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Drug repositioning of TANK-binding kinase 1 inhibitor CYT387 as an alternative for the treatment of Gram-negative bacterial sepsis

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ABSTRACT

There is currently no specific drug for the treatment of sepsis and antibiotic administration is considered the best option, despite numerous issues. Therefore, the development of drugs to control the pathogen-induced inflammatory responses associated with sepsis is essential. To address this, our study examined the transcriptomes of lipopolysaccharide (LPS)-induced dendritic cells (DCs), identifying TANK-binding kinase1 (Tbk1) as a key factor involved in the inflammatory response. These data suggested drug repositioning of the Tbk1 inhibitor CYT387, currently used for the treatment of myelofibrosis and some cancers, as a candidate for regulating the LPS-induced inflammatory response. CYT387 also inhibited pro-inflammatory cytokine and surface molecule expression by mature DCs after LPS exposure. These effects correlated with both Akt phosphorylation and IκBα degradation. Finally, CYT387 demonstrated therapeutic effects in LPS-induced endotoxemia and *Escherichia coli* K1-induced mouse models of sepsis and decreased the expression of pro-inflammatory cytokines. In conclusion, our study suggests that drug repositioning of CYT387 may serve as a potential therapeutic for sepsis.

1. Introduction

Sepsis is a life-threatening medical condition that can cause sudden death due to a wide range of clinical manifestations, particularly organ dysfunction due to a dysregulated host response [1]. This is often clinically diagnosed when patients with an infection meet a list of criteria consistent with systemic inflammatory response syndrome (SIRS). This led to the development in 2016 of the sequential (sepsis-related) organ failure assessment (SOFA) score that is currently recommended by the Sepsis-3 International Consensus Group. This score is based on an assessment of the patient's respiratory, cardiovascular, hepatic, coagulation, renal, and neurological performance and has substituted the conceptual and practical SIRS framework [1]. Various strategies have been considered for the treatment of sepsis, including several that target the disseminated intravascular coagulation that is often observed. Drotrecogin alfa (Xigris) is a typical example of an anti-thrombotic sepsis drug that is US Food and Drug Administration (FDA) approved. Xigris was designed and developed based on a prior study indicating that activated protein C provided protection from sepsis-induced death in an animal model, without increasing the risk of bleeding [2]. However, Xigris administration has subsequently been shown to have no significant survival benefits for patients with severe sepsis or septic shock, leading to its withdrawal from commercial markets [3]. It is also worthwhile mentioning that Eritoran [4,5] and Resatorvid [6–8], a pro-inflammatory cytokine inhibitor and a Toll-like receptor 4 (TLR4) antagonist respectively, were also proposed as candidates for sepsis drug development [9]. However, these too have not elicited the desired efficacies during the clinical tests.

TANK-binding kinase 1 (Tbk1) is a serine/threonine kinase that plays an essential role in regulating inflammatory responses to foreign components [10]. Activation of the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling pathway, shared exclusively by the TLR3 and TLR4 receptors, [11–14], recruits tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) associated NF-κB activator (TANK). This in-turn triggers the activation of non-canonical Tbk1 and IκB kinase ε (IKKε) that phosphorylates interferon regulatory factor 3 (IRF3) and IRF7. This phosphorylation allows their subsequent homodimerization and nuclear translocation, leading to

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transcriptional activation of pro-inflammatory and antiviral genes, including type I and type III interferons (IFNs). Furthermore, it has also been shown that IKKε/Tbk1 directly activates Akt by phosphorylation of hydrophobic motifs dependent on the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway [15,16]. This promotes KRAS-driven tumorigenesis by regulating autocrine C−C motif chemokine ligand 5 (CCL5) and interleukin (IL)-6. Due to this, Tbk1 inhibitor compounds, such as CYT387 (N-(cyanomethyl)-4-[2-[[4-(4-morpholinyl) phenyl] amino] 4-pyrimidinyl]-benzamide, Momelotinib, INN, or GS-0387), have been widely studied.

CYT387 has been routinely used for the treatment of myelofibrosis since 2011 and possesses an acceptable safety profile. Functionally, CYT387 may act as an ATP competitor, inhibiting Janus kinase 1 (JAK1) and JAK2, thereby interfering with the JAK-STAT signaling pathway and reducing the expression of pro-tumorigenic cytokines. It may also inhibit noncanonical IKK-ε and Tbk1 [17–19]. However, a recent report in 2016 warned of potential CYT387 treatment failures during phase 3 clinical trials aiming to alleviate myelofibrosis symptoms [20,21]. Despite this, it is still a promising molecule that has been shown to disrupt both metastatic KRAS-mutated non-small cell lung cancers and metastatic pancreatic ductal adenocarcinoma [22]. Additionally, there has been no examination of Tbk1 inhibition as a therapy for sepsis despite its effects on the immune response and various important pathways.

To identify potential targets for the treatment of sepsis, we performed a transcriptomic study of lipopolysaccharide (LPS)-activated DCs. This analysis revealed that Tbk1 is a critical upstream kinase of LPS-dependent activation of DCs. We therefore focused on whether the Tbk1 inhibitor CYT387 had a potential role in attenuating sepsis in LPS-induced endotoxemia and *Escherichia coli* K1-induced sepsis mouse models. These data suggested that CYT387 can indeed be repositioned for sepsis treatment and clinical application.

2. Materials and methods

2.1. Animals

Six week-old female C57BL/6 mice (H-2Kb and I-Ab) and 6-week-old Female BALB/c mice (20 mg) were purchased from Orient Bio (Daejeon, South Korea). The procedures used in the study were approved and monitored by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University (IACUC number: KU17044-2).

2.2. Bacteria

The *E. coli* K1 strain RS218 (O18:K1:H7) used in the *E. coli* K1-induced sepsis mouse model was kindly gifted by Dr. Jang-Won Yoon of Kangwon National University (Gangwon, South Korea).

