Correction of immunosuppression in aged septic rats by human ghrelin and growth hormone through the vagus nerve-dependent inhibition of TGF-β production

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Abstract

Background: Co-administration of human ghrelin and growth hormone (GH) reverse immunesuppression in septic aged animals, but the mechanism remains elusive. Here we hypothesize that ghrelin and GH co-treatment restores immune response in aged septic rats by inhibiting the production of transforming growth factor-β (TGF-β), an immunoregulatory cytokine through the vagus nerve.

Methods: Male aged Fischer rats (22-23-month-old) were made septic by cecal ligation and puncture (CLP) with or without dissecting the vagus nerve (vagotomy). Human ghrelin and GH or vehicle (PBS) were administrated subcutaneously at 5 h post CLP. After 20 h of CLP, serum and spleens were harvested.

Results: Serum TGF-β levels were increased in septic aged rats, while ghrelin and GH treatment significantly reduced its levels. Expression of TGF-β in the spleen was upregulated after sepsis, while ghrelin and GH treatment significantly inhibited its expression. TNF-α and IL-6 levels were significantly reduced after ex vivo LPS stimulation in splenocytes of CLP rats compared to sham rats, while these levels were significantly higher in splenocytes from ghrelin and GH-treated CLP rats compared to vehicle-treated CLP rats. Ghrelin and GH treatment reduced program death receptor-1 (PD-1) expression increased human leukocyte antigen (HLA) expression, attenuated lymphopenia and cleaved caspase-3 levels in splenocytes in septic aged rats. Vagotomy diminished those beneficial effects of ghrelin and GH treatment to sepsis rats. In vitro addition of ghrelin or GH or ghrelin and GH together had no effect on restoring immune response in splenocytes from CLP rats following LPS stimulation, indicating the requirement of the vagus nerve for ghrelin and GH's effect.

Conclusions: Ghrelin and GH attenuates immunosuppression in aged septic rats through the vagus nerve-dependent inhibition of TGF-β production.

Introduction

Sepsis is a life-threatening condition that arises due to a dysregulated immune response to infection leading to excessive inflammation and organ injury (1). Although uncontrolled inflammatory syndrome is the prevailing theory for sepsis, subsequent development of an immunosuppressive phenomenon is increasingly being recognized (2, 3). Studies with human septic patients demonstrate that immunosuppression is the predominant cause of morbidity and mortality in sepsis (3, 4). Immunosuppression is commonly encountered in elderly patients that develop sepsis, resulting in impaired efficacy in fighting against invading pathogens (3, 4). Persistent inflammation, T cell exhaustion, and lymphocytopenia are observed in elderly patients after sepsis (5). Those patients are susceptible to secondary infection with lower survival rate compared to young septic patients (5). Studies have shown that severe sepsis and septic shock are mainly observed in elderly patients (> 65 years of age), giving rise to significantly higher mortality rate of about 80% in septic death (4, 6–8). In-depth understanding of the pathophysiology and the development of novel therapeutics are urgently needed to protect elderly from sepsis injury.

The pleiotropic cytokine transforming growth factor-β (TGF-β) is a key regulator of the immune response to infection (9, 10). TGF-β is produced by a wide numbers of cells including leukocytes as well as epithelial cells (11). It controls the differentiation, proliferation and activation of immune cells (11, 12). There are 3 isoforms of TGF-β namely TGF-β1, -β2 and -β3 so far have been identified in mammals. These isoforms of TGF-β have similar biological function but are expressed in different tissues (13). Among these three isoforms, TGF-β1 is predominantly expressed by the cells of the immune system (13). TGF-β has multiple immunosuppressive properties as evidenced by the fact that TGF-β knockout mice develop multi-organ autoimmune inflammatory disease and die shortly after birth (13). Although the levels of TGF-β are elevated in patients with sepsis (14), its role in inducing immunosuppression is not fully understood.

Ghrelin is a small peptide predominantly produced by the gastrointestinal tract (15). Ghrelin binds to the growth hormone secretagogue receptor (GHSR)-1a to promote the release of growth hormone (GH) (16). Beyond its endocrine function, ghrelin has non-endocrine activities, including anti-inflammation and anti-apoptosis (17–20). Our previous studies showed that co-treatment of ghrelin and GH mitigated organ injury and improved survival in aged rats after sepsis (21, 22). Even though we recently demonstrated that the combined treatment with ghrelin and GH (GG) were able to reverse immunosuppression in aged septic rats, the underlying mechanism involving the potential role of TGF-β in GG-mediated restoration of immune response had not been studied (20). We therefore investigated whether the effect of GG on restoring immune response in aged animals with sepsis was mediated through the modulation of TGF-β production. We further aimed to determine the contribution of the vagus nerve for GG-mediated restoration of immune function in aged septic rats.

