ORIGINAL ARTICLE

Prophylactic uses of integrin CD18-βA peptide in a murine polymicrobial peritonitis model

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Abstract

AIM: To evaluate the prophylactic properties of integrin CD18-βA peptide in a murine model of abdominal polymicrobial peritonitis and sepsis.

METHODS: Bacterial sepsis was induced in Institute of Cancer Research (ICR) mice by cecal ligation and puncture (CLP) surgery. Inflicted mice were then injected with either sterile saline or CD18-βA peptide intraperitoneally at 2 h after surgery, and were sacrificed at 12 and 24 h after surgery. Blood samples were immediately collected, and analyzed for endotoxin activity and tumor necrosis factor (TNF)-α and interleukin (IL)-6. Lungs and liver were studied for CD45+ leukocyte and CD3 mRNA content. Pulmonary expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM) and E-selectin was also determined.

RESULTS: Intraperitoneal injection of CD18-βA peptide significantly suppressed circulating endotoxin activity ($P < 0.01$) at 24 h, as well as serum levels of TNF-α ($P < 0.05$ at 12 and 24 h) and IL-6 ($P < 0.01$ at 12 h, $P < 0.05$ at 24 h) in CLP-inflicted mice. CD18-βA peptide also abrogated leukocyte infiltration into liver and lungs as unveiled by reduced CD45+ leukocyte and CD3 mRNA contents. Furthermore, the peptide significantly reduced pulmonary expression of VCAM ($P < 0.01$ at 12 h, $P < 0.001$ at 24 h), E-selectin ($P < 0.01$ at 12 and 24 h), and ICAM-1 ($P < 0.01$ at 12 h, $P < 0.001$ at 24 h). These actions of CD18-βA peptide collectively protected septic mice against lethality ($P < 0.01$).

CONCLUSION: CD18-βA peptide is a potent endotoxin antagonist that can protect surgical patients against sepsis-associated lethality.

INTRODUCTION

Endotoxin [or lipopolysaccharide (LPS)] was first identified by Richard Pfeiffer from the late log phase culture of *Vibrio cholerae*, of which the heat inactivated bacterial

lysates could induce septic shock and death in experimental animals[1,2]. In clinical settings, circulating endotoxin acts as a pivotal initiator in the pathogenesis of bacterial sepsis and signals front-line immune host cells to trigger the "cytokine storm", resulting in systemic inflammatory response syndrome (SIRS)[3]. Given that the deleterious SIRS goes unchecked, patients may develop and die from acute respiratory distress, shock, and multiple organ dysfunction. Severely ill patients in surgical wards and intensive care units are at risk from sepsis-associated death, despite the use of broad-spectrum antibiotics, corticosteroids, and other new drugs like drotrecogin alfa[4]. To resolve the stringent clinical situation, many experimental therapeutics like cultured bone marrow stromal cells[5], cholinergic agonists[6] and lysophosphatidylcholine[7] have emerged as potential therapeutics. Studies on their therapeutic efficacies however still remain in pre-clinical stages.

Our previous finding has identified that leukocyte integrin CD18 βA peptide is able to bind bacterial LPS and to alleviate the pro-inflammatory responses in LPS-stimulated Jurkat cells[8]. In the present study, we evaluated the potential prophylactic use of CD18-βA peptide in a murine model of cecal ligation and puncture (CLP) that resembles abdominal polymicrobial peritonitis and sepsis in humans[9].

MATERIALS AND METHODS

CD18-*β*A peptide
We designed the CD18-βA peptide basing on the residues 266-318 of CD18 of leukocyte β2 integrins. We cloned the cDNA fragment into pET43.1B vector (Novagen, San Diego, CA, USA), expressed recombinant CD18-βA peptide in *E. coli*. BL21 (DE3) (Novagen), and subsequently purified the resulting protein using ion mobilized affinity chromatography following our published procedure[8,10]. Before we injected CD18-βA peptide into mice, we dialyzed the peptide against sterile phosphate buffered saline. We verified the purity using electrophoresis, and measured the concentration of CD18-βA peptide as described[11].

Polymicrobial CLP peritonitis model in mice
We obtained male Institute of Cancer Research (ICR) mice (30 g in weight) from the AAALAC-accredited Laboratory Animal Unit of the University of Hong Kong (Pokfulam, Hong Kong), and divided them randomly into three experimental groups of equal size. We then maintained all mice in a vivarium on a 12-h light/dark cycle at 21℃. Mice had ad libitum access to a sterilized animal chow and water. We performed all experimental procedures on mice in accordance with the ethical guidelines set forth by the University's Committee on Using Live Animals for Teaching and Research.

We performed CLP as previously described[5] in ICR mice. We anaesthetized mice with an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia), and opened the anterior abdomen by a 15 mm midline incision. Afterwards, we ligated the exposed cecum non-obstructively with surgical silk, punctured it twice with a 19-gauge needle, and then replaced it back to the peritoneal cavity. For sham-operated mice, we opened the abdomen, but the cecum was neither ligated nor punctured. Finally, we completed CLP surgery by closing the abdomen wall with nylon sutures, and gave no analgesics and antibiotics to mice. At 2 h after the completion of CLP surgery, we injected 0.1 mL sterile saline intraperitoneally into 12 mice, and 0.8 mg/kg CD18-βA peptide (this optimal dosage was prior determined in a pilot study ranging from 0.6-1.2 mg/kg) to another 12 mice. An additional injection of sterile saline or CD18-βA peptide, respectively, was given to mice at 14 h after the completion of CLP surgery. We gave no treatment to the sham-operated mice throughout the experiment. We killed six mice from each of the three experimental groups at 12 and 24 h after CLP. Therefore, mice that were sacrificed at 12 and 24 h received one and two injections of CD18-βA peptide, respectively. We collected blood from the interior vena cava, and harvested lung and liver. We obtained serum from the collected blood by centrifugation at 3000 rpm for 10 min at 4℃. For each harvested lung and liver, we minced it into two pieces that were formalin-fixed and snap-frozen separately.

