The Theta Defensin, Retrocyclin 101, Inhibits TLR4- and TLR2-dependent Signaling and Protects Mice against Influenza Infection

Daniel Prantner[*], Kari Ann Shirey[*], Wendy Lai[*], Wuyuan Lu[†‡], Alexander M. Cole[§], Stefanie N. Vogel[*¶], and Alfredo Garzino-Demo[*‡||]

[*]Department of Microbiology and Immunology, [†]Department of Biochemistry and Molecular Biology, and [‡]Institute for Human Virology,

University of Maryland, School of Medicine

[§] Division of Molecular Microbiology, Burnett School of Biomedical Sciences, UCF College of Medicine

[||] Department of Molecular Medicine, University of Padova, Italy

Summary: A member of the theta-defensin family protected mice during infection with influenza, suggesting a new strategy for viral therapy in humans.

¶Corresponding author: Stefanie N. Vogel, Ph.D., Dept. of Microbiology and Immunology, Univ. of Maryland, School of Medicine, 685 W. Baltimore St., Rm. 380, Baltimore, MD 21201 Tel: 410-706-4838; E-mail: svogel@som.umaryland.edu

Key Words: Innate immunity, inflammation, cytokine regulation, signal transduction

Running title: Protection from influenza infection by retrocyclin

Total Character count excluding spaces, Methods, and References: 27,727

Number of Figures: 7

Number of Color Figures: 0

Total number of References: 77

Total number of words in abstract: 250

Total number of words in summary sentence: 21

Abbreviations:

DAMP, Damage-Associated Molecular Patterns

DMXAA, 5,6-Dimethylxanthenone 4-acetic acid

HMGB1, High Mobility Group Box-1

IRAK, Interleukin-1 receptor-associated kinase

LAL, Limulus Amebocyte Lysate

LD, Lethal Dose

LPS, Lipopolysaccharide

MyD88, Myeloid differentiation primary response gene 88

Pam3CSK4, Palmitoyl-3-cysteine-serine-lysine-4

PR8, Influenza virus strain A/PR/8/34

RC, Retrocyclin

RIG-I, Retinoic acid-inducible gene I

RTD, Rhesus macaque θ-defensin

STING, Stimulator of interferon genes

TBK1, Tank binding kinase-1

TCID, Tissue culture infective dose

TLR, Toll-like Receptor

TRIF, TIR-domain-containing adapter-inducing interferon-β

WT, Wild-type

Abstract:

Despite widespread use of annual influenza vaccines, seasonal influenza-associated deaths number in the thousands each year, in part due to exacerbating bacterial superinfections. Therefore, discovering additional therapeutic options would be a valuable aid to public health. Recently, Toll-like Receptor 4 (TLR4) inhibition has emerged as a possible mechanism for protection against influenza-associated lethality and acute lung injury. Based on recent data showing that rhesus macaque θ-defensins could inhibit TLR4-dependent gene expression, we tested the hypothesis that a novel θ-defensin, retrocyclin (RC)-101, could disrupt TLR4-dependent signaling and protect against viral infection. In this study, RC-101, a variant of the humanized θ-defensin RC-1, blocked TLR4-mediated gene expression in mouse and human macrophages in response to LPS, targeting both MyD88- and TRIF-dependent pathways. In a cell-free assay, RC-101 neutralized the biological activity of LPS at doses ranging from 0.5 to 50 EU/ml, consistent with data showing that RC-101 binds biotinylated LPS. The action of RC-101 was not limited to the TLR4 pathway as RC-101 treatment of macrophages also inhibited gene expression in response to a TLR2 agonist, Pam3CSK4, but failed to bind this biotinylated agonist. Mouse macrophages infected *in vitro* with mouse-adapted A/PR/8/34 influenza A virus (PR8) also produced lower levels of proinflammatory cytokine gene products in a TLR4-independent fashion when treated with RC-101. Finally, RC-101 decreased both the lethality and clinical severity associated with PR8 infection of mice. Cumulatively, our data demonstrate that RC-101 exhibits therapeutic potential for the mitigation of influenza-related morbidity and mortality, potentially acting through TLR-dependent and TLR-independent mechanisms.

Introduction:

Influenza viruses A and B are negative sense, single strand RNA viruses in the *Orthomyxoviridae* family. To combat these pathogenic viruses, seasonal influenza vaccines are formulated and annual vaccination is recommended by the Center for Disease Control and Prevention (CDC) for everybody $\geq$ 6 months of age for the 2016-17 flu season [1]. During the 2014-2015 flu season , the CDC estimated that approximately 150 million doses of influenza vaccine were distributed in the United States [2]. Despite the availability of these seasonal vaccines, annual estimated mortality from 1977 to 2007 due to influenza ranged from 3,349 to 48,614 deaths [3], peaking when influenza A (H3N2) circulated in the population [4]. These vaccines are only protective when the circulating strains of influenza closely match the vaccine strains, and the fact that many people do not get immunized can explain these statistics. Importantly, these seasonal influenza vaccines also do not protect against newly emerging strains that could potentially cause large outbreaks or pandemics in the future. In addition to the mortality and morbidity associated with the viral infection, coinfections can exacerbate the outcome of influenza infection as exemplified by the 1918 pandemic where the majority of deaths were due to secondary bacterial pneumonia [5]. Secondary bacterial infection after influenza infection accounts for a significant number of deaths worldwide [6]. Therefore, it would be a valuable aid to public health to have therapeutic options for treating influenza-infected individuals. This was an unmet need in the 2014-15 influenza season, where the circulating influenza A (H3N2) strain was a drift variant of the seasonal vaccine strain, resulting in a vaccine with reduced efficacy [7].

In non-immunized individuals, the first barriers to influenza infection are cells of the innate immune system. These cells are capable of recognizing the invading virus and this recognition is essential for initiating an adaptive immune response. According to pattern recognition theory [8], cells of the innate immune system identify pathogens through germline encoded proteins called Pattern Recognition Receptors. These proteins identify pathogen-associated molecular patterns present in the invading microorganisms. In the case of influenza infection, the cytosolic RNA helicase retinoic acid-inducible gene I (RIG-I) plays an important role in recognition, triggering production of protective proinflammatory cytokines such as TNF-α and antiviral type I IFNs [9]. It is theorized that the host cell can distinguish the viral RNA from cellular RNA because of a panhandle structure in its 5' triphosphorylated terminus [10]. However, the influenza protein NS1 is capable of antagonizing RIG-I dependent signaling [11], highlighting the need for redundancy in innate immune recognition. TLR3 can also recognize viral RNA [12], and has been shown to be activated in response to influenza infection in human lung epithelial cells to induce proinflammatory cytokines [13]. In certain cell types like plasmacytoid dendritic cells, TLR7 can also recognize influenza genomic RNA [14].

Innate immune recognition does not exclusively occur through recognition of pathogen substructures. Influenza infection can induce cellular stress, leading to mitochondrial dysfunction which can culminate in NLRP3-dependent activation of caspase-1, leading to release of the pro-inflammatory mediators IL-1β and IL-18 [15]. Infected cells can also release damage associated molecular patterns (DAMPs) to alert neighboring cells of the danger. S100A9 has been shown to be produced during *in vitro* infection with influenza A virus [16], while HMGB1 is a DAMP that has been shown to

be released during *in vivo* influenza infections in both mice and humans [17, 18]. Reactive oxygen species released by cells in the lung of humans and animals during viral infection can cause oxidation of endogenous phospholipids to produce oxidized phosphatidylcholine [19, 20]. Importantly, oxidized phospholipids, HMGB1, and S100A9 have all been shown to signal through TLR4 [16, 21, 22]. Relevantly, we reported that TLR4$^{-/-}$ mice were resistant to infection with mouse-adapted influenza virus strain A/PR/8/34 (PR8) [20, 23], as well as a more potent mouse-adapted strain ma.Ca-04 [24]. The TLR4-specific antagonist, Eritoran (Eisai, Inc.), blocked lethality and clinical symptoms caused by PR8 infection when given starting two days after infection [20]. Blocking TLR4 signaling on day 2 or days 2 and 4 days post-PR8 infection with an anti-TLR4 specific antibody also conferred protection [25]. These and other TLR4 antagonists identified by our group [26, 27] indicate that TLR4 antagonists may represent a novel class of drugs for use in treating influenza patients.

Defensins are small, cysteine-rich cationic peptides that primarily serve as an innate immune defense mechanism against infectious microorganisms. Unlike the α- and β-defensins, θ-defensins are circular, formed by head-to-tail ligation of two truncated α-defensin-like precursor peptides and possess broad antimicrobial activity, targeting bacteria, viruses, and fungi [28-32]. All 6 human θ-defensin (*DEFT*) pseudogenes contain a premature stop codon, preventing translation [33, 34]. However, solid-phase peptide synthesis has allowed researchers to construct artificially humanized θ-defensins, called retrocyclins (RC) [35]. The individual RCs vary slightly in the non-cysteine amino residues, but have been shown to bind with high affinity to *N*- and *O*- linked glycosylated proteins such as HIV gp120 [36], and to prevent fusion mediated by influenza

hemagglutinin [37]. Consequently, RCs can effectively prevent *in vitro* infection of human cell lines with either HIV [35, 38] or influenza A virus [39]. Similarly, RCs are protective *in vitro* against HSV-1/2 through binding to its surface glycoprotein [40]. In addition to their role in fighting microorganisms, defensins might also play a key regulatory role to limit tissue damaging inflammation. Human β-defensin 3 can inhibit TLR4-dependent signaling *in vitro* and *in vivo* [41, 42]. Rhesus macaque θ-defensins (RTD) prevent cytokine secretion from human blood leukocytes treated with multiple TLR ligands [43], including the TLR4 agonist LPS [44], demonstrating that θ-defensins can also regulate host responses mediated by cellular glycoproteins. This also suggests a role for the structurally similar RCs in TLR antagonism. Clinically, RCs could represent a novel therapeutic against influenza infection, inhibiting the detrimental production of TLR4-dependent cytokines and blocking infection of the virus or co-infecting bacteria. The aims of our study were to determine if RC-101 could inhibit LPS-induced TLR4 signaling and if RC-101 could be used therapeutically against influenza-mediated disease.