Material And Methods

Animal model of sepsis and vagotomy

Aged male Fisher rats (22-23 month-old) were obtained from Charles River Laboratories via the National Institute for Aging, National Institutes of Health (NIH). Animals were housed in a temperature-controlled room with a 12 h light-dark cycle and fed a standard Purina rat chow diet. Rats were allowed to acclimate to the environment for at least 5 days before being used for experiments. All experiments were performed in accordance with the NIH guidelines for the use of experimental animals, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institutes for Medical Research.

Sepsis was induced in aged rats by cecal ligation and puncture (CLP) (22). Briefly, rats were anesthetized by 2% isoflurane inhalation. The abdomen was shaved and cleaned with iodine and alcohol solution, and a 2-cm midline incision was made. The cecum was then exposed and 70% of its length was ligated using 4-0 silk suture distal to the ileocecal valve. The cecum was punctured twice with an 18-G needle and a small amount of feces was squeezed out. The cecum then was returned to the abdominal cavity and abdominal incision was closed in layers and the animals were resuscitated with 30 ml/kg body weight

normal saline subcutaneously. In sham rats, same surgical procedure was performed with the exception that their cecum was not ligated or punctured.

Vagotomy procedure was performed on a group of rats at the time of CLP as reported previously (23, 24). The trunks of subdiaphragmatic vagus nerve were transected. Briefly, the dorsal and ventral branches of the vagus nerve were dissected and each branch of the nerve was tied with surgical sutures at 2 points separated by about 1 cm, and then severed between the sutures. After surgery, animals were allowed to eat and drink food and water, respectively.

Administration of human ghrelin and human growth hormone into aged septic rats

Human ghrelin (Phoenix Pharmaceuticals, Belmont, CA) and human GH (ProSpec, Ness Ziona, Israel) were dissolved in normal saline. A 500 µl mixture of human ghrelin and GH (GG) was prepared and injected subcutaneously to CLP animals at 5 h after CLP. Each GG treated animal received 80 nmol/kg human ghrelin and 50 µg/kg human GH in a single bolus dose.

Splenocyte isolation and stimulation

Spleens were harvested at 20 h after CLP or sham-operation and homogenized by gentle grinding between frosted glass slides, followed by passage through a 70-µm cell strainer to obtain single cell suspension (BD Biosciences, San Jose, CA). The suspension was centrifuged at 1500 rpm for 5 min and the pellet was suspended into 44% percoll solution (Sigma, St. Louis, MO), then careflly overlay on top of 66% percoll solution, centrifuge 2000 rpm for 30 min at room temperature. After density gradient centrifuge, leukocytes formed a white fluffy ring at the interface between 44% percoll and 66% percoll and were collected into RPMI-1640 medium (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin,10 mM HEPES and 0.5 µM 2-mercaptaethanol. Cell viability was greater than 90%. To evaluate immune responses of these cells, 2×10^6 cells were plated into a 24-well plate and stimulated with LPS (100 ng/ml, Sigma, St. Louis, MO) for 5 h. The released proinflammatory cytokines in the culture medium was measured.

Measurement of cytokines and analysis of lymphocytes, monocytes, and basophils number

Cytokine levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kits specific for rat TNF-α, IL-6 (BD Biosciences) and TGF-β1 (eBioscience, San Diego, CA). All measurements were performed according to manufacturer's instructions. Lymphocytes, monocytes, and basophils numbers in the blood were analyzed by using Cell-DYN 3700 analyzer (Abbott, Abbott Park, IL).

Western blot analysis

Spleen tissues were homogenized and lysed in RIPA buffer, 10 mM Tris buffer, pH 7.5 containing 0.1% Triton-X 100, 1 mM EDTA, 1 mM EGTA, protease inhibitor tablet (Thermo Fisher, Waltham, MA), phosphatase inhibitor tablet (Thermo Fisher). Protein concentration was determined by Bio-Rad DC

protein assay kit (Bio-Rad, Hercules, CA). Tissue lysates were electrophoresed on 4-12% NuPAGE Bis-Tris Gel (Life Technologies) and transferred to nitrocellulose membranes. The membranes were then blocked with 0.1% casein in Tris buffer saline and incubated with anti-TGF-β (Proteintech, Chicago, IL), cleaved caspase-3 (Cell Signaling Technologies, Danvers, MA), and β-actin (Sigma) overnight at 4°C. After washing, membranes were incubated with infrared dye-labeled respective secondary Abs (LI-COR, Lincoln, NE). The Odyssey infrared image system (LI-COR) was used to analyze the target bands and intensities of bands was qualified using Image Studio Lite software (LI-COR).