Survival study
We studied the survival of septic mice receiving different treatments after CLP in another group of ICR mice (*n* = 8 in each experimental group, in total 24 mice). We performed CLP as previously described, and injected mice intraperitoneally with either sterile saline or CD18-βA peptide at 2, 12 and 24 h after closure of abdomen. Sterile saline was used as a control according to our earlier studies and reported procedures[5,7,12]. We assessed survival after CLP continually for 48 h, and regarded mice that still remained alive beyond the observation period as survivors. Survival data were analyzed using the Kaplan-Meier module in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Limulus amoebocyte lysate assay of endotoxin activity
We determined the endotoxin activity in circulation using the Limulus amoebocyte lysate (LAL) pyrochrome kit (Associates of Cape Cod Inc, East Falmouth, MA, USA) as previously described[13,14]. All buffers for the LAL assay were prepared using endotoxin-free water according to manufacturer's instructions. We measured the resulting signal spectrophotometrically at 540 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA), and each sample was done in triplicate. We finally determined the endotoxin activity from the measured signals using a standard curve with a dynamic range from 0 to 0.623 Endotoxin Units (EU)/mL.

Tumor necrosis factor-*α* and interleukin-6 enzyme-linked immunosorbent assay
We measured the serum level of tumor necrosis factor (TNF)-α and interleukin (IL)-6 of mice using commercially available enzyme-linked immunosorbent assay kits

that were purchased from Dakewe (Shenzhen, China) and eBioscience (San Diego, CA, USA), respectively, in accordance with instructions previously reported[15,16].

Immunohistochemistry

We followed our published procedures to perform all immunohistochemical staining[17,18]. To study the CD45+ leukocyte content in lungs and liver of mice, we stained tissue paraffin sections with 10 μg/mL monoclonal antibody against CD45 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described[19]. We stained lung tissues with 20 μg/mL monoclonal antibody against VCAM (20 μg/mL; BD Biosciences, San Jose, CA, USA) and 20 μg/mL monoclonal antibody against E-selectin (BD Biosciences) to study pulmonary expression of the two adhesion molecules. We incubated tissue sections with respective antibodies at 4℃ overnight. After washing, we added a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). We visualized the resulting signals with a liquid DAB substrate kit (Invitrogen), and thereafter, we counterstained tissue sections with hematoxylin (Vector Laboratories, Burlingame, CA, USA).

Real-time polymerase chain reaction

We measured the level of CD3 mRNA in lung and liver of mice after CLP using real-time polymerase chain reaction (PCR). This method provides robust and quantitative measurement on tissue infiltration of inflammatory leukocytes[20]. To study the expression of ICAM-1, VCAM, and E-selectin in lungs, we measured their mRNA levels. We performed RNA extraction, first-strand cDNA synthesis, and real-time PCR in ABI PRISM 7700 sequence detector system (Applied Biosystems Inc., Forest Hill, CA, USA) as previously described[21-23].

Statistical analysis

We performed all statistical analysis using the GraphPad Prism (GraphPad Software Inc.); group means of the study parameters were compared by one-way ANOVA, and P values < 0.05 were considered to indicate statistical significance.

RESULTS

Survival of septic mice

Diagnosis of bacterial sepsis in most clinical scenarios is always delayed, therefore in this study we injected mice intraperitoneally with CD18-βA peptide (0.8 mg/kg) (or sterile saline) at 12-h intervals beginning two hours after the CLP surgery. We first looked at the survival rates after CLP surgery in saline- and CD18-βA peptide-treated mice. A statistically significant (P < 0.01) improvement in the survival of mice injected CD18-βA peptide intraperitoneally was noted (Figure 1A); about 40% of mice survived over the 48 h post-operative observation period, and remained alive thereafter despite discontinuation of therapy. In contrast, mice that received saline all died within the 36 post-operative hour period.

### Endotoxicity and pro-inflammatory cytokines in circulation

We next investigated whether the beneficial effect of CD18-βA peptide on the survival of septic mice was associated with reductions in endotoxin activity in circulation. To this end, we measured both biological activity of endotoxin and serum levels of TNF-α and IL-6 after we sacrificed mice at 12 and 24 h after CLP surgery and collected blood samples at the interior vena cava. Mice inflicted with CLP surgery showed up-regulation of the circulating endotoxin activity and TNF-α and IL-6 levels in serum samples, which could be remarkably modulated by treatment with CD18-βA peptide but not with saline control (Figure 1B). Intraperitoneal injection of CD18-βA peptide achieved a statistically significant reduction in circulating endotoxin activity (P < 0.01) at 24 h, as well as serum levels of TNF-α (P < 0.05 at 12 and 24 h) and IL-6 (P < 0.01 at 12 h, P < 0.05 at 24 h).

