Biomaterials 274 (2021) 120845

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Macrophage-preferable delivery of the leucine-rich repeat domain of NLRX1 ameliorates lethal sepsis by regulating NF-κB and inflammasome signaling activation

Ja-Hyun Koo [a,b], Sang-Hun Kim [c], Soung-Hoo Jeon [a,b], Min-Jong Kang [c], Je-Min Choi [a,b,d,*]

[a] Department of Life Science, College of Natural Sciences, Hanyang University, Seoul, 04763, Republic of Korea
[b] Research Institute for Natural Sciences, Hanyang University, Seoul, 04763, Republic of Korea
[c] Section of Pulmonary, Critical Care and Sleep Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, 06520, USA
[d] Research Institute for Convergence of Basic Sciences, Hanyang University, Seoul, 04763, Republic of Korea

ARTICLE INFO

Keywords:
Sepsis
NLRX1
Cell penetrating peptide
Macrophage
NF-κB
Inflammasome

ABSTRACT

Sepsis is an acute systemic inflammatory disease triggered by bacterial infection leading organ dysfunctions that macrophages are responsible for major triggering of systemic inflammation. Treatment options are limited to antibiotics and drugs to manage the symptoms of sepsis, but there are currently no molecular-targeted therapies. Here, we identified a novel macrophage-preferable delivery peptide, C10, which we conjugated to truncated domains of NLRX1 (leucine-rich repeat region (LRR), and nucleotide binding domain (NBD)) to obtain C10-LRR and C10-NBD. Leucine rich amino acid of C10 enables macrophage preferable moieties that efficiently deliver a cargo protein into macrophages *in vitro* and *in vivo*. C10-LRR but not C10-NBD significantly improved survival in an LPS-mediated lethal endotoxemia sepsis model. C10-LRR efficiently inhibited IL-6 production in peritoneal macrophages via prevention of IκB degradation and p65 phosphorylation. In addition, C10-LRR negatively regulated IL-1β production by preventing caspase-1 activation with a sustained mitochondrial MAVS level. Finally, co-treatment with anti-TNFα antibody and C10-LRR had a synergistic effect in an LPS-induced sepsis model. Collectively, these findings indicate that C10-LRR could be an effective therapeutic agent to treat systemic inflammation in sepsis by regulating both NF-κB and inflammasome signaling activation.

1. Introduction

Sepsis affects more than 50 million people worldwide every year, potentially leading to 11 million deaths with around 20% mortality, with the highest mortality observed in intensive care units [1,2]. It is mainly triggered by bacterial infection, in which pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs) [3]. Macrophages are the main cell types that express PRRs. Upon ligand recognition by these receptors, NF-κB and inflammasome signaling pathways are activated to produce pro-inflammatory cytokines including TNFα, IL-1β, and IL-6 [4]. However, excessive production of these cytokines contributes to a severe inflammatory response and tissue damage [5,6]. Corticosteroids are used as immunosuppressive drugs to decrease this response, but they have many side effects and their efficacy in sepsis is controversial [7,8]. Although excessive inflammation and tissue malfunction are obvious pathologic problems of sepsis, antibiotics are the only reliable treatment. However, the efficacy of antibiotics decreases significantly with delays in treatment, after which symptom-managing approaches such as fluid boluses or vasopressors remain the only options [8,9]. Therefore, there is huge unmet need for therapy targeting macrophages in sepsis to effectively regulate excessive inflammation as soon as possible. For this reason, TNFα, IL-1R, and TLR4 are considered major targets for sepsis control [7]. However, inhibitors of these molecules have not been successful in clinical trials thus far, indicating that identification and investigation of other targets are required.

Nucleotide-binding oligomerization domain-like receptor family member X1 (NLRX1) is a mitochondrial protein initially identified as a negative regulator of antiviral responses by inhibiting mitochondrial antiviral-signaling protein (MAVS)-mediated production of type I interferon [10,11]. Later studies demonstrated that NLRX1 suppresses

* Corresponding author. Department of Life Science, Hanyang University, 222 Wangsimni-ro, Seongdong-gu, Seoul, 04763, Republic of Korea.
E-mail address: jeminchoi@hanyang.ac.kr (J.-M. Choi).

https://doi.org/10.1016/j.biomaterials.2021.120845
Received 4 December 2020; Received in revised form 17 April 2021; Accepted 18 April 2021
Available online 4 May 2021

IL-6 production of activated macrophages by regulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling; NLRX1 knock down (KD)-mice were more susceptible to LPS-mediated lethal sepsis than wild type (WT) mice [12,13]. In addition, NLRX1 knock-out (KO) mice produced more IL-1β in a chronic obstructive pulmonary disease (COPD) model via MAVS-dependent inflammasome regulation [14,15]. Based on these reports, we identified NLRX1 as a potentially potent negative regulator of innate immune responses due to its ability to regulate cytokine production and macrophage activation upon recognition of PAMPs like LPS.

In this study, we first identified the novel macrophage-preferable cell-penetrating peptide, C10, which efficiently and preferentially delivers cargo molecules into macrophages *in vitro* and *in vivo*. We then generated fusions proteins comprising C10 conjugated to the leucine-rich repeat (LRR) domain or nucleotide binding domain (NBD) of NLRX1 (C10-LRR and C10-NBD, respectively). C10-LRR treatment significantly improved survival in an LPS-mediated lethal endotoxemia mouse model, which was associated with reduced serum IL-6 and IL-1β levels. C10-LRR inhibited IL-6 and IL-1β production by negatively regulating IκB degradation and caspase-1 activation of inflammasome signaling. Furthermore, C10-LRR and TNFα neutralizing antibodies interacted synergistically, resulting in 100% recovery from sepsis in our mouse model. Our study demonstrates that C10-LRR can prevent both NF-κB and inflammasome activation and regulate the overwhelming inflammation associated with sepsis.

2. Materials and methods

2.1. Purification of recombinant proteins

6-His tagged recombinant proteins were expressed in *E. coli* and purified by Ni-NTA based affinity chromatography as described previously [16,17]. Briefly, bacterial pellets were suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and cell walls were disrupted by sonication. After centrifugation, the supernatant was incubated with Ni-NTA agarose (Qiagen) beads. Bound proteins were then washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), and desalted using a PD-10 Sephadex G-25 column (GE Healthcare). Bacterial endotoxins were further removed by Triton X-114 separation as described previously [16].

2.2. Mice

Mice (C57BL/6J) were maintained in a specific pathogen-free facility at Hanyang University. Animal experiments were approved by the Animal Experimentation Ethics Committee of Hanyang University. Experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Hanyang University.

2.3. Cell lines and cell culture

HeLa, THP-1, and Jurkat cells were purchased from ATCC and maintained in DMEM or RPMI (Corning). Media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator.

2.4. Isolation of primary cells from the spleens of mice

To isolate splenocytes, spleens was harvested, ground, and then filtered using a 40 μm cell strainer (SPL) to isolate single cells. To isolate peritoneal macrophages, 5 ml of PBS was injected into the mouse peritoneum and fluid was aspirated. Harvested cells were seeded in culture plates that were then incubated at 37 °C in a 5% CO_2 incubator for 2 h. After incubation, the supernatant was removed and cells were washed with PBS to eliminate unattached cells. Attached cells were considered to be peritoneal macrophages.

2.5. Flow cytometry

Jurkat, HeLa, or primary peritoneal macrophages (2×10^5 cells) were incubated with recombinant proteins in 96- or 12-well plates at 37 °C for 1 h in a 5% CO_2 incubator. After incubation, cells were harvested by centrifugation and washed twice with PBS. To remove proteins still attached to the cell membrane, cells were incubated with trypsin for 10 min at 37 °C and DMEM with 10% FBS was applied to neutralize the trypsin. Cells were then washed with PBS and analyzed by flow cytometry (FACS Canto II, BD Bioscience). Data were analyzed by Flowjo software (Tree Star, Ver. 10). Mouse splenocytes (1×10^6 cells per well) were seeded into 24-well plates and incubated with 5 μM recombinant proteins for 1 h. After incubation, cells were harvested and washed with PBS. Cells were stained with α-CD11c, MHCII, CD11b, F4/80, CD4, NK1.1, or CD19 (Biolegend, 1:1000 diluted). Intracellular fluorescence was analyzed by flow cytometry.