Materials and Methods:

Synthesis of RC-101: RC-101, containing an arginine to lysine substitution from the parent RC peptide [38], was made by solid phase synthesis. Backbone cyclization of RC101 is achieved through intra-molecular native chemical ligation [45, 46] of a thioester-ending linear peptide synthesized on solid phase using Boc chemistry. Disulfide bonds formed under oxidative conditions in the presence of DMSO. The peptide was purified by reversed-phase (RP) HPLC to homogeneity (>98% purity) and verified by electro-spray ionization mass spectrometry (ESI-MS). Shown in Supplemental Figure 1 is the final product analyzed by RP-HPLC on a Waters XBridge C18 column (4.6x150 mm, 3.5 μm) running at 40 °C a 30-min gradient of 5-65% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min. The observed molecular mass of 1889.7 Da is within experimental error of the expected value of 1890.4 Da calculated based on the average isotopic compositions of the peptide. RC-101 was solubilized in PBS prior to use.

Reagents: Protein-free *E. coli* K235 LPS was prepared as described [47]. *E. coli* 0111:B4 labeled with a Biotin tags (LPS-Biotin) (InvivoGen, San Diego, CA), 5,6-Dimethylxanthenone 4-acetic acid (DMXAA) (Sigma-Aldrich, St. Louis, MO), synthetic triacylated lipoprotein, palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) (InvivoGen, San Diego, CA), Pam3CSK4- Biotin (InvivoGen, San Diego, CA) and recombinant mouse TNF-α (eBioscience, San Diego, CA) were purchased from the indicated vendors. Mouse monoclonal antibodies were used to visualize TLR4 proteins with the tags Flag (M2 Clone) (Sigma Aldrich, St. Louis, MO) and eCFP (Clone OTI8A6) (Origene Technologies, Inc., Rockville, MD) by Western blot analysis. An anti-biotin-HRP linked

antibody (Cell Signaling Technology, Danvers, MA) was used to visualize LPS-Biotin. Rabbit-specific polyclonal antibody against RC-101 has been previously described [34]. All other primary and secondary antibodies used in the study have been previously described [48]. The LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, #88282) was used according to the manufacturer's instructions to test the biological activity of *E. coli* 0111:B4 LPS (included in the kit as a positive control). Chromo-LAL and control standard endotoxin (*E. coli* 0113:H10) were purchased from Associates of Cape Cod Incorporated (E. Falmouth, MA) and used according to the Manufacturer's instructions to measure the ability of RC-101 to antagonize the biological activity of LPS at doses higher than 1 EU/ml. Recombinant High Mobility Group Box-1 (HMGB1) protein was kindly provided by Dr. Kevin Tracey (Feinstein Institute for Medical Research, NY).

Macrophage isolation, treatment, and infection: $TRIF^{-/-}$ mice were kindly provided by Dr. Donald Cook (NIH) and Interleukin-1 receptor-associated kinase 4 (IRAK4) kinase-dead knock-in ($IRAK4^{KDKI}$) mice were obtained from Lilly Research Laboratories (Indianapolis, IN). IRAK4 is the first enzyme recruited to MyD88 and $IRAK4^{KDKI}$ macrophages behave similarly to $MyD88^{-/-}$ macrophages [49]. $TLR4^{-/-}$ mice (N≥10 on a C57BL/6 background) were originally obtained from Dr. Shizuo Akira (Osaka University, Osaka, Japan) for breeding at UMB. Thioglycollate-elicted, mouse peritoneal macrophages were isolated from wild-type (WT) C57BL/6J (Jackson Laboratories), $TLR4^{-/-}$, $TRIF^{-/-}$, and $IRAK4^{KDKI}$ and cultured as previously described [50]. Human monocyte-derived macrophages were matured from elutriated peripheral blood mononuclear cells of healthy donors as described [51]. Mouse and human macrophages

were pretreated with either PBS control or RC-101 within 30 seconds of stimulation. *In vitro* infection with PR8 was carried out as previously described [20, 23]. Macrophage lysates were harvested at the indicated times in TriPure (Roche Diagnostics Corporation, Indianapolis, IN) for subsequent gene expression analysis.

Gene expression analysis by quantitative qRT-PCR. cDNA was synthesized from RNA as previously described [50]. qRT-PCR reactions were monitored with the 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA) using the 2x *Power* SYBR green mix PCR master mix (Life Technologies Corporation, Carlsbad, CA). Individual genes were analyzed using cytokine gene-specific human [23] and mouse primers [50] that have previously been published. The total amount of mRNA was calculated using the Comparative ΔΔCt method [52] and expressed as fold-induction compared to untreated or uninfected cells.

Transfections and pulldown assays: HEK293T cells were grown in DMEM that was supplemented with 2 mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum as previously described [53]. One night prior to transfection, 2 x 10^5 cells were plated in a 12-well tissue culture dish. The following day, these cells were transfected with expression plasmids using the transfection reagent SuperFect (Qiagen, Valencia, CA) according to the manufacturer's supplied instructions. Specifically, 1 μg of total plasmid (Empty vector pcDNA3.1, TLR4-Flag [53], or TLR4-CFP [54]) was mixed with SuperFect at a 1:7 ratio. For dual transfections, 0.5 μg of each plasmid was used and the amount of SuperFect kept constant. After transfection, the cells were allowed to incubate in growth media for 48h before monitoring transgene expression. After 48 h, cells were washed twice with ice cold PBS and lysed (20 mM

HEPES, 0.5% Triton X-100, 150 mM NaCl and 1 mM PMSF). Whole-cell lysates were incubated on ice for 30 min and centrifuged at 12,000 rpm for 10 min at 4^{O} C to pellet cellular debris. Supernatants were withdrawn and analyzed by Western blot directly (input samples) or immunoprecipitated. In the latter case, 50 μg of whole-cell lysates (in 100 μl) were incubated overnight at 4^{O} C with gentle rotation in the presence of 1 μg of Flag-specific antibody. Protein G Agarose (Thermo Fisher Scientific, Waltham, MA) bead slurries (100 μl) was added to mixtures and incubated for a further 60 min with constant rotation at room temperature. Beads were harvested by centrifugation and washed a total of 5 times with binding buffer (25 mM Tris, 150 mM NaCl; pH 7.2). To harvest the bound proteins, beads were re-suspended in 50 μl Laemmli buffer, vortexed, boiled for 10 min at 95^{O} C, and centrifuged. Supernatants were separated by SDS-PAGE on a 10% gel and transferred to a PVDF membrane. For LPS pulldowns, LPS-biotin and RC-101 were incubated for 30 min at room temperature in the presence or absence of excess unlabeled *E. coli* K235 LPS. Pierce Streptavidin Ultralink Resin beads (Thermo Fisher Scientific, Waltham, MA) (50 μl slurry) were added to the samples and incubated for a further 60 min with constant rotation at room temperature. Beads were harvested by centrifugation and washed a total of 5 times with binding buffer (0.1 M phosphate, 0.15 M NaCl; pH 7.2). To harvest the bound proteins, beads were re-suspended in 50 μl Laemmli buffer, vortexed, boiled for 10 min at 95^{O} C, and centrifuged. Supernatants were withdrawn and serially diluted. Samples (5 μl) were dot blotted onto nitrocellulose membrane (0.2 μM pore size), air dried, blocked, and incubated in parallel for 1 h when probing for biotin or overnight when probing for RC-101.

Mouse infection model and clinical scoring: All mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore. On day 0, female, 6-8 week old WT C57BL/6J mice were infected intranasally (i.n.) with ~7500 $TCID_{50}$ PR8 as we described [20]. Mice were injected daily on days 2-6 post-infection intravenously with 100 μl of saline, or RC-101 (100 μg/mouse). A similar dosing regimen with the TLR4 antagonist Eritoran was previously shown to protect mice against PR8 infection [20]. Mice were monitored daily for survival, clinical score, and weight loss for a total of 14 days. Clinical scores ranging from 0 (no symptoms) to 5 (moribund) takes into account piloerection, hunched posture, ruffled fur, audible lung crackling, and lethargy as previously detailed [20]. Mice were euthanized as soon as they became moribund to prevent suffering of the animal.

Statistical analysis: Experiments were performed in duplicate for analysis of gene expression. The numerical values obtained from these assays were analyzed using GraphPad Prism 4 (GraphPad software Inc, La Jolla, CA) with either a one-way ANOVA or a two-way ANOVA, depending on the number of experimental variables. Significance was determined to be $p < 0.05$. For the one-way ANOVA, a Tukey's post-hoc test was used to test for significance between experimental groups within the data set. Survival curves of mice were analyzed for significance by a Log Rank test.

Online Supplemental Materials: Supplemental material to this manuscript includes one figure. This supplemental figure shows the peptide sequence of RC-101 and a representative example of the purity of RC-101 after peptide synthesis.

Results:

RC-101 inhibits TLR4-mediated cytokine expression in human and mouse macrophages. RTDs have been shown to decrease cytokine secretion in response to multiple TLR agonists [43]. As RC-101 is structurally similar to these RTDs, we hypothesized that RC-101 would antagonize TLR4-mediated signaling leading to cytokine induction. Consistent with this prediction, RC-101 inhibited LPS-induced IFN-β in a dose-dependent manner (Fig. 1A). Induction of IFN-β was decreased in the presence of RC-101 as early as 60 min post-LPS treatment (Fig. 1B). Signal transduction in response to LPS is initiated by homodimerization of TLR4 [55]. TLR4 signal transduction contains a unique bifurcation, utilizing both myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) as adaptor proteins [56, 57]. IFN-β is considered a MyD88-independent gene induced downstream of the TLR4-TRIF pathway [56, 58]. In contrast to IFN-β, TNF-α and IL-1β represent two MyD88- dependent cytokines [56]. In order to determine if MyD88 pathways were also affected by RC-101 treatment, expression of these MyD88-dependent genes were analyzed. Both LPS-dependent TNF-α and IL-1β were inhibited by RC-101 (Fig. 1C, D), albeit to a lesser extent than was evident for IFN-β mRNA. To determine whether RC-101-mediated TLR4 antagonism was a species-specific response, primary human monocyte-derived macrophages were also stimulated with LPS in the presence of RC-101. Similar to that observed in mouse macrophages, RC-101 also inhibited TRIF- and MyD88-dependent cytokine gene expression in response to LPS stimulation (Fig. 1E-H).

Decreased LPS-dependent cytokine gene expression due to RC-101 treatment correlated with a RC-101-mediated block in activation-induced phosphorylation of the TLR4-proximal signaling elements Tank binding kinase-1 (TBK1) and NF-κB p65 at 10 and 30 min after LPS stimulation (Fig. 2). Similarly, activation of MAPK pathways, ERK, JNK, and p38, were also decreased at those time points in the presence of RC-101 (Fig. 2). TBK1 phosphorylation remained decreased at 60 min post-LPS treatment in the presence of RC-101 (Fig. 2), while phosphorylation of MAPK and NF-κB was increased at this time point (Fig. 2), indicating that RC-101 blockade of TLR4 only delays signal transduction at this dose of LPS and does not ablate it entirely.