2.3. Reagents and antibodies

Recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF) was purchased from BioLegend (San Diego, CA, USA). To maintain DCs, RPMI 1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin solution were used, purchased from Biowest (Nouaille, France). *E. coli* O111:B4 LPS used in the *in vitro* experiments was purchased from Invivogen (San Diego, CA, USA) and *E. coli* O127:B8 LPS used for the *in vivo* experiments was purchased from Sigma-Aldrich (St. Louis, MO, USA). CYT387 was purchased from Selleckchem (Houston, TX, USA). An MTT Cell viability kit was purchased from Promega (Madison, WI, USA). For western blotting, anti-p-p38 antibody, anti-p38 antibody, anti-p-ERK antibody, anti-ERK antibody, anti-p-JNK antibody, anti-JNK antibody, anti-p-Akt antibody, anti-Akt antibody, anti-IκBα antibody, anti-p-IRF3, and anti-p-Tbk1 antibody were purchased from Cell Signaling Technology (CST; Danvers, MA, USA). Anti-α-tubulin and anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Protease inhibitor cocktail Luria-Bertani (LB) broth powder and LB agar powder were both purchased from Biobasic (Amherst, NY, USA) and an LAL Endotoxin Detection Kit was purchased from Lonza (Basel, Switzerland).

2.4. Generation of murine bone marrow DCs (BMDCs)

Bone marrow from the tibias and femurs was used to isolate DCs after euthanasia of C57BL/6 mice following IACUC guidelines. Red blood cells were removed from the bone marrow using red blood cell lysis buffer, leaving only progenitor cells. These progenitor cells were placed into cell culture plates with RPMI 1640 (containing 10% FBS, 1% penicillin/streptomycin, and 10 ng·mL^{-1} of GM-CSF) and incubated for 6 d at 37 °C under a 5% CO_2 atmosphere. After six days, precursor cells had differentiated into immature DCs. To produce mature DCs, immature DC were treated with 50 ng·mL^{-1} LPS and incubated overnight at 37 °C under 5% CO_2.

2.5. Microarray assay

RNA purity and integrity were evaluated using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA labeling and hybridization were performed using an Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). Briefly, 100 ng of total RNA was linearly amplified from each sample and labeled with Cy3-dCTP. The labeled cRNAs were purified using an RNAeasy Mini Kit (Qiagen, Hilden, Germany). The concentration and specific activities of the labeled cRNAs (pmol Cy3·μg cRNA^{-1}) were measured using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). Next, 600 ng of each labeled cRNA sample was fragmented by adding 5 μL 10× blocking agent and 1 μL of 25× fragmentation buffer before being heated at 60 °C for 30 min. Finally, 25 μL 2× GE hybridization buffer was added to dilute the labeled cRNA. A total of 40 μL of hybridization solution was dispensed into the gasket slide and inserted into the Agilent SurePrint G3 Mouse GE 8X60K Microarrays (Agilent Technologies, Santa Clara, CA, USA). These slides were then incubated for 17 h at 65 °C in an Agilent Hybridization Oven (Agilent Technologies, Santa Clara, CA, USA) and then washed at room temperature, following the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). The hybridized array was immediately scanned with an Agilent Microarray Scanner D (Agilent Technologies, Santa Clara, CA, USA). Raw data were extracted using Agilent Feature Extraction Software v11.0.1.1 (Agilent Technologies, Santa Clara, CA, USA) and then summarized automatically using the Agilent feature extraction protocol to generate a raw data text file. This provided the expression data for each gene included on the array for further analysis. Array probes flagged with Flag A were removed. Each selected gProcessedSignal value was transformed logarithmically and normalized using the quantile method. Statistical significance within the expression data was determined by fold change and an LPE test in which the null hypothesis was that there was no difference between the two groups. Hierarchical cluster analysis was performed using complete linkage and Euclidean distances as a measure of similarity. Gene-Enrichment and Functional Annotation analysis for significant probe list were performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp). All data analysis and the visualization of differentially expressed genes were conducted using R 3.0.2 (http://www.r-project.org).

2.6. Data access

Gene expression microarray of LPS-induced DC maturation; GSE108289.

2.7. Real time-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent, digested with DNase I, and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Amplification of the cDNA was performed using a LightCycler 480 II (Roche, Basel, Switzerland) and LightCycler 480 SYBR Green I Master mix (Roche, Basel, Switzerland), according to the manufacturer's recommended conditions.

2.8. Cell viability assay

Immature DCs were treated with various concentrations of CYT387 and incubated overnight. H_2O_2 was used as a positive control. After incubation, the cell viability assay kit solution was added to the cells. These were then incubated at room temperature for 12 min and luminescence was measured using a Veritas Luminometer (Turner Biosystems, Inc., Sunnyvale, CA, USA).

2.9. ELISA

The levels of various pro-inflammatory cytokines, including tumor necrosis factor-α (TNF), interferon β (IFN-β), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin (IL-12p70) were measured by sandwich ELISA (eBioscience, San Diego, CA, USA). Optical density at 450 nm was measured using a Sunrise Spectrophotometer (TECAN, Männedorf, Switzerland).

2.10. Flow cytometry

Immature DCs were treated with CYT387 at various concentrations (0, 0.1, 0.25, or 0.5 μM). After 30 min, the cells were treated with LPS and incubated overnight. Afterwards, the cells were harvested and washed with phosphate-buffered saline (PBS). Cells were initially stained with FITC anti-CD11c antibody and then stained with PE anti-CD86 antibody, PE anti-H-2Kd/H-2Dd antibody, and PE anti-I-A/I-E antibody for 30 min. The stained cells were washed with PBS and analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.11. Western blotting

CYT387/LPS-treated DCs and LPS-treated DCs were harvested at the same time and washed with PBS. The cells were then lysed using RIFA buffer (pH 8.0, containing 0.5% NP-40, 0.01% protease inhibitor cocktail, 1 mM EDTA, 0.5 M NaF, 120 mM NaCl, 50 mM Tris–HCl, and 0.5 mM PMSF) for 30 min at 4 °C. The lysates were next centrifuged at 13,000 rpm for 15 min and aliquots of protein lysate were quantified and separated by SDS-PAGE using a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane and the membrane blocked with 5% skimmed milk for 1 h before washing three times, 10 min each, with Tris-buffered saline with 0.1% Tween-20 (TBS-T). Antibodies specific for p-p38, p-38, p-ERK, ERK, p-JNK, JNK, p-Akt, Akt, IκBα, or α-tubulin were incubated with the membrane overnight at 4 °C. After this step, the membrane was washed with TBS-T and incubated with either a HRP-tagged anti-rabbit or anti-mouse secondary antibody at room temperature for 1 h. ECL solution was added to the membranes and then washed with TBS-T. Bands were detected using a LAS4000 (Fuji Film, Tokyo, Japan).