2.6. Microscopy

2×10^5 peritoneal macrophages were incubated with recombinant proteins in 6-well plates at 37 °C for 1 h in a 5% CO_2 incubator. After incubation, cells were washed three times with PBS and fixed in 4% paraformaldehyde phosphate buffer solution (Wako) for 10 min. Nuclei were stained with 0.01% Hoechst in PBS for 10 min and cells were washed twice with PBS. Five micrograms of CPP-dTomato was injected intraperitoneally into mice. After 2 h, mice were sacrificed, and blood vessels were perfused with 10 ml of PBS to eliminate remnant blood in tissues. Tissues were harvested, washed, and fixed in 4% paraformaldehyde. Fixed tissues were frozen in optimal cutting temperature compound. Frozen tissues were sliced to 7–10 μm thickness and stained with α-F4/80 or α-CD3 rat monoclonal antibody (Abcam, 1:200 diluted) and α-rat IgG Alexa Fluor 488 antibody (Invitrogen, 1:200 diluted). Nuclei were stained using 0.01% Hoechst 33342 in PBS for 10 min. Fluorescence signals in the cytoplasm and nucleus were analyzed using a C2si confocal microscope (Nikon) or fluorescence microscopy (Leica DMi8).

2.7. ELISA

Peritoneal macrophages were seeded in 96- or 12-well plates and incubated at 37 °C in a 5% CO_2 incubator for attachment. Floating cells were removed by washing with PBS and attached macrophages were stimulated with 1 mg/mL LPS (O55:B5, Sigma L2880) and then incubated with recombinant protein for 4 h. After stimulation, culture supernatants were collected for ELISA. To assess inflammasome activation, seeded macrophages were primed with 1 mg/ml of LPS for 4 h. After priming, cells were stimulated with 5 μM nigericin for 2 h. After stimulation, the culture supernatants were collected for ELISA. Blood was harvested by eye bleeding of mice. Serum was then isolated by centrifugation of the collected blood and analyzed using ELISA. Levels of IL-6, TNFα, and IL-1β in culture supernatants or serum were measured using ELISA kits (BioLegend) according to the manufacturer's instructions.

2.8. Western blot

Peritoneal macrophages or THP-1 cells were stimulated with 1 mg/ml of LPS (O55:B5, Sigma L2880) for 15 min to 5 h. These cells were then treated with nigericin and recombinant proteins such as C10-LRR for 1–2 h. Cells were then lysed with RIPA buffer (Cell Signaling Technology) on ice for 30 min. Protein amounts in lysates were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). After SDS-PAGE, proteins were transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with 5% skim milk in Tris-buffered saline

containing 0.1% Tween-20 (TBS-T) and incubated with primary antibodies including IκBα (Cell Signaling, 1:1000 diluted), pIκBα Ser32 (Cell Signaling, 1:1000 diluted), p65 (Cell Signaling, 1:1000 diluted), p65 Ser536 (Cell Signaling, 1:1000 diluted), PI3K p110β (Cell Signaling, 1:1000 diluted), pAKT Ser473, caspase (Adipogen, 1:1000 diluted), MAVS (Cell Signaling, 1:1000 diluted), NLRP3 (Adipogen, 1:1000 diluted), or VDAC (Cell Signaling, 1:1000 diluted) antibodies overnight at 4 °C. Membranes were washed with TBS-T and incubated with secondary antibody for 1 h at RT. Membranes were washed with TBS-T and EZ-Western Lumi Pico or Femto reagent (DoGen) were added as substrates. Band intensity was measured by Fusion-Solo software (Vilber). Relative intensity of IκB normalized to β-actin level by analyzing with densitometer of ImageJ (Ver. 1.49).

2.9. Cytotoxicity assay

1×10^5 peritoneal macrophages were seeded on 96-well plates and incubated with recombinant proteins including C10-LRR for 24 h. After incubation, 10 μl of cell counting kit-8 (CCK-8) reagent (Dojindo) was added to each well and absorbance at 450 nm was monitored every 30 min for 4 h using a microplate reader (iMark, Bio-Rad) to determine the number of live cells.

2.10. Isolation of mitochondrial fraction

Mitochondrial fraction was isolated using the Qproteome mitochondrial isolation kit (Qiagen) according to the manufacturer's instructions. Briefly, peritoneal macrophages were harvested in lysis buffer and then centrifuged at 1000 g for 10 min at 4 °C. Pellets were disrupted by 10 passages through a 23-gauge needle and then centrifuged at 1000 g for 10 min at 4 °C. The supernatant was further centrifuged at 8000 g for 15 min at 4 °C. The pellet was then washed and kept as the mitochondrial fraction.

2.11. Mouse sepsis model

Seven-to 8-week-old male or female C57BL/6 mice were primed by intraperitoneal injection of LPS (2 mg/kg) with co-injection of recombinant proteins (1 mg/kg) including C10-LRR. After 5 h, mice were challenged by intraperitoneal injection of LPS (5 mg/kg) and co-injection of recombinant proteins (1 mg/kg). α-IL-6, α-TNFα (Biolegend), or α-IL-1β neutralizing antibodies (Invitrogen) were then injected intraperitoneally. Survival rate was monitored for 7 days in addition to measuring weight changes. Blood was harvested 2 h or 24 h after challenge to analyze cytokine levels and AST activity.

2.12. Public single-cell RNA sequencing (scRNA-seq) data analysis

Public scRNA-seq data were downloaded from the Broad Institute Single Cell Portal (https://singlecell.broadinstitute.org/single_cell) using the identifier SCP548 (subject PBMCs) [18]. PBMC samples from three cohorts (healthy controls, $n = 19$; sepsis, $n = 4$; sepsis – ICU, $n = 8$) were analyzed.

2.13. Statistics

Data were analyzed using Prism 7 software (GraphPad). Significance was evaluated by Non-parametric Kruskal-Wallis test or two-way ANOVA. To generate survival curves, data were analyzed using the Mantel-Cox test. Results with *P*-values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Identification of a novel macrophage-preferable peptide, C10

In our previous study, the serendipitous discovery of a novel human origin cell-penetrating peptide (CPP) in cytokines like IL-1α [17] led us to further analyze the amino acid sequences of various human cytokines to identify other CPPs. We filtered all sequences using the support vector machines (SVM) score, which is the CPP score provided by the *in silico* CPP prediction tool CellPPD (available online: https://webs.iiitd.edu.in/raghava/cellppd/index.html) [19,20], and the ratios of arginine, lysine, or leucine in the sequences, as these are the most frequent amino acids in CPPs (Fig. 1a). Finally, three candidate CPPs were identified using this screening strategy: C10 (in IL-10), TP (in TSLP), and TB (in TGFβ). We designed DNA constructs encoding CPP candidate sequences with a 6xHis tag and enhanced green fluorescent protein (EGFP) (Fig. 1b). Recombinant proteins were expressed in *E. coli* and purified by affinity chromatography (Fig. 1c). To examine the cell-penetrating efficiency of the recombinant CPP proteins, we incubated CPP-EGFP proteins with Jurkat cells for 1 h. Intracellular fluorescence intensity of C10- and TP-EGFP treated cells was significantly higher than that of PBS, TB-EGFP, or EGFP-treated cells, but lower than that of the TAT-EGFP signal (Fig. 1d). Surprisingly, in HeLa cells, delivery efficiency of C10 was superior to that of the other CPP-EGFPs (Fig. 1e), implying a cell-type preference for C10. Next, we examined the cell type preference of C10 in isolated mouse splenocytes (Fig. 1f). C10 showed much stronger EGFP delivery efficiency than TAT in macrophages and its delivery efficiency was comparable to that of dNP2, a previously identified superior cell-penetrating peptide [21], in macrophages (Fig. 1g). However, C10-mediated intracellular delivery to lymphoid cells was poor (Fig. 1h). The delivery efficiency of C10 was greater than that of dNP2 and TAT in macrophages (Fig. 1i and j), but not neutrophils (Fig. 1k). From these results, we concluded that C10 had macrophage-preference with regard to intracellular delivery. Next, we analyzed the *in vivo* distribution of C10 in mice. C10-dTomato was injected intraperitoneally and frozen-sectioned tissues were stained with anti-F4/80 antibody and analyzed by microscopy (Fig. 1l). C10-dTomato signal was mostly co-localized with $F4/80^+$ macrophages in various organs with a significantly stronger intensity than that obtained when TAT was used for delivery (Fig. 1m), indicating that C10 can efficiently deliver cargo molecules into macrophages *in vivo*. These results collectively demonstrate that we identified a novel macrophage-preferable CPP, C10 from human IL-10, which is able to specifically deliver a cargo protein to macrophages *in vivo*.

