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(54) Title: LYSOZYME-MODIFIED PROBIOTIC COMPONENTS AND USES THEREOF

(57) Abstract: The present invention relates to methods and compositions for treating sepsis in a subject in need thereof. The method comprises administering effective amounts of at least one lysozyme-modified probiotic component. Also provided are kits that find use in practicing the subject methods. The subject methods and compositions find use in a variety of different applications, including but not limited to the treatment of sepsis.

LYSOZYME-MODIFIED PROBIOTIC COMPONENTS AND USES THEREOF

RELATED APPLICATIONS

[0001] This application claims the benefit of provisional U.S. Patent Application Serial No. 60/777,991, filed February 28, 2006, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The present invention relates to methods of treatment using effective components derived from probiotics.

BACKGROUND

[0003] Sepsis is a major and extremely costly medical problem. Each year in the United States, over 750,000 individuals develop severe sepsis, which can rapidly lead to organ dysfunction and, ultimately, death. The sepsis syndrome is associated with an initially overwhelming innate immune response, characterized by unabated activation and release of pro-inflammatory mediators, i.e. humoral effectors. Subsequently, the exaggerated systemic inflammatory response is counterbalanced by a sustained expression of potent anti-inflammatory mediators, which often results in the desensitization of effector cells (such as phagocytes) and the development of immunosuppression. Both the excessive inflammation and the profound immunosuppression are major determinants to an adverse clinical outcome in sepsis. Furthermore, physiological functions of cellular effectors of the innate immune system such as macrophages/monocytes and neutrophils are altered in sepsis.

[0004] Current methods of severe sepsis treatment include antibiotics, surgical drainage of infected fluid collections, fluid replacement, and drugs to raise blood pressure. Dialysis may be necessary in the event of kidney failure, and mechanical ventilation is often required if respiratory failure occurs. Recently, several investigations suggested that modulation of immune cell function might be a novel therapeutic strategy for attenuation of sepsis. However, no single agent or treatment strategy has shown sufficient success for

the management of patients with sepsis. Although therapeutic interventions have been shown to slightly improve survival of septic patients, the disease remains associated with a high mortality rate.

[0005] Cathelicidin is a protein stored in granules as inactive propeptide precursors in polymorphonuclear leukocytes and monocytes/macrophages. Upon stimulation, cathelicidin is released from phagocytes. After release, the C-terminal end of cathelicidin is processed into active peptides. It has been demonstrated that phagocytes of human and rodent express a single cathelicidin peptide, namely, hCAP-18/LL-37 in humans and CRAMP (cathelicidin-related antimicrobial peptide) in rodents. hCAP-18/LL-37 and CRAMP are effective killers of a variety of bacteria, including *E. coli*, *P. aeruginosa*, and *S. aureus*. CRAMP has been shown to impair intracellular replication of pathogens. Apart from its antimicrobial properties, hCAP-18/LL-37 has also been demonstrated to neutralize lipopolysaccharide (LPS) and protect mice from LPS lethality. CRAMP-deficient mice are susceptible to severe bacterial infection. Administration of hCAP-18/LL-37 protects against sepsis in neonatal rats. Thus, cathelicidin-related peptides play an important role in the maintenance of protective innate immunity.

[0006] Probiotics are non-pathogenic microorganisms, which confer benefits to the host when administrated in sufficient amounts. Previous studies have shown that oral administration of probiotics modulates intestinal immunity, improves the balance of the gut microflora, enhances the recovery of the disturbed gut mucosal barrier, and prevents microbial translocation. Experimental and clinical studies have provided evidence for the possible use of probiotics in several inflammatory diseases including inflammatory bowel disease, enteritis, diarrhea, and pancreatitis. In addition, studies have shown that the protective effect of probiotics is not limited to the gut. However, enteral delivery of intact probiotic bacteria has not been shown to prevent sepsis. Although probiotics have been suggested to enhance natural and acquired immunity, it is unclear whether their effects are associated with cathelicidin-related innate immunity.

SUMMARY OF THE INVENTION

[0007] In one aspect, the invention comprises a method for enhancing macrophage antimicrobial activity. At least one probiotic is selected and digested with a lytic enzyme, such that the at least one probiotic cell wall is broken releasing probiotic components. The probiotic components are then administered to a subject in need thereof. Preferably, the lytic enzyme comprises lysozyme, and the probiotic is *lactobacillus sp.*

[0008] In another aspect, the invention provides a method of treating sepsis. A subject in need thereof is administered an amount of at least one probiotic component which is effective in targeting the subject's cellular effectors.

[0009] In a further aspect, a pharmaceutical composition is provided that comprises at least one probiotic component and a pharmaceutically acceptable carrier.

[0010] In yet another aspect, the invention provides a method of enhancing Interleukin-1 receptor-associated kinase-M expression in a subject in need thereof. The method comprises administering to the subject an amount of at least one probiotic component which is effective for induction of Interleukin-1 receptor-associated kinase-M. Desirably, the at least one probiotic component comprises peptidoglycan or a component of *lactobacillus sp.* and is administered orally.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1 depicts the survival rate of rats treated with lysozyme-modified probiotic component ("LzMPC"), vehicle, viable Lactobacillus, or lysozyme-modified *E. coli* component following cecal ligation and puncture.

[0012] FIG. 2 depicts the effect of LzMPC treatment on bacterial clearance in the liver of septic rats.

[0013] FIGS. 3A – 3E are images of rat liver tissues counterstained with DAPI examined under a fluorescent microscope.

[0014] FIGS. 4A and 4B are images of rat peritoneal macrophages examined under a microscope at X 400 magnification. FIG. 4A rats were treated with LzMPC and FIG. 4B rats were treated with vehicle.

[0015] FIG. 4C depicts the intracellular killing of bacteria by macrophages pretreated with LzMPC or vehicle.

[0016] FIG. 5A illustrates the effect of bacterial components on CRAMP expression.

[0017] FIG. 5B depicts an increase in CRAMP mRNA expression in macrophages treated by LzMPC *in vitro.*

[0018] FIG. 5C depicts an increase in TNF production in macrophages with LPS and LzMPC *in vitro.*

[0019] FIG. 6 depicts the effects of surgical stress, CLP, of LzMPC on CRAMP expression in rat liver.

[0020] FIGS. 7A and 7B depict the effect of LzMPC treatment on CRAMP gene expression in phagocytes *in vivo.*

[0021] FIG. 8A illustrates the protocol for delivery of LzMPC, induction of sepsis by CLP, and administration of anti-CRAMP antibodies (Abs).

[0022] FIG. 8B depicts the survival rate of rats treated with LzMPC and anti-CRAMP Abs following cecal ligation and puncture.

[0023] FIG. 9 depicts an increase in cytokine production capacity by phagocytes in rats treated with LzMPC.

[0024] FIG. 10 depicts the effect of LzMPC on bacterial growth in rat cecum.

[0025] FIG. 11 depicts the effect of LzMPC treatment on serum TNF level during CLP-induced sepsis.

[0026] FIG. 12 depicts the survival rate of mice treated with probiotic component peptidoglycan following cecal ligation and puncture.

[0027] FIG. 13 depicts the effect of probiotic component peptidoglycan treatment on expression of Interleukin-1 receptor-associated kinase-M in mouse liver.

DETAILED DESCRIPTION

[0028] The present invention relates to methods for treatment of sepsis in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of lysozyme-modified probiotic component (LzMPC). Also provided are compositions and kits useful in practicing the subject methods.