2.12. LPS-induced endotoxemia mouse model

For the endotoxemia model, mice were injected with CYT387, LPS, or both. To evaluate the serum levels of inflammatory cytokines, blood was collected 2 h after injection. Serum was then isolated, diluted with PBS, and inflammatory cytokines were determined using an ELISA. At the indicated time points, mice were sacrificed, and blood and lung tissue were harvested. Lungs were divided in two and half stained with hematoxylin and eosin (H&E) while the other half was homogenized with stainless beads and centrifuged at 3000 rpm for 5 min to obtain a supernatant used to measure the pro-inflammatory cytokines levels. Serum was also isolated from the blood and diluted with PBS. Liver and renal damage indices were measured using medical laboratory equipment, including a Total Laboratory Automation system (Hitachi, Tokyo, Japan) and a TBA-200FR NEO (Toshiba, Tokyo, Japan).

2.13. E. coli K1-induced sepsis mouse model

Experiments similar to those performed in the LPS-induced endotoxemia mouse model were also performed in an *E. coli* K1-induced sepsis mouse model. Blood, lung, liver, kidney, and spleen samples were removed from mice sacrificed after injection of *E. coli* K1 that were left overnight. Serum endotoxin levels were measured using the same LAL assay kit as before (Lonza, Basel, Switzerland). To assess bacterial loads, the major organs, including the lung, liver, kidney, and spleen, were homogenized with stainless beads and the homogenates diluted in PBS. The homogenates were then incubated overnight on LB agar plates at 37 °C. The numbers of colonies were then counted to measure the relative bacterial populations in the major organs.

2.14. Statistical analysis

All experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as means ± SEM. A Student's *t*-test was performed to compare experimental groups and controls and a Tukey's multiple comparison test using Prism v3.0 (GraphPad Software, La Jolla, CA, USA) was used to compare multiple groups. Kaplan-Meier curves for survival rates were analyzed using a log rank test. The threshold of statistical significance was set at $P < 0.05$.

3. Results

3.1. Tbk1 downstream signaling is a major pathway affected by the LPS treatment of DCs

RNA was isolated from immature DCs (Day 6) and LPS-induced mature DCs (Day 7) for microarray examination to understand the transcriptomic changes induced by LPS (Fig. 1 and Supplementary Fig. 1). LPS treatment induced a relatively large number of changes in gene expression in DCs, so we used cutoffs of a 4-fold expression change and a 0.05 significance threshold to identify the more critical mediators (Supplementary Fig. 1A). We next examined the effects on canonical pathways using Ingenuity Pathway Analysis (IPA), revealing that the “dendritic cell maturation” pathway was significantly altered, as expected (Supplementary Fig. 1B). To further examine the effects of LPS exposure on DCs, we next performed Gene-Enrichment and Functional Annotation analysis on the most significant differentially expressed genes. This revealed multiple Gene Ontology (GO) categories that associated with LPS exposure, with the top eight clusters involved in glycoproteins, hydrolases, the immune response, the cell cycle, lipoproteins, and cytokines (Fig. 1A). Finally, network analysis using IPA suggested several cytokines and transcription regulators known to be involved in the LPS response were present (Fig. 1B), indicating that we had generated a true representative LPS-dependent transcriptome in the DCs. To identify key signaling molecules that may be involved, we also performed upstream regulator analysis using IPA. The serine/threonine kinase Tbk1 was identified as the most relevant kinase among the signaling enzymes (Fig. 1C). Examination of our data revealed that the expression of genes downstream of Tbk1 were upregulated in DCs after LPS treatment (Fig. 1D). To validate these predictions, the expression changes of two downstream genes (*Il6* and *Irf7*) were examined by real