3.2. Generation of NLRX1 protein domains conjugated with C10 for macrophage-preferred targeting

NLRX1 negatively regulates both NF-κB signaling and inflammasome activation [12–14]. Based on analysis of public single-cell RNA sequencing (scRNA-seq) data (PBMC samples from three cohorts (healthy control, $n = 19$; sepsis, $n = 4$; sepsis – ICU, $n = 8$) [18]), we identified significant down-regulation of NLRX1 in sepsis patients relative to healthy controls (Fig. 2a). We hypothesized that regulation of both NF-κB and inflammasome signaling by utilizing NLRX1 and C10 would allow effective treatment of sepsis-associated inflammation (Fig. 2b). To test this hypothesis, we designed DNA constructs encoding two domains of NLRX1 (LRR, NBD) conjugated to C10; EGFP was used as a control. All proteins were expressed in *E. coli* and purified by affinity chromatography (Fig. 2c) as described previously [16,17]. In mouse peritoneal macrophages, LRR proteins were efficiently delivered by C10, but not by TAT, suggesting that C10 is required for macrophage-specific delivery (Fig. 2d). C10-LRR was localized in the cytoplasm and even in the nucleus, demonstrating diffusion in the cells after endocytosis (Fig. 2e). These results indicated that C10-conjugated NLRX1 proteins were successfully delivered into primary macrophages.

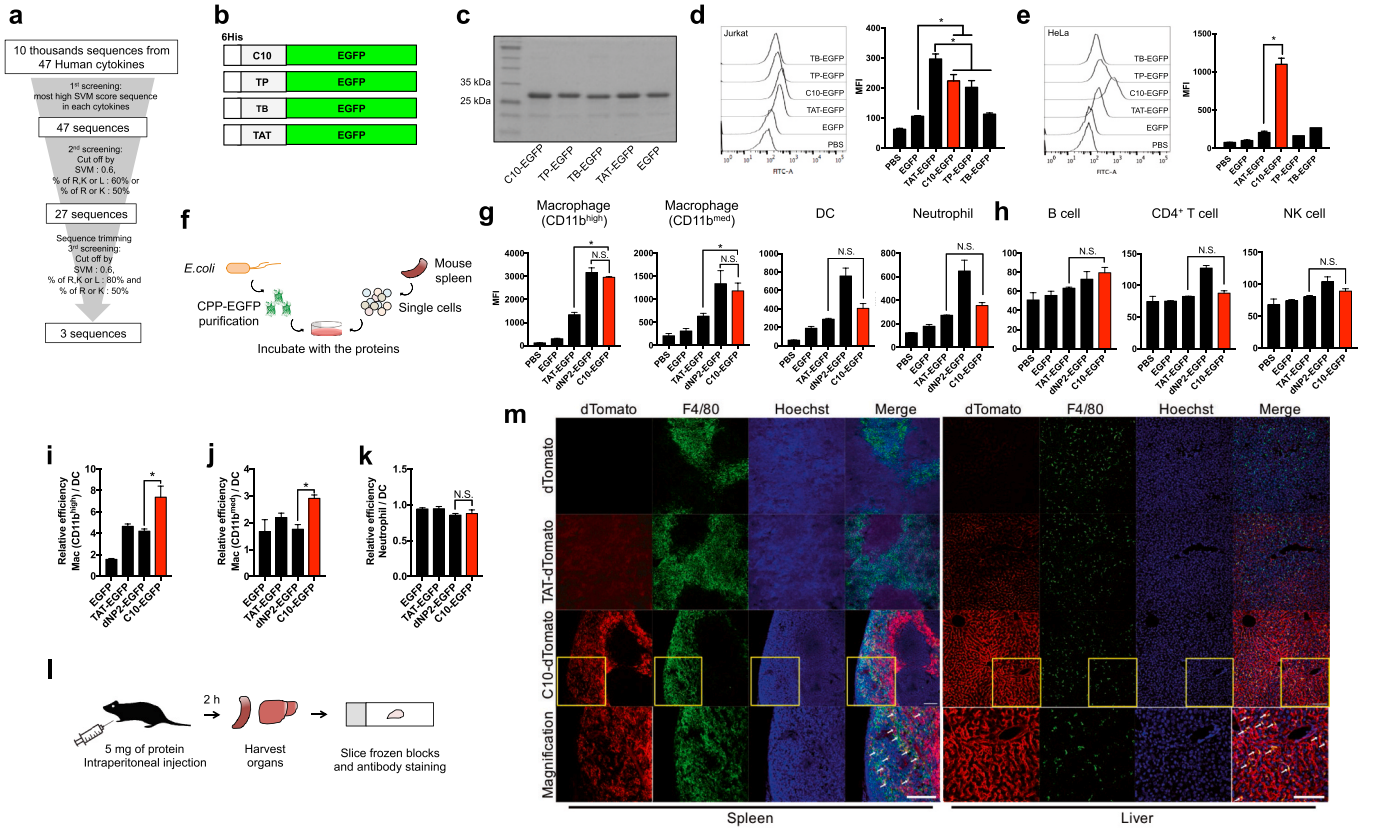

Fig. 1. Identification of a human IL-10-derived peptide C10 with target specificity for macrophages. (a) Screening for CPP candidates in human cytokines. CPP candidate sequences were identified using SVM score and the percentages of arginine, leucine, or leucine in candidate sequences by *in silico* screening. (b) Structure of candidate sequence-conjugated EGFP and TAT-EGFP. (c) SDS-PAGE of purified proteins. (d) Intracellular delivery efficiency in Jurkat or (e) HeLa cells was analyzed after incubation with 5 μM of recombinant protein for 1 h. Intracellular fluorescence was analyzed by flow cytometry. (f) Experimental scheme of protein delivery into splenocytes. Single cells were isolated from the spleen and incubated with 5 μM recombinant protein for 1 h. (g) Splenocytes were classified as $CD11b^{high}F4/80^{+}$ macrophages, $CD11b^{med}F4/80^{+}$ macrophages, $MHCII^{high}CD11c^{+}$ classic DCs, $Ly6G^{+}$ neutrophils as myeloid cells or (h) $CD19^{+}$ B cells, $CD4^{+}$ T cells, and $NK1.1^{+}$ cells as lymphoid cells. Intracellular fluorescence was analyzed by flow cytometry. (i) Relative intracellular delivery efficiency into $CD11b^{high}$ macrophages, (j) $CD11b^{med}$ macrophages, or (k) neutrophils. (l) Experimental scheme of *in vivo* tissue localization of C10-dTomato. 5 mg of the proteins were injected intraperitoneally into mice and tissues were harvested after 2 h. (m) Fluorescence signals in the tissues were analyzed by confocal microscopy. *Error bars indicate S.D.* $*P < 0.05$. *N.S., not significant. MFI, median fluorescence intensity. Scale bar indicates* 100 μm.

3.3. C10-LRR ameliorates lethal sepsis and decreases levels of pro-inflammatory cytokines

Next, we examined the *in vivo* functional effects of C10-LRR treatment in an LPS-induced lethal sepsis mouse model. We used a prime/challenge LPS injection model as a lethal sepsis model to induce caspase-11- and caspase-1-mediated inflammasome signaling [22,23] based on a recent study that demonstrated that inflammasome signaling and pyroptosis are crucial factors in the initial stage of sepsis and sepsis-associated mortality [24]. Mice were primed with 2 mg/kg of LPS via intraperitoneal injection and then challenged with 5 mg/kg of LPS after 5 h. Proteins (1 mg/kg of C10-LRR, -NBD, -EGFP) or PBS were also injected at the same time as the LPS was injected, and mice were monitored (Fig. 3a). C10-LRR-treated mice, but not C10-NBD or -EGFP treated mice, showed significantly improved survival (Fig. 3b) and weight recovery 48 h after the last LPS challenge, implying successful regulation of abnormal acute inflammation and physiology (Fig. 3c). 2 h after the LPS challenge, serum IL-1b levels were significantly lower in C10-LRR treated mice than the other mouse groups (Fig. 3d), which indicated inhibition of IL-6 production 24 h post-LPS challenge (Fig. 3e). Levels of the enzyme AST in the blood increased during LPS challenge, presumably due to liver toxicity; however, C10-LRR treatment prevented elevation of AST levels in C10-LRR-treated mice with levels comparable to those in sham mice (Fig. 3f). The effects of C10-LRR treatment in our LPS-induced sepsis model were dose-dependent (Fig. 3g). Collectively, these results suggest that C10-LRR can treat LPS-induced sepsis in mice via regulation of both IL-1β and IL-6 levels.

