Coupling killing to neutralization: combined therapy with ceftriaxone/Pep19-2.5 counteracts sepsis in rabbits

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Sepsis, which is induced by severe bacterial infections, is a major cause of death worldwide, and therapies combating the disease are urgently needed. Because many drugs have failed in clinical trials despite their efficacy in mouse models, the development of reliable animal models of sepsis is in great demand. Several studies have suggested that rabbits reflect sepsis-related symptoms more accurately than mice. In this study, we evaluated a rabbit model of acute sepsis caused by the intravenous inoculation of Salmonella enterica. The model reproduces numerous symptoms characteristic of human sepsis including hyperlactatemia, hyperglycemia, leukopenia, hypothermia and the hyperproduction of several pro-inflammatory cytokines. Hence, it was chosen to investigate the proposed ability of Pep19-2.5—an anti-endotoxic peptide with high affinity to lipopolysaccharide and lipoprotein—to attenuate sepsis-associated pathologies in combination with an antibiotic (ceftriaxone). We demonstrate that a combination of Pep19-2.5 and ceftriaxone administered intravenously to the rabbits (1) kills bacteria and eliminates bacteremia 30 min post challenge; (2) inhibits Toll-like receptor 4 agonists in serum 90 min post challenge; (3) reduces serum levels of pro-inflammatory cytokines (interleukin-6 and tumor necrosis factor α); and (4) reverts to hypothermia and gives rise to temperature values indistinguishable from basal levels 330 min post challenge. The two components of the combination displayed synergism in some of these activities, and Pep19-2.5 notably counteracted the endotoxin-inducing potential of ceftriaxone. Thus, the combination therapy of Pep19-2.5 and ceftriaxone holds promise as a candidate for human sepsis therapy.

INTRODUCTION

Sepsis is one of the leading causes of death in intensive care units worldwide, with mortality rates ranging from 10% to over 40% depending on the clinical associations.1 Triggered by an infection and characterized by an inflammatory state affecting the patient’s whole body, sepsis accounts for 300–1000 cases per 100 000 persons in the United States.2 Thus, the urgency for an effective therapy against sepsis cannot be overemphasized.

The established animal models of sepsis differ in their response to the triggering factor of inflammation and consequently in their reaction to medications. This may partly explain the failure of multiple clinical trials investigating drug strategies for sepsis therapy during recent years. Moreover, the difficulties encountered are likely due to major differences in immune system functioning between animal species and humans.3 Animal sepsis models widely vary and include intraperitoneal (i.p.)/intravenous (i.v.) injection of lipopolysaccharide (LPS) or dead bacteria (endotoxemia), inoculation of live bacteria or specific surgeries such as cecal ligation and puncture, colon ascendens stent peritonitis and polymicrobial

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peritoneal contamination and infection. Although surgical models are known to reflect more accurately the clinical scenario, they usually require a long-term evaluation, and their mortality rates vary depending on surgical efficiency. On the other side, non-surgical models (for example, inoculation of cells or antigens into the animal) are widely used for acute sepsis evaluation, being highly controlled and less laborious than models relying on the animal microbiota as the infectious agent.

Furthermore, most tested therapies aim at killing the bacteria or modulating the immune response. Yet, these treatments do not address the major underlying cause of sepsis—that is, the release of toxins (pathogenicity factors) by the bacteria. Thus, besides the standard supportive intensive care treatment, no satisfactory specific therapeutic option exists to date. Current therapies with antibiotics aim at killing bacteria, but this frequently leads to the release of the pathogenicity factors, hence aggravating the patient’s inflammatory response. Considering the rapid increase in multiresistant strains and the lack of newly approved antibiotics, the situation of the most severely ill patients in intensive care units becomes more and more threatening.

One approach alternative to conventional antibiotic-based therapy entails the use of antimicrobial peptides (AMPs). Until now, there are only a limited number of approved AMP drugs available, although daptomycin (against skin and skin structure infections) and colistin (against multiresistant Gram-negative bacteria) are two relevant agents. Thus, the recent development of a synthetic anti-LPS peptide, Pep19-2.5 (Aspidasept), appears promising also for a broader application.