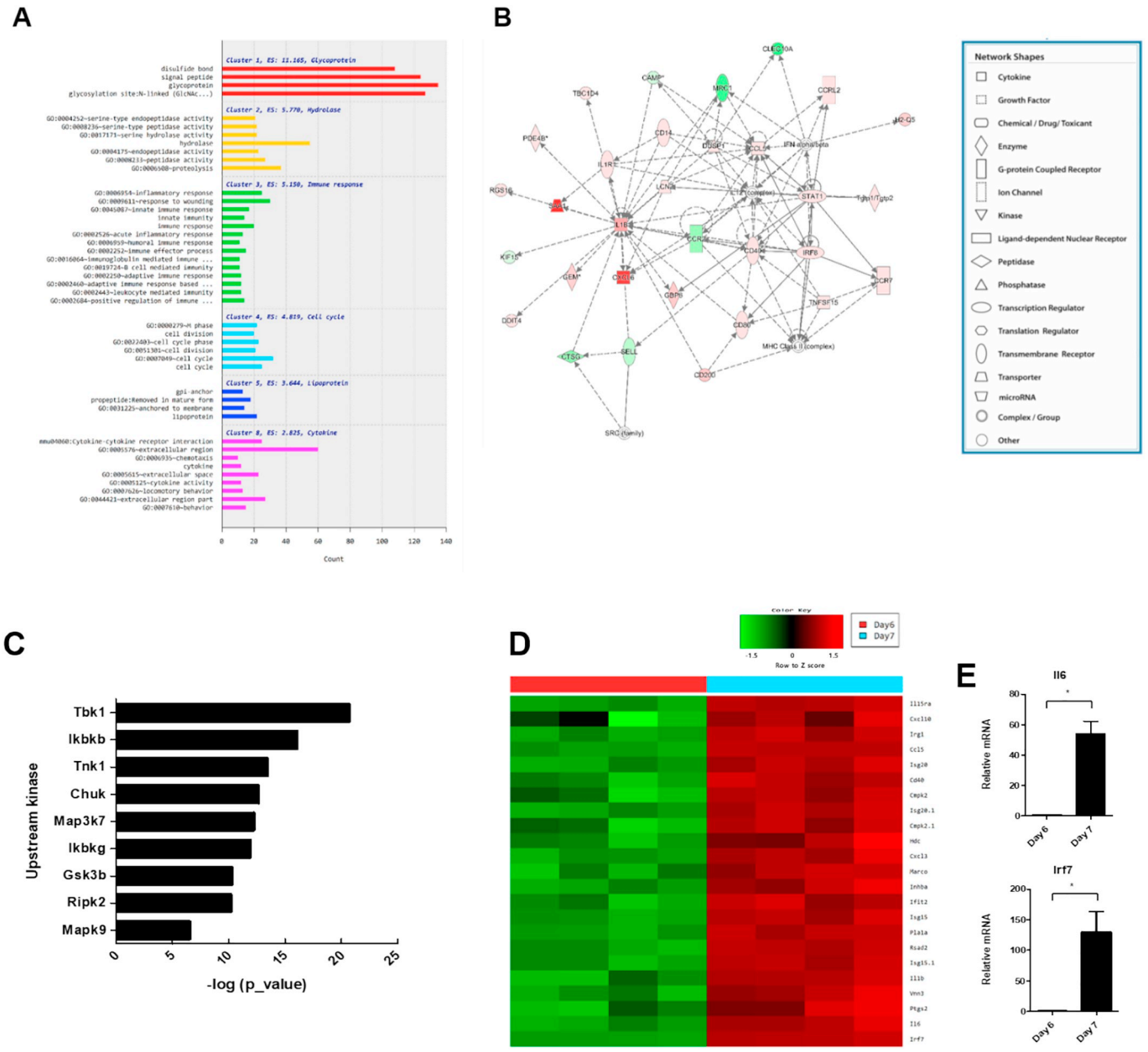

Fig. 1. Tbk1 is a key mediator of LPS-induced transcriptomic changes in DCs.
(A) The graphs show the Gene Ontology (GO) clusters that significantly associate with LPS treatment of mouse DCs. The bar heights correspond with the gene counts for each GO term. (B) Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response Network were identified as the Top networks that associate with LPS treatment of DCs using Ingenuity Pathway Analysis (IPA). Pink or red colored nodes in each network indicate a gene that is up regulated between LPS-treated (mature DCs, Day 7) and untreated groups (immature DCs, Day 6), whereas green indicates genes that are down regulated by LPS treatment. (C) Predicted upstream regulators after LPS treatment of DCs. The listed kinases were all predicted to be more active by IPA. (E) A heatmap showing the gene expression of Tbk1 downstream targets. (E) Real time RT-PCR analysis of *Il6* and *Irf7* expression performed after LPS treatment (mature DCs, Day 7) compared to control DCs (immature DCs, Day 6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time PCR, confirming our transcriptome data (Fig. 1E). In summary, these data demonstrated that Tbk1 likely plays a critical role in the transcriptional changes induced by LPS in DCs.

3.2. CYT387 impedes the LPS-induced maturation of bone marrow derived DCs

As our transcriptome analysis suggested that Tbk1 may be a key molecule involved in LPS-induced DC maturation (Fig. 1), we decided to test whether Tbk inhibitors could affect DC maturation. Among the Tbk inhibitors, CYT387 (Fig. 2A) was selected due to relatively high potency and selectivity [22]. We first verified the cytotoxicity of CYT387 on DCs using an MTT assay and H_2O_2, a strong inducer of apoptosis inducer, as a positive control. We tested a range of CYT387 concentrations up to 5 μM, with no toxicity observed in DCs up to 1 μM (Fig. 2B). We therefore set the maximum concentration of CYT387 to be used in our experiments at 1 μM.

We next examined the release of several important cytokines by ELISA. These DC-released cytokines play a pivotal role in both the adaptive and the innate immune responses. In particular, IL-12 released by DCs drives the differentiation of naïve T cells towards an IFN-γ-producing Th1 phenotype [23,24]. Conversely, pro-inflammatory cytokines, such as TNF and IL-6, induce an innate immune response [25,26]. In our study, we found that CYT387 did not alter the levels of

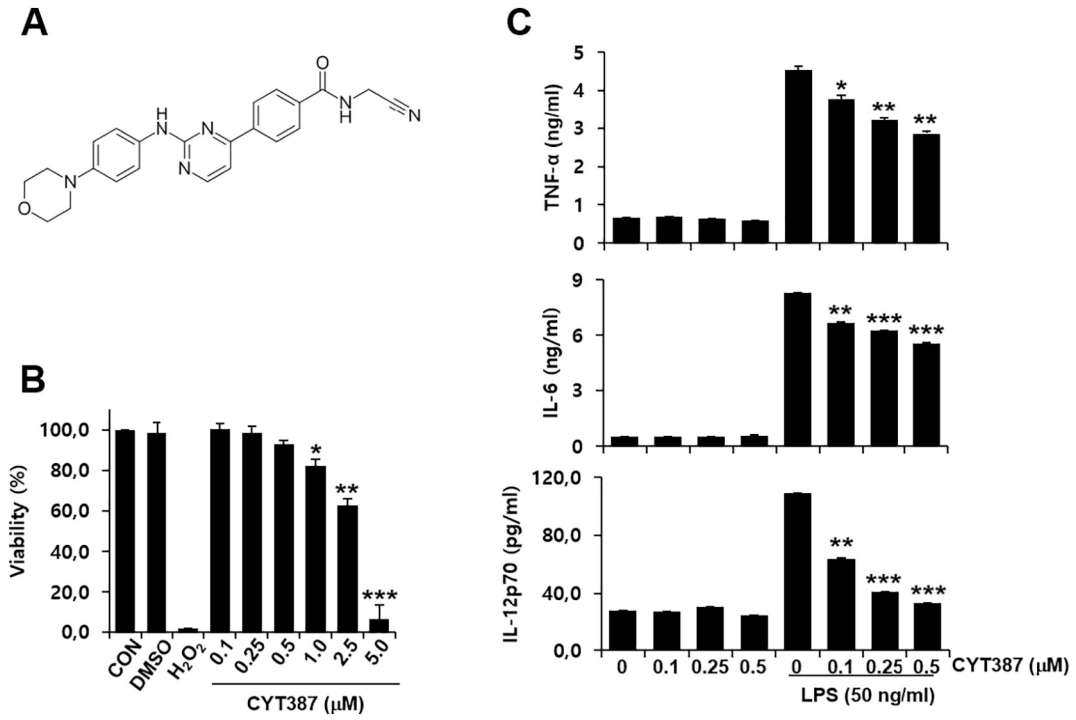

Fig. 2. The effects of CYT387 on LPS-induced DC maturation.
(A) The chemical structure of CYT387. (B) DCs were treated with CYT387, DMSO, and H_2O_2 overnight and the cytotoxicity of CYT387 on DCs was analyzed using a luminescent cell viability kit. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared to control (CON) DCs. (C) DCs were pretreated with the indicated concentrations of CYT387 for 30 min before LPS treatment (50 ng·mL^{-1}) and incubated overnight. The supernatants were collected and the levels of TNF, IL-6, and IL-12p70 were measured by ELISA. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared to DCs treated with LPS only. n.s., no significance.