3.4. C10-LRR inhibits NF-κB signaling and IL-6 production in macrophages

Based on the reduced serum IL-6 level in C10-LRR-treated mice upon LPS challenge, we examined its functions in macrophages *in vitro*. Murine peritoneal macrophages were isolated and then co-incubated with LPS and C10-LRR, C10-NBD, or C10-EGFP for 4 h. IL-6 in the culture supernatant was significantly reduced in C10-LRR but not -NBD or -EGFP-treated cells in a dose-dependent manner (Fig. 4a). TNFα level was not significantly different among groups (Fig. 4b) and there were no significant differences in toxicity among groups (Fig. 4c). Pre-treatment with C10-LRR significantly inhibited IκB degradation upon LPS stimulation (Fig. 4d). These results were confirmed in human macrophage cells, namely THP-1 cells stimulated with phorbol 12-myristate 13-acetate (PMA); C10-LRR treatment inhibited IL-6 production (Fig. 4e) but not that of TNFα (Fig. 4f). Activation of NF-κB signaling including phosphorylation of IκB Ser32, degradation of IκB, and phosphorylation of p65 Ser536 were significantly attenuated by C10-LRR treatment (Fig. 4g). Collectively, these results suggest that C10-LRR is a negative regulator of NF-κB signaling upon LPS challenge of macrophages, which

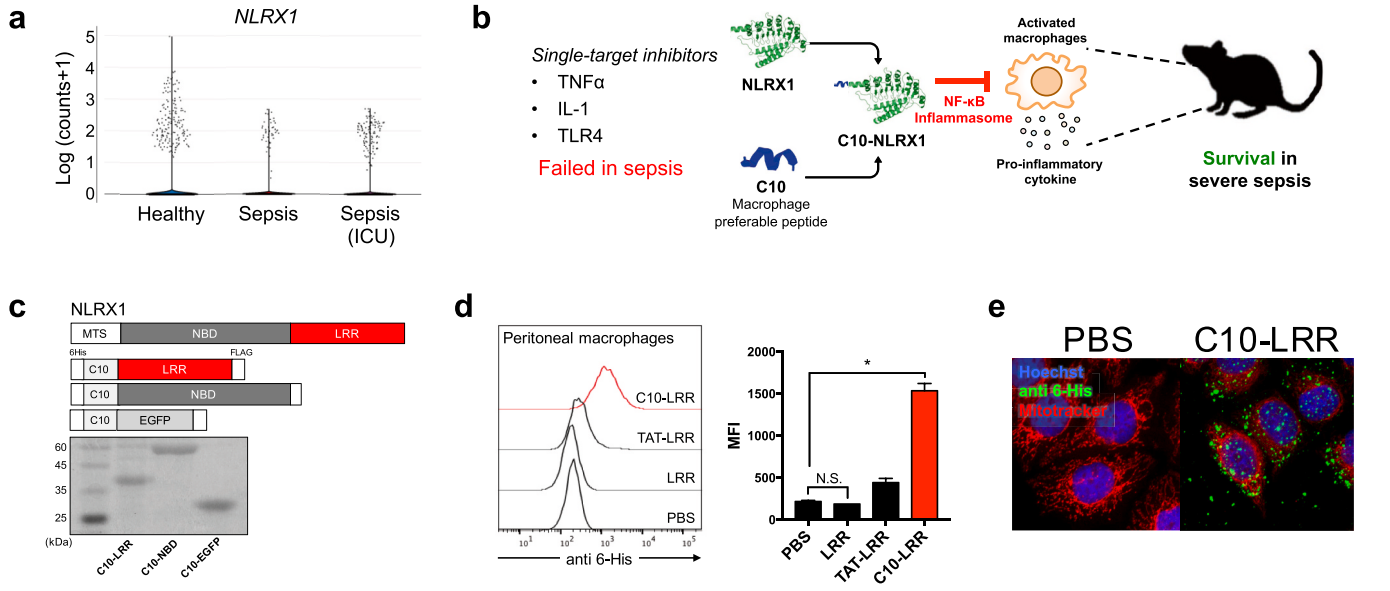

Fig. 2. Generation of a C10-conjugated NLRX1-LRR fusion protein and its intracellular delivery into macrophages. (a) Violin plot of NLRX1 expression in PBMC samples from healthy or sepsis patients. scRNA-seq data were from Reyes et al., 2020 and the plots were created using the Single Cell Portal (https://singlecell.broadinstitute.org/single_cell). (b) Research hypothesis. (c) Structure of DNA constructs encoding recombinant proteins. Purified proteins were analyzed by 12% SDS-PAGE. (d) Intracellular delivery efficiency in peritoneal macrophages was analyzed after incubation with 2 μM of recombinant protein for 1 h. (e) Intracellular localization of C10-LRR in HeLa cells. Fluorescence was visualized by confocal microscopy. *Error bars indicate S.D. *P < 0.05. N.S., not significant.*

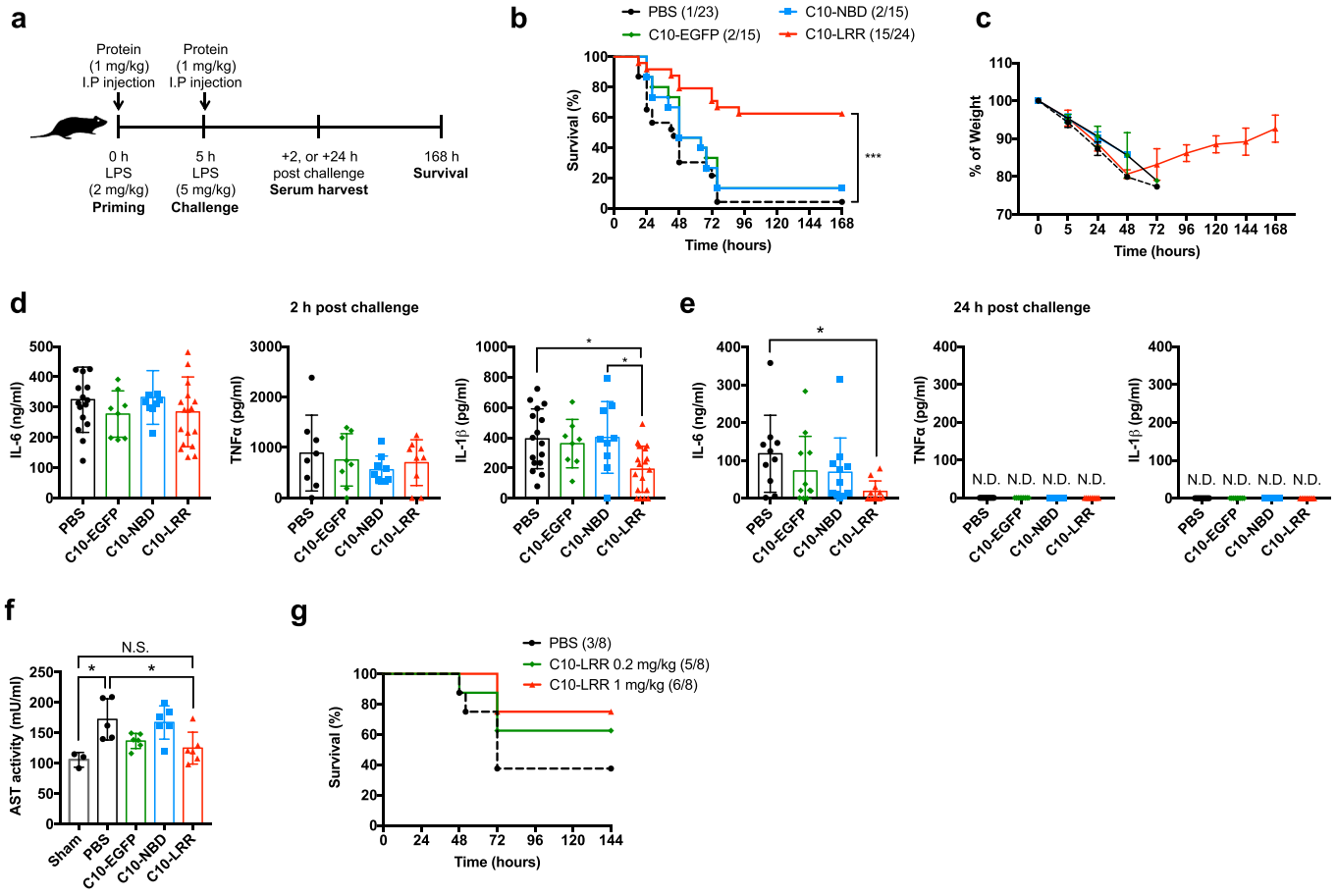

Fig. 3. Systemic administration of C10-LRR ameliorates lethal sepsis. (a) The sepsis model scheme. (b) Survival rate and (c) weight changes are summarized. (d) 2 h post-LPS challenge, serum IL-6, TNFα, and IL-1β levels were evaluated by ELISA. (e) 24 h post LPS challenge, serum IL-6 level and (f) AST activity were evaluated. (g) Survivals rate of C10-LRR injected mice treated with 0.2 mg/kg or 1 mg/kg of C10-LRR. *Error bars indicate S.D. *P < 0.05, and ***P < 0.001. N.S., not significant.*