Inhibition of TLR4 signaling by RC-101 in human and mouse cells could possibly be accounted for by sequestration of LPS from the host TLR4 signaling complex, so we sought to test this possibility in a cell-free system. The biological activity of LPS was measured in the Limulus Amebocyte Lysate (LAL) assay in the presence or absence of RC-101 in order to determine if our cytokine expression results were likely due to a interaction between RC-101 and LPS. The biological activity of 1 Endotoxin Unit/ml (approx. 0.1 ng/ml) of LPS was partially inhibited by 30 μg/ml RC-101 and almost completely inhibited by 100 μg/ml RC-101 (Fig. 3A). The dose of LPS used in the cell-free LAL assay represents a one-thousand-fold lower dose of LPS than used for macrophage TLR4 stimulation (*e.g.*, see Fig. 1). To test more relevant doses of LPS, we used a different LAL assay that had a linear range up to 50 EU/ml, while maintaining the dose of RC-101 at 30 μg/ml. Under these conditions, RC-101 (30 μg/ml) strongly inhibited the biological activity of all LPS doses tested, including 50 EU/ml (Fig. 3B). RC-101 also inhibited the LAL activity of biotinylated-LPS at doses ranging from 1-100

ng/ml (Figure 3C), suggesting that it is likely that RC-101 inhibits TLR4 signaling, in part, by sequestration of LPS. To examine this possibility further, we performed an *in vitro* pull-down assay with biotinylated-LPS in the presence or absence of RC-101. RC-101 did not impair the ability of Streptavidin beads to pulldown LPS-Biotin (Figure 3D). Additionally, RC-101 was recovered upon pull-down of biotinylated LPS with streptavidin beads, suggesting direct binding between RC-101 and LPS (Figure 3D). To verify that this binding was specific, an excess of unlabeled LPS *E. coli* K235 was also added to the mix prior to pull-down. Excess unlabeled LPS prevented the pull-down of RC-101 with biotinylated-LPS, suggesting that the binding of LPS and RC-101 is specific (Figure 3D). In contrast to its interaction with LPS, RC-101 was not pulled down by the biotinylated TLR2 agonist Pam3CSK4 (Figure 3E).

Although our data shows evidence that RC-101 may mediate inhibition of TLR4-dependent signaling though sequestration of LPS, it must be noted that even 90% inhibition of LPS activity due to sequestration might not affect TLR4-mediated cytokine expression in our system because the LPS dose we used to stimulate macrophages (100 ng/ml) was not in the linear portion of the cytokine dose response curve (Fig. 4A, B). For that reason, we also sought to explore the capacity of RC-101 to disrupt TLR4 dimers, using a model of TLR4 oligomerization that occurs in the absence of ligand. Our lab has previously shown that overexpression of Flag-tagged TLR4 in HEK293T cells can facilitate TLR4-dependent signaling in the absence of LPS [53]. Co-transfection of TLR4-Flag and TLR4-CFP induced a physical interaction between the two TLR4 constructs as evidenced by co-immunoprecipitation (co-IP) of the two epitope tags (Figure 4C). Using this model, we examined whether RC-101 could destabilize this

interaction. Addition of RC-101 failed to inhibit the co-IP of TLR4-CFP with TLR4-Flag (Fig 4D).

After describing the inhibitory role of RC-101 in LPS-dependent signal transduction, we sought to test whether its role was similar in other innate immune signaling pathways. TLR2-dependent cytokine secretion has been shown to be regulated by RTDs in human blood leukocytes [43], so TLR2 might be expected to be regulated by RC-101 similar to TLR4. In our hands, RC-101 also inhibited pro-inflammatory TLR2-dependent gene induction in mouse macrophages (Fig. 5A, B), demonstrating that RC-101-mediated inhibition is not restricted to TLR4; however, RC-101 failed to bind to biotinylated-Pam3CSK4 (Fig. 3E). Therefore, inhibition of signaling is not restricted to agonists that RC-101 can bind directly. To further determine the degree of pathway specificity of RC-101-mediated inhibition, mouse macrophages were also stimulated with non-TLR agonists in its absence or presence. RC-101 failed to inhibit cytokine gene induction in response to either recombinant TNF-α or DMXAA (Fig. 5C-F). DMXAA is a potent inducer of TBK1-dependent IFN-β in mouse macrophages through intracellular activation of Stimulator of Interferon Genes (STING) [50], unlike TLR4 and TLR2 that initiate signaling extracellularly. Note that the apparent lack of induction of IFN-ß in response to LPS in Figure 5E is due to a sub-optimal time point for its expression (see Figure 1). Collectively, these latter results demonstrate that RC-101 is not a universal inhibitor of innate immune signaling pathways.

RC-101 protects mice therapeutically from infection with PR8. The recent discovery that Eritoran protects mice from lethal infection with PR8 [20] suggested that treatment of mice with other TLR4 antagonists, like RC-101, may also protect against

lethal influenza infection. The effect of RC-101 on PR8 infection was first examined in an *in vitro* infection model. In WT macrophages infected for 8 and 24 h *in vitro*, RC-101 inhibited PR8-induced expression of the pro-inflammatory genes encoding pro-IL-1β and TNF-α (Fig. 6A, B). Although TNF-α secretion by PR8-infected macrophages has been shown to be partially TLR4-dependent [16], we observed that induction of pro-IL-1β and TNF-α mRNA were not significantly impaired in PR8-infected $TLR4^{-/-}$ macrophages at 8 h post-infection (Fig. 6C, D). Similarly, PR8-infected $TRIF^{-/-}$ or $IRAK4^{KDKI}$ macrophages induced similar amounts of IL-1β and TNF-α mRNA in response to PR8 as WT controls (Fig. 6E, F). IRAK4 is the first kinase recruited to MyD88 during signal transduction and $IRAK4^{KDKI}$ macrophages behave comparably to $MyD88^{-/-}$ macrophages [49]. This suggests that the DAMPs that activate TLR4-dependent or other TLR-dependent signaling *in vivo* are likely not present in our *in vitro* model at the time points tested. This result also indicates that RC-101 influences cytokine induction during *in vitro* infection independently of TLR4, possibly by inhibiting the signaling of some other non-TLR PRR such as RIG-I that is also capable of recognizing PR8 [9]. HMGB1 is a DAMP that has been shown to be released during influenza infection of mice and humans [17, 18]. Importantly, HMGB1 is also known to activate TLR4-dependent signaling [22]. RC-101 suppressed cytokine gene induction in response to HMGB1 (Fig. 6G, 6H), demonstrating that it can also block TLR4-mediated gene expression mediated by DAMPs.

To determine whether RC-101 could be used as an anti-viral and/or anti-inflammatory therapeutic *in vivo*, mice were infected with PR8 (~LD_{90}) and given 5 daily i.v. injections of RC-101 (100 μg/mouse), starting 48 h after PR8 infection, and

continuing until day 6 post infection. We had previously reported that Eritoran protects 90-100% of mice using this identical dosing regimen [20]. In this study, RC-101 treatment of mice also protected from PR8-induced lethality (Fig. 7A) and significantly lessened PR8-induced clinical symptoms (Fig. 7B). This latter significant decrease in clinical symptoms suggests that RC-101 is not merely delaying the time to death, as the survivors are typically healthy at the end of 2 weeks. Due to the timing of drug administration, RC-101 is not hypothesized to mediate its effect by blocking initial uptake of the virus. Together, these data suggest that RC-101 and other θ-defensins are strong candidates for future human clinical trials in the search for novel viral therapeutics.

Discussion:

Although the innate immune system plays a crucial role in initiating an immune response against an invading microorganism, overexuberant signaling may actually contribute to pathology during the same infection. This double-edged sword effect of inflammation in response to innate immune signaling is illustrated during infection with influenza virus, the TLR adaptor, MyD88, is required for mouse survival following infection with PR8 [59]. Further illustrating the beneficial role of innate signaling pathways, pretreatment of mice with TLR4 or TLR2 agonists prior to *in vivo* infection with influenza has been shown to be protective [60-62]. However, the timing of these signaling pathways is crucial as oxidative stress in the lung during infection with PR8 has been linked to the development of oxidized phospholipids [20] and other DAMPs that mediate TLR4 signaling-dependent ALI [19]. The detrimental role of inflammation can be exacerbated further by bacterial co-infection [63]. As a consequence of the role of TLR4 during PR8 infection, $TLR4^{-/-}$ mice are refractory to lethality associated with PR8 infection [20, 23]. In further support of the role of oxidized phospholipids in mediating TLR4-dependent lethality, mortality can be prevented by inhibiting oxidized phospholipid formation by administering the antioxidant N-acetyl cysteine [64]. Additionally, we reported that mortality can be prevented by inhibiting TLR4 signaling after PR8 infection with a potent synthetic TLR4 antagonist, Eritoran [20], an anti-TLR4 antibody [25], and other agents that block TLR4 signaling [26, 27]. Thus, we hypothesized that drugs that target host cell TLR4-dependent signaling pathways could potentially function as a novel anti-influenza therapy.

In our study, RC-101 was fully capable of inhibiting LPS-dependent signaling in both mouse and human macrophages. The impairment of both MyD88- and TRIF-signaling pathways suggests that RC-101 could function by preventing the initial interaction of LPS with TLR4, either through sequestration of LPS, or blockade of the signaling event itself. Based on our study, we cannot rule out the strong possibility that a significant part of the TLR4 inhibition by RC-101 is mediated by LPS sequestration from the TLR4 signaling complex since RC-101 could both bind LPS in *in vitro* pull-down assays and inhibit a broad range of LPS bioactivity in cell-free LAL assays. However, the dose of LPS used to stimulate macrophages in our study was in the saturation range where only strong antagonists would be expected to have a significant effect on cytokine gene expression. Signal transduction in response to LPS is initiated at the plasma membrane by homodimerization of TLR4 molecules [55]. The ectodomain of human TLR4 contains 9 N-linked glycosylation sites that are required for cell surface expression and function [65]. Removal of these sialic acid residues increases LPS-dependent signaling [66], suggesting that these sialic residues may be at the interface of TLR4 dimerization. Since RCs bind with high affinity to proteins exhibiting *N*- and *O*-linked glycosylation [36], we hypothesize that RC-101 could antagonize TLR4-mediated signaling by binding to the glycans on TLR4 to prevent ligand-induced dimerization. In line with this theory, Human β-defensin 3 can also inhibit TLR4-dependent signaling through direct binding to TLR4 [67]. Using a model of ligand-independent oligomerization of TLR4 in HEK293T cells, we tested if RC-101 could prevent or disrupt homotypic TLR4 interactions. Addition of RC-101 failed to disrupt oligomers of TLR4 in this assay. This could mean that RC-101 does not target TLR4 directly or that it's

binding sites were unavailable in this conformation. Unfortunately, we could not decrease the input concentrations of the TLR4 plasmids used for transfection to a level that permitted ligand-dependent signaling yet still permit detection of co-immunoprecipitated TLR4-Flag and TLR4-CFP molecules. The MD-2 protein is a crucial part of the LPS recognition complex [68] and is also glycosylated [65], so it is also possible that MD-2, and not TLR4, might be the primary target of RC-101 in this pathway.