Recently, it has been demonstrated that this compound has high affinity not only for Gram-negative LPS but also for Gram-positive lipoprotein (LP) in various in vitro and in vivo mouse experiments. Pep19-2.5 has also been shown to be protective in mice. In this model, bacteremia was induced in the animals by injecting live cells of Gram-negative (Salmonella) bacteria. The combined treatment of the antibiotic ceftriaxone to kill the bacteria and Pep19-2.5 to neutralize released LPS was evaluated. The data showed that this combination is able to drastically reduce the strong inflammation as evidenced by a drop in the levels of interleukin 6 (IL-6) and tumor-necrosis-factor α (TNFα). Endotoxin detection in the blood of the animals showed that this decrease in cytokine concentrations is directly correlated with a marked reduction of the detectable LPS concentration in serum. Essentially, we demonstrate that Pep19-2.5 and ceftriaxone work in conjunction to kill bacteria and reduce inflammation.

MATERIALS AND METHODS
Bacterial strains and culture conditions
Salmonella enterica serovar Minnesota (SF114, smooth form) was grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) at 37°C with orbital shaking. For inoculum preparation, an overnight liquid culture was centrifuged (4000 g, 5 min), and the pellet was suspended in sterile saline and adjusted to OD600nm = 1.0, approximately corresponding to 3.7 × 10^9 colony-forming units (CFUs) per ml. For viable cell counting, aliquots were plated onto TSA agar and were incubated for 24 h.

Peptide synthesis
The synthesis and purification of Pep19-2.5 (Lot 1053821) was previously described, and the batch used in the present work was produced under GMP conditions by BACHEM (Bubendorf, Switzerland). The purity was >95%, and the amino-acid sequence of this 20’mer is GCKKYRFRWKFGKFWFWG, containing a C-terminal amidation.

Animal experiments
All experiments were approved by the Animal Ethical Committee of the University of Navarra (protocol number: 182-12). Care and handling of the animals were in accordance with the ICH and OECD guidelines. Male New Zealand rabbits (2–2.5 kg in weight; Granja San Bernardo S.I., Tulebras, Spain) were housed under standard conditions with free access to food and water. The animals were granted a 7-day acclimatization period prior to experimentation, and they were food fasted 12–14 h before inoculation. Each day, three animals were challenged, treated and killed. The inoculum consisted of 1 ml of sterile saline approximately containing 3.7 × 10^8 CFU of S. enterica serovar Minnesota (Supplementary Figure S1). Animals (n = 6 per experimental group) were intravenously (i.v.) inoculated by the marginal ear vein. Treatments were administered i.v. with a total volume of 2 ml immediately after the inoculum by the marginal ear and consisted of ceftriaxone (15 mg kg^-1; Sigma-Aldrich, Madrid, Spain) dissolved in saline and/or Pep19-2.5 (5 mg kg^-1) dissolved in saline. A group of animals was left untreated and received only vehicle. Blood samples were extracted from the marginal ear vein at different time points post challenge (0, 2, 30, 90 and 180 min). Fifteen minutes before each blood extraction, acetpromazine (1 mg kg^-1; Sigma-Aldrich) was i.v. administered for sedative and vasodilator purposes. All the procedures were performed aseptically and using sterile labware. The rectal temperature was monitored at 0, 90, 150, 210 and 330 min post challenge. Six hours after inoculation, the
animals were killed by the administration of a lethal dose of T-61 (Sigma-Aldrich).

**Blood and serum testing**

For bacteremia analysis, whole blood was serially diluted (1:10 in sterile saline) and plated in TSB Agar plates. Viable counts were determined after 16–18 h of incubation at 37 °C. For serum preparation, blood samples (4 ml) were taken under sterile conditions, centrifuged (3000 r.p.m., 10 min, 4 °C) using serum-separating tubes and stored at −80 °C for subsequent analysis. Fluoride/EDTA and EDTA-containing vacutainer tubes were used for lactate quantification and hematological analysis, respectively (Sysmex XT-1800i; Sant Just Desvern, Spain). Biochemical parameters were determined using a Hitachi-911 automatic analyzer (Hitachi Medical Systems, Madrid, Spain).