[0029] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting. Methods recited herein may be carried out in any order of the recited events which is logically possible, as well as the recited order of events.

Definitions

[0030] The terms "about" or "substantially" used with reference to a quantity includes variations in the recited quantity that are equivalent to the quantity recited, such as an amount that is insubstantially different from a recited quantity for an intended purpose or function.

[0031] The term "therapeutically effective amount" refers to an amount high enough to significantly positively modify the condition to be treated but low enough to avoid serious side effects (at reasonable benefit/risk ratio) within the scope of sound medical judgment. The therapeutically effective amount will vary with the particular condition being treated and the patient's physical condition.

[0032] The term "pharmaceutically acceptable," as used herein, refers to those compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower mammals without undue toxicity, irritation, and allergic response, are commensurate with a reasonable benefit/risk ratio, and are effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention.

[0033] The term "administered with," as used herein, means that a given pharmacological agent and at least one other adjuvant (including one or more other different pharmacological agents) are administered at times sufficiently close that the results observed are indistinguishable from those achieved when the pharmacological agent and at least one other adjuvant are administered at the same point in time. The pharmacological agent and at least one other adjuvant may be administered simultaneously (i.e., concurrently) or sequentially. Simultaneous administration may be carried out by mixing a given pharmacological agent and at least one other adjuvant prior to administration, or by administering a given pharmacological agent and at least one other adjuvant at the same point in time. Such administration may be at different anatomic sites or using different routes of administration. The phrases "concurrent administration," "administration in combination," "simultaneous administration" or "administered simultaneously" may also be used interchangeably and mean that a given pharmacological agent and at least one other adjuvant are administered at the same point in time or immediately following one another. In the latter case, the pharmacological agent and at least one other adjuvant are administered at times sufficiently close that the results produced are synergistic and/or are indistinguishable from those achieved when the at least one pharmacological agent and at least one other adjuvant are administered at the same point in time. Alternatively, a pharmacological agent may be administered separately from the administration of an adjuvant, which may result in a synergistic effect or a separate effect.

[0034] Though the following detailed description describes and illustrates various exemplary embodiments of the invention with reference to lysozyme, any lytic enzyme may be utilized in the present invention. "Lytic enzyme" includes any substance capable of degrading the bacterial wall resulting in lysis (and death) of the cell. For example, the lytic enzyme may be glucosaminidase, amidase, chitinase, and endopeptidase.

Lysozyme

[0035] Lysozyme is a natural antimicrobial enzyme found in a number of secretions in humans, animals, and plants. Lysozyme can be isolated from the tear fluid, saliva and nasal mucus of humans. It is found in the milk and the colostrum of cows. It has also been possible to isolate the lysozyme from cauliflower juice. On an industrial scale, lysozyme is typically extracted from chicken albumen.

[0036] Lysozyme's antimicrobial action is responsible for cleaving peptidoglycan in the walls of many kinds of bacteria. The enzyme destroys bacterial walls by catalyzing the insertion of a water molecule at a glycosidic bond. This hydrolysis breaks up the peptidoglycan at that point. By degrading the bacterial wall, lysozyme not only functions as a potent antibacterial molecule, but also has the ability to release components from within bacteria which modulate the activity of host immune cells.

Priobiotics

[0037] Probiotics are non-pathogenic microorganisms or components thereof capable of a therapeutically beneficial effect in vertebrate subjects (i.e., members of the subphylum cordata), including mammals such as cattle, sheep, pigs, goats, horses, dogs, cats and humans. For example, probiotics that may be used in the present invention include, but are not limited to, Bifidobacteria (such as Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, amd Bifidobacterium longum), Lactobacilli (such as Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus plantarum,

Lactobacillus rhamnosus, Lactobacillus GG, and Lactobacillus reuteri), Streptococci (such as streptococcus thermophilus), and yeast (such as Saccaromyces boulardii).

[0038] Any single probiotic or combination of probiotics and extracts or by-products thereof may be employed in creating LzMPC. Desirably, the LzMPC is created by employing one or more species of Lactobacilli or Bifidobacteria or combinations thereof. Even more desirably, the LzMPC is created utilizing Lactobacillus rhamnosus or Lactobacillus acidophilus.

LzMPC

[0039] LzMPC is produced by treating one or more probiotics with lysozyme. Lysozyme cleaves the probiotic cell wall, killing the probiotic while releasing probiotic components from within. These LzMPC may modulate the activity of the host immune cells. For example, administering LzMPC to an individual may target cellular effectors such as phagocytes, thereby enhancing protective immune capacity of phagocytes and protecting against sepsis.

[0040] Phagocytes, including neutrophils and macrophages, are cellular effectors of the innate immune system. They play an important role in regulation of innate immunity and protection of a host from invading microbe. During the development and resolution of sepsis, the protective innate immune capacity of phagocytes undergoes a dynamic change. In the early phase, pro-inflammatory mediators and bacterial components, such as lipopolysaccharide, enhance phagocyte activity, which contributes to efficient regulation of the antibacterial response. Subsequently, the innate immune capacity/activity of phagocytes is suppressed, which is associated with the state of immunoparalysis in sepsis. In this stage, monocytes/macrophages and neutrophilic polymorphonuclear leukocytes are deactivated. They have depressed-cytokine productivity and a poor ability to eliminate bacteria. The desensitization of phagocytes appears to be mediated by anti-inflammatory mediators, which presumably leads to impaired bactericidal activity in phagocytes and cause patients with sepsis to be at a high

risk for bacterial infection. LzMPC treatment, by improving phagocyte function, may enhance the innate immune capacity, thus protecting against sepsis.

[0041] For example, in one embodiment, administration of peptidoglycan (PGN) modulates macrophage protein expression thereby having a protective effect in sepsis. PGN, a complex polymer present in bacterial cell walls, is released from *lactobacillus sp.* via treatment with lysozyme. Bacterial infections such as sepsis are often mediated by activation of toll like receptors (TLR) in inflammatory cells, which plays an important role in the pathogenesis of infection-induced inflammation. Administration of probiotic-PGN, for example via oral and/or enteral delivery, therapeutically modifies expression of Interleukin-1 receptor-associated kinase-M (IRAK-M), a protein expressed by macrophages. The protective effect of probiotic-PGN and LzMPC in sepsis could be mediated by up-regulation of IRAK-M in macrophages. In addition, the strategy of induction of IRAK-M by administration, for example oral or enteral administration, of probiotic components such as probiotic-PGN and LzMPC can be applied to prevention and treatment of several other diseases requiring up-regulation of IRAK-M.

[0042] LzMPC may be prepared by any suitable method known in the art. For example, in one embodiment, fresh cultured probiotic bacteria are washed and suspended in a buffer containing lytic enzyme. Preferably, the bacteria are washed multiple times in a phosphate buffered saline (PBS) with a pH between about 5.0 to about 8.0. Even more preferably, the PBS has a pH of about 7.0.

[0043] Preferably, the probiotic is suspended in a buffer containing lytic enzyme and having a pH between about 5.0 to about 8.0. The lytic enzyme is preferably a glucosamindase or amidase or a combination thereof. Even more preferred, the buffer is a phosphate buffer with a pH about 7.0 containing lysozyme (Sigma# L-6876, 2mg/ml).

[0044] The probiotic suspended in a buffer containing lytic enzyme may be incubated at between about 20 Celsius to about 50 Celsius until at least the probiotic is digested. Preferably, the suspension is incubated for between about

30 minutes to about 90 minutes at about 35 Celsius to about 40 Celsius. More preferably, the suspension is incubated at about 37 Celsius for about 60 minutes.