In addition to inhibition of LPS-dependent signaling, we also found that Pam3CSK4-dependent activation of TLR2 signaling was inhibited by RC-101. In contrast to its direct interaction with LPS, RC-101 was unable to interact with Pam3CSK4 *in vitro*. This suggests that RC-101 has the capacity to act in more ways than just ligand sequestration. Human TLR2 also contains glycosylation sites [69] which could be a putative target of RC-101. Alternatively, TLR-dependent signal transduction are sensitive to the architecture of lipid membrane microdomains [70]. Addition of a phosphatidylserine species can impair both TLR4 and TLR2 signaling pathways [70]. Given that the positively charged RC-101 has been found to bind to anionic membranes such as dipalmitoyl phosphatidylglycerol [71], it is tempting to speculate that the ability of RC-101 to block both TLR4- and TLR2-dependent signaling is caused by an effect on the cell membrane itself. However, such a mechanism seems less likely since TNF-α-mediated signaling, that also requires receptor oligomerization [72], was not perturbed by RC-101.

The fusion protein for Respiratory Syncytial Virus can be recognized in a TLR4-dependent manner [73], but no influenza protein has been identified that can function as a TLR4 ligand. In contrast, DAMPs like HMGB1 are released during *in vivo* infection and

signal through TLR4. It is less clear whether these DAMPs are produced *in vitro* and how much of an effect they have on gene regulation. Secretion of proinflammatory genes at 24 h post infection with PR8 has been shown to be MyD88-dependent in bone marrow-derived and peritoneal macrophages [16, 26]. However, TNF-α protein secretion at 12 h and 24 h was only decreased by approximately 20-30% in the absence of TLR4, exhibiting only a partial TLR4 dependence [16]. Our data, using the IRAK4^{KDKI} macrophages, indicates that the difference in cytokine production mediated by a lack of MyD88-dependent signaling is not due to a defect in transcriptional upregulation of MyD88-dependent genes like TNF-α or IL-1β at 8 h post-infection. As part of its role as an innate immune adaptor protein, MyD88 has also been shown to be involved in stabilization of mRNA species containing Adenosine- and Uridine- rich elements, which are common to many cytokines [74]. So, we surmise that the TLR4-MyD88 pathway may be mediating its effect on cytokine secretion in response to PR8 through alterations in mRNA stability and not through altered transcription.

RCs offer the potential to limit inflammation mediated by DAMPs and also to target the virus itself. Although RC-101 may act by blocking initial viral entry as previously described [37], it has been shown to be less effective against the mouse-adapted PR8 than other influenza strains [39]. For instance, pre-incubating the virus with various different RCs for 30 min only achieved approximately 60% decrease in viral infectivity as measured by a fluorescent focus formation assay [39]. Importantly, none of our experiments featured preincubation of the virus with RC-101, which would further limit the ability of RC101 to block virus directly. As a proof of concept that RCs can be used therapeutically, RC-101 was tested in a mouse model of influenza infection.

Treatment with RC-101, starting 2 days post-infection, was shown to lessen the severity of PR8-associated disease, resulting in a highly significant increase in survival (Fig. 8). Our results on inhibition of TLR4-mediated cytokine expression are consistent with the possibility that antagonism of TLR4 signaling accounts at least, in part, for protection in PR8-infected mice. That the protective therapeutic effect of RC-101 against PR8 is similar to what we have previously reported for the TLR4 antagonist Eritoran [20] suggests that RC-101 may act *in vivo*, at least partially, through TLR4 antagonism. However, we cannot rule out the possibility of an additional direct effect on the virus itself as has been observed by others [37, 39]. While Eritoran does not directly kill influenza *in vitro* [20], Eritoran therapy was associated with decreased influenza titers *in vivo*, but not until day 6 after infection [20]. This highlights an important connection between inflammation and viral replication in this infection model. It is quite possible that the effect of RC-101 in this system is both antiviral and anti-inflammatory.

Overall, our results in the mouse model are especially encouraging because the mouse-adapted PR8 strain used in our study lacks *N*-linked glycosylation on its cell envelope hemagglutinin protein [75], making it more resistant than other influenza strains to RC-101 *in vitro* [39]. Therefore, RC-101 would be predicted to be more effective against human strains with increased glycosylation [76]. Similar to the efficacy demonstrated by RC-101 in our study, RTDs protected mice from a lethal lung infection with a mouse adapted SARS-coronavirus and limited the potentially overexuberant cytokine response occurring in the lung [77]. Together, these data suggest that θ-defensins, and specifically, RC-101, are strong candidates for future human clinical trials in the search for novel viral therapeutics.

Authorship:

DP designed and coordinated the experiments in the study, performed and analyzed the experiments, and wrote the manuscript. KAS designed and carried out the experiments outlined in Figure 7. WL1 provided technical assistance involving PR8 infection described in Figure 7. WL2 provided technical assistance for all figures by synthesizing RC-101. AMC provided technical assistance for detection of RC-101 by Western Blot in Figure 3. AGD conceived the study and helped in drafting of the manuscript. SNV helped with the design and conception of experiments, analysis of the data, and in drafting of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgements:

This work has been supported by the following NIH grants: AI-18797 and AI-125215 (SNV), NS-066842 (AGD), and AI-072732 (WL). The authors would like to thank Dr. Kevin Tracey (Feinstein Institute for Medical Research, NY) for the kind gift of High Mobility Group Box-1 (HMGB1) protein.

Conflict of Interest Disclosure:

The authors declare no conflict of interest. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the National Institute of Allergy and Infectious Diseases.

References:

1. Grohskopf, L.A., Sokolow, L.Z., Broder, K.R., Olsen, S.J., Karron, R.A., Jernigan, D.B., Bresee, J.S. (2016) Prevention and Control of Seasonal Influenza with Vaccines. *MMWR Recomm Rep* **65**, 1-54.
2. (2014) Seasonal Influenza Vaccine & Total Doses Distributed. In *http://www.cdc.gov/flu/professionals/vaccination/vaccinesupply.htm*.
3. (2010) Estimates of deaths associated with seasonal influenza --- United States, 1976-2007. *MMWR Morb Mortal Wkly Rep* **59**, 1057-62.
4. Thompson, W.W., Moore, M.R., Weintraub, E., Cheng, P.Y., Jin, X., Bridges, C.B., Bresee, J.S., Shay, D.K. (2009) Estimating influenza-associated deaths in the United States. *Am J Public Health* **99 Suppl 2**, S225-30.
5. Morens, D.M., Taubenberger, J.K., Fauci, A.S. (2008) Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis* **198**, 962-70.
6. Chertow, D.S., Memoli, M.J. (2013) Bacterial coinfection in influenza: a grand rounds review. *Jama* **309**, 275-82.
7. (2014) Early Data Suggests Potentially Severe Flu Season. In *http://www.cdc.gov/media/releases/2014/p1204-flu-season.html*.
8. Janeway, C.A., Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* **54 Pt 1**, 1-13.
9. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.S., Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S. (2006)

Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101-5.

10. Weber, M., Gawanbacht, A., Habjan, M., Rang, A., Borner, C., Schmidt, A.M., Veitinger, S., Jacob, R., Devignot, S., Kochs, G., Garcia-Sastre, A., Weber, F. (2013) Incoming RNA virus nucleocapsids containing a 5'-triphosphorylated genome activate RIG-I and antiviral signaling. *Cell Host Microbe* **13**, 336-46.
11. Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., Reis e Sousa, C. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997-1001.
12. Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-8.
13. Le Goffic, R., Pothlichet, J., Vitour, D., Fujita, T., Meurs, E., Chignard, M., Si-Tahar, M. (2007) Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol* **178**, 3368-72.
14. Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529-31.
15. Ichinohe, T., Yamazaki, T., Koshiba, T., Yanagi, Y. (2013) Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. *Proc Natl Acad Sci U S A* **110**, 17963-8.

16. Tsai, S.Y., Segovia, J.A., Chang, T.H., Morris, I.R., Berton, M.T., Tessier, P.A., Tardif, M.R., Cesaro, A., Bose, S. (2014) DAMP molecule S100A9 acts as a molecular pattern to enhance inflammation during influenza A virus infection: role of DDX21-TRIF-TLR4-MyD88 pathway. *PLoS Pathog* **10**, e1003848.

17. Alleva, L.M., Budd, A.C., Clark, I.A. (2008) Systemic release of high mobility group box 1 protein during severe murine influenza. *J Immunol* **181**, 1454-9.

18. Momonaka, H., Hasegawa, S., Matsushige, T., Inoue, H., Kajimoto, M., Okada, S., Nakatsuka, K., Morishima, T., Ichiyama, T. (2014) High mobility group box 1 in patients with 2009 pandemic H1N1 influenza-associated encephalopathy. *Brain Dev* **36**, 484-8.

19. Imai, Y., Kuba, K., Neely, G.G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y.H., Wang, H., Liu, H., Sun, Y., Pasparakis, M., Kopf, M., Mech, C., Bavari, S., Peiris, J.S., Slutsky, A.S., Akira, S., Hultqvist, M., Holmdahl, R., Nicholls, J., Jiang, C., Binder, C.J., Penninger, J.M. (2008) Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* **133**, 235-49.

20. Shirey, K.A., Lai, W., Scott, A.J., Lipsky, M., Mistry, P., Pletneva, L.M., Karp, C.L., McAlees, J., Gioannini, T.L., Weiss, J., Chen, W.H., Ernst, R.K., Rossignol, D.P., Gusovsky, F., Blanco, J.C., Vogel, S.N. (2013) The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature* **497**, 498-502.