Immunohistochemical analysis

Spleen tissues were fixed in 10% buffered formalin solution for 2 days and processed for paraffin sections using standard histology procedures. Paraffin sections were dewaxed and rehydrated in xylenes and series concentrations of alcohols, followed by antigen retrieval using antigen unmasking solution (Vector Labs, Burlingame, CA). Slides were then incubated with rabbit anti-rat PD-1 (1:50, Abcam, Cambridge, MA) and rabbit anti-rat HLA-DR (1:50 dilution, Proteintech, Chicago, IL) antibodies overnight at 4^{o}C. Slides were washed with Tris-buffered saline containing 0.02% triton x-100 and further reacted with biotinylated anti-rabbit IgG (Vector Labs). Then, the slides were incubated with peroxidase conjugated avidin followed by reaction with substrate, DAB (Vector Labs). Slides were counterstained with hematoxylin and evaluated using a Nikon microscope.

Statistical analysis

All data are expressed as mean ± SEM and compared by one-way ANOVA and Student-Newman-Keuls (SNK) test and Student's t-test. Differences in values were considered significant when $P < 0.05$.

Results

Combined treatment with ghrelin and GH reduces TGF-β production in aged septic rats through the vagus nerve

We found that the protein levels of TGF-β1 in the serum and spleen were increased by 2.1 and 2.0 folds, respectively in aged rats at 20 h after CLP as compared to the sham-operated animals (Figs. 1A, B). Treatment with GG significantly lowered the levels of TGF-β in the serum and spleen of septic aged rats by 52% and 25%, respectively at 20 h after CLP (Figs. 1A, B). On the other hand, vagotomy eliminated the inhibitory effect of GG on serum and spleen levels of TGF-β in septic aged rats (Figs. 1A, B). Thus, GG modulates the production of TGF-β in serum and spleen through the vagus nerve.

Combined treatment of ghrelin and GH improves the immune response of splenocytes from aged septic rats

The immune response of aged rats at 20 h after CLP was evaluated by the release of cytokines from isolated splenocytes in response to *ex vivo* LPS stimulation. After 5 h of incubation with LPS, the levels of

TNF-α and IL-6 in the culture supernatants of splenocytes from sham animals were 520 ± 10 and 218 ± 9 pg/ml, respectively, while the levels of these cytokines were dramatically lower in vehicle-treated septic aged rats (Figs. 2A, B). On the other hand, the culture supernatants of splenocytes isolated from GG-treated aged septic rats showed significantly increased levels of TNF-α and IL-6 at the levels of 287 ± 10 and 149 ± 11 pg/ml, respectively, in response to *ex vivo* LPS-stimulation as compared to the vehicle treated rats (Figs. 2A, B). Interestingly, the GG-treated vagotomized animals were not able to restore the levels of TNF-α and IL-6 in the culture supernatants after *ex vivo* stimulation with LPS (Figs. 2A, B). These results demonstrate the restoration of immune response of aged septic animals by GG treatment was mediated through the vagus nerve.

Co-treatment with ghrelin and GH corrects lymphopenia and reduces cleaved caspase-3 levels in the spleen

The loss of lymphocytes often resulting in diminished capacity of the host to fight against pathogens is a primary feature of immune suppression in the critically ill septic patients (3), rendering them to develop secondary infections (3, 4, 20). Hematological analysis of the blood from sham and septic aged rats showed that lymphocyte count and percentage dramatically reduced from 2.57×10^3/μl and 37.8% in sham, respectively, to 0.52×10^3/μl and 14.4% in aged septic rats, respectively (Figs. 3A, B). GG treatment significantly increased lymphocyte count and percentage to 1.05×10^3/μl and 27.0%, respectively, while vagotomy in septic rats diminished the GG's effect on increasing the count and percentage of lymphocytes in the blood (Figs. 3A, B). The levels of an apoptosis inducing protein cleaved caspase-3 were upregulated in the spleen of septic aged rats at 20 h after CLP (Fig. 3C), suggesting that apoptosis was involved in the loss of lymphocytes in sepsis. The GG treatment significantly downregulated the expression of cleaved-caspase-3 in the spleen of septic aged animals compared to vehicle treated aged septic rats (Fig. 3C). By contrast, the beneficial effects of GG were diminished in vagotomy animals, suggesting the action of GG for reversing lymphocytopenia and inhibiting the production of cleaved caspase-3 protein was mediated through the vagus nerve dependent mechanism (Fig. 3C).