[0045] Following incubation and digestion, the suspension may be separated. For example, the suspension may be separated by centrifuge at about 7000 X g for about 30 minutes at between about 2 Celsius to about 10 Celsius. The supernatant is collected and boiled to inactivate the lysozyme. Preferably, the supernatant is boiled for about 30 minutes at about 100 Celsius. Further processing may be performed if desired.

[0046] For example, after boiling the supernatant may be cooled to room temperature and centrifuged at about 10000 X g for about 30 minutes at about 4 Celsius. The resulting supernatant is collected and may be processed through a chromatography with Detoxi-Gel (Pierce, Cat#20344) to remove any endotoxin. If further preferred, LzMPC may be filtered before use, for example through a .2 µm filter.

Method of Treatment

[0047] A method of treatment involves administering to a subject in need a therapeutically effective amount of LzMPC. LzMPC may be administered in any form by any effective route, including, for example, oral, parenteral, enteral, intraperitoneal, topical, transdermal (e.g., using any standard patch), ophthalmic, nasally, local, non-oral, such as aerosal, spray, inhalation, subcutaneous, intravenous, intramuscular, buccal, sublingual, rectal, vaginal, intra-arterial, and intrathecal, etc. LzMPC can be administered alone, or in combination with any ingredient(s), active or inactive. Desirably, LzMPC is administered orally.

[0048] LzMPC may be administered alone or in the form of a pharmaceutical composition that contains the LzMPC in admixture with a pharmaceutically acceptable carrier. For example, the pharmaceutical composition may be in dosage forms such as tablets, coated tablets, capsules, granules, fine granules, powders, syrups, suppositories, injections, or the like. These preparations can be prepared by conventional methods well known in the art.

[0049] Examples of suitable carriers are well known in the art and can include, but are not limited to, all organic or inorganic carrier materials that are usually used for the pharmaceutical preparations and are inert to LzMPC. Examples of suitable carriers suitable for the preparation of tablets capsules, granules and fine granules are diluents such as lactose, starch, sucrose, D-mannitol, calcium sulfate, or microcrystalline cellulose; disintegrators such as sodium carboxymethylcellulose, modified starch, or calcium carboxymethylcellulose; binders such as methylcellulose, gelatin, acacia, ethylcellulose, hydroxypropylcellulose, or polyvinylpyrrolidone; lubricants such as light anhydrous silicic acid, magnesium stearate, talc, or hydrogenated oil; or the like. When formed into tablets, they may be coated in a conventional manner by using conventional coating agents such as calcium phosphate, carnauba wax, hydroxypropyl methylcellulose, macrogol, hydroxypropyl methylphthalate, cellulose acetate phthalate, titanium dioxide, sorbitan fatty acid ester, or the like.

[0050] Examples of carriers suitable for the preparation of syrups are sweetening agents such as sucrose, glucose, fructose, or D-sorbitol; suspending agents such as acacia, tragacanth, sodium carboxymethylcellulose, methylcellulose, sodium alginate, microcrystalline cellulose, or veegum; dispersing agents such as sorbitan fatty acid ester, sodium lauryl sulfate, or polysorbate 80; or the like. When formed into syrups, the conventional flavoring agents, aromatic substances, preservatives, or the like may optionally be added thereto. The syrups may be in the form of dry syrup that is dissolved or suspended before use.

[0051] Examples of bases used for the preparation of suppositories are cacao butter, glycerin saturated fatty acid ester, glycerogelatin, macrogol, or the like. When formed into suppositories, the conventional surface active agents, preservatives or the like may optionally be admixed.

[0052] For solid compositions, conventional non-toxic carriers that may be utilized include, for example mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and. Liquid pharmaceutically administerable compositions can, for example, be

prepared by dissolving, dispersing, etc. LzMPC and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like to thereby form a solution or suspension. If desired, LzMPC may also contain minor amounts of non-toxic auxiliary pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 15th Edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of LzMPC in a therapeutically effective amount to alleviate the symptoms of the subject being treated.

[0053] For delayed release, the compounds of the invention may be formulated in a pharmaceutical composition, such as in microcapsules formed from biocompatible polymers, or in liposomal carrier systems according to methods known in the art.

[0054] As noted above, some embodiments include administering an effective amount of LzMPC and an effective amount of at least a second, different pharmacological agent, e.g., concurrently administered, where the two may differ in one or more of a variety of aspects, e.g., dosage, type, route of administration, etc. For example, embodiments may include administering LzMPC and at least one other type of pharmacological agent to provide an enhanced therapeutic effect. By "enhanced therapeutic effect" is meant that at least the initial relief of the particular condition being treated by LzMPC occurs more quickly with a combination of LzMPC and at least one other different pharmacological agent, as compared to the same doses of each component given alone, or that doses of one or all component(s) are below what would otherwise be a minimum effective dose (a "sub-MED").

[0055] Although exemplary embodiments of the invention have been described with respect to the treatment of sepsis, delivery of LzMPC may be used to treat a wide variety of conditions. For example, LzMPC may be used to treat inflammation or any number of bacterial infections.

Dose Levels of LzMPC

[0056] The therapeutically effective amount of LzMPC that is provided in connection with the various embodiments ultimately depends upon the condition and severity of the condition to be treated; the type and activity of the specific therapeutic agent employed; the method by which the medical device is administered to the patient; the age, body weight, general health, gender and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts. Desirably, LzMPC is administered in doses between about 1 ml LzMPC per kg of subject's body weight to about 30 ml LzMPC per kg of subject's body weight.

EXAMPLES

Example 1: LzMPC preparation

[0057] LzMPC was prepared from *lactobacillus sp.* (ATCC#53103), a probiotic strain isolated from human feces. In addition, a component named LzMEcC (i.e. lysozyme-modified E. coli component) using commensal bacteria strain (ATCC#25922) was prepared. LzMEcC was used as the control for LzMPC in some experiments.

[0058] For *in vivo* experiments, fresh cultured 10^{11} CFU bacteria were washed with phosphate buffer solution (PBS; pH 7.0) three times, suspended in 100 ml PBS (pH 7.0) containing lysozyme (Sigma# L-6876; 2mg/ml) and incubated at 37°C for 60 min. After digestion, the cells were centrifuged at 7000 X g for 30 min at 4°C. The supernatant was collected and the remaining cell pellets were discarded. To inactivate lysozyme, the supernatants were boiled at 100°C for 30 min, then cooled to room temperature and centrifuged at 10,000 X g for 30 min and 4°C. The resulting supernatant (i.e. LzMPC or LzMEcC) was used in various *in vivo* experiments. In the *in vivo* preparations, 1ml of LzMPC contains probiotic components derived from 10^9 bacteria.

[0059] For *in vitro* experiments, fresh cultured bacteria (1gm) were washed with PBS and digested with lysozyme (2mg/ml) in PBS, as described above. After digestion, the cells were centrifuged at 7000 X g for 30 min at 4°C. The supernatant was collected and the remaining cell pellets were discarded. To inactivate lysozyme, the supernatants were boiled at 100°C for 30 min. The boiled supernatants (i.e. LzMPC or LzMEcC) were cooled to room temperature and centrifuged at 10,000 X g for 30 min and 4°C. The supernatant was collected, processed for column chromatography on a Detoxi-gel to remove endotoxin from the prep, and filtered through a 0.2 µm filter before applyint to *in vitro* experiments. In preparations used for cell culture (i.e. *in vitro*) experiments, 1 ml of LzMPC stock solution contains probiotic products derived from 100 mg of bacteria.