21. Walton, K.A., Hsieh, X., Gharavi, N., Wang, S., Wang, G., Yeh, M., Cole, A.L., Berliner, J.A. (2003) Receptors involved in the oxidized 1-palmitoyl-2-

arachidonoyl-sn-glycero-3-phosphorylcholine-mediated synthesis of interleukin-8. A role for Toll-like receptor 4 and a glycosylphosphatidylinositol-anchored protein. *J Biol Chem* **278**, 29661-6.

22. Yang, H., Hreggvidsdottir, H.S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., Rosas-Ballina, M., Al-Abed, Y., Akira, S., Bierhaus, A., Erlandsson-Harris, H., Andersson, U., Tracey, K.J. (2010) A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci U S A* **107**, 11942-7.

23. Nhu, Q.M., Shirey, K., Teijaro, J.R., Farber, D.L., Netzel-Arnett, S., Antalis, T.M., Fasano, A., Vogel, S.N. (2010) Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors in vitro and in vivo. *Mucosal Immunol* **3**, 29-39.

24. Ye, J., Sorrell, E.M., Cai, Y., Shao, H., Xu, K., Pena, L., Hickman, D., Song, H., Angel, M., Medina, R.A., Manicassamy, B., Garcia-Sastre, A., Perez, D.R. (2009) Variations in the hemagglutinin of the 2009 H1N1 pandemic virus: potential for strains with altered virulence phenotype? *PLoS Pathog* **6**, e1001145.

25. Shirey, K.A., Lai, W., Patel, M.C., Pletneva, L.M., Pang, C., Kurt-Jones, E., Lipsky, M., Roger, T., Calandra, T., Tracey, K.J., Al-Abed, Y., Bowie, A.G., Fasano, A., Dinarello, C.A., Gusovsky, F., Blanco, J.C., Vogel, S.N. (2016) Novel strategies for targeting innate immune responses to influenza. *Mucosal Immunol* **9**, 1173-82.

26. Piao, W., Shirey, K.A., Ru, L.W., Lai, W., Szmacinski, H., Snyder, G.A., Sundberg, E.J., Lakowicz, J.R., Vogel, S.N., Toshchakov, V.Y. (2015) A Decoy Peptide that

Disrupts TIRAP Recruitment to TLRs Is Protective in a Murine Model of Influenza. *Cell Rep* **11**, 1941-52.

27. Perrin- Cocon, L., Aublin- Gex, A., Sestito, S.E., Shirey, K., Patel, M.C., Andre, P., Blanco, J.C., Vogel, S.N., Peri, F., Lotteau, V. (2017) TLR4 antagonist FP7 inhibits LPS-induced cytokine production and glycolytic reprogramming in dendritic cells, and protects mice from lethal influenza infection. *Scientific Reports* **7**, 40791.

28. Tran, D., Tran, P.A., Tang, Y.Q., Yuan, J., Cole, T., Selsted, M.E. (2002) Homodimeric theta-defensins from rhesus macaque leukocytes: isolation, synthesis, antimicrobial activities, and bacterial binding properties of the cyclic peptides. *J Biol Chem* **277**, 3079-84.

29. Tang, Y.Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C.J., Ouellette, A.J., Selsted, M.E. (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286**, 498-502.

30. Leonova, L., Kokryakov, V.N., Aleshina, G., Hong, T., Nguyen, T., Zhao, C., Waring, A.J., Lehrer, R.I. (2001) Circular minidefensins and posttranslational generation of molecular diversity. *J Leukoc Biol* **70**, 461-4.

31. Arnett, E., Lehrer, R.I., Pratikhya, P., Lu, W., Seveau, S. (2011) Defensins enable macrophages to inhibit the intracellular proliferation of Listeria monocytogenes. *Cell Microbiol* **13**, 635-51.

32. Rothan, H.A., Han, H.C., Ramasamy, T.S., Othman, S., Rahman, N.A., Yusof, R. (2012) Inhibition of dengue NS2B-NS3 protease and viral replication in Vero cells by recombinant retrocyclin-1. *BMC Infect Dis* **12**, 314.

33. Nguyen, T.X., Cole, A.M., Lehrer, R.I. (2003) Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **24**, 1647-54.

34. Venkataraman, N., Cole, A.L., Ruchala, P., Waring, A.J., Lehrer, R.I., Stuchlik, O., Pohl, J., Cole, A.M. (2009) Reawakening retrocyclins: ancestral human defensins active against HIV-1. *PLoS Biol* **7**, e95.

35. Cole, A.M., Hong, T., Boo, L.M., Nguyen, T., Zhao, C., Bristol, G., Zack, J.A., Waring, A.J., Yang, O.O., Lehrer, R.I. (2002) Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. *Proc Natl Acad Sci U S A* **99**, 1813-8.

36. Wang, W., Cole, A.M., Hong, T., Waring, A.J., Lehrer, R.I. (2003) Retrocyclin, an antiretroviral theta-defensin, is a lectin. *J Immunol* **170**, 4708-16.

37. Leikina, E., Delanoe-Ayari, H., Melikov, K., Cho, M.S., Chen, A., Waring, A.J., Wang, W., Xie, Y., Loo, J.A., Lehrer, R.I., Chernomordik, L.V. (2005) Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. *Nat Immunol* **6**, 995-1001.

38. Owen, S.M., Rudolph, D.L., Wang, W., Cole, A.M., Waring, A.J., Lal, R.B., Lehrer, R.I. (2004) RC-101, a retrocyclin-1 analogue with enhanced activity against primary HIV type 1 isolates. *AIDS Res Hum Retroviruses* **20**, 1157-65.

39. Doss, M., White, M.R., Tecle, T., Gantz, D., Crouch, E.C., Jung, G., Ruchala, P., Waring, A.J., Lehrer, R.I., Hartshorn, K.L. (2009) Interactions of alpha-, beta-,

and theta-defensins with influenza A virus and surfactant protein D. *J Immunol* **182**, 7878-87.

40. Yasin, B., Wang, W., Pang, M., Cheshenko, N., Hong, T., Waring, A.J., Herold, B.C., Wagar, E.A., Lehrer, R.I. (2004) Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J Virol* **78**, 5147-56.

41. Semple, F., MacPherson, H., Webb, S., Cox, S.L., Mallin, L.J., Tyrrell, C., Grimes, G.R., Semple, C.A., Nix, M.A., Millhauser, G.L., Dorin, J.R. (2011) Human beta-defensin 3 affects the activity of pro-inflammatory pathways associated with MyD88 and TRIF. *Eur J Immunol* **41**, 3291-300.

42. Semple, F., Webb, S., Li, H.N., Patel, H.B., Perretti, M., Jackson, I.J., Gray, M., Davidson, D.J., Dorin, J.R. (2010) Human beta-defensin 3 has immunosuppressive activity in vitro and in vivo. *Eur J Immunol* **40**, 1073-8.

43. Schaal, J.B., Tran, D., Tran, P., Osapay, G., Trinh, K., Roberts, K.D., Brasky, K.M., Tongaonkar, P., Ouellette, A.J., Selsted, M.E. (2012) Rhesus macaque theta defensins suppress inflammatory cytokines and enhance survival in mouse models of bacteremic sepsis. *PLoS One* **7**, e51337.

44. Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085-8.

45. Dawson, P.E., Kent, S.B. (2000) Synthesis of Native Proteins by Chemical Ligation. *Annu Rev Biochem* **69**, 923-960.

46. Dawson, P.E., Muir, T.W., Clark- Lewis, I., Kent, S.B. (1994) Synthesis of Proteins by Native Chemical Ligation. *Science* **266**, 776-779.

47. McIntire, F.C., Sievert, H.W., Barlow, G.H., Finley, R.A., Lee, A.Y. (1967) Chemical, physical, biological properties of a lipopolysaccharide from Escherichia coli K-235. *Biochemistry* **6**, 2363-72.

48. Perkins, D.J., Polumuri, S.K., Pennini, M.E., Lai, W., Xie, P., Vogel, S.N. (2013) Reprogramming of murine macrophages through TLR2 confers viral resistance via TRAF3-mediated, enhanced interferon production. *PLoS Pathog* **9**, e1003479.

49. Pennini, M.E., Perkins, D.J., Salazar, A.M., Lipsky, M., Vogel, S.N. (2013) Complete dependence on IRAK4 kinase activity in TLR2, but not TLR4, signaling pathways underlies decreased cytokine production and increased susceptibility to Streptococcus pneumoniae infection in IRAK4 kinase-inactive mice. *J Immunol* **190**, 307-16.

50. Prantner, D., Perkins, D.J., Lai, W., Williams, M.S., Sharma, S., Fitzgerald, K.A., Vogel, S.N. (2012) 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) activates stimulator of interferon gene (STING)-dependent innate immune pathways and is regulated by mitochondrial membrane potential. *J Biol Chem* **287**, 39776-88.

51. Lafferty, M.K., Sun, L., DeMasi, L., Lu, W., Garzino-Demo, A. (2010) CCR6 ligands inhibit HIV by inducing APOBEC3G. *Blood* **115**, 1564-71.

52. Schmittgen, T.D., Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3**, 1101-8.

53. Rallabhandi, P., Nhu, Q.M., Toshchakov, V.Y., Piao, W., Medvedev, A., Hollenberg, M.D., Fasano, A., Vogel, S.N. (2008) Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. *J Biol Chem* **283**, 24314-25.

54. Toshchakov, V.Y., Szmacinski, H., Couture, L.A., Lakowicz, J.R., Vogel, S.N. (2011) Targeting TLR4 Signaling by TLR4 Toll/IL-1 Receptor Domain-Derived Decoy Peptides: Identification of the TLR4 Toll/IL-1 Receptor Domain Dimerization Interface. *J Immunol* **186**, 4819-4827.

55. Saitoh, S., Akashi, S., Yamada, T., Tanimura, N., Kobayashi, M., Konno, K., Matsumoto, F., Fukase, K., Kusumoto, S., Nagai, Y., Kusumoto, Y., Kosugi, A., Miyake, K. (2004) Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. *Int Immunol* **16**, 961-9.

56. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**, 115-22.

57. Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S.O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., Beutler, B. (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* **424**, 743-8.

58. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., Akira, S. (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* **169**, 6668-72.