CD45+ leukocyte and CD3 mRNA contents in lungs and liver

In view of the fact that infiltrating leukocytes usually injure tissues, we first studied histologically the effect of CD18-βA peptide on CD45+ leukocyte infiltration in lungs and liver of septic mice (Figure 2A). Intense CD45 reactivity was detected in the lungs and liver organs from the placebo group. Strikingly, a bolus of CD18-βA peptide injection drastically diminished CD45+ cells in liver, and almost completely eradicated the CD45+ signals in lungs. Further to confirm this observation, we measured the level of CD3 mRNA that is expressed by infiltrating leukocytes using real-time PCR (Figure 2B). Compared to the sham control, CD3 mRNA levels of lungs and liver were up-regulated by over 10-20 fold by CLP surgery, and these elevations were reduced significantly by treatment with the CD18-βA peptide (P < 0.05). Taken together, we suggest that CD18-βA peptide can be used to prevent leukocyte infiltration in bacterial sepsis.

ICAM-1, VCAM and E-selectin expression in lungs

Adhesion molecules facilitate leukocyte trafficking, and are implicated in mediating organ injuries[24]. To study the effect of CD18-βA peptide on adhesion molecule expression, we first determined the pulmonary expression of VCAM and E-selectin by immunohistochemistry (Figure 2C). Intense immunoreactivity toward VCAM and E-selectin was detected in lung paraffin sections prepared from saline-treated septic mice, whereas the corresponding immunoreactivities were markedly diminished from the CD18-βA peptide-treated mice. Expression of ICAM-1, VCAM, and E-selectin mRNA in lungs has also been determined by real-time PCR. Similarly, mRNA levels of the three adhesion molecules surged in lungs of saline-treated septic mice, whereas such elevations were significantly suppressed after CD18-βA peptide treatment: VCAM (P < 0.01 at 12 h, P < 0.001 at 24 h), E-selectin (P < 0.01 at 12 and 24 h), and ICAM-1 (P < 0.01 at 12 h, P < 0.001 at 24 h) (Figure 2C).
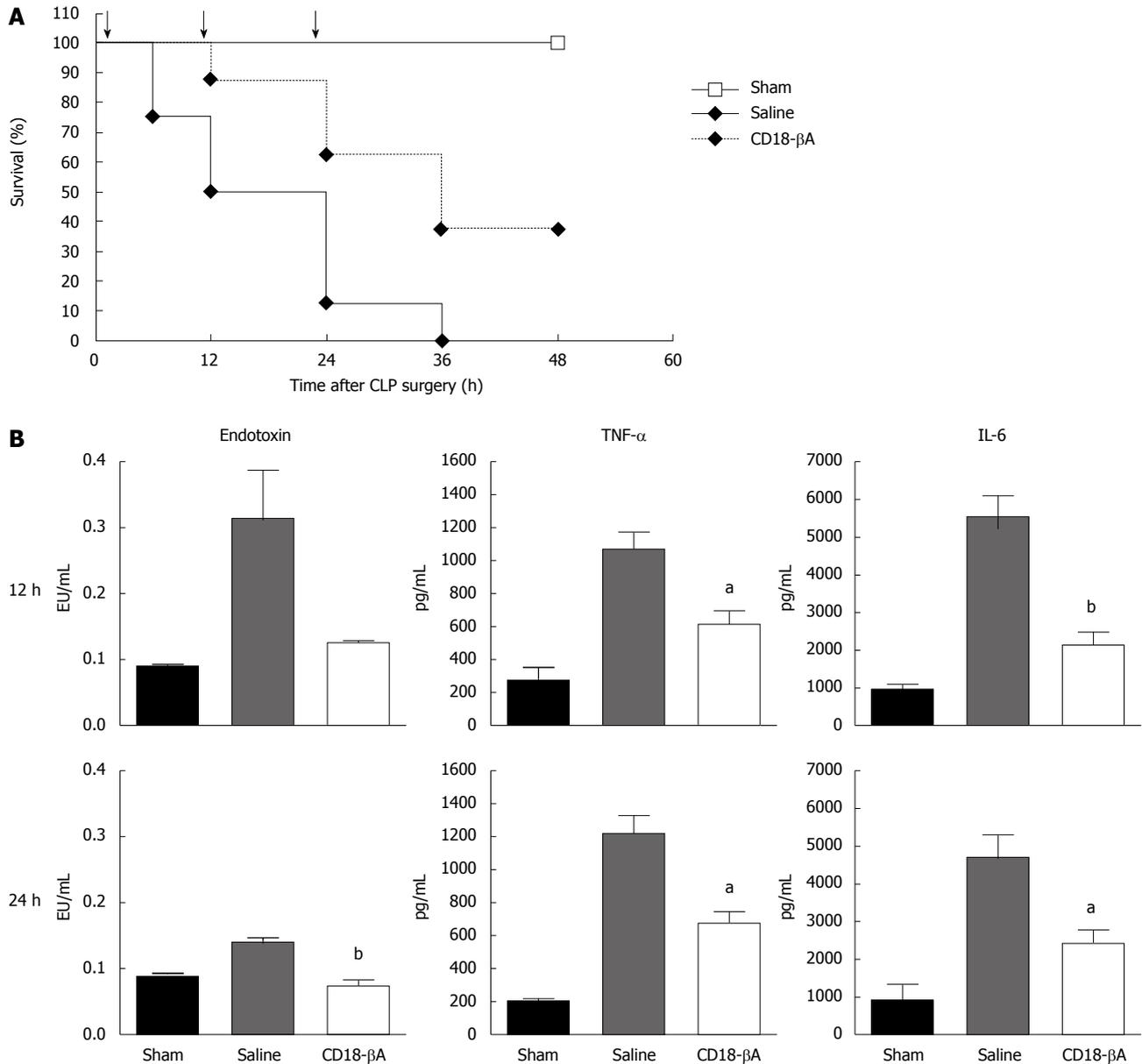
Figure 1 CD18-βA peptide protects mice against cecal ligation and puncture (CLP)-induced lethality and inflammatory responses. A: Mice were injected with either sterile saline or CD18-βA peptide intraperitoneally at 12 h intervals beginning 2 h after CLP (as indicated by arrows), and were monitored continually over 48 h. There was statistically significant improvement in survival of septic shock mice that received CD18-βA injection (*P* < 0.01), compared to those that received saline. *n* = 8 for each experimental groups; B: Measurement of endotoxin activity and tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in blood circulation. Injection of CD18-βA peptide achieved significant reductions in endotoxin activity at 24 h, and reductions in TNF-α and IL-6 at 12 and 24 h after CLP. 12 h, *n* = 5 and 24 h, *n* = 6 for each experimental group. [a]*P* < 0.05, [b]*P* < 0.01 *vs* saline group (one-way ANOVA). Error bars represent mean ± SD.