Example 2: LzMPC protects against sepsis-induced lethality in rats and mice

[0060] The role of LzMPC in sepsis *in vivo*, using a polymicrobial sepsis model was investigated. Initially, a cecal ligation and puncture (CLP) model in rats was set up. The CLP procedure involves surgical stress and triggers disseminated infection, leading to development of peritonitis, bacteremia, and polymicrobial sepsis. To examine the effect of LzMPC, rats and mice were subjected to LzMPC treatment and challenged with CLP. Animals were randomly assigned to four groups, including: 1) LzMPC + CLP (i.e. experimental group, enteral administration of LzMPC and operation with CLP); 2) Vehicle + CLP (enteral administration of vehicle and operation with CLP); 3) Viable Lactobacillus + CLP (enteral administration of lactobacillus (10^9 CFU/gavage) and operation with CLP); and 4) LzMEcC + CLP (enteral administration of LzMEcC and operation with CLP).

[0061] LzMPC was administered to rats starting at 5 days before CLP and continuing until 9 days following CLP. Survival was monitored for 9 days after CLP. Shown in FIG. 1, administration of LzMPC resulted in protection of rats against CLP-induced death, whereas delivery of LzMEcC or viable lactobacillus (LB, i.e. probiotic bacteria) fails to protect against sepsis. In "Vehicle + CLP" and "LzMEcC + CLP" groups survival was 83% and 80% respectively, 24 hours after CLP. This diminished progressively each day until day 6, at which time only 33% and 40% were alive respectively in these groups. LzMPC markedly improved the survival. In the "LzMPC + CLP" group, about 15% of rats died by day 1 and 75% of animals survived by day 9 after the CLP challenge. In contrast, 60% of animals in "viable Lactobacillus + CLP" group died within 9 days after CLP. All rats in the sham-operated group survived during the 9-day interval (data not shown).

Example 3: Oral administration of LzMPC enhances bacterial clearance

[0062] Invasion by enteral commensal bacteria contributes to the development of sepsis in the CLP model. The level of the bacterial count in tissues is known to be associated with the severity of CLP-induced inflammatory response.

Because the liver is a major organ responsible for bacterial clearance in abdominal infection, it was examined whether the survival benefit afforded by LzMPC was functionally related to bacterial elimination function in the liver.

[0063] Briefly, rats were subjected to "Vehicle + CLP" or "LzMPC + CLP" treatment as described above. Livers were harvested 72 hours after CLP and processed for measurement of the bacterial load. As shown in FIG. 2, oral administration of LzMPC enhances bacterial clearance in the liver during the post-CLP period. Livers isolated from animals in "Vehicle + CLP" group contained a substantial amount of bacteria. In contrast, the bacterial load in the liver of "LzMPC + CLP" group was markedly reduced ($P<0.01$). The data indicate that LzMPC treatment reduces bacterial load in sepsis.

Example 4: Orally administered LzMPC is engulfed by cells in the liver

[0064] As demonstrated in Example 3, oral administration of LzMPC results in the enhancement of bactericidal activity in the liver. It was thus further investigated whether LzMPC is translocated into the liver. To this end, rats were gavaged with BacLight Green-labeled LzMPC. Cryosections of the liver were examined from rats 16 hours after enteral feeding with the labeled LzMPC. As illustrated in FIGS. 3A – 3E, particles with green fluorescence were found in the liver sinusoids and cells in the liver sections from rats fed with LzMPC labeled with BacLight Green stain (FIG. 3A). Some of these cells displayed a distinct morphology of Kupffer cells (FIG. 3B), the residential macrophages in the liver. In contrast, the green fluorescent signal was barely found in the liver sections from normal control animals (FIG. 3C). The data indicated the uptake of LzMPC into the liver and macrophages.

[0065] Furthermore, cryosections of the small intestine from rats 90 min after enteral feeding with the labeled LzMPC were examined. This allowed identification of labeled LzMPC at the early stages after it crossed the intact mucosal barrier. When comparing sections from rats fed with labeled LzMPC to sections from controls, cells throughout the lamina propria contained particles with a strong degree of green fluorescence (FIG. 3D), whereas they exhibited

only a weak auto-fluorescence in the controls (FIG. 3E). The data suggests translocation of LzMPC from the gut lumen into intestinal lamina propria.

Example 5: LzMPC activates macrophage's bacterial activity

[0066] Macrophages play an essential role in the innate immune response against bacterial invasion. They eliminate bacteria from tissues during sepsis. Because LzMPC enhances bacterial clearance, it was further examined whether LzMPC directly targeted the innate immune activity of macrophages. First, freshly isolated rat residential peritoneal macrophages were treated with LzMPC (10 μl/ml) or vehicle (control) for 6 hrs and examined under a microscope. As illustrated in FIG. 4A, LzMPC profoundly induced pseudopod formation in rat macrophages, as compared to the control group (FIG. 4B).

[0067] Furthermore, rat residential peritoneal macrophages were pre-treated with LzMPC (10 μl/ml) or vehicle (control) overnight then processed for a bacterial killing assay. As shown in FIG. 4C, macrophages from the control group killed approximately 50% of ingested bacteria within 90 min. Pretreatment with LzMPC led to a significant increase in intracellular killing of bacteria by macrophages ($P<0.05$). Together, the data suggests that LzMPC activates bactericidal activity of macrophages *in vitro*.

Example 6: LzMPC induces expression of CRAMP in macrophages

[0068] To understand the mechanism whereby LzMPC stimulates protective innate immunity of macrophages against invasion of bacteria induced by CLP, it was determined whether LzMPC modulated the expression of CRAMP gene, which encodes an important anti-microbial peptide in rat phagocytes. Residential peritoneal macrophages were stimulated with LzMPC (10 μl/ml) for 2 hours. In control groups, cells were treated with LPS (1 μg/ml) or medium. Total cellular RNA was then isolated and CRAMP gene expression was determined with semi-quantitative conventional RT-PCR. As shown in FIG. 5A, CRAMP gene was constitutively expressed in rat macrophages. LzMPC but not LPS enhanced CRAMP gene expression.

[0069] Next, macrophages were stimulated with LPS (1μg/ml) or LzMPC (10 μl/ml) for 6 hours, and CRAMP gene expression was determined quantitatively with real-time RT-PCR. As shown in FIG. 5B, LPS had no effect on CRAMP expression in macrophages. In contrast, the gene expression was increased more than 14-fold within 6 hours in response to LzMPC stimulation ($P<0.01$ compared to control).

[0070] To confirm that both LPS and LzMPC activated macrophages, the culture supernatants were assayed for TNF using ELISA. As shown in FIG. 5C, TNF production was increased by approximately 50-fold in LPS-stimulated cells and 10-fold in LzMPC-treated cells, indicating that cells were activated by both stimulations, although only LzMPC induced CRAMP gene expression. Together, these observations suggested that LzMPC specifically up-regulated CRAMP-associated innate immune capacity of macrophages.