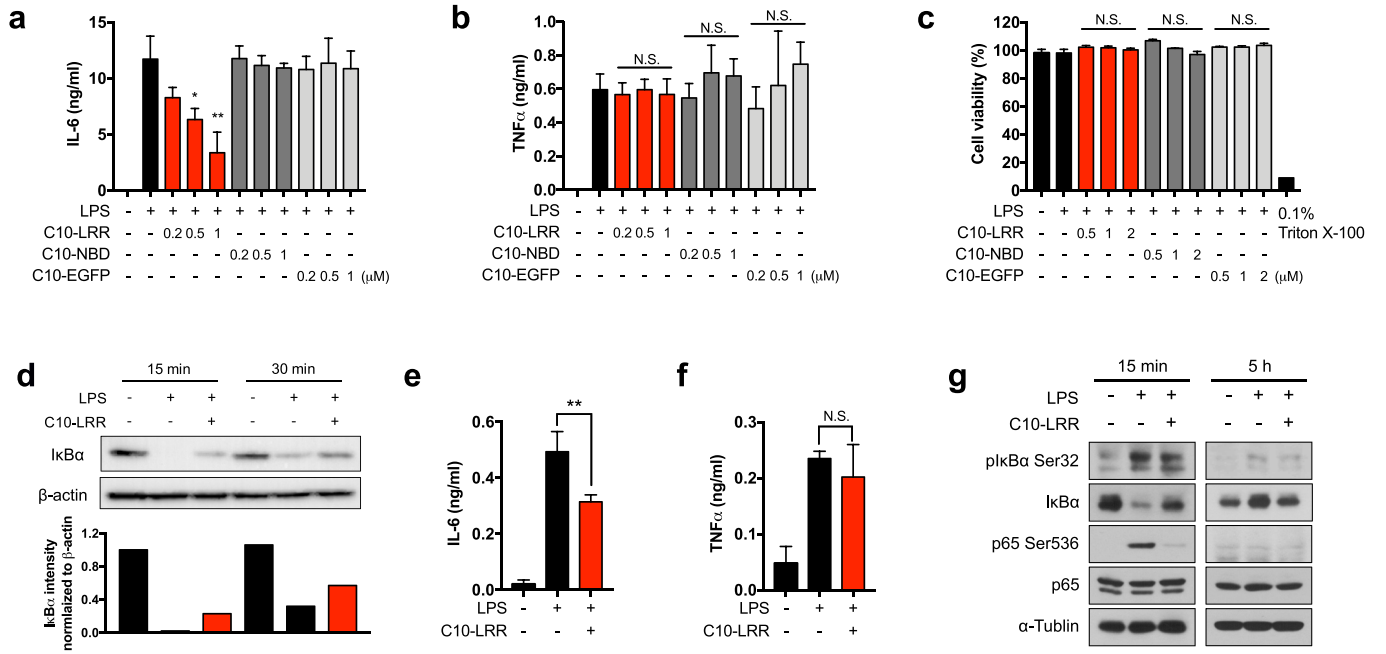

Fig. 4. C10-LRR inhibits NF-κB activation of macrophages. (a) Peritoneal macrophages were incubated with the indicated concentrations of each protein for 4 h with LPS stimulation. (a) IL-6 or (b) TNFα levels in cultured supernatant were evaluated by ELISA. (c) Peritoneal macrophages were incubated with the indicated concentrations of recombinant proteins for 24 h with LPS stimulation. Cellular viability was analyzed by CCK-8 assay. (d) Peritoneal macrophages were pre-incubated with 1 μM of C10-LRR for 30 min and stimulated with LPS for the indicated times. Cells were lysed and cellular protein levels were analyzed by Western blot. (e) PMA-stimulated THP-1 cells were incubated with the indicated concentration of each protein for 4 h with LPS stimulation. IL-6 or (f) TNFα levels in culture supernatant were evaluated by ELISA. (g) THP-1 cells were pre-incubated with 1 μM C10-LRR for 30 min and stimulated with LPS for the indicated time. Cells were lysed and cellular protein levels were analyzed by Western blot. *Error bars indicate S.D. *P < 0.05, and **P < 0.01. N.S., not significant.*

inhibits IL-6 production.

3.5. C10-LRR inhibits inflammasome signaling and IL-1β production in macrophages

To determine whether reduced serum IL-1β expression in our mouse sepsis model was related to regulation of inflammasome activation by C10-LRR, we stimulated peritoneal macrophages with LPS and nigericin. After LPS priming, nigericin treatment significantly increased IL-1β levels in macrophages; however, only C10-LRR treatment inhibited IL-1β significantly compared to control groups (Fig. 5a). Next, we designed three different protein treatment schemes, including treatment in the priming step (NF-κB activation), stimulation step (inflammasome activation), or both steps (Fig. 5b). We utilized mouse peritoneal macrophages (Fig. 5c) and embryonic fibroblast cells (Fig. 5d) and found that IL-1β production induced by LPS and nigericin treatment was significantly reduced by C10-LRR treatment in a dose-dependent manner, presumably due to the inhibition of both NF-κB and inflammasome signaling. This result prompted us to examine the level of caspase-1 p20, which can cleave the N-terminal of pro-IL-1β to generate a secretable form of mature IL-1β. Level of caspase-1 p20 was significantly decreased upon C10-LRR treatment in a dose-dependent manner (Fig. 5e). In previous study, MAVS was reported to regulate the NLRP3-inflammasome in the outer membrane of the mitochondria [25]. Therefore, we examined NLRP3 and MAVS levels in mitochondria and cytoplasm. Upon nigericin stimulation, mitochondrial MAVS level increased significantly, but C10-LRR treatment almost completely prevented MAVS expression or localization in mitochondria. Levels of both MAVS and NLRP3 in whole cell lysate were also significantly reduced by C10-LRR treatment (Fig. 5f). Together, these results suggest that C10-LRR negatively regulates inflammasome signaling and IL-1β production.

3.6. C10-LRR and TNFα neutralizing antibody co-treatment synergistically ameliorate sepsis

As C10-LRR negatively regulated IL-6 and IL-1β production but not that of TNFα upon LPS challenge, we hypothesized that C10-LRR co-treatment with a TNFα neutralizing antibody (anti-TNFα Ab) would have a synergistic effect. In mice challenged twice with LPS (Fig. 6a), C10-LRR or anti-TNFα Ab treatment significantly improved survival rate (Fig. 6b). Treatment of these mice with C10-LRR and/or anti-TNFα Ab resulted in significantly faster weight recovery than in the control group (Fig. 6c). As a semi-therapeutic treatment scheme, C10-LRR and/or anti-TNFα Ab were administered at the time of challenge with LPS (Fig. 6d). Compared to treatment with either of these agents alone, co-treatment with C10-LRR and anti-TNFα Ab resulted in 100% survival (Fig. 6e) and weight recovery (Fig. 6f) from systemic lethal endotoxemia, suggesting C10-LRR and TNFα neutralizing antibodies have a synergistic effect in protecting against LPS-induced sepsis.

Our results indicate that recombinant C10-LRR is efficiently delivered into macrophages and negatively regulates production of IL-1 and IL-6 via regulation of NF-κB and inflammasome signaling, which leads to successful inhibition of systemic inflammation associated with LPS-induced sepsis (Fig. 6g). Thus, C10-LRR and anti-TNFα Ab combination treatment in addition to antibiotic treatment is a promising approach for the treatment of sepsis (Fig. 6h).

4. Discussion

Although antibiotics are widely used to treat sepsis, there are as yet no effective drugs that can target molecular pathways downstream of pathogen receptor signaling to control inflammation. Single-target inhibitors have not shown effectiveness in sepsis [7], but nanoparticles (multiple pro-inflammatory cytokine blocker and endotoxin neutralizer), TLR inhibitory peptide and super-repressor IκBα have been reported to be effective in preventing organ damage in animal models of