TNF, IL-6, nor IL-12p70 in the untreated DC population, but did dose-dependently repress the production of these cytokines in LPS-activated DCs (Fig. 2C).

Together, these results confirmed that CYT387 could inhibit LPS-induced DC maturation, leading to a reduction in the inflammatory response of matured DCs.

3.3. LPS-induced Akt and NF-κB activities in bone marrow derived DCs are reduced by CYT387

We next sought to further investigate the mechanism of Tbk1 inhibition by CYT387. Tbk1 and IKK-i have both been shown to activate IRF3, the primary transcription factor that regulates type I IFNs. Tbk1 is particularly important to IRF3 activation and IFNβ production in response to LPS due to TLR4 ligand stimulation. Our data revealed that CYT387 treatment with LPS repressed the production of IFNβ, confirming our previous findings that CYT387 is a potential inhibitor of Tbk1 (Fig. 3A). LPS has also been shown to potently induce DC maturation and the production of pro-inflammatory cytokines by TLR4-Myd88 activation that depends on the NF-κB and MAPK signaling pathways [27-30]. To examine whether the effects of CYT387 on LPS-induced DCs are associated with the NF-κB and MAPK signaling pathways, matured DCs were treated with LPS in the presence or absence of CYT387. The phosphorylation of several MAPKs was then examined by western blot analysis, including ERK, JNK, and p38. Unexpectedly, this showed that CYT387 did not inhibit phosphorylation of ERK, p38, nor JNK in LPS-activated DCs (Fig. 3B). To the contrary, we found that CYT387 significantly inhibited the phosphorylation of Akt and modestly affected the degradation of IκBα in LPS-activated DCs in a time-dependent manner (Fig. 3B). These results indicated that CYT387 inhibits pro-inflammatory gene expression by interfering with the Akt and NF-κB signaling cascades in LPS-activated DCs, independent of the MAPK signaling pathway.

3.4. CYT387 has a therapeutic effect in an LPS-induced lethal endotoxemia mouse model

Tbk1 inhibition has been shown to effectively suppress polyinosinic-polycytidylic acid-induced immune activation both *in vitro* and *in vivo*, implicating the TLR4 signaling pathway [31,32]. Additional experimental evidence has shown that inhibiting Tbk1 with compound II may serve as an effective treatment for TREX1-associated autoimmune diseases and other interferonopathies [32]. These studies provide further support for Tbk1 inhibition as a therapeutic approach for inflammatory response-related diseases. Given our *in vitro* data demonstrating that CYT387 suppresses inflammatory responses by reducing the pro-inflammatory cytokine levels in dendritic cells, we considered the possibility that CYT387 may counteract inflammatory responses during sepsis and limit pathology *in vivo*. To address this, we first examined the effects of CYT387 on overall mortality in an LPS-induced endotoxemia mouse model. This confirmed that CYT387 treatment improved survival rates (Fig. 4A). We next conducted an ELISA to demonstrate the effects that CYT387 had on serum cytokine levels in the LPS-induced endotoxemia mouse model. Consistent with our *in vitro* observations, CYT387 treatment suppressed the increases in TNF, IL-6, and IL-12p70 levels found in the serum of LPS-induced endotoxemia mice (Fig. 4B). Treatment also resulted in a reduction in LPS-triggered lung internal pro-inflammatory IL-1β, IL-6, and TNF levels (Fig. 4D) and LPS-triggered spleen TNF, IL-1β and IL-6 levels (Fig. 4E).

To more broadly assess the effects that CYT387 had on inflammatory cell migration into the lung, we performed H&E staining on prepared specimens. These data showed that polymorphonuclear (PMN) cell infiltration into the lungs of LPS-injected was reduced by CYT387 treatment (Fig. 4C). Next, we demonstrated the effects of CYT387 treatment on liver and renal damage caused by LPS by measuring the levels of released AST, ALT, and BUN in the bloodstream. The levels of all of these markers were mitigated by CYT387 treatment, with