Example 7: Surgical stress or CLP decreases CRAMP expression whereas LzMPC restores CRAMP gene expression

[0071] In order to assess the *in vivo* contribution of LzMPC treatment on CRAMP expression during the development of sepsis triggered by CLP, rats were divided into groups of (1) normal control, (2) sham-surgery, (3) CLP, and (4) "LzMPC+CLP". The enteral feeding of LzMPC was conducted using the protocol described above in *Example 2* (LzMPC was administered to rats starting at 5 days before CLP and continuing until 9 days following CLP). Animals were sacrificed 72 hourrs after surgery, total cellular RNA of liver was isolated, and CRAMP gene expression was determined with real-time RT-PCR. As shown in FIG. 6, CRAMP gene was constitutively expressed in the rat liver. The gene expression was down-regulated in the liver 72 hours after sham-surgery or CLP. Oral administration of LzMPC prevented or reversed the down-regulation of CRAMP gene expression in the liver of septic rats. Taken together, the data suggests that (1) surgical stress or CLP inhibits CRAMP-associated innate immune capacity in tissues, and (2) LzMPC restores the capacity of CRAMP.

Example 8: Oral administration of LzMPC enhances expression of CRAMP and mRNA and protein in macrophages

[0072] To examine whether LzMPC modulates CRAMP gene expression in macrophages *in vivo*, rats were gavaged with LzMPC for 5 days. Peritoneal macrophages were isolated, total cellular RNA and protein were extracted respectively. Then, CRAMP mRNA was measured with real-time RT-PCR and CRAMP protein was analyzed with western blotting. As demonstrated in FIG. 7A, CRAMP mRNA was constitutively expressed in macrophages. Treatment with LzMPC *in vivo* resulted in a marked up-regulation of CRAMP mRNA in the peritoneal residential macrophages. Furthermore, LzMPC up-regulated the expression of CRAMP protein in macrophages (FIG. 7B).

Example 9: Antibody against CRAMP blocks the protective effect of LzMPC on sepsis

[0073] Because the survival benefit of administrating LzMPC is associated with induction of CRAMP, the role of cathelicidin-related innate immunity in LzMPC-induced protection against sepsis was examined by utilizing an antibody against CRAMP. To accomplish this, rats were divided into groups of (1) LzMPC + CLP; (2) LzMPC + CLP + control IgG; and (3) LzMPC + CLP + anti-CRAMP pAb. Animals were subjected to treatment with LzMPC and challenged with lethal CLP using a protocol described in FIG. 8A. As stated above, LzMPC treatment resulted in a marked decrease in mortality two days after CLP challenge (FIG. 1). Animals who survived two days after CLP were then treated with goat anti-CRAMP antibody (0.75 mg/kg, i.p.). The normal goat IgG was used as a control. The animals were continuously treated with LzMPC and monitored for an additional 8-day after administration of antibodies. As illustrated in FIG. 8B, survival in "LzMPC + CLP + control IgG" group was similar to that in "LzMPC + CLP" group, indicating that administration of control goat IgG (i.e. IgG prepared from non-immunized animals) did not result in any change in survival rate. In contrast, rats in "LzMPC + CLP + anti-CRAMP pAb" group had worse

survival after CLP. The data suggest that endogenous CRAMP is a mediator for LzMPC action.

Example 10: Effect of repeated enteral delivery of LzMPC on cytokine production by peritoneal macrophages and enteral flora in cecum

[0074] Pretreatment with LPS (a bacterial component) could result in the development of tolerance, which may lead to protection against septic peritonitis. Because LzMPC is also derived from bacteria, it was investigated whether treatment with LzMPC would alter cytokine production by phagocytes. Briefly, rats were subjected to LzMPC or vehicle treatment for five days. Peritoneal macrophages were then isolated, stimulated with LzMPC (10 μl/ml) for 6 hours, and TNF secretion was determined by ELISA. As shown in FIG. 9, macrophages spontaneously secreted TNF *in vitro*. Oral administration of LzMPC had no effect on the basal level of TNF production in macrophages. However, TNF secretion by macrophages was increased by LzMPC stimulation *in vitro*. Macrophages from LzMPC treated animals had an increased capacity of TNF production in response to LzMPC stimulation *in vitro*. In addition, cells were also stimulated with LPS (1 μg/ml) for 6 hours and TNF secretion was determined. It was found that LPS induced TNF production at 954 $\pm$ 26.35 pg/10^6 cells in macrophages of Vehicle group and 1033 $\pm$ 16.95 pg/10^6 cells in LzMPC group (P<0.05, Vehicle vs. LzMPC). Together, the data indicates that enteral treatment with LzMPC causes an increase in the capacity of cytokine production by macrophages rather than tolerance against LzMPC or cross-tolerance against bacterial component stimulation.

[0075] Enteral delivery of probiotics may influence the status of the gut flora. To examine the effect of LzMPC on bacterial growth in the cecum, rats were subjected to LzMPC or vehicle treatment for 5 days, collected lumenal contents from the cecum by needle-puncture, and processed samples for measurement of bacterial count. As shown in FIG. 10, cecal bacterial colony count in the LzMPC group was similar to the count in the control, indicating that LzMPC did not modulate bacterial growth in the gut. This observation suggests

that LzMPC-induced protective effect in sepsis is derived from mechanisms other than reduction of the amount of commensal bacteria in the intestine.

Example 11: LzMPC treatment effect on serum TNF level during CLP-induced sepsis

[0076] As described above, LzMPC induces the capacity of TNF production by macrophages (FIG. 9). Therefore, the effect of LzMPC treatment on systemic TNF level during the development of sepsis was examined. Rats were divided into the following groups: (1) CLP alone; (2) "LzMPC + CLP" (i.e. enteral feeding LzMPC for 5 days followed by CLP); and (3) "LzMPC + CLP + LzMPC" (i.e. enteral feeding LzMPC for 5 days, challenging with CLP, and enteral feeding LzMPC 2 hours after CLP). All rats were sacrificed 6 hours after CLP. ELISA analysis of serum TNF level of rats in each group demonstrated a similar degree of TNF production 6 hours after CLP stimulation (FIG. 11). The data suggested that LzMPC treatment did not lead to the alteration of systemic TNF level in response to acute polymicrobial sepsis.

Example 13: Probiotic-PGN is an effective component of LzMPC which protects against sepsis

[0077] Peptidoglycan (PGN) is a complex polymer existed in bacterial cell walls. PGN is released from bacteria when cells are treated with lysozyme. In this experiment, it was examined whether PGN derived from *lactobacillus sp.* (ATCC#53103), namely, probiotic-PGN mimics the protective effect on LzMPC on sepsis. First, PGN was extracted from lactobacillus using a standard protocol. Mice were randomly assigned to the following groups: 1) "probiotic-PGN + CLP" (i.e. experimental group, enteral administration of probiotic-PGN and operation with CLP); and 2) "Vehicle + CLP" (enteral administration of vehicle and operation with CLP). Probiotic-PGN was delivered enterally to mice starting at 5 days before CLP and coninuing until 10 days following CLP. The protocol descibed above was also used for enteral admininstration of vehicle and conducting CLP. Survival was monitored for 10 days after CLP. Shown in FIG.

12, oral administration of probiotic-PGN resulted in protection of mice against CLP-induced death, whereas orally delivery of vehicle fails to protect against sepsis.

Example 14: Orally administered probiotic-PGN induces IRAK-M expression

[0078] It was examined whether orally administration of probiotic-PGN modulates IRAK-M expression in mouse liver. Briefly, mice were fed with probiotic-PGN for 4 days and total liver proteins were processed for immunobloting with an anti-IRAK-M antibody. Shown in FIG 13, IRAK-M is constitutively expressed in mouse liver. Enterally administration of probiotic-PGN markedly induced IRAK expression in the liver. The data suggests that therapeutic modification of IRAK-M can be accomplished by enteral delivery of probiotic-PGN. The protective effect of probiotic-PGN and LzMPC in sepsis could be mediated by up-regulation of IRAK-M in macrophages.