**Post-mortem analyses**

Post-mortem samples of the spleen, liver, kidneys and lungs were extracted and weighed, and 1 g of each was homogenized and serially diluted for plating and viable counting. The remainder of the organ was fixed by immersion in 4% formaldehyde and was subsequently prepared for inclusion, cutting and hematoxylin–eosin staining. All the procedures were performed aseptically and using sterile labware. Anatomopathological analyses of histological preparations were carried out using the Anatomopathologic Diagnostic Service for Laboratory Animals (University of Zaragoza, Spain).

**Endotoxin and cytokine quantification**

Endotoxin was indirectly quantified by measuring IL-8 in a Toll-like receptor (TLR4)-expressing cell line that responds to LPS by secreting this cytokine (HEK-293/TLR4; kindly provided by Dr Juan José Lasarte). To account for the presence of potential IL-8 inducing compounds other than LPS in rabbit serum, IL-8 levels were measured in a Toll-like receptor (HEK-293/LacZ). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g l⁻¹ glucose, 10% heat-inactivated fetal bovine serum, penicillin/streptomycin and blasticidin (5 µg ml⁻¹ for each; GibCO; Thermo Fisher Scientific, Alcobendas, Spain) at 37 °C in 5% CO₂. In the case of HEK-293/TLR4, cells were supplemented with 25 µg ml⁻¹ hygromycin B (Invivogen Ultrapure; InvivoGen, San Diego CA, USA). Cells (5 × 10⁵) were exposed to rabbit serum (1:10 dilution) for 24 h, and IL-8 was then quantified in the supernatants by solid-phase sandwich enzyme-linked immunosorbent assay (R&D Systems; Madrid, Spain). Assays were performed in triplicate. The serum levels of IL-6 and TNFα were determined using solid-phase sandwich enzyme-linked immunosorbent assay (R&D Systems) following the manufacturer’s instructions.

**Statistics**

The data were statistically analyzed using SPSS V15.0 software (IBM España, Madrid, Spain). Analysis of variance was applied to study the potential differences in the bacterial load, endotoxin levels, cytokine concentrations and biochemical parameters. If there was homogeneity of variances, parametric Student–Neuman–Keuls post hoc test was conducted. Otherwise, the Tamhane test was applied. For the group comparisons of temperatures at different time points, repeated measures factorial design-analysis of variance was applied, followed by Student's t-test for related measures, adjusting the average s.d. Graphics were prepared using GraphPad Prism V6.01 software (GraphPad Software, La Jolla, CA, USA).
immunosorbent assay. In control experiments, we demonstrated that LPS quantitatively induced IL-8 secretion in these cells (data not shown). To account for the presence of potential IL-8-inducing compounds other than LPS, a duplicate measurement was performed in an identical cell line lacking the TLR4 receptor (HEK-293/LacZ). Cells were exposed to rabbit serum for 24 h, and IL-8 was quantified in the supernatants by solid-phase sandwich enzyme-linked immunosorbent assay. Assays were performed in triplicate, and the results were analyzed using analysis of variance followed by post hoc tests (*P<0.05; **P<0.01; ***P<0.001). The horizontal line within the boxes represents the median, whereas the lower part represents the 25th percentile, and the upper part represents the 75th percentiles. The whiskers represent the range of the values.

Concentration of pro-inflammatory cytokines

Based on the ceftriaxone-related release of TLR4 agonist, we next investigated the pro-inflammatory response of rabbits on *S. enterica* challenge. As shown in Figure 3, we measured the serum concentrations of TNFα and IL-6 at 90 min and 180 min post challenge, respectively, coinciding with the peak concentration of each cytokine in serum, as determined in preliminary assays (Supplementary Figure S2). Ceftriaxone treatment displayed a trend to increase both the IL-6 and TNFα serum levels compared with untreated animals. By contrast, Pep19-2.5 not only lacked pro-inflammatory activity when given alone but also counteracted the IL-6- and TNFα-inducing ability of ceftriaxone. Notably, animals receiving the combined therapy had levels of IL-6 even lower than those measured in untreated animals (Figure 3a), suggesting that the two components of the combination act in synergy to neutralize this cytokine.