59. Seo, S.U., Kwon, H.J., Song, J.H., Byun, Y.H., Seong, B.L., Kawai, T., Akira, S., Kweon, M.N. (2010) MyD88 signaling is indispensable for primary influenza A virus infection but dispensable for secondary infection. *J Virol* **84**, 12713-22.

60. Shinya, K., Okamura, T., Sueta, S., Kasai, N., Tanaka, M., Ginting, T.E., Makino, A., Eisfeld, A.J., Kawaoka, Y. (2011) Toll-like receptor pre-stimulation protects mice against lethal infection with highly pathogenic influenza viruses. *Virol J* **8**, 97.

61. Shinya, K., Ito, M., Makino, A., Tanaka, M., Miyake, K., Eisfeld, A.J., Kawaoka, Y. (2012) The TLR4-TRIF pathway protects against H5N1 influenza virus infection. *J Virol* **86**, 19-24.

62. Tan, A.C., Mifsud, E.J., Zeng, W., Edenborough, K., McVernon, J., Brown, L.E., Jackson, D.C. (2012) Intranasal administration of the TLR2 agonist Pam2Cys provides rapid protection against influenza in mice. *Mol Pharm* **9**, 2710-8.

63. Chen, W.H., Toapanta, F.R., Shirey, K.A., Zhang, L., Giannelou, A., Page, C., Frieman, M.B., Vogel, S.N., Cross, A.S. (2012) Potential role for alternatively activated macrophages in the secondary bacterial infection during recovery from influenza. *Immunol Lett* **141**, 227-34.

64. Zhang, R.H., Li, C.H., Wang, C.L., Xu, M.J., Xu, T., Wei, D., Liu, B.J., Wang, G.H., Tian, S.F. (2014) N-acetyl-l-cystine (NAC) protects against H9N2 swine influenza virus-induced acute lung injury. *Int Immunopharmacol* **22**, 1-8.

65. da Silva Correia, J., Ulevitch, R.J. (2002) MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. *J Biol Chem* **277**, 1845-54.

66. Feng, C., Stamatos, N.M., Dragan, A.I., Medvedev, A., Whitford, M., Zhang, L., Song, C., Rallabhandi, P., Cole, L., Nhu, Q.M., Vogel, S.N., Geddes, C.D., Cross, A.S. (2012) Sialyl residues modulate LPS-mediated signaling through the Toll-like receptor 4 complex. *PLoS One* **7**, e32359.

67. Zhu, C., Bao, N., Chen, S., Zhao, J. (2016) HBD-3 regulation of the immune response and the LPS/TLR4-mediated signaling pathway. *Exp Ther Med* **12**, 2150-54.

68. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., Miyake, K. (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* **3**, 667-72.

69. Weber, A.N., Morse, M.A., Gay, N.J. (2004) Four N-linked glycosylation sites in human toll-like receptor 2 cooperate to direct efficient biosynthesis and secretion. *J Biol Chem* **279**, 34589-94.

70. Parker, L.C., Prestwich, E.C., Ward, J.R., Smythe, E., Berry, A., Triantafilou, M., Triantafilou, K., Sabroe, I. (2008) A phosphatidylserine species inhibits a range of TLR- but not IL-1beta-induced inflammatory responses by disruption of membrane microdomains. *J Immunol* **181**, 5606-5617.

71. Abuja, P.M., Zenz, A., Trabi, M., Craik, D.J., Lohner, K. (2004) The cyclic antimicrobial peptide RTD-1 induces stabilized lipid-peptide domains more efficiently than its open-chain analogue. *2004* **566**, 301-6.

72. Bigda, J., Beletsky, I., Brakebusch, C., Varfolomeev, Y., Engelmann, H., Bigda, J., Holtmann, H., Wallach, D. (1994) Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. *J Exp Med* **180**, 445-60.

73. Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J., Finberg, R.W. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* **1**, 398-401.

74. Datta, S., Novotny, M., Li, X., Tebo, J., Hamilton, T.A. (2004) Toll IL-1 receptors differ in their ability to promote the stabilization of adenosine and uridine-rich elements containing mRNA. *J Immunol* **173**, 2755-61.

75. Tate, M.D., Brooks, A.G., Reading, P.C. (2011) Specific sites of N-linked glycosylation on the hemagglutinin of H1N1 subtype influenza A virus determine sensitivity to inhibitors of the innate immune system and virulence in mice. *J Immunol* **187**, 1884-94.

76. Zhang, M., Gaschen, B., Blay, W., Foley, B., Haigwood, N., Kuiken, C., Korber, B. (2004) Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology* **14**, 1229-46.

77. Wohlford-Lenane, C.L., Meyerholz, D.K., Perlman, S., Zhou, H., Tran, D., Selsted, M.E., McCray, P.B., Jr. (2009) Rhesus theta-defensin prevents death in a mouse model of severe acute respiratory syndrome coronavirus pulmonary disease. *J Virol* **83**, 11385-90.

Figure Legends:

Figure 1. *RC-101 inhibits TLR4-dependent gene expression in mouse and human macrophages*. (A-D) Peritoneal macrophages were treated with mock (PBS) control or RC-101 (30 μg/ml, if not otherwise indicated) immediately prior to treatment with LPS (100 ng/ml). Expression of IFN-β mRNA (A, B) and TNF-α mRNA (C), and IL-1β mRNA (D) were determined by qRT-PCR. (E-H) Human monocyte-derived macrophages isolated from peripheral blood mononuclear cells were treated with mock control (PBS) or the indicated concentration of RC-101 immediately prior to stimulation with LPS (100 ng/ml). Gene expression of the following human cytokines were determined using qRT-PCR: IL-6 (E), IFN-β (F), TNF-α (G), IL-1β (H). The data represents the mean $\pm$ standard deviation for experimental conditions performed in duplicate. A representative of 3 independent experiments is shown. An * denotes $P < 0.05$. An ** denotes $P < 0.001$. For (A), significance is compared to LPS treatment alone (0 μg/ml RC-101).

Figure 2. *RC-101 inhibits TLR4-dependent signaling in mouse macrophages*. Peritoneal macrophages were treated with the indicated dose of RC-101 or mock control (PBS) immediately prior to treatment with LPS (100 ng/ml). Peritoneal macrophages lysates were harvested at the indicated times, separated by denaturing SDS-PAGE and probed with antibodies directed against the following phosphorylated proteins and loading controls: TBK1, NF-κB p65, ERK1/2, JNK, p38. The housekeeping protein β- Tubulin and unphosphorylated proteins were used as loading controls. A representative blot from 3 independent experiments is shown.

Figure 3. *RC-101 is an antagonist of LPS biologic activity.* (A) Increasing doses of RC-101 were incubated with 1 Endotoxin Unit/ml (~0.1 ng/ml) of LPS for 10 min at 37^{O} C prior to measurement of the biological activity of LPS using the Limulus Amebocyte Lysate (LAL) assay. Each dot represents the mean from an individual experiment and the bar represents the mean biological activity calculated from the results of 2 or 3 independent experiments. (B, C)) Increasing doses of LPS (0.05- 50 EU/ml) or LPS-Biotin (1- 100 ng/ml) were incubated with 30 μg/ml RC-101 for 10 min at 37^{O} C prior to measurement of the biological activity of LPS using the Chromo- Limulus Amebocyte Lysate (LAL) assay. For panels (B) and (C), each dot represents the mean from an individual experiment and the bar represents the mean biological activity calculated from the results of the 3 independent experiments. (D) LPS-Biotin, RC-101, and unlabeled LPS were incubated in the indicated combinations for 30 min before pull-down with Streptavidin-coated beads. Eluted samples were isolated, diluted, and dot blotted onto nitrocellulose membrane and probed for biotin or RC-101. (E) LPS-Biotin, RC-101, and Pam3CSK4- Biotin were incubated in the indicated combinations for 30 min before pull-down with Streptavidin-coated beads. Eluted samples were isolated, diluted, and dot blotted onto nitrocellulose membrane and probed for biotin or RC-101. A representative pulldown from three independent experiments is shown.

Figure 4. *RC-101 fails to destabilize the ligand-independent TLR4 oligomerization in HEK293T cells*. (A, B) Mouse macrophages were stimulated with increasing doses of LPS for 60 min and analyzed for expression of IL-1β (A) and TNF-α (B). The arrow represents the dose of LPS used throughout the study. The data represents the mean $\pm$ standard deviation for experimental conditions performed in duplicate. A representative

of 4 independent experiments is shown. (C) HEK293T cells were transfected with the indicated expression plasmids and incubated for 48 hours. Whole-cell lysates were separated by SDS-PAGE and blotted for transgene expression (input) or immunoprecipitated with anti-Flag specific antibody. Immunoprecipitated samples were separated by SDS-PAGE and probed with anti-Flag or anti-CFP specific antibodies. (D) Same as (C) except that RC-101 (30 ug/ml) was added where noted 30 min prior to harvesting of the whole-cell lysates. For (C) and (D), representative blots from one of three independent immunoprecipitation reactions are shown.

Figure 5. *RC-101 inhibits TLR2 signaling but is not a global inhibitor of innate signaling pathways*. Peritoneal macrophages were treated with RC-101 (30 μg/ml) or mock control (PBS) immediately prior to treatment with LPS (100 ng/ml) and either Pam3CSK4 (20 ng/ml) (A, B), TNF-α (100 ng/ml) (C, D), or DMXAA (100 μg/ml) (E, F). Expression of IFN-β and TNF-α at 30 min post DMXAA treatment or IL-1β and TNF-α at 30 min post-TNF-α or Pam3CSK4 treatment were determined by qRT-PCR. The data represents the mean +/- standard deviation for experimental conditions performed in duplicate. A representative of 3 independent experiments is shown. An * denotes $P < 0.01$. NS denotes there was not a significant difference.

Figure 6. *RC-101 inhibits upregulation of PR8-dependent and HMGB1-dependent proinflammatory cytokines in mouse macrophages*. (A, B) Peritoneal macrophages from WT mice were treated with RC-101 (30 μg/ml) or mock control (PBS) immediately prior to infection with PR8 at a MOI of 1. At the indicated times post-infection, cells were harvested and analyzed for expression of IL-1β and TNF-α. (C, D) WT and $TLR4^{-/-}$ peritoneal macrophages were infected as in (A) for 8 h or treated with LPS (100 ng/ml)

for 60 min and analyzed for IL-1β and TNF-α mRNA. (E, F) WT, $TRIF^{-/-}$, and $IRAK4^{KDKI}$ peritoneal macrophages were infected as in (A) and harvested at 8 h post-infection and analyzed for IL-1β and TNF-α mRNA. (G, H) Peritoneal macrophages from WT mice were stimulated with recombinant HMGB1 (2.5 μg/ml) immediately following treatment with RC-101 (30 μg/ml) or mock control and harvested at the indicated times for expression of (G) IL-1β and (H) TNF-α mRNA. Data for all panels represent the mean ± standard deviation for experimental conditions performed in duplicate. A representative of 3 independent experiments is shown.