CLAIMS

1. A method for enhancing macrophage activity, comprising the steps of:

selecting at least one probiotic;

digesting the at least one probiotic with lytic enzyme such that the at least one probiotic cell wall is broken releasing probiotic components;

administering at least one of the probiotic components to a subject in need thereof.

2. The method of claim 1, wherein the lytic enzyme is selected from the group consisting of glucosaminidases, amidases, and endopeptidases.

3. The method of claim 1, wherein the lytic enzyme is lysozyme.

4. The method of claim 1 or 2, wherein the probiotic is selected from the group consisting of Lactobacilli and Bifidobacteria.

5. The method of claim 4, wherein the probiotic is selected from the group consisting of lactobacillus acidophilus and lactobacillus rhamnosus.

6. The method of any of the preceding claims, wherein the at least one of the probiotic components administered to a subject in need thereof comprises peptidoglycan.

7. The method of any of the preceding claims, wherein the at least one of the probiotic components is administered to a subject in need thereof orally or enterally, or a combination thereof.

8. A method of treating sepsis in a subject in need thereof, the method comprising administering to the subject an amount of at least one probiotic component which is effective in targeting said subject's cellular effectors.

9. The method of claim 8, wherein the at least one probiotic component comprises a component of Lactobacilli or Bifidobacteria, or a combination thereof.

10. The method of claim 8, wherein the at least one probiotic component comprises a component of lactobacillus acidophilus or lactobacillus rhamnosus, or a combination thereof.

11. The method of claim 8, wherein the at least one probiotic component comprises peptidoglycan.

12. The method of any one of claims 8-11, wherein the amount of the at least one probiotic component administered to the subject in need thereof is between 1 ml and 3ml of probiotic component per kilogram of subject body weight.

13. The method of any one of claims 8-12, wherein the probiotic component is administered to a subject in need thereof orally or enterally, or a combination thereof.

14. A pharmaceutical composition comprising at least one probiotic component and a pharmaceutically acceptable carrier.

15. The composition of claim 14, wherein the at least one probiotic component comprises a component of Lactobacilli or Bifidobacteria, or a combination thereof.

16. The composition of claim 14, wherein the at least one probiotic component comprises a component of lactobacillus acidophilus or lactobacillus rhamnosus, or a combination thereof.

17. The composition of claim 14, wherein the at least one probiotic component comprises peptidoglycan.

18. The composition of any one of claims 14-17, wherein the pharmaceutically acceptable carrier is selected from the group consisting of diluents, disintegrators, binders, and lubricants.

19. The composition of any one of claims 14-17, wherein the pharmaceutically acceptable carrier is selected from the group consisting of sweetening agents, suspending agents, and dispersing agents.

20. The composition of any one of claims 14-19, wherein the composition further comprises a second pharmacological agent different from the at least one probiotic component.

21. A method of enhancing Interleukin-1 receptor-associated kinase-M expression in a subject in need thereof, the method comprising administering to the subject an amount of at least one probiotic component which is effective for induction of Interleukin-1 receptor-associated kinase-M.

22. The method of claim 21, wherein the at least one probiotic component comprises a component of Lactobacilli or Bifidobacteria, or a combination thereof.

23. The method of claim 21, wherein the at least one probiotic component comprises a component of lactobacillus acidophilus or lactobacillus rhamnosus, or a combination thereof.

24. The method of claim 21, wherein the at least one probiotic component comprises peptidoglycan.

25. The method of any one of claims 21-24, wherein the amount of the at least one probiotic component administered to the subject in need thereof is between 1 ml and 3ml of probiotic component per kilogram of subject body weight.

26. The method of any one of claims 21-25, wherein the probiotic component is administered to a subject in need thereof orally or enterally, or a combination thereof.

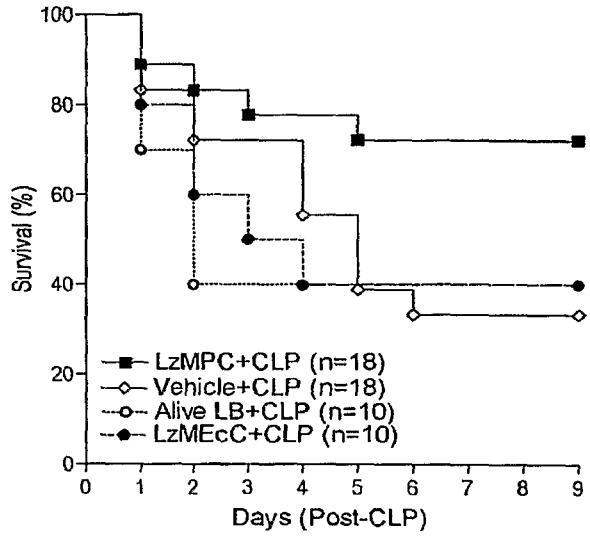

100
80
60
40
20
0
Survival (%)
0 1 2 3 4 5 6 7 8 9
Days (Post-CLP)
LzMPC+CLP (n=18)
Vehicle+CLP (n=18)
Alive LB+CLP (n=10)
LzMEcC+CLP (n=10)