Co-treatment with ghrelin and GH corrects monocytosis and basophilia in sepsis

Monocytes are an essential part of cellular innate immune system and play an important role in host defense against pathogen (25). Recruitment of monocytes is essential for effective control and clearance of bacterial infection, but monocytes can also contribute to tissue destruction during some infection and inflammatory diseases (25). Peripheral monocytosis associates with respiratory symptoms, later infection and higher mortality in the patients admitted to emergency room (26). Hematological analysis of the blood from sham and septic aged rats showed that monocytes count and percentage markedly increased from 0.43×10^3/μl and 9.1% in sham, respectively, to 0.75×10^3/μl and 18.9% in aged septic rats, respectively (Figs. 4A, B). GG treatment significantly reduced monocytes count and percentage to 0.57×10^3/μl and 13.7%, respectively, while vagotomy in septic rats diminished the GG's effect on reducing the count and percentage of monocytes in the blood (Figs. 4A, B). Interestingly, similar result was obtained from circulating basophils. The blood from sham and septic aged rats showed that

basophils count and percentage dramatically increase from 0.18×10^3/μl and 3.1% in sham, respectively, to 0.36×10^3/μl and 10.8% in aged septic rats, respectively (Figs. 4C, D). GG treatment significantly decreased basophil count and percentage to 0.227×10^3/μl and 6.3%, respectively, while vagotomy in septic rats diminished the GG's effect on decreasing the count and percentage of basophils in the blood (Figs. 4C, D).

Co-treatment with ghrelin and GH decreases PD1 and restores HLA expression in spleen in sepsis

Programmed death-1 (PD-1) protein expressed in immune cells negatively regulates the immune response of immune cells to antigen (27). We assessed the expression of PD-1 in the spleen tissues of sepsis rats treated with or without GG. The expression of PD-1 in spleen of septic aged rats was upregulated at 20 h after CLP compared to aged sham rats (Fig. 5). By contrast, treatment with GG markedly downregulated the PD-1 expression in the spleen in aged septic rats compared to vehicle-treated animals, while vagotomy reduced the effect of GG on inhibiting PD-1 expression in the spleen of aged septic rats (Fig. 5). Human leukocyte antigen-DR (HLA-DR) is an major histocompatibility complex (MHC) class II cell surface receptor expressed on antigen presenting cells like macrophages presents processed antigen to the T cells to activate them (28).We assessed the expression of HLA-DR in the spleen tissues of sepsis rats treated with or without GG. The expression of HLA-DR in spleen of septic aged rats was decreased at 20 h after CLP compared to aged sham rats (Fig. 5). On the other hand, treatment with GG restored the HLA-DR expression in the spleen in aged septic rats compared to vehicle-treated animals, while vagotomy minimized the effect of GG on upregulating HLA-DR expression in the spleen of aged septic rats (Fig. 5).

In vitro treatment of ghrelin and GH do not alter the immune response of splenocytes from septic aged rats

Splenocytes were isolated from sham and septic aged rats at 20 h after CLP and the cells were stimulated with LPS for cytokines release. Ghrelin and GH were added independently or in combination into the culture medium to determine their effect on the response of cells to LPS stimulation. As expected, the splenocytes from sham animals released significantly higher levels of TNF-α and IL-6 in response to LPS stimulation, as compared to the splenocytes isolated from septic aged rats (Figs. 6A, B). *In vitro* treatment of ghrelin or GH alone or their combination, GG, didn't markedly increase the immune response of these splenocytes from septic aged animals (Figs. 6A, B). In addition, ghrelin, GH alone or GG also has no effect on cytokines release from splenocytes of sham-operated animals (Figs. 6A, B). These results further suggested that the increase in immune response by GG after sepsis was mediated through the vagus nerve dependent manner.