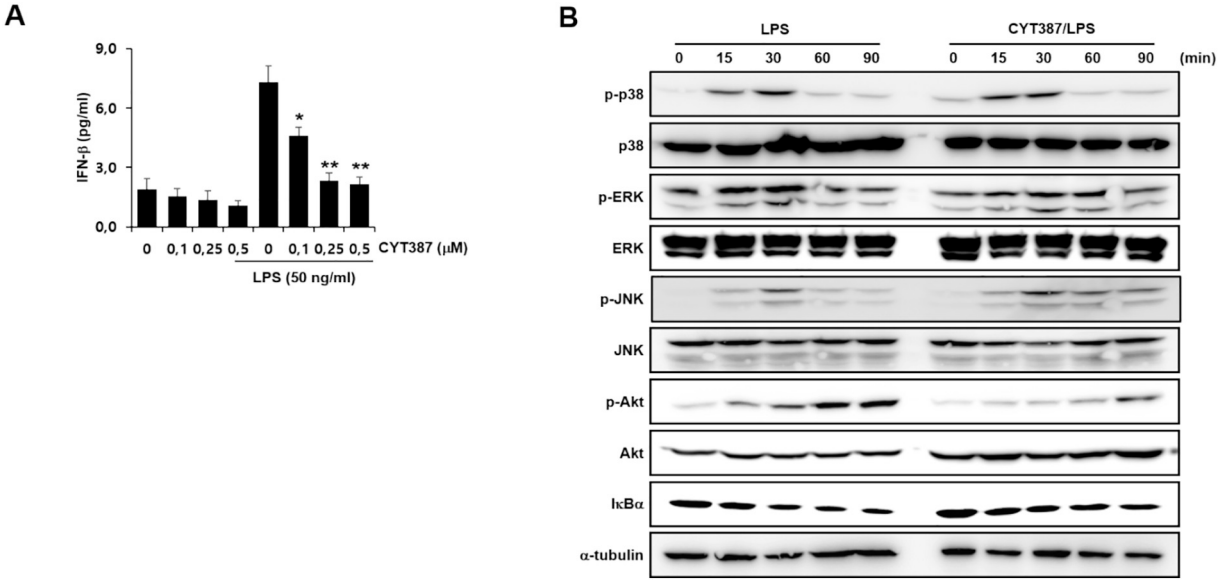

Fig. 3. The effects of CYT387 on the TLR4-mediated signaling pathway in LPS-activated DCs.
(A) DCs were pretreated with the indicated concentrations of CYT387 for 30 min before LPS treatment (50 ng·mL^{-1}) and incubated overnight. The supernatants were collected and the levels of IFN-β were measured by ELISA. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared to DCs treated with LPS only. n.s., no significance. (B) DCs were pretreated with CYT387 (0.5 μM) for 30 min before LPS stimulation (50 ng·mL^{-1}) for the indicated time points. Protein expression of p-p38, p38, p-ERK, ERK, p-JNK, JNK, p-Akt, Akt, IκBα, and α-tubulin in the cell lysates was detected by western blotting.

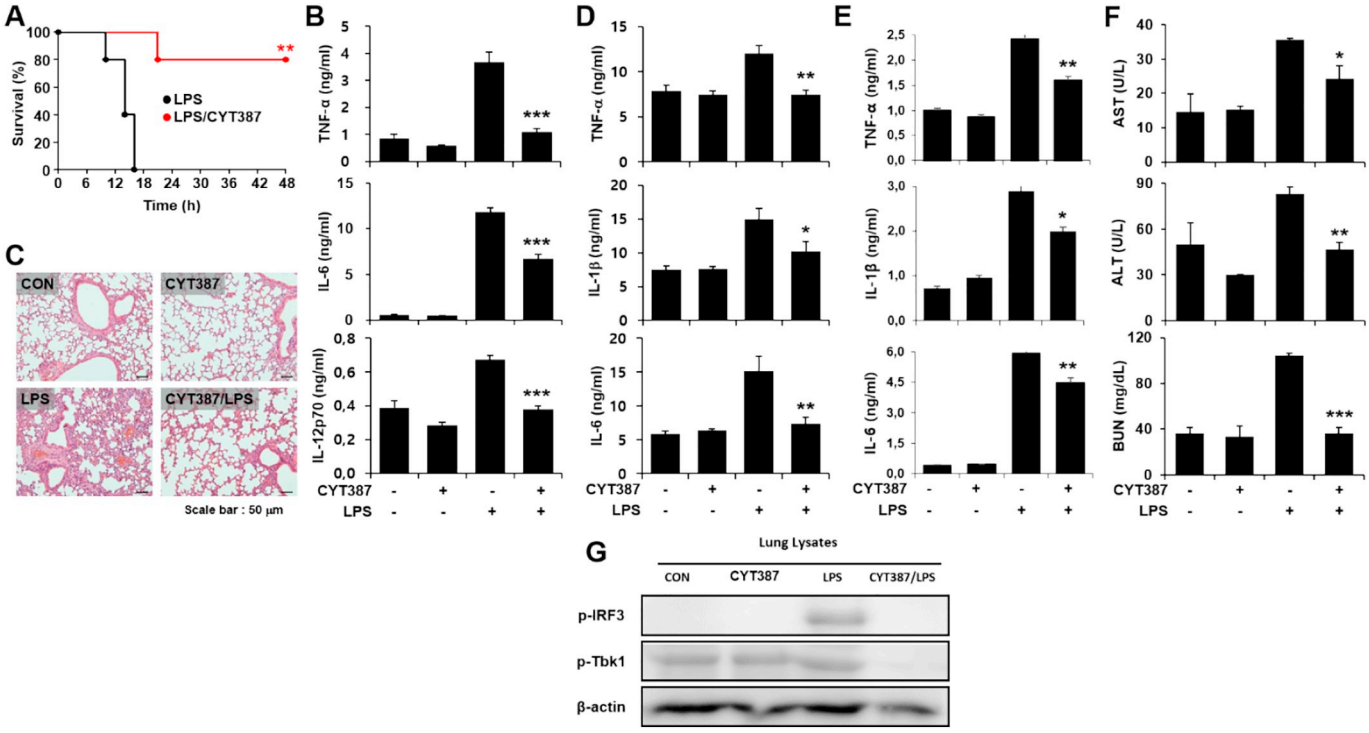

Fig. 4. CYT387 has a therapeutic effect in an LPS-induced endotoxemia model.
(A) Mice (n = 5 per group) were intraperitoneally injected with CYT387 (50 mg·kg^{-1}) 1 h before an intraperitoneal injection with LPS (20 mg·kg^{-1}) and the survival rate was observed over 72 h. (B) Mice (n = 5 per group) were intraperitoneally injected with CYT387 (50 mg·kg^{-1}) 1 h before an intraperitoneal injection of LPS (10 mg·kg^{-1}) and left for 2 h. The serum cytokine levels of TNF, IL-6, and IL-12p70 were measured by ELISA. (C) Mice (n = 5 per group) were intraperitoneally injected with CYT387 (50 mg·kg^{-1}) 1 h before an intraperitoneal injection of LPS (10 mg·kg^{-1}) and then left overnight. The mice were then euthanized and the lungs perfused and fixed with formalin. These were then stained with hematoxylin and eosin (H&E). Representative images of three independent experiments are shown. (D) The cytokine levels of TNF, IL-1β, and IL-6 in the lung lysates from the same mice were measured by ELISA. (E) The cytokine levels of TNF, IL-1β, and IL-6 in the spleen lysates from the same mice were measured by ELISA. (F) The serum levels of AST, ALT, and BUN were measured by ELISA to establish liver and renal damage. (G) Finally, Protein expression of p-Tbk1, p-IRF-3, and β-actin in the lung lysates from the same mice was detected by western blotting. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared to the LPS-injected group.