FIG. 1

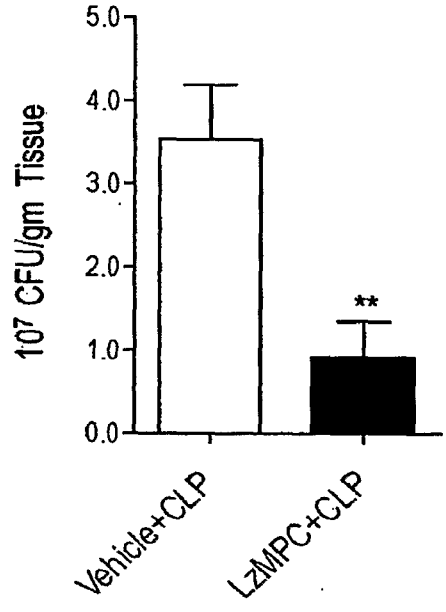

5.0
4.0
3.0
2.0
1.0
0.0
10⁷ CFU/gm Tissue
**
Vehicle+CLP
LzMPC+CLP

FIG. 2

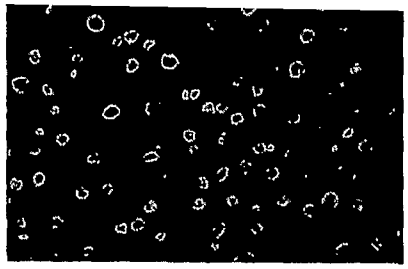


FIG. 3A

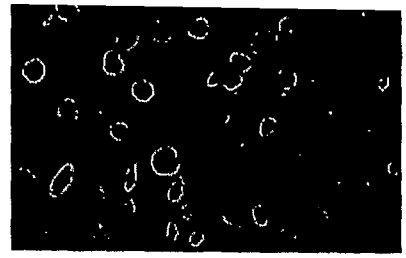


FIG. 3B

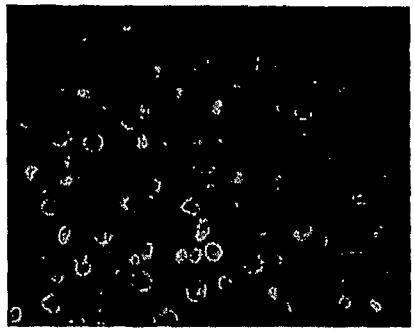


FIG. 3C

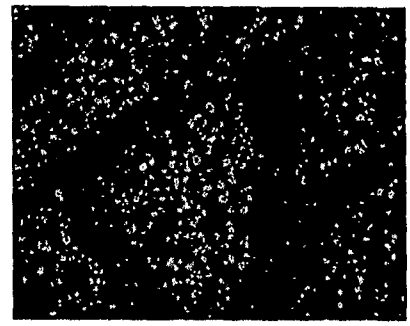


FIG. 3D

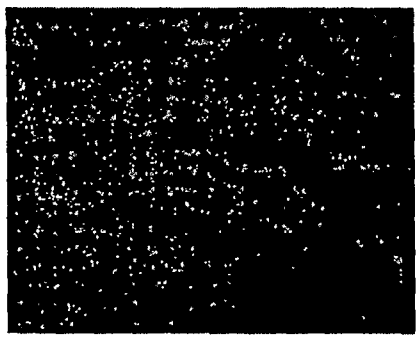


FIG. 3E

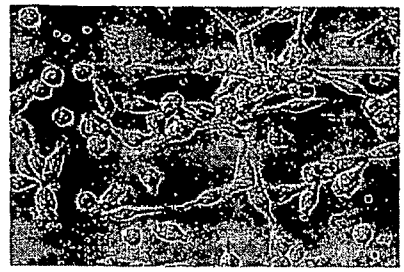


FIG. 4A

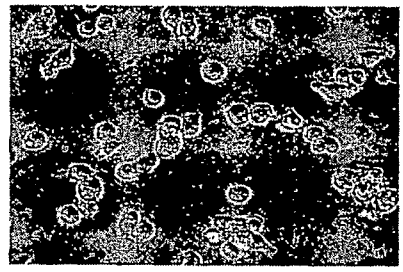


FIG. 4B

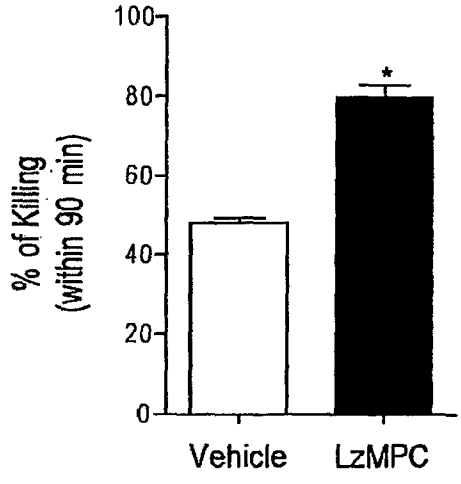


FIG. 4C

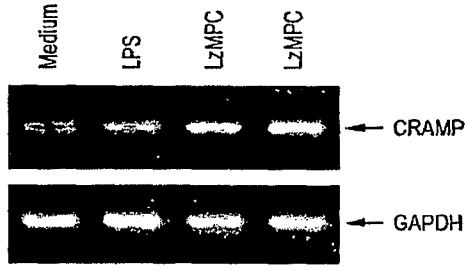

Medium
LPS
LzMPC
LzMPC
CRAMP
GAPDH

FIG. 5A

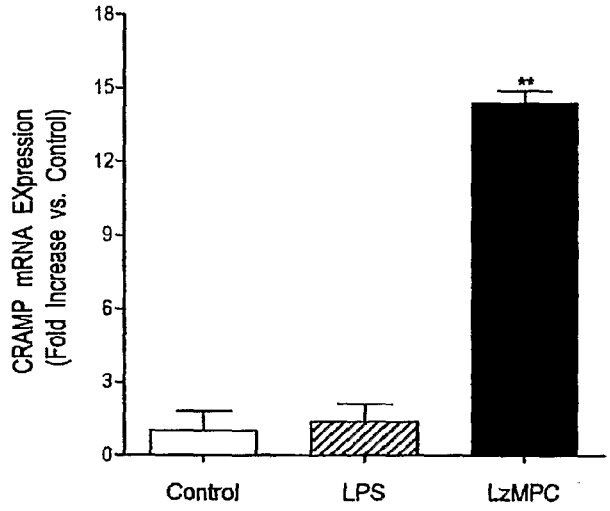

CRAMP mRNA EXpression
(Fold Increase vs. Control)
18
15
12
9
6
3
0
**
Control
LPS
LzMPC

FIG. 5B

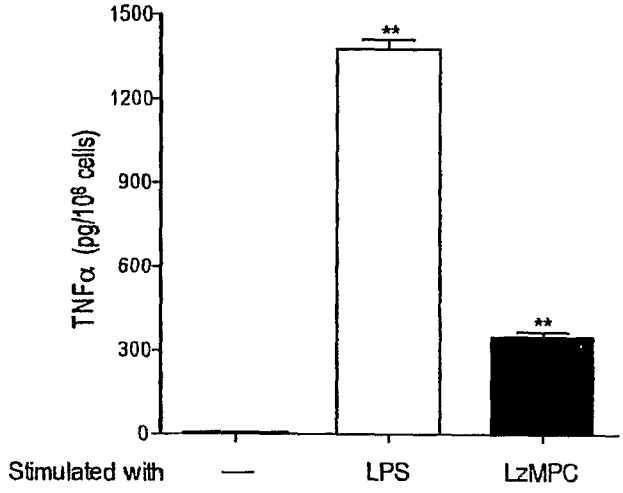

1500
1200
900
600
300
0
TNFα (pg/10⁶ cells)
**
**
Stimulated with
—
LPS
LzMPC

FIG. 5C

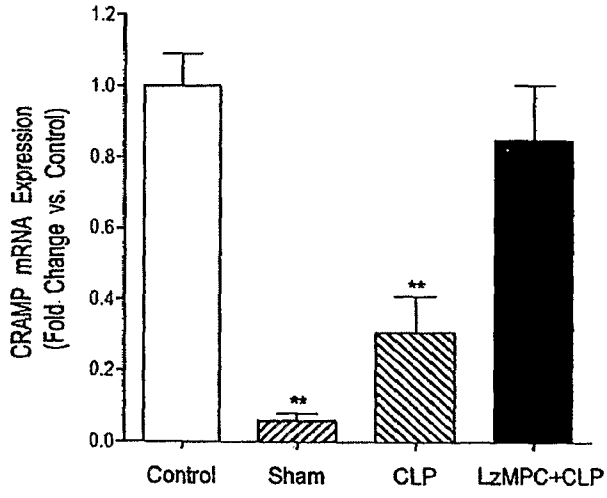

1.2
1.0
0.8
0.6
0.4
0.2
0.0
CRAMP mRNA Expression
(Fold Change vs. Control)
**
**
Control
Sham
CLP
LzMPC+CLP

FIG. 6

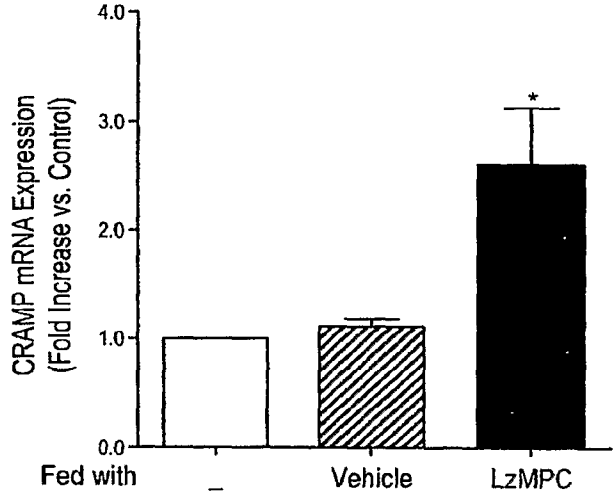

CRAMP mRNA Expression
(Fold Increase vs. Control)
4.0
3.0
2.0
1.0
0.0
*
Fed with
_
Vehicle
LzMPC