Metabolic parameters

Abnormally high serum values of both glucose and lactate (that is, hyperglycemia and hyperlactatemia, respectively) are common during acute infection, particularly in patients with sepsis. To investigate whether our rabbit model reproduces these symptoms, we measured the serum levels of glucose and lactate throughout the course of the experiment. Inoculation of *S. enterica* caused a significant and progressive increase in glucose and lactate levels that was even more severe for the latter marker (Supplementary Figure S3). Neither the single nor combined treatment, however, was found to significantly reverse these increments at any time point (Supplementary Figure S3).

Hematological markers

Infections are known to induce leukocytosis, whereas leukopenia is characteristic of acute sepsis due to overwhelming infections. As shown in Supplementary Figure S4, the inoculation of *S. enterica* caused a severe decrease in white blood cells, which was of similar magnitude in both the untreated and treated groups (~60% of the initial value).

Temperature monitoring

Sepsis in humans is known to induce either hypothermia or hyperthermia. To study whether any of these symptoms could be detected in our animal model, we monitored the rectal temperature of all the animals throughout the assay. As shown in Figure 4, the inoculation of *S. enterica* markedly reduced the rabbit temperature from 38°C at the beginning of the experiment to ~ 35.5°C at the late time points post challenge (330 min). The kinetics of hypothermia in animals treated with Pep19-2.5 tended to be more rapid and to reach lower temperature values, but this trend was not significant. However, the apparent pro-inflammatory activity exhibited by ceftriaxone was not associated with aggravated hypothermia compared with untreated animals. Remarkably, when ceftriaxone was administered combined with Pep19-2.5, the two compounds acted jointly, leading to less-intense hypothermia (Figure 4). This convergent activity reverted hypothermia and resulted in a rise of the temperature 330 min after *S. enterica* challenge that was indistinguishable from basal levels (Figure 4).
remained within the normal weight ranges at the end of the experiment with the exception of the spleen, which showed increased weight in all groups (data not shown).

**Histological analysis of the spleen**

The presence of splenomegaly prompted us to perform a more detailed histological study to assess potential differences between the groups. In general, microscopic findings were similarly independent of the treatment, but with different intensity (Supplementary Figure S5). Splenitis was confirmed in samples from all groups and was due to the presence of an inflammatory infiltrate of heterophils (that is, rabbit cells equivalent to polynuclear neutrophils). Abundant bacteria were present in this infiltrate. Bacteria were detected both inside macrophages and free in the extracellular space. Bacterial cells frequently appeared distorted likely because of the activation of immune system effectors (Supplementary Figure S5). The previous findings were accompanied by various degrees of hemorrhage in the splenic tissue. Although there were marked individual responses, in general, treatment with Pep19-2.5 either alone or combined with ceftriaxone, was associated with milder alterations and with a lower bacterial burden than in the untreated controls and in animals that received ceftriaxone single treatment (Table 1).

**DISCUSSION**

The current study demonstrated the therapeutic potential of Pep19-2.5, a synthetic peptide, in combination with the antibiotic ceftriaxone in a rabbit model of sepsis. The dual therapy of Pep19-2.5 and ceftriaxone reduced both the bacterial load (Figures 1 and 5) and endotoxin levels in serum (Figure 2), causing a considerable drop in the production of IL-6 (Figure 3a and Supplementary Figure S2A) and TNF-α (Figure 3b and Supplementary Figure S2B). Moreover, we found an abrogation of hypothermia and a re-establishment of normal rectal temperature (Figure 4), as well as milder alterations and a lower bacterial burden in the spleens of Pep19-2.5/ceftriaxone-treated animals (Table 1).