Figure 7. *RC-101 increases survival and improves clinical outcome in mice infected with PR8*. C57BL/6J mice were infected with ~7500 $TCID_{50}$ PR8 (~LD_{90}) and injected intravenously with saline, or retrocyclin (RC-101; 100 μg/mouse) at days 2, 3, 4, 5, and 6 post-infection. Mice were monitored until day 14 post-infection. Survival (A) and mean clinical score (B) are shown. Data in (B) represents the mean clinical score ± SEM with a range of 0 (no symptoms) to 5 (moribund). N = 10 mice per experimental group. The data graphed represents the pooled results from 2 separate independent *in vivo* studies.

Figure 1

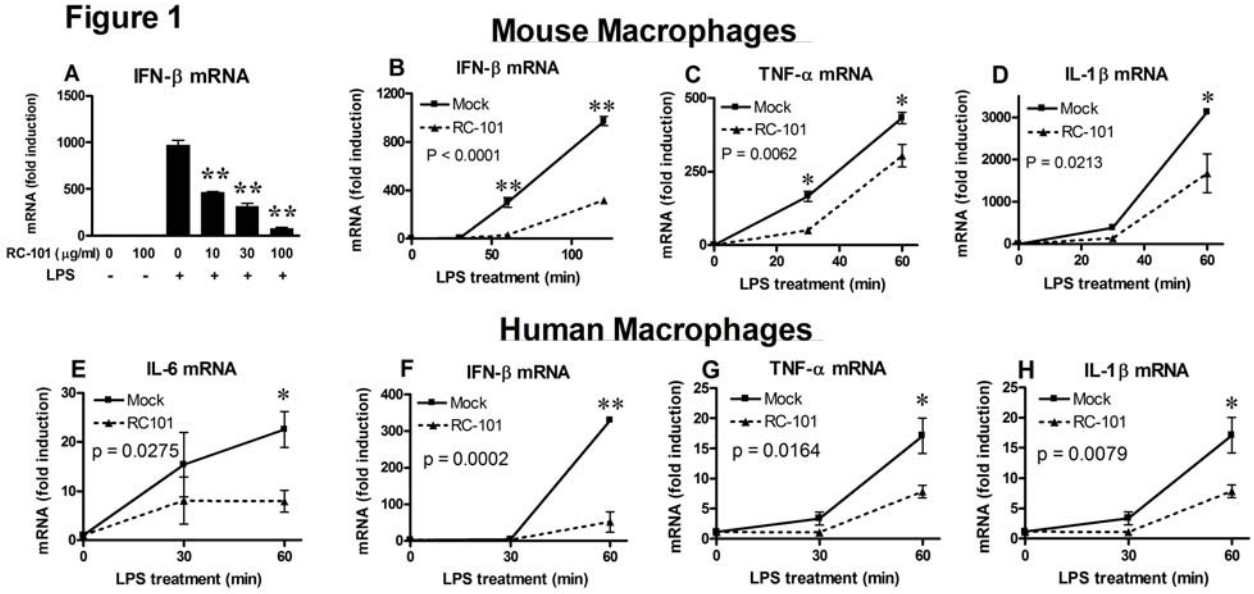

Mouse Macrophages
A
IFN-β mRNA
mRNA (fold induction)
RC-101 (μg/ml) 0 100 0 10 30 100
LPS - - + + + +
B
IFN-β mRNA
Mock
RC-101
P < 0.0001
LPS treatment (min)
C
TNF-α mRNA
Mock
RC-101
P = 0.0062
LPS treatment (min)
D
IL-1β mRNA
Mock
RC-101
P = 0.0213
LPS treatment (min)
Human Macrophages
E
IL-6 mRNA
Mock
RC101
p = 0.0275
LPS treatment (min)
F
IFN-β mRNA
Mock
RC-101
p = 0.0002
LPS treatment (min)
G
TNF-α mRNA
Mock
RC-101
p = 0.0164
LPS treatment (min)
H
IL-1β mRNA
Mock
RC-101
p = 0.0079
LPS treatment (min)

Figure 2

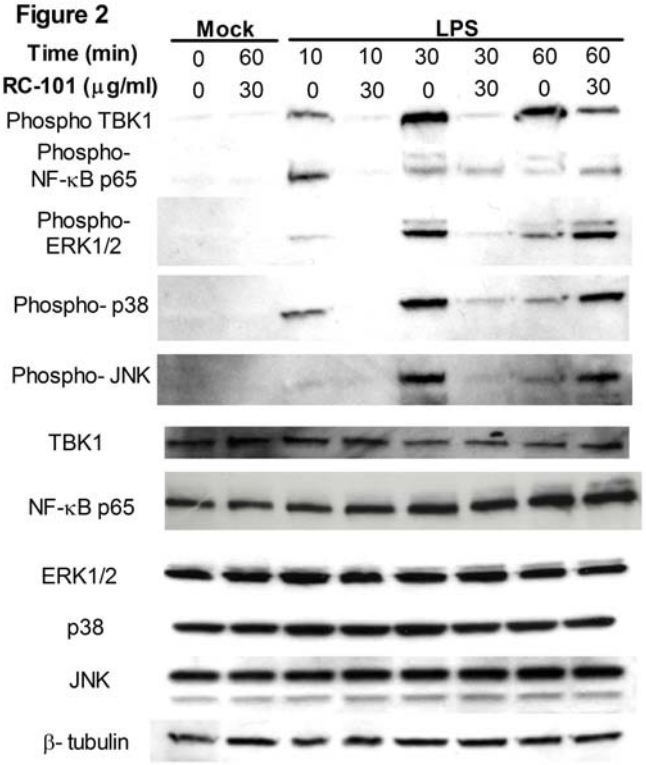
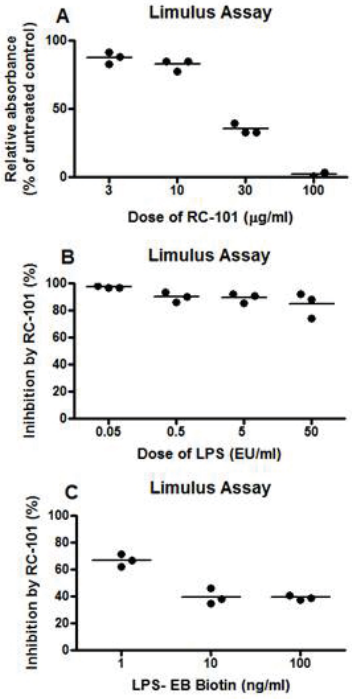

Mock
LPS
Time (min)
0 60 10 10 30 30 60 60
RC-101 (μg/ml)
0 30 0 30 0 30 0 30
Phospho TBK1
Phospho-NF-κB p65
Phospho-ERK1/2
Phospho- p38
Phospho- JNK
TBK1
NF-κB p65
ERK1/2
p38
JNK
β- tubulin

Figure 3


A
Limulus Assay
Relative absorbance (% of untreated control)
100
50
0
3
10
30
100
Dose of RC-101 (µg/ml)
B
Limulus Assay
Inihbition by RC-101 (%)
100
80
60
40
20
0
0.05
0.5
5
50
Dose of LPS (EU/ml)
C
Limulus Assay
Inhibition by RC-101 (%)
100
80
60
40
20
0
1
10
100
LPS- EB Biotin (ng/ml)

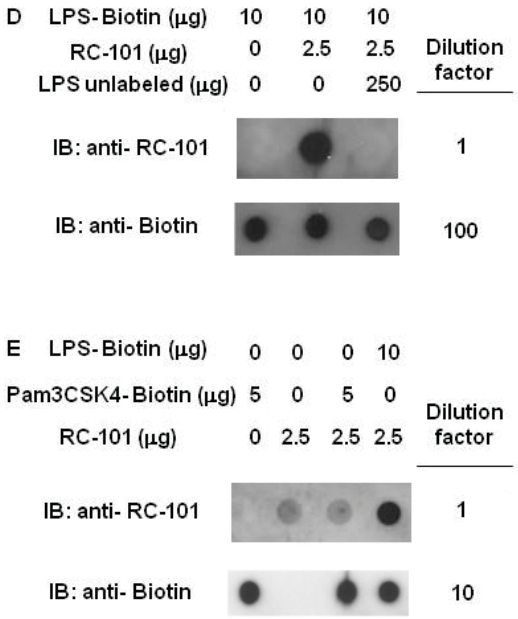

D
LPS-Biotin (µg) 10 10 10
RC-101 (µg) 0 2.5 2.5
LPS unlabeled (µg) 0 0 250
Dilution factor
IB: anti- RC-101
1
IB: anti- Biotin
100
E
LPS-Biotin (µg) 0 0 0 10
Pam3CSK4-Biotin (µg) 5 0 5 0
RC-101 (µg) 0 2.5 2.5 2.5
Dilution factor
IB: anti- RC-101
1
IB: anti- Biotin
10