FIG. 7A


Fed with
Vehicle
LzMPC
CRAMP
β-actin

FIG. 7B

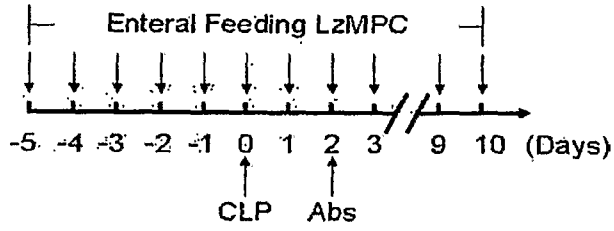

Enteral Feeding LzMPC
-5 -4 -3 -2 -1 0 1 2 3 9 10 (Days)
CLP
Abs

FIG. 8A

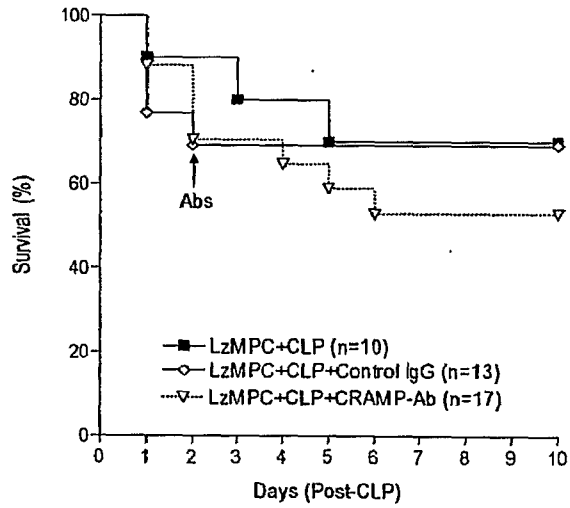

Survival (%)
100
80
60
40
20
0
Abs
LzMPC+CLP (n=10)
LzMPC+CLP+Control IgG (n=13)
LzMPC+CLP+CRAMP-Ab (n=17)
0 1 2 3 4 5 6 7 8 9 10
Days (Post-CLP)

FIG. 8B

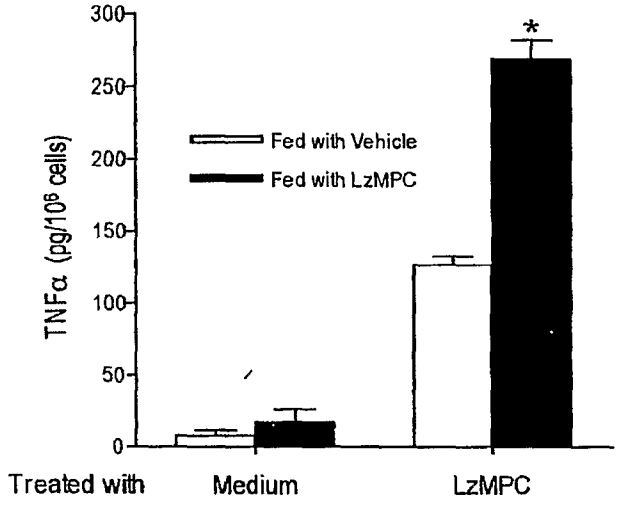

300
250
200
150
100
50
0
TNFα (pg/10⁶ cells)
*
Fed with Vehicle
Fed with LzMPC
Treated with
Medium
LzMPC

FIG. 9

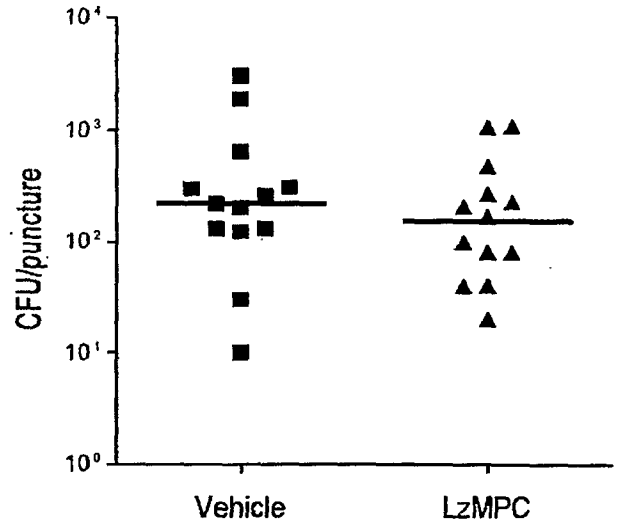

10⁴
10³
10²
10¹
10⁰
CFU/puncture
Vehicle
LzMPC

FIG. 10

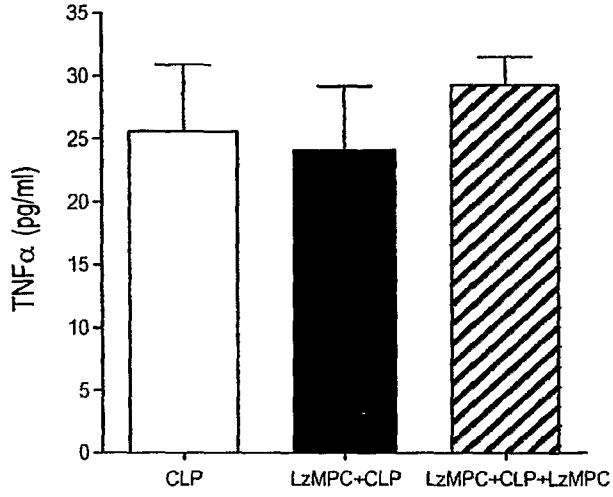


FIG. 11

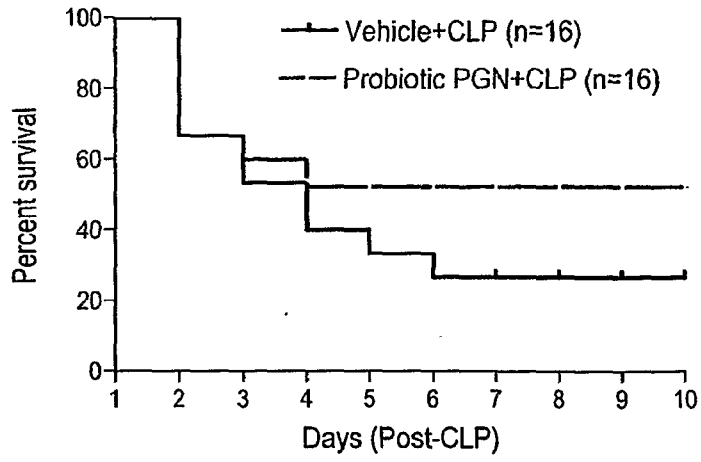


FIG. 12

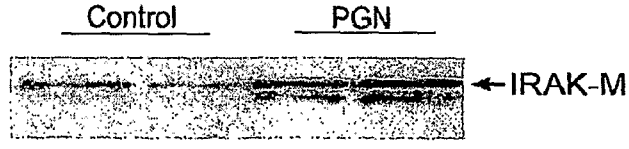


FIG. 13