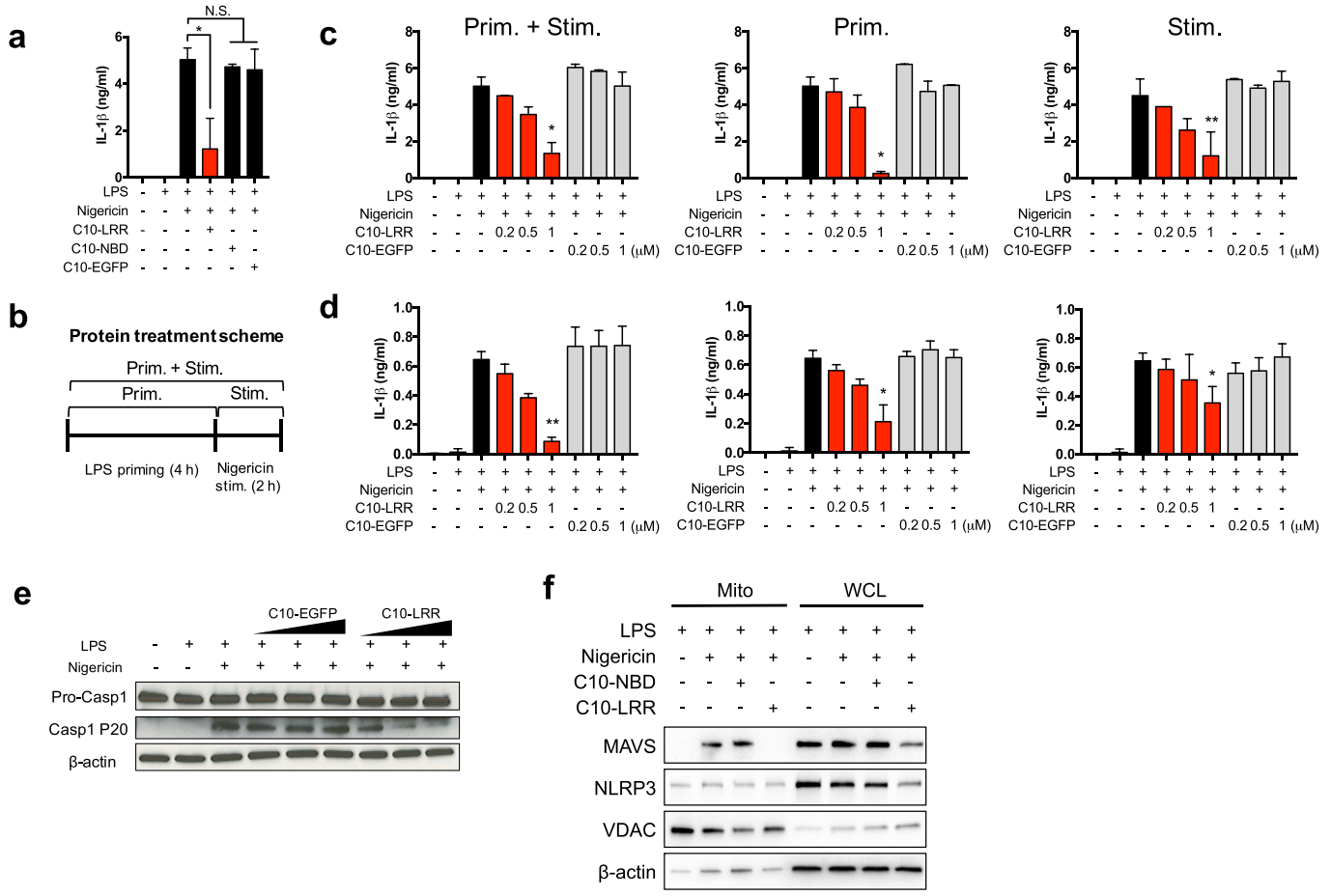

Fig. 5. C10-LRR inhibits NLRP3-inflammasome activation of macrophages. (a) Peritoneal macrophages were incubated with proteins during LPS priming for 4 h and nigericin stimulation for 2 h. IL-1β levels in culture supernatant were evaluated by ELISA. (b) Protein treatment scheme of inflammasome activation in peritoneal macrophages. (c) Peritoneal macrophages or (d) murine embryonic fibroblasts (MEF) were incubated with recombinant proteins during LPS priming for 4 h and nigericin stimulation for 2 h. (e) LPS-primed peritoneal macrophages were incubated with proteins during nigericin stimulation for 1 h. Whole cells were lysed or the (f) mitochondrial fraction was isolated. Protein levels were analyzed by Western blot. *Error bars indicate S.D. *P < 0.05, and **P < 0.01. N.S., not significant.*

sepsis [26–29]. NF-κB signaling mediators have been intensively studied as targets to regulate inflammation in sepsis [30]. More recently, inflammasome activation by DAMPs or PAMPs has been demonstrated to cause pyroptosis and blood coagulation during inflammation [24,31,32]. Activation of the inflammasome induces HMGB1 signaling, which can activate NF-κB signaling, and crosstalk between these two signaling pathways has been suggested to result in hyper-inflammation [33,34]. Unfortunately, molecular-targeted therapy based on these signaling pathways has not yet been approved in clinical trials, but the therapeutic efficacy of inhibiting those signaling pathways has been reported in animal models of sepsis [28,35]. We propose here that a single drug that can control both NF-κB and inflammasome signaling would be more efficient at successfully inhibiting systemic inflammation and facilitating recovery from sepsis than using a drug that targets only one of these signaling pathways.

NLRX1 has several domains: a mitochondrial targeting sequence (MTS), a nucleotide binding domain (NBD), and a leucine rich repeat (LRR) [10]. In previous studies, LRR was reported to be important for regulation of NF-κB signaling upon LPS stimulation [12,13]. LPS triggers MyD88/TRAF6 dependent NF-κB activation which leads inflammatory gene expressions. IκBα degradation is a key process for nuclear entry of NF-κB transcription factor. p65 is a NF-κB subunit which should be phosphorylated to be localized into the nucleus and it leads transcription of inflammatory genes such as IL-6 and TNFα [36]. In this study, only IL-6 production was affected by NLRX1-LRR, not TNFα production despite regulation of NF-κB. A possible reason for specific regulation of IL-6 production is that C10-LRR interacts physically with PI3K. Although there are several NF-κB binding sites such as, κ1, κ2, κB1, and κB2 in the promoter region of human and murine TNFα [37,38], deletion or site-directed mutation of those binding sites [39,40] or deletion of NF-κB-regulated genes including p50, p52, cRel, and RelB [41,42] had little or no effect on TNFα transcription and expression. This suggests fine-tuned molecular regulation by NF-κB of target genes. Because transcription of pro-IL-1β was inhibited by C10-LRR-mediated regulation of NF-κB, IL-1β production by macrophages could be regulated independently from the inflammasome. Recent studies reported that NLRX1 regulated NLRP3-inflammasome activation and IL-1β production [14,15]. Because NLRP3 could be regulated by interaction with MAVS [25,43], interaction between NLRX1 and MAVS has been proposed to regulate the NLRP3-inflammasome. In this study, we observed sustained mitochondrial MAVS levels upon C10-LRR treatment during LPS and nigericin activation, suggesting that C10-LRR can inhibit MAVS-mediated NLRP3 inflammasome activation.

Excessive production of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 IL-12, IFNγ, and IL-18 is a key feature of sepsis [5,6,9]. These cytokines can induce tissue damage, vasodilation with low blood pressure, and increased blood vessel permeability. This unusual production of cytokines is called a “cytokine storm” or “cytokine release syndrome (CRS)”. Among these cytokines, TNFα was first targeted to treat sepsis. Anti-TNFα, anti-TNFR antibodies, and TNFα inhibitors were effective in an animal model [44] but unfortunately not in human patients, and clinical Phase II or III studies failed [45,46]. One of the reasons proposed