Despite the intense research and innumerable efforts made in the last decades to develop therapies against sepsis, only modest improvements were introduced in the management of septic patients. The standard therapy of sepsis comprises an antimicrobial treatment in conjunction with supportive therapies (for example, hemodynamic stabilization and airway management). In many cases, however, this treatment does not prevent the heavy and fast onset of inflammation characteristic of sepsis. Possible reasons for this failure involve the release of inflammation-inducing toxins (that is, LPS and LP) from the cell envelopes of bacteria triggered by various antibiotics, which may worsen the patient’s pro-inflammatory response. Thus, to be efficient, sepsis therapy must combat the bacterial infection while simultaneously being capable to decrease the inflammatory reaction. To achieve this double objective, we used a classical antibiotic, ceftriaxone, to kill the bacteria and Pep19-2.5 to neutralize endotoxin.

Bacterial load in organs

The post-mortem quantification of the bacterial load in selected organs revealed high numbers of viable bacteria in the liver, spleen, lungs and kidneys of all studied rabbits (Figures 5a–d). Animals receiving ceftriaxone (either alone or combined with Pep19-2.5) showed lower levels of CFUs in the kidneys and liver (Figures 5a and c, respectively). However, the efficiency of the combined treatment to reduce bacterial colonization in the lungs was lower than that of the ceftriaxone single treatment (Figure 5b). When Pep19-2.5 was administered as a single treatment, it increased the bacterial load in the liver compared with that in untreated animals (Figure 5c) and showed a non-significant trend to do so in the kidneys and lungs (Figures 5a, b and d). This phenomenon was not detected in the spleen.

To indirectly quantify the extent of the inflammatory response in animals, we measured the weight of the liver, spleen, lung and kidney of the rabbits. All organs studied

![Figure 3](image-url)
respectively). Nevertheless, it is possible that the immunomodulating activity displayed by Pep19-2.5 in the present work is not only due to the peptide’s ability to bind and inactivate LPS but also to its inherent capacity to downregulate the inflammatory response. Using mouse models of endotoxemia and septicemia, we previously demonstrated the therapeutic efficacy of Pep19-2.5 in combination with antibiotics, including ceftriaxone. In the current study, the rabbit was chosen as the model organism because it is assumed to more closely resemble the human immune system than mice, which are the most commonly used animals in sepsis research. In fact, rabbits are notably less resistant to LPS than mice, although these non-rodents are still far from the human immune system that is characteristic of an infectious process. By contrast, surgical models, such as cecal ligation and puncture, do reflect the pathophysiological features of human sepsis; however, these models use uncharacterized mixtures of bacterial populations as the endotoxic stimulus, thereby hindering the intra-assay and inter-assay reproducibility. For these reasons, sepsis was induced in our model by the i.v. inoculation of live S. enterica cells to resemble an infectious process, while simultaneously ensuring the necessary reproducibility. Furthermore, the induction of bacteremia in our model is of importance because, in one-third of human sepsis cases, it is possible to obtain a blood-positive culture. Finally, patients with blood-positive cultures have a higher risk of experiencing cardiovascular events (for example, myocardial infarction) even years after infection.

Similar to our findings, other authors using different rabbit models of sepsis reported metabolic disorders such as hyperglycemia and hyperlactatemia, and showed that the experimental treatment reduced lactate levels. However, in these studies, animals received therapy before being infected or continuous infusion of the agent, unlike in our study. Similarly, Garcia et al. reported amelioration of acidosis in a rabbit model in which animals were pre-treated with the experimental drug.
The release of endotoxin from antibiotic-treated bacteria is well documented both in vitro and during active infection in mice, rats and rabbits. The administration of AMPs alone or combined with antibiotics has been reported to reduce the LPS levels and counteract sepsis-associated symptoms, leading to survival benefits in several animal models, including mice and rats. Using rabbit models, other authors have reported that polymyxin B alleviated pathophysiological disorders caused by endotoxemia or septicemia. Similarly, Lin et al. showed that a combination of an AMP (a fragment of the bactericidal/permeability-increasing protein \( \text{bPI21} \)) and cefamandole accelerated bacterial clearance, improved cardiopulmonary dysfunction and prevented rabbit death; however, in this model, the antibiotic treatment was given before bacterial challenge. Finally, other groups reported that a recombinant endotoxin neutralizing protein from \( \text{Limulus polyphemus} \) combined with antibiotics partially protected rabbits from lethal peritonitis or from lethal endotoxemia if animals received antiendotoxic therapy before being infected.