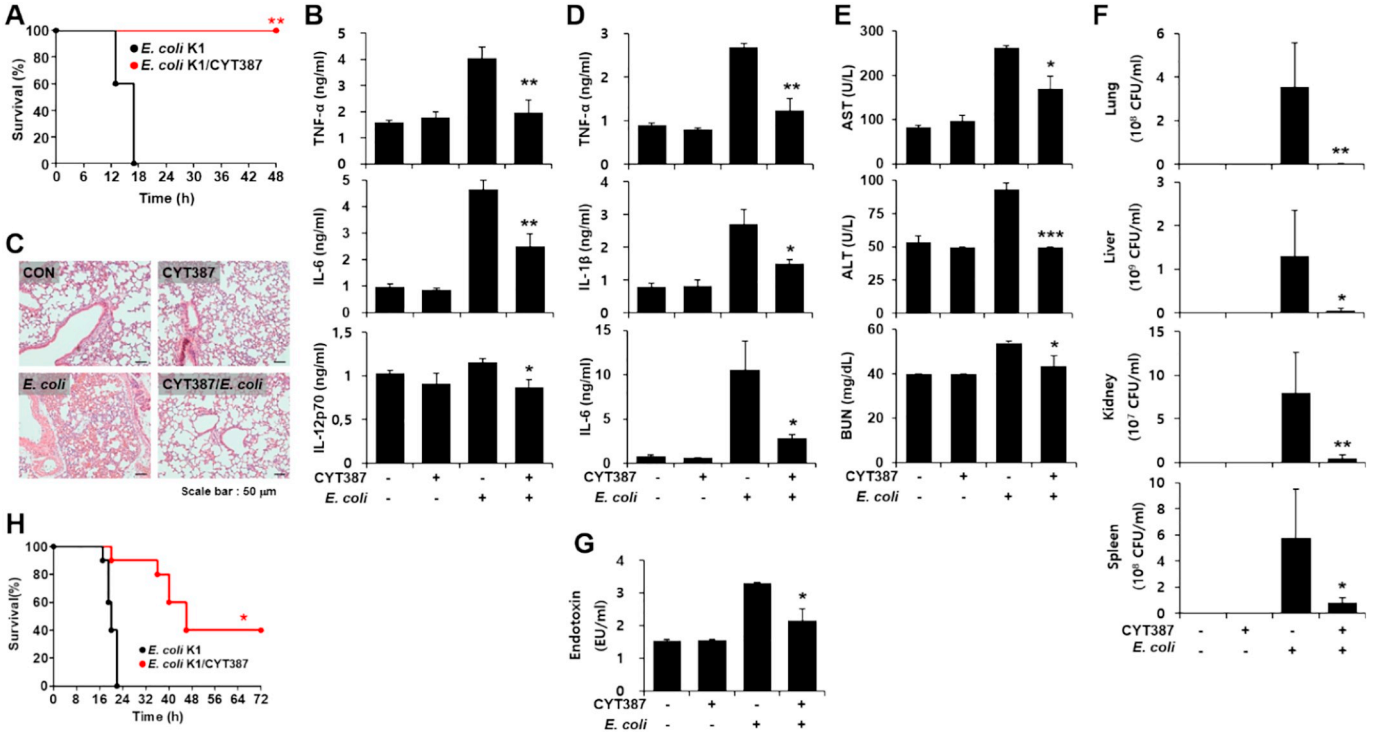

Fig. 5. CYT387 has a therapeutic effect in an *E. coli* K1-induced sepsis model.
(A) Mice (n = 5 per group) were intraperitoneally injected with CYT387 (50 mg·kg^{-1}) 1 h before an intraperitoneal injection with *E. coli* K1 (1 × 10^{7} CFU·mice^{-1}). Survival rates were observed over 72 h. (B) Mice (n = 5 per group) were intraperitoneally injected with CYT387 (50 mg·kg^{-1}) 1 h before an intraperitoneal injection of *E. coli* K1 (1 × 10^{6} CFU·mice^{-1}) and left for 2 h. Serum cytokine levels of TNF, IL-6, and IL-12p70 were measured by ELISA. (C) Mice (n = 5 per group) were intraperitoneally injected with CYT387 (50 mg·kg^{-1}) 1 h before an intraperitoneal injection of *E. coli* K1 (1 × 10^{6} CFU·mice^{-1}) and incubated overnight. The mice were euthanized and the lungs perfused and fixed with formalin. Lung sections were then stained with hematoxylin and eosin (H&E). Shown are representative images from three independent experiments. (D) Cytokine levels of TNF-α, IL-1β, and IL-6 in the lung lysates from the same mice were measured by ELISA. (E) The serum levels of AST, ALT, and BUN were measured by ELISA to establish liver and renal damage in the *E. coli* K1-induced sepsis model. (F) The colony counts of bacteria isolated from the lung, liver, kidney, and spleen were measured using LB agar plates between different the treatments. (G) Endotoxin levels in the serum were measured using an LAL assay kit. (H) Mice (n = 10 per group) were intraperitoneally injected with *E. coli* K1 (1 × 10^{7} CFU mice^{-1}).
1 h before an intraperitoneal injection with CYT387 (50 mg kg^{-1}). Survival rates were observed over 72 h. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared to the *E. coli* K1-injected group.

levels similar to those detected in the control mice (Fig. 4F). To confirm that the effects of CYT387 are in a Tbk1-dependent manner, we detected the phosphorylation of IRF3 and Tbk1 in the lung lysates. As expected, we found that CYT387 significantly inhibited the expression of p-IRF3 and p-Tbk1 (Fig. 4G). Together, these findings support the hypothesis that Tbk1 inhibition by CYT387 directly attenuates an inflammatory state that promotes severe sepsis.

3.5. CYT387 has a net therapeutic effect in an E. coli K1-induced sepsis mouse model

As sepsis is often caused by bacterial infection, especially Gram-negative bacteria; next, we studied the clinical efficacy of CYT387 using a Gram-negative *E. coli* K1 sepsis mouse model rather than an LPS model. Initially, we examined the effects that CYT387 treatment had on survival rates, demonstrating that CYT387 had a positive effect, both in a pre-treatment (Fig. 5A) and post mice challenge (Fig. 5H). As previously stated, we performed an ELISA to analyze the serum levels of various pro-inflammatory cytokines in the *E. coli* K1-induced sepsis model. This again showed that CYT387 attenuated the expression of TNF, IL-6, and IL12-p70 in the sepsis model (Fig. 5B). CYT387 also decreased the expression of pro-inflammatory cytokines in lung lysates from mice with sepsis induced by *E. coli* K1 (Fig. 5D). H&E staining of lung samples confirmed that CYT387 mitigated PMN infiltration into the lungs of mice with *E. coli* K1-induced sepsis (Fig. 5C) and an ELISA suggested that CYT387 treatment reduced lung damage by decreasing the levels of TNF, IL-1β, and IL-6 in the lung (Fig. 5D). Finally, we examined the levels of AST, ALT, and BUN damage markers in *E. coli* K1-induced mice, finding that CYT387 treatment reduced each of them (Fig. 5E).