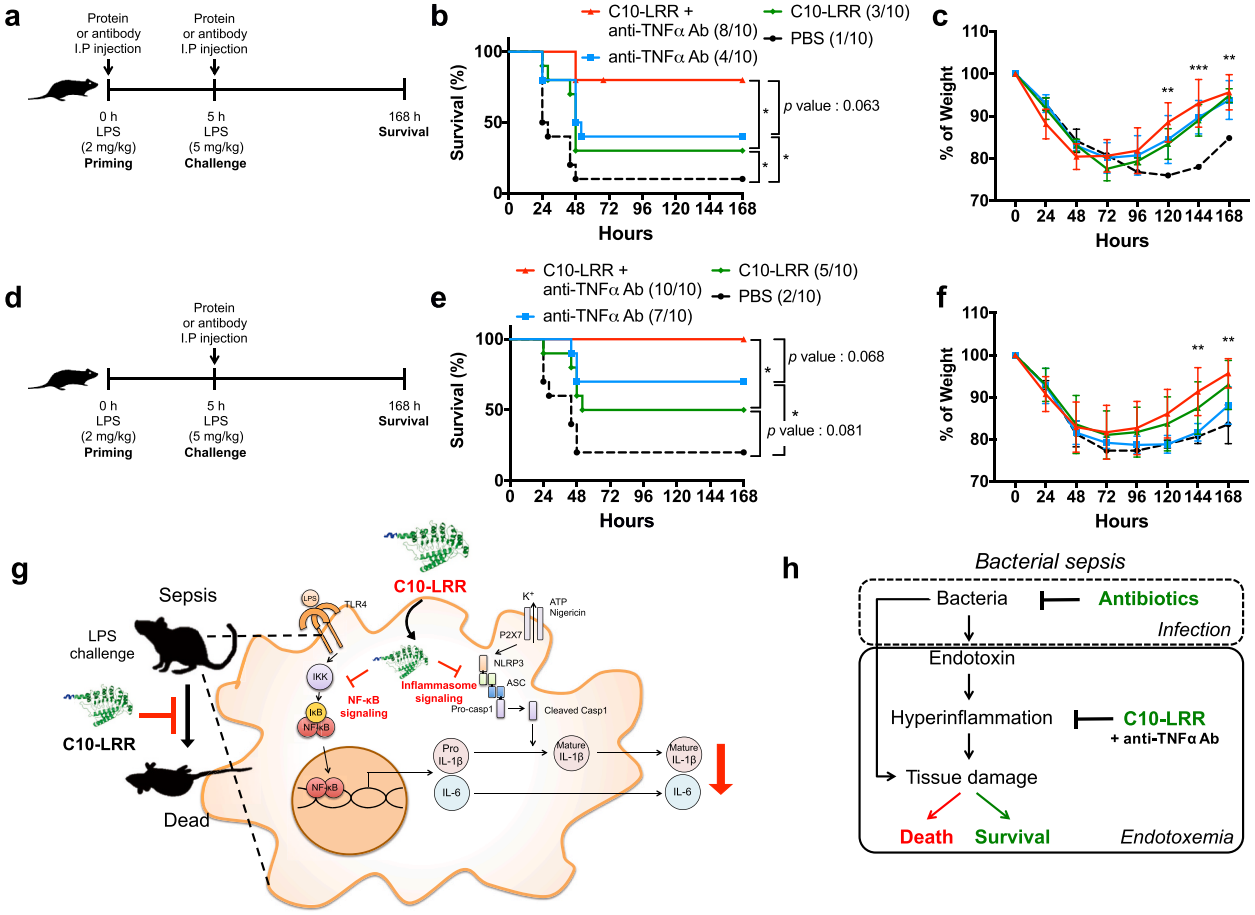

Fig. 6. Administration of TNFα neutralizing antibodies synergistically ameliorates lethal sepsis. (a) Co-treatment of a mouse model of lethal sepsis with C10-LRR and TNFα neutralizing antibody (anti-TNFα Ab). Experimental scheme, (b) survival rate, and (c) weight changes are summarized. (d) Semi-therapeutic treatment scheme of C10-LRR protein or anti-TNFα antibody in lethal sepsis. Experimental scheme, (e) survival rate, and (f) weight change are summarized. (g) Summary of the research. (h) Mechanism of sepsis and our suggesting strategy to treat sepsis. *S.D. *P < 0.05, **P < 0.01, and ***P < 0.001.*

to explain this discrepancy is that TNFα is produced in the very early stage of sepsis but is not significant when the patient is diagnosed. Anakinra is an IL-1R antagonist that also failed to treat sepsis in a Phase III study [47]. In a recent study, however, the efficacy of Anakinra in sepsis patients with macrophage activation syndrome (MAS) was re-analyzed and Anakinra was shown to improve the survival rate of these patients [48]. Toclizumab is an anti-IL-6R antibody and there is Phase II study of this antibody in MAS, but not sepsis [49]. Recent clinical trials suggest blockade of pro-inflammatory cytokines or their signaling could be used to treat sepsis in patients with MAS. C10-LRR is a promising candidate for this purpose. Although IL-6 inhibition by prevention of NF-κB signaling might be concerned by side effects of long-term treatment such as infection or cancer, for life threating urgent condition of sepsis, it is critical to be treated as early as possible to control overwhelmed inflammation.

In previous studies, CPPs were shown to deliver cargo non-specifically into various cell types. We reported that CPPs can deliver cargo molecules into myeloid cells more efficiently than into lymphocytes [50]. In this study, we found that the amino acid sequence 'C10' from human cytokine IL-10 results in delivery of cargo molecules into macrophages in preference to other cell types. Most known CPPs contain positively charged amino acids that bind to negatively charged molecules on cell membranes such as heparan sulfate proteoglycans (HSPGs) [51]. Unlikely other CPPs, Xentry (LCLRPVG) is not positive charge rich but hydrophobic, and has been shown to have significant delivery efficiency in adherent cell lines [52]. Similarly, C10 can deliver cargo molecules with better efficiency in adherent cells like macrophages, HeLa, HaCaT, or NIH3T3 cells than suspension-type cells like T cells. Leucine could increase preference for adherent cells; this should be investigated in future studies.

5. Conclusions

We previously demonstrated a potential role for NLRX1 (dNP2-LRR) in regulating T cell function and experimental autoimmune encephalomyelitis (EAE) [16]. Here, we firstly proved the ability of a C10-conjugated LRR fusion protein to modulate macrophage-mediated NF-κB signaling and inflammasome activation. C10-LRR can potentially be further developed as a novel therapeutic agent to regulate excessive pro-inflammatory cytokine production by macrophages and to treat severe inflammatory diseases like sepsis in combination with other treatments such as antibiotics or anti-TNFα Ab.

Credit author statement

Ja-Hyun Koo: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - Original Draft, Sang-Hun Kim: Investigation, Soung-Hoo Jeon: Investigation, Min-Jong Kang: Writing - Review & Editing, Funding acquisition, Je-Min Choi: Conceptualization, Writing – Original Draft, Review & Editing, Supervision, Funding acquisition, Project administration.

Data availability

The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors (Ja-Hyun Koo and Je-Min Choi) are pending patent approval regarding C10 peptide application and C10-LRR protein in sepsis treatment.

Acknowledgments

This research was supported by grants from the Basic Science Research Program (NRF-2019R1A2C3006155, NRF-2020R1A4A1018398), Bio and Medical Technology Development Program (NRF-2017M3A9C8027972), and Original Technology Research Program for Brain Science (NRF-2019M3C7A1032655) of the National Research Foundation funded by Korean government to J.-M.C. and in part by the grant from the National Institutes of Health (NIA R01AG053495) to M.-J.K.

