberculosis due to insufficient recruitment, activation or survival of macrophages at the site of infection [52].

Also, while all of these agents neutralize TNF- $\alpha$  effectively, there are fundamental differences in molecular structures, binding specificities and the way they neutralize bioactivity that may explain their dissimilar clinical efficacy in treating Crohn's disease [11,53] or psoriasis [12,54]. Furthermore, use of protein-based anti-TNF- $\alpha$  agents may have unintended consequences as when infliximab-treated progressive multiple sclerosis patients exhibited [33-35,55] accelerated disease progression, forcing the discontinuation of the clinical trial [39]. Also, administration of etanercept for treatment of juvenile rheumatoid arthritis was associated with the onset of multiple sclerosis [56]. Moreover, treatment of systemic lupus erythematosus patients with a TNF- $\alpha$  antagonist preserved kidney function, but was accompanied by a rise in anti-dsDNA antibody titers [57,58]. This has led some to suggest that protein-based anti-TNF- $\alpha$  therapies may prolong survival of autoreactive T cells and therefore might increase autoimmune disease activity.

In light of the shortcomings of protein-based anti-TNF- $\alpha$  drugs, several laboratories, including our own, have studied pyridone-based small molecules with the intent of targeting excessive TNF- $\alpha$  production in inflammatory diseases. These orally-effective compounds might offer significant relief from acute and chronic inflammation without the unpredicted side effects of some protein-based anti-TNF- $\alpha$  therapies. In this regard, pirfenidone, fluorofenidone and 5-EPP have demonstrated potent anti-TNF- $\alpha$  activity in several animal models by protecting against either LPS, LPS + D-GalN, CLP or staphylococcal enterotoxin B-induced septic shock in mice via down-regulation of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12 and IFN- $\gamma$  [24,25,27-29]. While the mechanism of action for fluorofenidone and 5-EPP are not yet known, it is possible that these newer compounds may also inhibit TNF- $\alpha$  synthesis at the translational level similar to pirfenidone [26].

In the current study, both etanercept and 5-EPP limited the bio-available TNF- $\alpha$  secreted by LPS-stimulated THP-1 cells in a dose-dependent manner. However, they had strikingly contradictory effects on cell-associated

TNF- $\alpha$  levels when co-incubated with LPS. Etanercept increased cell associated TNF- $\alpha$  levels by several-fold at non-toxic concentrations, while 5-EPP inhibited TNF- $\alpha$  in a dose-dependent manner as compared with the LPS treatment alone. An intriguing *in vivo* study by Mohler, *et al.* reported elevated serum TNF- $\alpha$  levels in mice treated with monomeric sTNFR or low doses of a synthetic dimeric sTNFR:Fc (a structure similar to etanercept) following LPS treatment as compared to LPS treatment only [59]. Our current findings are consistent with those of Mohler *et al.*, lending credence to our findings that etanercept somehow further enhances LPS-stimulated levels of cell-associated TNF- $\alpha$ .

The mechanism responsible for enhanced effect of etanercept on cell-associated TNF- $\alpha$  in LPS-treated cells is not clearly understood. It is possible that etanercept-TNF- $\alpha$  complexes are endocytosed making them resistant to repeated washes and resulting in elevated intracellular TNF- $\alpha$ . Alternatively, etanercept only binds trimeric forms of TNF- $\alpha$  and does not maintain a stable complex with either mTNF or soluble TNF- $\alpha$ . Thus the etanercept/TNF- $\alpha$  complex instability may result in a relatively larger pool of unbound and bio-available TNF- $\alpha$  [43], which is then available to stimulate TNFRs and potentially contribute to adverse events. Also, Xin *et al.* showed that cells previously pre-treated with soluble TNFR (analogous to etanercept treatment) became primed for subsequent activation by TNF- $\alpha$  through reverse signaling [60]. Thus, in the present study, simultaneous treatment with both etanercept and LPS might allow etanercept-mTNF- $\alpha$  reverse signaling to further prime these cells for additional activation from LPS-stimulated soluble TNF- $\alpha$  release. Lastly, further study is warranted to demonstrate that this *in vitro* effect seen in the transformed cell line also occurs in human macrophages isolated from blood.

The combination of LPS + etanercept that produced elevated levels of cell-associated TNF- $\alpha$  did not have any detectable effects on cell viability as revealed by our cytotoxicity experiments. However, other cell types may be more sensitive to the cytotoxic effects of mTNF and might exhibit increased adverse effects if exposed to etanercept-treated cells. *In situ* cells laden with TNF- $\alpha$  on their membranes are capable of altering the physiology of neighboring cells via mTNF-mediated reverse signaling [17,18], which is known to trigger a myriad of enhanced pro-inflammatory responses. For example, reverse signaling through TNF- $\alpha$  induces the pro-inflammatory cytokines interleukin-2 and interferon gamma [18] and cell adhesion molecules [22], none of which would be reversed by protein-based TNF- $\alpha$  antagonists. Moreover, the near-complete suppression of soluble TNF- $\alpha$  release by TACE inhibition did not prevent hepatocellular

necrosis and apoptosis induced by LPS + D-GalN or ConA. In fact, TACE inhibition exacerbated ConA-induced liver injury and did not reduce ConA-elevated cell-associated TNF- $\alpha$  [61]. Thus, a protein-based TNF- $\alpha$  antagonist might also cause unexpected adverse effects via up-regulated mTNF-mediated cytotoxicity or cytokine release.

The elevation of both TNF- $\alpha$  secretion and cell-associated TNF- $\alpha$  levels by LPS were clearly suppressed by 5-EPP. In addition, 5-EPP was also able to significantly suppress the rise in cell-associated TNF- $\alpha$  induced by a combined LPS + etanercept treatment. The ability of 5-EPP to reduce both LPS-stimulated TNF- $\alpha$  release and cell-associated TNF- $\alpha$  *in vitro* is entirely consistent with earlier studies with pirfenidone [24,62]. Five-EPP also protects mice from a lethal dose of LPS + D-GalN and completely blocks the simultaneous rise in LPS + D-GalN-stimulated serum TNF- $\alpha$  [28]. Similar protective effects in rodent models have been reported for the related pyridones, pirfenidone and fluorofenidone [24,29]. In contrast to the actions of protein-based TNF- $\alpha$  antagonists or TACE inhibitors, inhibiting the synthesis of this cytokine via pyridone-based compounds would likely reduce both forward and reverse signaling and suppress the multifactorial inflammatory cascade at its origin.

In conclusion, our results confirm that pyridone compounds such as 5-EPP can effectively reduce both LPS-induced soluble and cell-associated TNF- $\alpha$  bioactivity *in vitro*. Etanercept also reduced soluble TNF- $\alpha$  bioactivity in LPS-stimulated cells, but in marked contrast, elevated cell-associated bioactivity. Thus, inhibition of TNF- $\alpha$  production by pyridone compounds such as 5-EPP may provide significant advantages over some of the currently available protein-based anti-TNF- $\alpha$  therapies and may offer a viable therapeutic strategy for the management of TNF- $\alpha$  driven acute and chronic inflammation. However, further research with these and related compounds is required to determine if our findings can be substantiated for these classes of compounds.

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