DISCUSSION

We illustrated in a stringent murine model of bacterial sepsis that CD18-βA peptide was able to neutralize circulating endotoxin, to reduce serum TNF-α and IL-6 levels, to inhibit leukocyte infiltrating into lungs and liver, and to suppress endotoxin-induced adhesion molecule expression. All these protective actions of CD18-βA peptide might contribute to survival benefits of the animals in the sepsis model.

We found the protection of CD18-βA peptide on septic mice was in some ways impressive. Notably, CD18-βA peptide still afforded protection on septic mice even

when it was administered after onset of bacterial sepsis. This is in contrast to many other therapeutic interventions that are incapable of halting the already-initiated super-inflammatory responses in surgical patients. In view of this, CD18-βA peptide can serve as an adjunctive therapeutic particularly for patients who are being challenged by high concentration of endotoxin, which usually results with a delayed diagnosis of bacterial sepsis. One of the possible underlying mechanisms for the effective endotoxin neutralization could be attributed to the presence of an amphipathic helix motif (VGQLAHKLAENNIQPI-FA) at the C-terminal end (Figure 3). Such structural moiety favors endotoxin binding and neutralization of

A

Lung CD45+

B

C

Lung VCAM

Isotype control   Sham   Saline   CD18-βA

Lung E-selectin

Isotype control   Sham   Saline   CD18-βA

ICAM-1   VCAM   E-selectin
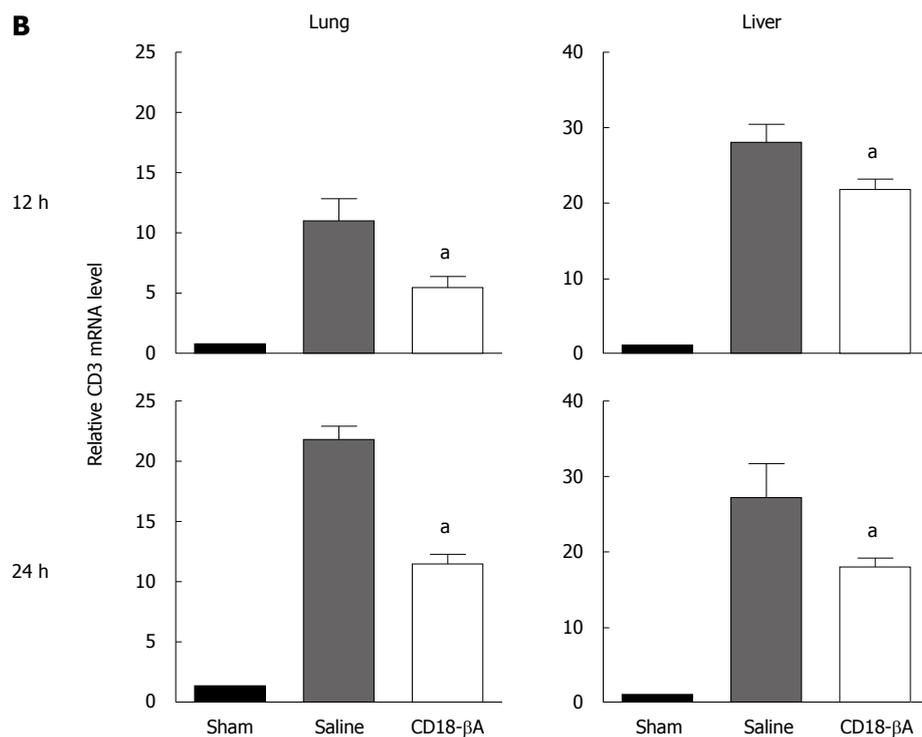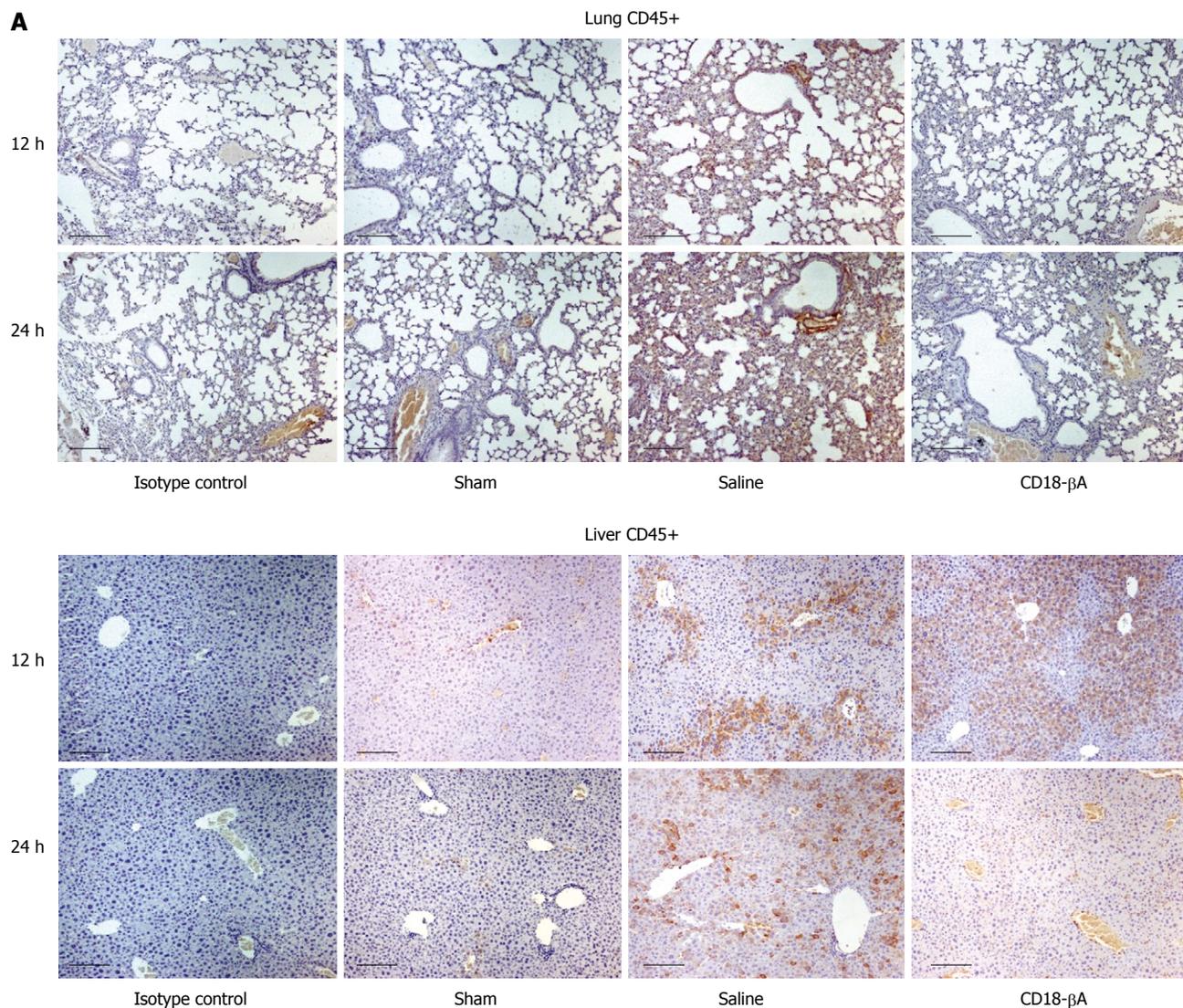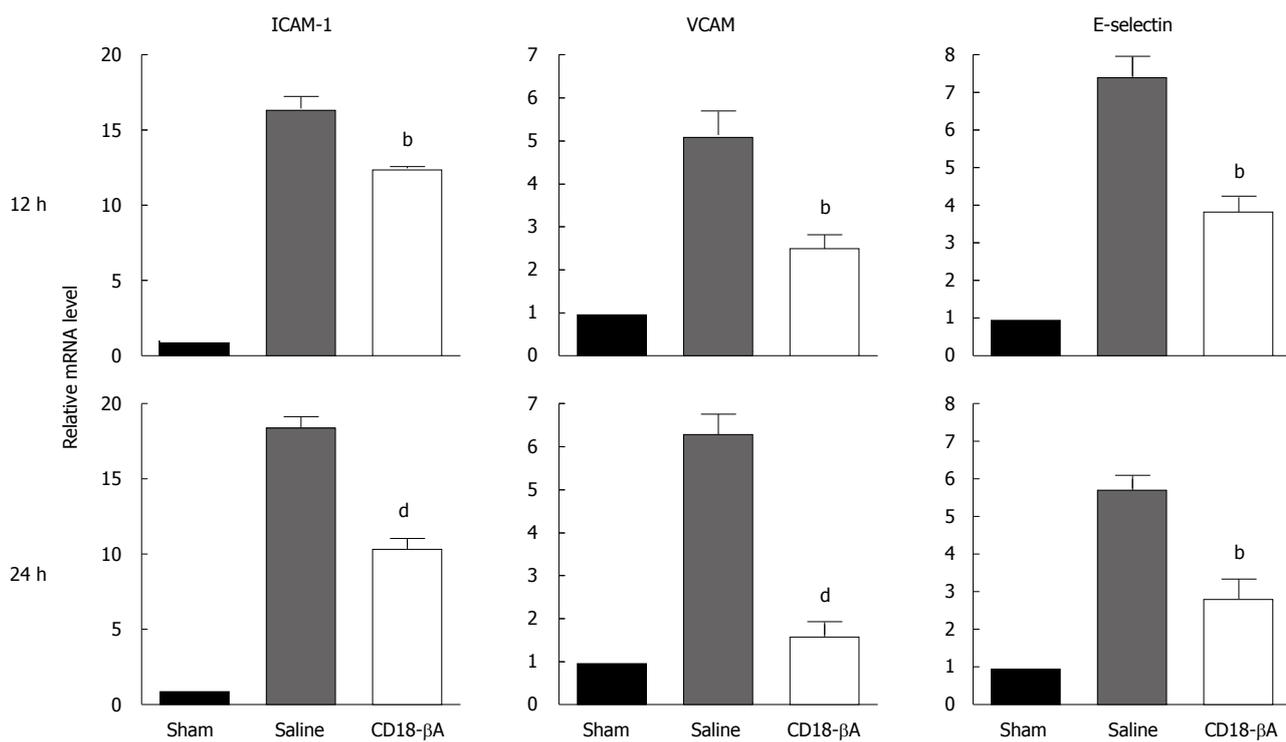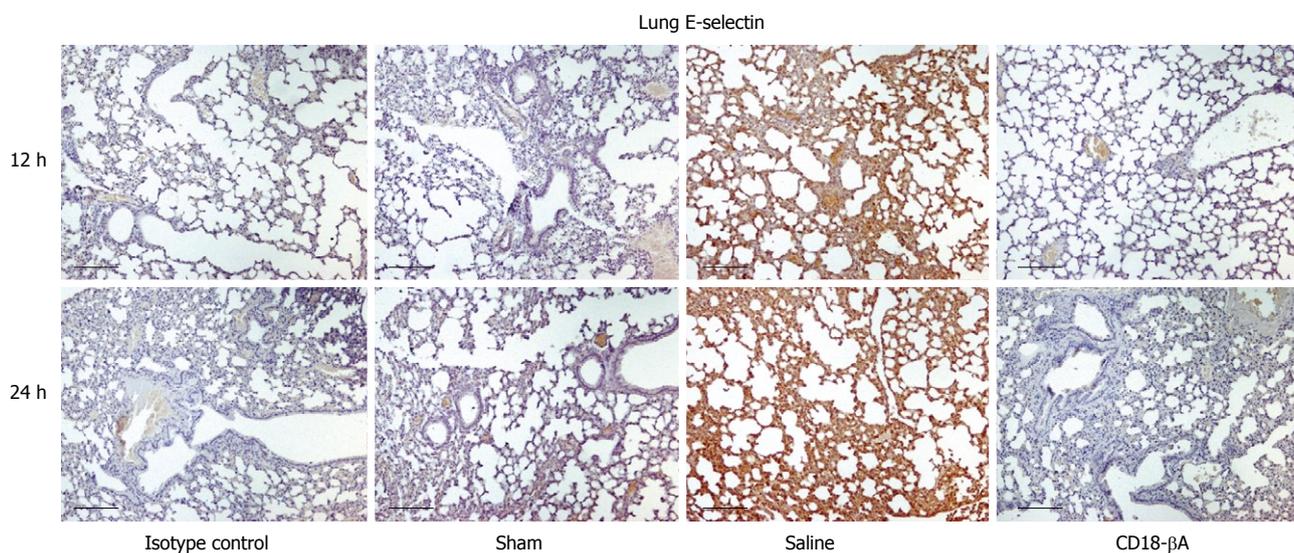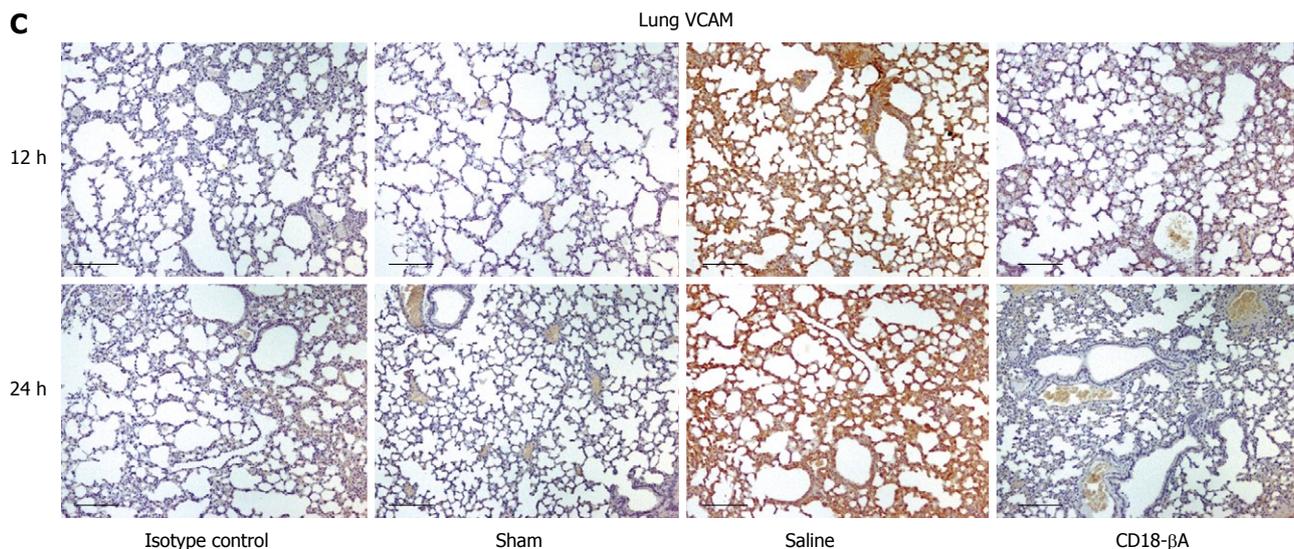
Figure 2 CD18-βA peptide inhibits CD45+ leukocyte infiltration into lung and liver in CLP-induced mice. A: CD45+ leukocyte content in lung and liver after CLP was visualized by immunohistochemistry. Images are representative of *n* = 6 for each experimental group. Scale bar, 80 μmol/L; B: Real-time polymerase chain reaction (PCR) analysis of CD3 mRNA level in lungs and liver, normalized to β-actin, at 12 and 24 h after CLP. Significant reductions in CD3 mRNA contents were observed in lungs and liver of septic mice that have been treated with CD18-βA peptide. [a]*P* < 0.05 *vs* saline group (one-way ANOVA); C: Pulmonary expression of adhesion molecules was studied using immunohistochemistry and real-time PCR. Lung sections were stained with VCAM and E-selectin antibody, and images are representative of *n* = 6 for each experimental group. Scale bars, 80 μm. Real-time PCR was employed to study mRNA expression of ICAM-1, VCAM, and E-selectin in lungs. Significant reductions in adhesion molecule mRNA contents were observed in lungs of septic mice that were treated with CD18-βA peptide. [b]*P* < 0.01, [d]*P* < 0.001 *vs* saline group (one-way ANOVA). For all real-time PCR study, *n* = 6 for each experimental group at 12 and 24 h. Error bars represent mean ± SD.