References

[1] Y. Sakr, U. Jaschinski, X. Wittebole, T. Szakmany, J. Lipman, S.A. Namendys-Silva, et al., Sepsis in intensive care unit patients: worldwide data from the intensive care over nations audit, Open Forum Infect Dis 5 (2018) ofy313.
[2] J.L. Vincent, G. Jones, S. David, E. Olariu, K.K. Cadwell, Frequency and mortality of septic shock in Europe and North America: a systematic review and meta-analysis, Crit. Care 23 (2019) 196.
[3] T. van der Poll, F.L. van de Veerdonk, B.P. Scicluna, M.G. Netea, The immunopathology of sepsis and potential therapeutic targets, Nat. Rev. Immunol. 17 (2017) 407–420.
[4] M.G. Netea, F. Balkwill, M. Chonchol, F. Cominelli, M.Y. Donath, E.J. Giamarellos-Bourboulis, et al., A guiding map for inflammation, Nat. Immunol. 18 (2017) 826–831.
[5] P.M. Honore, E. Hoste, Z. Molnar, R. Jacobs, O. Joannes-Boyau, M. Malbrain, et al., Cytokine removal in human septic shock: where are we and where are we going? Ann. Intensive Care 9 (2019) 56.
[6] H. Chaudhry, J. Zhou, Y. Zhong, M.M. Ali, F. McGuire, P.S. Nagarkatti, et al., Role of cytokines as a double-edged sword in sepsis, In Vivo 27 (2013) 669–684.
[7] M.P. Fink, H.S. Warren, Strategies to improve drug development for sepsis, Nat. Rev. Drug Discov. 13 (2014) 741–758.
[8] J. Rello, F. Valenzuela-Sanchez, M. Ruiz-Rodriguez, S. Moyano, Sepsis: a review of advances in management, Adv. Ther. 34 (2017) 2393–2411.
[9] D.C. Angus, T. van der Poll, Severe sepsis and septic shock, N. Engl. J. Med. 369 (2013) 2063.
[10] C.B. Moore, D.T. Bergstralh, J.A. Duncan, Y. Lei, T.E. Morrison, A.G. Zimmermann, et al., NLRX1 is a regulator of mitochondrial antiviral immunity, Nature 451 (2008) 573–577.
[11] H. Guo, R. Konig, M. Deng, M. Riess, J. Mo, L. Zhang, et al., NLRX1 sequesters STING to negatively regulate the interferon response, thereby facilitating the replication of HIV-1 and DNA viruses, Cell Host Microbe 19 (2016) 515–528.
[12] I.C. Allen, C.B. Moore, M. Schneider, Y. Lei, B.K. Davis, M.A. Scull, et al., NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-kappaB signaling pathways, Immunity 34 (2011) 854–865.
[13] X. Xia, J. Cui, H.Y. Wang, L. Zhu, S. Matsueda, Q. Wang, et al., NLRX1 negatively regulates TLR-induced NF-kappaB signaling by targeting TRAF6 and IKK, Immunity 34 (2011) 843–853.
[14] M.J. Kang, C.M. Yoon, B.H. Kim, C.M. Lee, Y. Zhou, M. Sauler, et al., Suppression of NLRX1 in chronic obstructive pulmonary disease, J. Clin. Invest. 125 (2015) 2458–2462.
[15] H. Li, S. Zhang, F. Li, L. Qin, NLRX1 attenuates apoptosis and inflammatory responses in myocardial ischemia by inhibiting MAVS-dependent NLRP3 inflammasome activation, Mol. Immunol. 76 (2016) 90–97.
[16] J.H. Koo, D.H. Kim, D. Cha, M.J. Kang, J.M. Choi, LRR domain of NLRX1 protein delivery by dNP2 inhibits T cell functions and alleviates autoimmune encephalomyelitis, Theranostics 10 (2020) 3138–3150.
[17] J.H. Koo, H. Yoon, W.J. Kim, S. Lim, H.J. Park, J.M. Choi, Cell membrane penetrating function of the nuclear localization sequence in human cytokine IL-1alpha, Mol. Biol. Rep. 41 (2014) 8117–8126.
[18] M. Reyes, M.R. Filbin, R.P. Bhattacharyya, K. Billman, T. Eisenhaure, D.T. Hung, et al., An immune-cell signature of bacterial sepsis, Nat. Med. 26 (2020) 333–340.
[19] A. Gautam, K. Chaudhary, R. Kumar, A. Sharma, P. Kapoor, A. Tyagi, et al., In silico approaches for designing highly effective cell penetrating peptides, J. Transl. Med. 11 (2013) 74.
[20] A. Gautam, K. Chaudhary, R. Kumar, G.P. Raghava, Computer-aided virtual screening and designing of cell-penetrating peptides, Methods Mol. Biol. 1324 (2015) 59–69.
[21] S. Lim, W.J. Kim, Y.H. Kim, S. Lee, J.H. Koo, J.A. Lee, et al., dNP2 is a blood-brain barrier-permeable peptide enabling ctCTLA-4 protein delivery to ameliorate experimental autoimmune encephalomyelitis, Nat. Commun. 6 (2015) 8244.
[22] J.A. Hagar, D.A. Powell, Y. Aachoui, R.K. Ernst, E.A. Miao, Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock, Science 341 (2013) 1250–1253.
[23] I. Zanoni, Y. Tan, M. Di Gioia, J.R. Springstead, J.C. Kagan, By capturing inflammatory lipids released from dying cells, the receptor CD14 induces inflammasome-dependent phagocyte hyperactivation, Immunity 47 (2017) 697–709 e3.
[24] C. Wu, W. Lu, Y. Zhang, G. Zhang, X. Shi, Y. Hisada, et al., Inflammasome activation triggers blood clotting and host death through pyroptosis, Immunity 50 (2019) 1401–14011 e4.
[25] N. Subramanian, K. Natarajan, M.R. Clatworthy, Z. Wang, R.N. Germain, The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation, Cell 153 (2013) 348–361.
[26] C. Shi, X. Wang, L. Wang, Q. Meng, D. Guo, L. Chen, et al., A nanotrap improves survival in severe sepsis by attenuating hyperinflammation, Nat. Commun. 11 (2020) 3384.
[27] S. Shen, F. Han, A. Yuan, L. Wu, J. Cao, J. Qian, et al., Engineered nanoparticles disguised as macrophages for trapping lipopolysaccharide and preventing endotoxemia, Biomaterials 189 (2019) 60–68.
[28] H. Choi, Y. Kim, A. Mirzaaghasi, J. Heo, Y.N. Kim, J.H. Shin, et al., Exosome-based delivery of super-repressor IkappaBalpha relieves sepsis-associated organ damage and mortality, Sci Adv 6 (2020), eaaz6980.
[29] M. Shah, G.Y. Kim, A. Achek, E.Y. Cho, W.Y. Baek, Y.S. Choi, et al., The alphaC helix of TIRAP holds therapeutic potential in TLR-mediated autoimmune diseases, Biomaterials 245 (2020) 119974.
[30] S.F. Liu, A.B. Malik, NF-kappa B activation as a pathological mechanism of septic shock and inflammation, Am. J. Physiol. Lung Cell Mol. Physiol. 290 (2006) L622–L645.
[31] K.V. Swanson, M. Deng, J.P. Ting, The NLRP3 inflammasome: molecular activation and regulation to therapeutics, Nat. Rev. Immunol. 19 (2019) 477–489.
[32] A. Trzeciak, A.P. Pietropaoli, M. Kim, Biomarkers and associated immune mechanisms for early detection and therapeutic management of sepsis, Immune Netw 20 (2020) e23.
[33] L. Vande Walle, T.D. Kanneganti, M. Lamkanfi, HMGB1 release by inflammasomes, Virulence 2 (2011) 162–165.
[34] M. Deng, Y. Tang, W. Li, X. Wang, R. Zhang, X. Zhang, et al., The endotoxin delivery protein HMGB1 mediates caspase-11-dependent lethality in sepsis, Immunity 49 (2018) 740–753 e7.
[35] D.C. Cornelius, O.K. Travis, R.W. Tramel, M. Borges-Rodriguez, C.H. Baik, M. Greer, et al., NLRP3 inflammasome inhibition attenuates sepsis-induced platelet activation and prevents multi-organ injury in cecal-ligation puncture, PloS One 15 (2020), e0234039.
[36] K. Taniguchi, M. Karin, NF-kappaB, inflammation, immunity and cancer: coming of age, Nat. Rev. Immunol. 18 (2018) 309–324.
[37] A.E. Goldfeld, C. Doyle, T. Maniatis, Human tumor necrosis factor alpha gene regulation by virus and lipopolysaccharide, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 9769–9773.
[38] D.V. Kuprash, I.A. Udalova, R.L. Turetskaya, D. Kwiatkowski, N.R. Rice, S. A. Nedospasov, Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide, J. Immunol. 162 (1999) 4045–4052.
[39] E.Y. Tsai, J.V. Falvo, A.V. Tsytsykova, A.K. Barczak, A.M. Reimold, L.H. Glimcher, et al., A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo, Mol. Cell Biol. 20 (2000) 6084–6094.
[40] J.V. Falvo, A.M. Uglialoro, B.M. Brinkman, M. Merika, B.S. Parekh, E.Y. Tsai, et al., Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter, Mol. Cell Biol. 20 (2000) 2239–2247.
[41] J. Wang, X. Wang, S. Hussain, Y. Zheng, S. Sanjabi, F. Ouaaz, et al., Distinct roles of different NF-kappa B subunits in regulating inflammatory and T cell stimulatory gene expression in dendritic cells, J. Immunol. 178 (2007) 6777–6788.
[42] S. Sanjabi, A. Hoffmann, H.C. Liou, D. Baltimore, S.T. Smale, Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12705–12710.
[43] S. Park, C. Juliana, S. Hong, P. Datta, I. Hwang, T. Fernandes-Alnemri, et al., The mitochondrial antiviral protein MAVS associates with NLRP3 and regulates its inflammasome activity, J. Immunol. 191 (2013) 4358–4366.
[44] J.A. Lorente, J.C. Marshall, Neutralization of tumor necrosis factor in preclinical models of sepsis, Shock 24 (Suppl 1) (2005) 107–119.
[45] E. Abraham, P.F. Laterre, J. Garbino, S. Pingleton, T. Butler, T. Dugernier, et al., Lenercept (p55 tumor necrosis factor receptor fusion protein) in severe sepsis and early septic shock: a randomized, double-blind, placebo-controlled, multicenter phase III trial with 1,342 patients, Crit. Care Med. 29 (2001) 503–510.
[46] E. Abraham, A. Anzueto, G. Gutierrez, S. Tessler, G. San Pedro, R. Wunderink, et al., Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. NORASEPT II Study Group, Lancet 351 (1998) 929–933.

[47] S.M. Opal, C.J. Fisher Jr., J.F. Dhainaut, J.L. Vincent, R. Brase, S.F. Lowry, et al., Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group, Crit. Care Med. 25 (1997) 1115–1124.

[48] B. Shakoory, J.A. Carcillo, W.W. Chatham, R.L. Amdur, H. Zhao, C.A. Dinarello, et al., Interleukin-1 receptor blockade is associated with reduced mortality in sepsis patients with features of macrophage activation syndrome: reanalysis of a prior phase III trial, Crit. Care Med. 44 (2016) 275–281.

[49] S. Kang, T. Tanaka, M. Narazaki, T. Kishimoto, Targeting interleukin-6 signaling in clinic, Immunity 50 (2019) 1007–1023.

[50] S. Lim, J.A. Lee, J.H. Koo, T.G. Kang, S.J. Ha, J.M. Choi, Cell type preference of a novel human derived cell-permeable peptide dNP2 and TAT in murine splenic immune cells, PloS One 11 (2016), e0155689.

[51] A. Ziegler, J. Seelig, Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters, Biophys. J. 86 (2004) 254–263.

[52] K. Montrose, Y. Yang, X. Sun, S. Wiles, G.W. Krissansen, Xentry, a new class of cell-penetrating peptide uniquely equipped for delivery of drugs, Sci. Rep. 3 (2013) 1661.