Figure 4

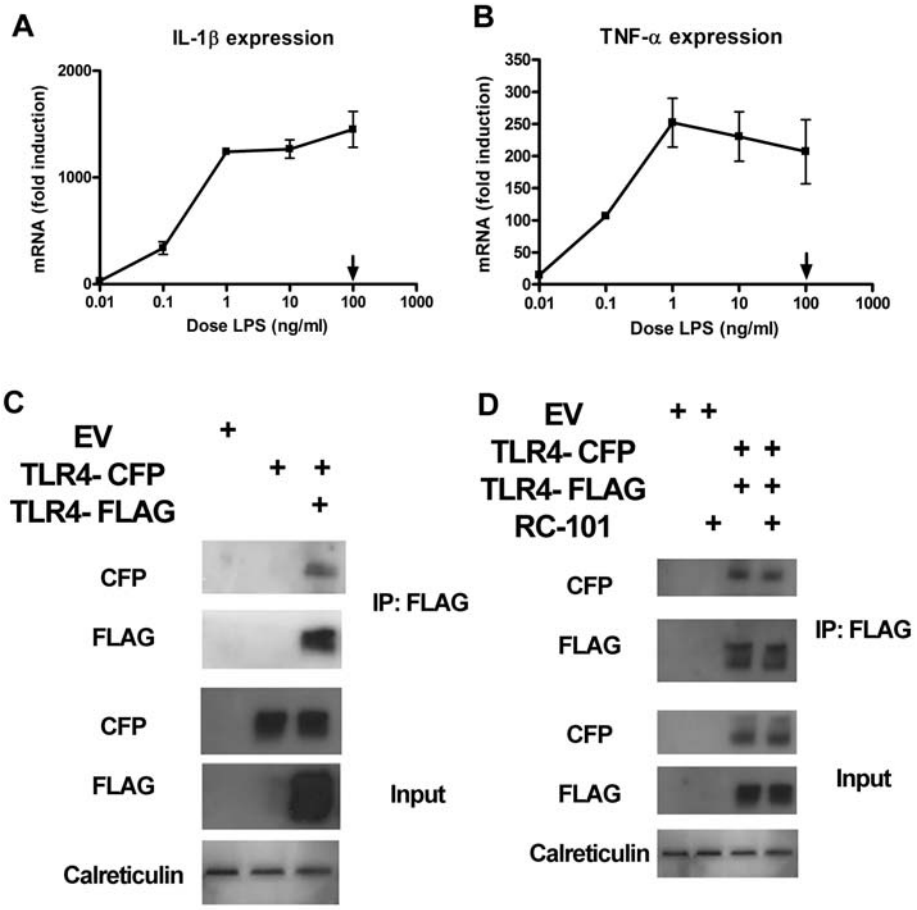

A
IL-1β expression
mRNA (fold induction)
2000
1000
0
0.01
0.1
1
10
100
1000
Dose LPS (ng/ml)
B
TNF-α expression
mRNA (fold induction)
350
300
250
200
150
100
50
0
0.01
0.1
1
10
100
1000
Dose LPS (ng/ml)
C
EV
TLR4- CFP
TLR4- FLAG
CFP
FLAG
IP: FLAG
CFP
FLAG
Input
Calreticulin
D
EV
TLR4- CFP
TLR4- FLAG
RC-101
CFP
FLAG
IP: FLAG
CFP
FLAG
Input
Calreticulin

Figure 5

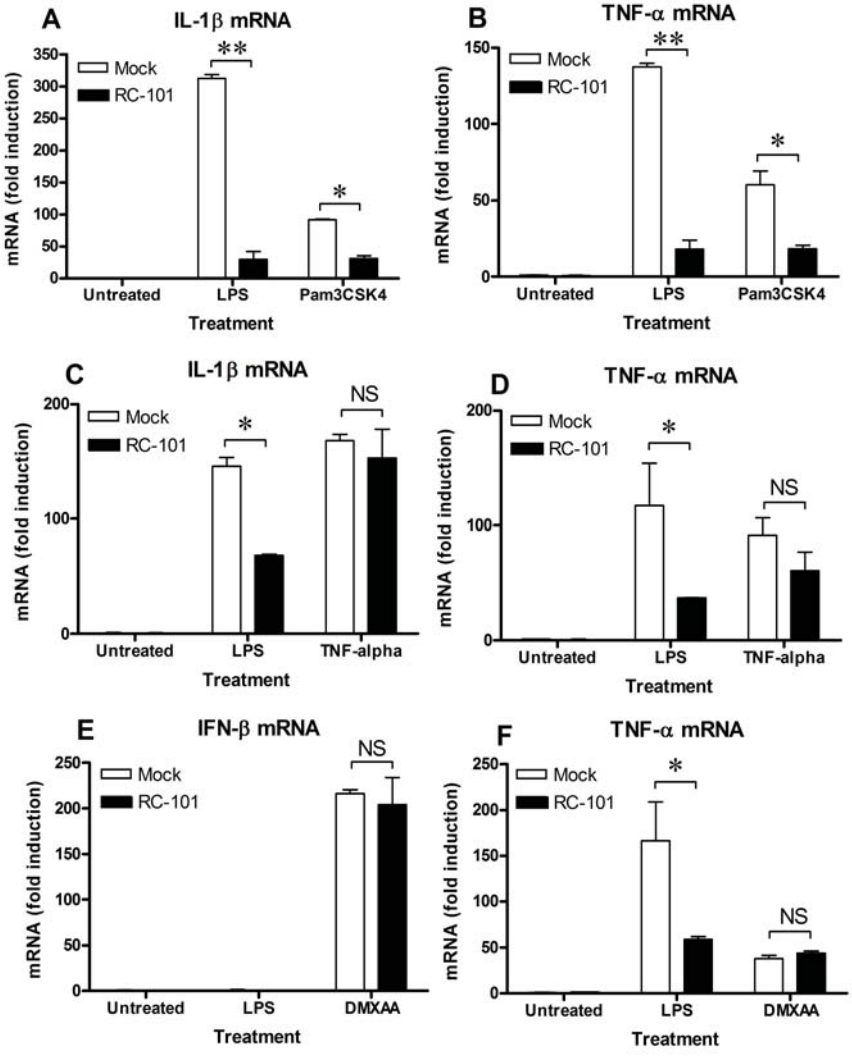

A
IL-1β mRNA
Mock
RC-101
mRNA (fold induction)
Untreated
LPS
Pam3CSK4
Treatment
B
TNF-α mRNA
Mock
RC-101
mRNA (fold induction)
Untreated
LPS
Pam3CSK4
Treatment
C
IL-1β mRNA
Mock
RC-101
NS
mRNA (fold induction)
Untreated
LPS
TNF-alpha
Treatment
D
TNF-α mRNA
Mock
RC-101
NS
mRNA (fold induction)
Untreated
LPS
TNF-alpha
Treatment
E
IFN-β mRNA
Mock
RC-101
NS
mRNA (fold induction)
Untreated
LPS
DMXAA
Treatment
F
TNF-α mRNA
Mock
RC-101
NS
mRNA (fold induction)
Untreated
LPS
DMXAA
Treatment

Figure 6

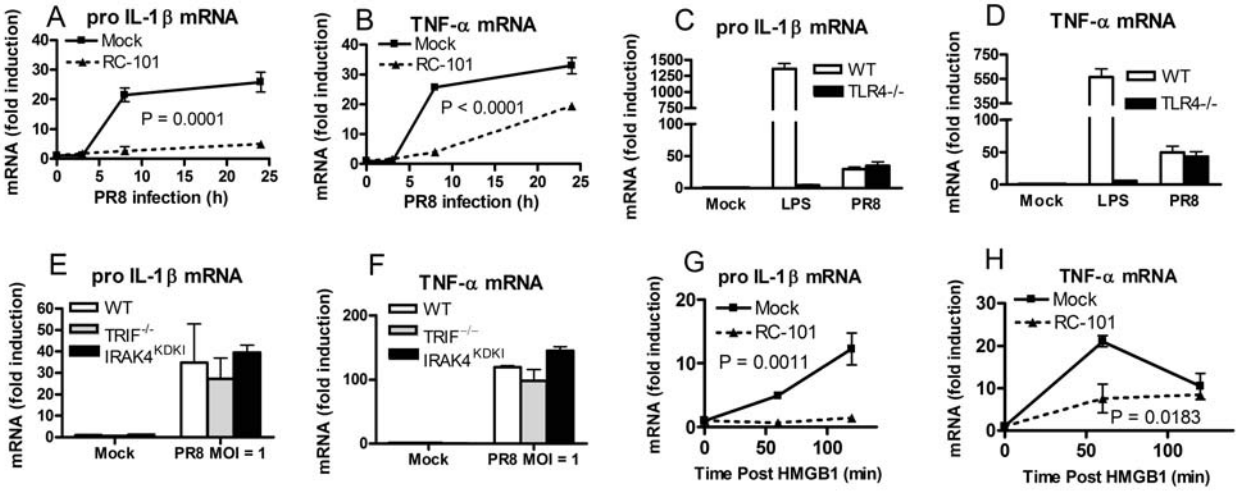
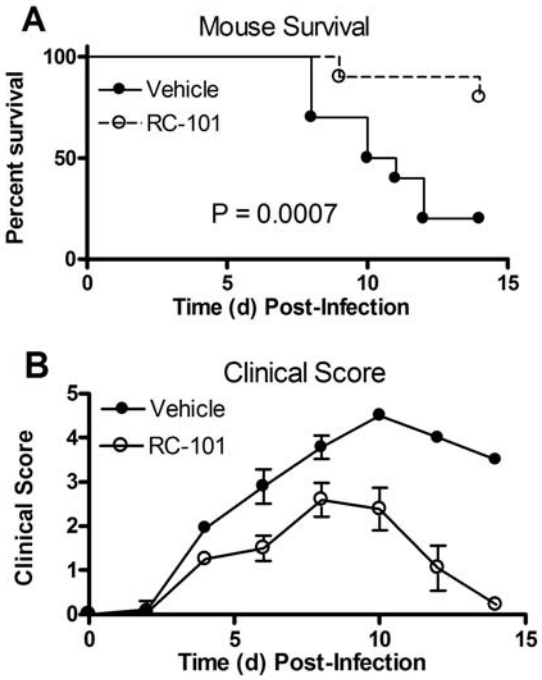

A
pro IL-1β mRNA
Mock
RC-101
P = 0.0001
mRNA (fold induction)
PR8 infection (h)
B
TNF-α mRNA
Mock
RC-101
P < 0.0001
mRNA (fold induction)
PR8 infection (h)
C
pro IL-1β mRNA
WT
TLR4-/-
mRNA (fold induction)
Mock
LPS
PR8
D
TNF-α mRNA
WT
TLR4-/-
mRNA (fold induction)
Mock
LPS
PR8
E
pro IL-1β mRNA
WT
TRIF-/-
IRAK4KDKI
mRNA (fold induction)
Mock
PR8 MOI = 1
F
TNF-α mRNA
WT
TRIF-/-
IRAK4KDKI
mRNA (fold induction)
Mock
PR8 MOI = 1
G
pro IL-1β mRNA
Mock
RC-101
P = 0.0011
mRNA (fold induction)
Time Post HMGB1 (min)
H
TNF-α mRNA
Mock
RC-101
P = 0.0183
mRNA (fold induction)
Time Post HMGB1 (min)

Figure 7


A
Mouse Survival
Percent survival
100
50
0
Vehicle
RC-101
P = 0.0007
0
5
10
15
Time (d) Post-Infection
B
Clinical Score
Clinical Score
5
4
3
2
1
0
Vehicle
RC-101
0
5
10
15
Time (d) Post-Infection