endotoxicity[25]. In addition, we have previously shown that the positively charged residues (e.g. arginine, lysine) along the CD18-βA peptide are contributing to the effective binding to LPS of various bacterial strains[8].

We showed CD18-βA peptide is an effective agent to suppress the "cytokine storm" in bacterial sepsis, given that the peptide significantly reduced TNF-α and IL-6 levels in the circulation of septic mice. This aspect of CD18-βA peptide is essential to sepsis treatment because pro-inflammatory cytokines can stimulate phagocytes to release reactive oxygen species, increasing vascular permeability and leading to organ injury[26]. In fact, therapeutic agents like anti-cytokine antibodies and receptor antagonists that are aiming to abolish cytokine functions have long been developed. Unfortunately, none of these agents proved efficacious in protecting sepsis patients[27,28], apparently because of the redundancy of cytokine functions. Unlike these therapeutic agents, use of CD18-βA peptide can circumvent this problem because in our recent study, we have shown CD18-βA peptide could down-modulate the NF-κB-mediated signaling that is central to the release of many pro-inflammatory cytokines.

The clinical utility of CD18-βA peptide as a sepsis therapeutic is further enhanced by its ability to inhibit leukocyte infiltration into lungs and liver, given that leukocyte-mediated injury on these two organs cause the high mortality rate of sepsis patients[29,30]. The inhibition is likely attributed to the suppression of adhesion molecules in lungs. In addition to this mechanism, it was apparent that CD18-βA peptide could block the integrin binding sites on adhesion molecules (e.g. ICAM-1) because CD18-βA peptide is homologous to the CD18 antigen of leukocyte β2 integrins. Indeed, this hypothesis is supported by our early study showing that a recombinant CD18 subunit could inhibit the binding of leukocytes to immobilized adhesion molecules (Luk JM, unpublished observation). We also hypothesized that CD18-βA peptide could antagonize direct activation of leukocytes by bacterial endotoxin as our recent study has illustrated that CD18-βA peptide could prevent the sensing of Jurkat cells by bacterial LPS *in vitro*[8].

Furthermore, a recent *in vivo* animal study of necrotizing enterocolitis has readily demonstrated the potential therapeutic efficacy of CD18-βA peptide against LPS-induced proinflammatory reactions[31]. In that study, rat intestine was distended and then challenged by bacterial LPS, culminating in profound TNF-α release from immune cells, intense leukocyte influx into the intestine, and severe intestinal tissue damage. CD18-βA peptide was able to neutralize the LPS endotoxicity and modu-