To the best of our knowledge, this is the first report showing evidence in rabbits of AMP-mediated inactivation of endotoxin (Figure 2), leading not only to the neutralization of pro-inflammatory cytokine production (Figure 3) but also to the recovery of normal temperature values (Figure 4). Our results suggest that Pep19-2.5 and ceftriaxone act in synergy to combat bacteremia (Figure 1) and neutralize IL-6 production (Figure 3). This phenomenon is particularly relevant because any anti-sepsis drug candidate is expected to be administered in combination with one or several antibiotics. Keeping in mind that we administered the drugs as a bolus, it is likely that a constant infusion might result in an even more pronounced therapeutic effect. Taken together, our results indicate that the therapy of Pep19-2.5 and ceftriaxone is a promising drug combination for controlling the crucial causes of sepsis.

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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### Table 1 Summary of anatomopathological findings in the histological preparations of the rabbit spleen

| Sample from the indicated animal group | Hemorrhage | Degree of necrosis | Degree of splenitis | Size of follicles | Number of bacteria |
|---------------------------------------|------------|--------------------|---------------------|------------------|-------------------|
| Untreated 1                           | ++++       | +++                | +++                 | Reduced          | ++++              |
| Untreated 2                           | +++        | +++                | +++                 | Reduced          | ++++              |
| Untreated 3                           | ++         | +++                | +++                 | Normal           | ++++              |
| Untreated 4                           | ++++       | +++                | +++                 | Normal           | ++++              |
| Untreated 5                           | +++        | +++                | +++                 | Normal           | ++++              |
| Untreated 6                           | +++        | +++                | +++                 | Normal           | ++++              |
| Ceftriaxone 1                         | ++         | +++                | +++                 | Normal           | ++               |
| Ceftriaxone 2                         | ++++       | +++                | +++                 | Normal           | ++               |
| Ceftriaxone 3                         | ++++       | ++                 | +++                 | Normal           | +                |
| Ceftriaxone 4                         | ++++       | ++                 | +++                 | Normal           | ++               |
| Ceftriaxone 5                         | +++        | +++                | +++                 | Normal           | +++               |
| Ceftriaxone 6                         | +++        | +++                | +++                 | Normal           | +++               |
| Pep19-2.5 1                           | +          | +                  | +++                 | Normal           | +                |
| Pep19-2.5 2                           | +          | +                  | +                   | Normal           | +                |
| Pep19-2.5 3                           | ++         | +                  | +++                 | Normal           | ++               |
| Pep19-2.5 4                           | ++         | +                  | ++                  | Increased        | ++               |
| Pep19-2.5 5                           | +++        | +                  | +                   | Normal           | +                |
| Pep19-2.5 6                           | ++         | +                  | +                   | Normal           | +                |
| Pep19-2.5+ceftriaxone 1               | ++         | ++                 | +++                 | Normal           | +++              |
| Pep19-2.5+ceftriaxone 2               | +          | +                  | ++                  | Normal           | +                |
| Pep19-2.5+ceftriaxone 3               | ++         | +                  | +                   | Reduced          | +                |
| Pep19-2.5+ceftriaxone 4               | +          | +                  | +                   | Normal           | +                |
| Pep19-2.5+ceftriaxone 5               | ++         | ++                 | ++                  | Normal           | +                |
| Pep19-2.5+ceftriaxone 6               | ++         | +                  | ++                  | Normal           | +                |

Anatomopathological evaluation of all samples was carried out in a blinded manner by the same pathologist, according to the institutional guidelines.

*Post-mortem samples of the spleen were fixed by immersion in 4% formaldehyde and were subsequently prepared for inclusion, cutting and hematoxylin–eosin staining.*
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