During sepsis, bacteremia can occur when microbial agents enter the blood stream from one or more infected sites. This can occur regardless of location, leading to the colonization of other tissues and forming secondary sites of infection [33]. A correlation has been found between the degree of bacteremia and mortality in patients with sepsis across several studies [34]. We therefore used the *E. coli* K1-induced sepsis mouse model to perform quantitative bacterial population counts to determine if CYT387 could contribute to reducing this lethal manifestation during sepsis. Our data indicated that CYT387 treatment did indeed lead to a reduction in the numbers of bacteria in the major internal organs, including a total suppression of bacteremia in both the lung and liver (Fig. 5F).

In addition, the morbidity of sepsis is not always due to an excessive inflammatory response and there is substantial evidence suggesting that most septic patients become immunosuppressed, unable to eradicate the infectious agent. Potential mechanisms for this immunosuppression include the inability of antigen presenting cells (APCs) to activate T cells but most important is the generation of tolerance to endotoxins, leading to leukocytes unable to respond to a secondary endotoxic challenge [35,36]. Endotoxins are molecules closely associated with the cytoplasm or cell walls of micro-organism that induce inflammation through interactions with specific high-affinity receptors found on

leukocytes. In clinical Gram-negative bacterial sepsis, endotoxemia often occurs secondary to infection when the causative bacteria are eliminated by the immune system or antibiotic administration, leading to the release of large amounts of endotoxins into the bloodstream [37]. We therefore used the *E. coli* K1-induced mouse model to examine whether CYT387 treatment could reinforce the immune response against endotoxin and affect levels in the serum of septic mice. An LAL assay verified that CYT387 treatment could reduce the serum endotoxin levels that were originally increased by *E. coli* K1-induced sepsis (Fig. 5G).

Taken together, the findings gathered from the *E. coli* K1-induced mouse model suggest that Tbk1 is a major factor in the pathology and immunosuppression found in sepsis and suggests that CYT387 could serve as a potential therapeutic intervention.

4. Discussion

Sepsis is a deadly disease that is caused by many pathogens, including bacteria, fungi, parasites, and viruses [38]. Of these, Gram-negative bacterial infection account for the greatest proportion of septic patients [37,39]. Related to sepsis is septic shock, caused by severe organ dysfunction due to systemic coagulation in the major organs following excessive pro-inflammatory gene expression and a subsequent cytokine storm [40,41]. Early control of cytokine production is therefore key to developing an effective therapy for sepsis. During an infection, DCs are stimulated to develop into mature cells by pathogen-associated molecular patterns or PAMPs [42]. These mature DCs acquire the ability to serve as professional APCs that play a vital role in the immune response. This is primarily mediated through activation of T lymphocytes and adaptation of T cell polarization specific to the threat [43,44]. As a key mediator of the immune response, DCs are also important to modulating abnormal immune responses during the progression of sepsis, contributing to survival [45–48]. A full understanding of the role that DCs play during the pathology of sepsis will likely make an important contribution to therapeutic-oriented research for sepsis and septic shock.

In our study, we performed an initial transcriptome analysis to identify critical mediators of LPS-induced DC maturation. These data suggested that Tbk1 was the most important variable modulator differentiating LPS-treated DCs from control cells. Tbk1 is a key signaling molecule involved in triggering transcriptional activation of various pro-inflammatory genes that can lead to the induction of a cytokine storm *via* TRIF-dependent inflammatory responses. LPS initiates these signaling pathways through TLR4 and dsRNA-initiated TLR3 signaling [49–51]. Based on these data, we next established whether Tbk1 inhibition using CYT387 could affect various factors associated with sepsis. CYT387 is currently used for the treatment of myelofibrosis and other tumors and, importantly, has been routinely used on patients with little evidence of cumulative toxicity. Some previously described immunomodulatory effects of various Tbk1 pathway inhibitors supported our hypothesis that Tbk1 inhibition would affect sepsis [17], although the anti-inflammatory effects that Tbk1 inhibitors may possess have been relatively poorly studied. Of the few studies performed, there is some support for a causal relationship between the Tbk1 pathway and sepsis although there had been no comprehensive examination until our present study.

Our data also provide some details concerning the mechanisms by which Tbk1 inhibition by CYT387 can affect sepsis, particularly the inhibitory effects of CYT387 on the expression of several inflammatory mediators *in vitro*. This highlights the potential use of CYT387 as a therapeutic agent for treating LPS-induced sepsis. Supporting our *in vitro* study, treatment with CYT387 also reduced the expression of markers associated with damage to the major organs in both an LPS-induced endotoxemia model and an *E. coli* K1-induced sepsis model. This indicates that CYT387 may also have a role in preventing the organ dysfunction that can arise during severe sepsis. Interestingly, CYT387 treatment also reduced the levels of released endotoxins in the sepsis mouse model, as well as stopping the formation of bacterial populations in the major organs that often occur during the disease. Other studies have indicated that high levels of endotoxin correlate with the degree of cardiovascular failure in sepsis and also with the acute physiology and chronic health evaluation (APACHE) II and SOFA scores [52–54]. While Gram-negative pathogens from the primary site of infection are the main source of endotoxins released during septic shock, another source of endotoxin release can be due to the transmucosal passage of either Gram-negative bacteria or LPS from the intestines to sterile tissues [55,56]. Our observation that CYT387 effectively mitigated endotoxemia by affecting this pathophysiology suggests that CYT387 has major therapeutic potential for the treatment septic shock. Through our study we clearly demonstrated that CYT387 has *in vivo* efficacy in *E. coli* K1-induced sepsis and LPS-induced endotoxemia. This is likely to form the basis for future clinical development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.05.051.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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