CD18-βA domain

PIDLYYLMDLSYSMLDDLRNVKKLGGDLLRALNEITESGRIGFGSFVDK
TVLPFVNTHPDKLRNPCPNKEKECQPPFAFRHVLKLTNNSNQFQTEVGK
QLISGNLDAPEGGLDAMMQVAACPEEIGWRNVTRLLVFATDDGFHFAGD
GKLGAILTPNDGRCHLEDNLYKRSNEFDYPSVGQLAHKLAENNIQPIFA
VTSRMVKTYEKLTEIIPKSAVGELSEDSSNVVQLIKNAYNKLS
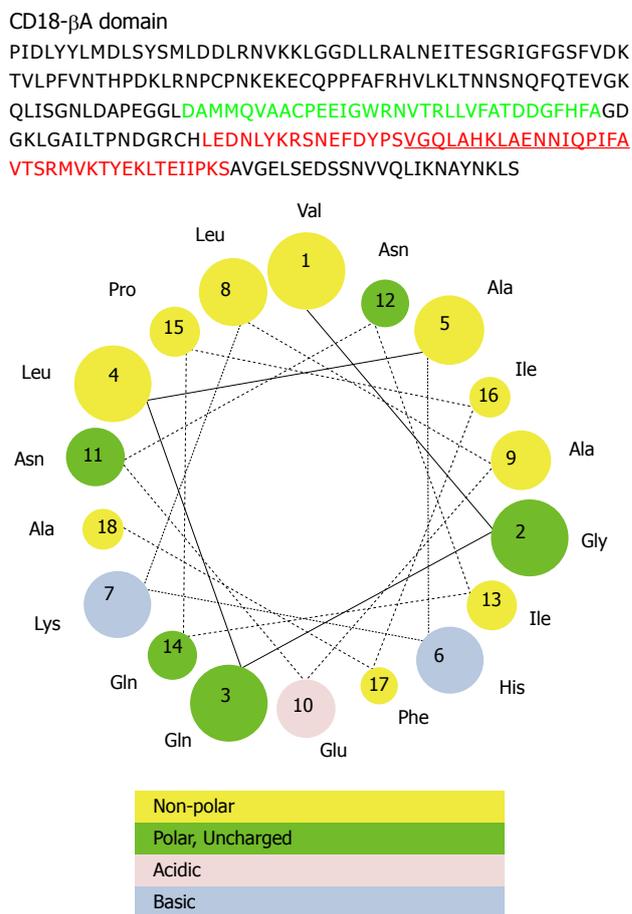
Figure 3 A predicted model of the amphipathic helix sequence at CD18-βA that binds bacterial endotoxin. Shown is the polypeptide sequence of the CD18-βA domain of leukocyte β2 integrin, and the two LPS-binding sites are colored green and red. CD18-βA peptide is derived from the red binding site. The underlined portion of CD18-βA peptide demonstrates a feature of the amphipathic helix, as revealed by the helical wheel diagram.

late the above-mentioned deleterious immune responses.

In summary, CD18-βA peptide is a promising therapeutic for intervention of bacterial sepsis. Unlike anti-LPS antibodies that failed to protect septic patients because of their inability to inhibit pro-inflammatory cytokine releases from immune cells[32], CD18-βA peptide can halt the deleterious "cytokine storm" in bacterial sepsis. To further assess the therapeutic efficacy of CD18-βA peptide, we are going to study the protective action in a porcine model of endotoxemia[33].

ACKNOWLEDGMENTS

for critical review and professional editing of the manuscript. The use of CD18-βA peptide in treating sepsis has been filed for US patent (application 61/080,558) and PCT Patent Application No. PCT/CN2009/000791.

COMMENTS

Background

Patients severely infected with Gram-negative bacteria can die of endotoxemia and sepsis. Indeed, despite the emergence of broad-spectrum antibiotics, corticosteroids, and advanced surgical resuscitative supports, sepsis remains one of the leading causes of death in intensive care units. In this context, many experimental therapeutic agents have been developed over the last few decades. Disappointingly, only a few of these agents were shown to improve survival, and nearly none of them could halt the unchecked inflammatory response of sepsis.

Research frontiers

CD18-βA peptide is a novel endotoxin antagonistic peptide derived from a lipopolysaccharide (LPS)-binding site of leukocyte integrins, and was found able to suppress the stimulation of LPS on leukocytes *in vitro*. The present work is the first published data concerning the use of CD18-βA peptide in the treatment of mice with polymicrobial peritonitis.

Innovations and breakthroughs

The key pathogenesis of sepsis is the super-inflammatory response (or "cytokines storm") that can ultimately lead to multiple organ failure and inevitably death. To date, clinical uses of corticosteroids and antibiotics, and experimental uses of cytokine antibodies all fail to halt the deleterious immunological cascades. In this study, CD18-βA peptide was shown to antagonize endotoxicity in circulation, preventing the pro-inflammatory "cytokines storm" from initiation. This mitigation of the inflammatory response reduced the influx of leukocytes from the bloodstream into lungs and liver, protecting both organs from leukocyte-mediated damage. All these beneficial effects of CD18-βA peptide collectively protected septic mice against death.

Applications

The indication of CD18-βA peptide as a potent endotoxin antagonist promises to be an effective therapeutic agent to alleviate the deleterious superinflammatory responses in patients with acute septic conditions.

Terminology

Polymicrobial peritonitis model is a clinically relevant model that resembles sepsis resulting from endotoxemia. Endotoxin (e.g. LPS) is a structural component of the Gram-negative bacteria cell wall, and once been liberated to circulation, can stimulate the innate immunity to release pro-inflammatory cytokines, culminating in systemic inflammatory response syndrome and sepsis.

Peer review

This is a paper that evaluates the prophylactic properties of integrin CD18, and the results are very important. The authors show integrin CD18 suppressed circulating endotoxin activity and levels of tumor necrosis factor-α and interleukin-6.

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