Discussion

Sepsis often causes immunosuppression, thereby increasing the susceptibility to secondary infection (3). Here, we demonstrated that TGF-β played a critical role in sepsis-induced immunosuppression in aged septic rats. Although TGF-β has been shown to play an important role in cell growth and differentiation (9,

10, 29), subsequent study revealed its inhibitory role in lymphocyte proliferation and activation through the generation of T regulatory (Treg) cells (30). TGF-β can induce lymphocytes apoptosis in pathological condition (31). Tumor cells produce high levels of TGF-β that inhibits immune cells' function to eliminate cancer cells and foster tumor growth and metastasis (30). The role of TGF-β has been demonstrated in cancer and other diseases (32), but its role in the development of sepsis-associated immunosuppression has not been fully revealed. Our study determined the increased levels of TGF-β in the serum and spleen which were associated with the decreased lymphocytes, increased monocytes and basophils in the blood. We found increased expression of cleaved caspase-3, and immune co-inhibitory molecule, PD-1 and decreased expression of HLA-DR in the spleen. We also revealed that the splenocytes from septic aged rats exhibited a poor immune response to LPS stimulation *ex vivo*. We found that the combined treatment of ghrelin and GH inhibited TGF-β production, attenuated lymphopenia, corrected monocytosis and basophilia in the blood, downregulated the expression of cleaved caspase-3, and PD-1, and upregulated the expression of HLA-DR in the spleen. Similarly, the splenocytes from GG treated septic animals demonstrated a marked improvement in their immune responses to the *ex vivo* LPS stimulation. Moreover, our results indicated that the protective effect of GG in restoring the lymphocyte contents and their immune response was mediated through the vagus nerve.

Sepsis-induced immunosuppression is resulted from the loss of lymphocytes, increasing the expression of co-inhibitory molecules and the differentiation of Treg (3, 4, 33). These alterations can be linked to increased TGF-β production. Elevated levels of TGF-β have been reported in patients with sepsis (14, 34). High levels of circulating TGF-β during sepsis caused by pneumonia have been shown to correlate with higher tissue injury score and mortality (34). Increased levels of TGF-β are associated with the reduced bacterial clearance and increased organ injury (12). It has been shown that bacteria-induced immunosuppression is mediated through the induction of TGF-β, followed by the decrease in the expression of surface MHCII on the dendritic cells (DC), thereby inhibiting DC activation (35). TGF-β plays a prominent role in the differentiation of immunoparalyzed DC, leading to immunosuppression after lung infection by *E. coli* (36). TGF-β can suppress the release of proinflammatory mediators such as TNF-α and IL-1β from monocytes and macrophage (32, 37). The patients with sepsis can have both increased or decreased levels of TGF-β (14, 37, 38), which could be due to the variant sepsis stages of these patients.

In sepsis, host response to infection is associated with sustained inflammation to eliminate pathogens in early phase and immune suppression in later phase (39). The patients with sepsis show the signs of both excessive inflammation and immune suppression, although the extent of which may vary between individuals (39). The TGF-β signaling is important to maintain immune homeostasis in sepsis and other infectious conditions. The dysregulation of TGF-β has been associated with the tissue injury and higher mortality (34, 40). The sepsis survivors have higher levels of TGF-β in the early time point and lower levels of TGF-β at 10 days after hospitalization (40). In contrast, the non-survivors have lower levels of TGF-β in early stage and higher levels of TGF-β at 10 days after their admission in the hospital (40). Elderly are particularly susceptible to infections due to insufficient immunity. Aged septic patients have features consistent with immunosuppression that results in an inability to clear infection, and predisposition to nosocomial infections (3, 4, 33). The gene expression profile showed that the TGF-β expression in elderly

septic patients is higher as compared to young septic patients (41). The higher TGF-β in elderly septic patients correlates with their suppressed immune response, higher mobility and mortality (3, 4). Immunosuppression was observed in aged rats at 20 h after CLP. These animals demonstrated apoptosis in T cells, increase of co-inhibitory molecules and Treg differentiation (20). Our current study demonstrates the upregulation of TGF-β is associated with the immunosuppression in aged animal with sepsis. It has been shown that immunosuppression occurs at much later time point after sepsis in young animals (42). Although the circulating TGF-β was increased in aged septic rat at 20 h after CLP, but such increase was not observed in 3-month-old young rats at 20 h after CLP (data not shown). Thus, the levels of TGF-β between young and aged animals reflect the differentiated immune response between different age groups during sepsis.

TGF-β can be used as a prognostic marker of sepsis-induced immunosuppression in elderly population. Inhibition of TGF-β have been shown to ameliorate organ injury and improve the survival in sepsis (12, 43). Blockade of TGF-β attenuated immunosuppression and reduced the susceptibility to secondary infection following sepsis (36). Thus, modulating TGF-β could be a potential therapeutic target for elderly patients with sepsis. Administration of ghrelin was found to reduce TGF-β expression and exert an anti-fibrotic effect in patients with systemic sclerosis (44). Here, we demonstrated that ghrelin reduced TGF-β levels in the serum in septic aged animals and ameliorated sepsis-induced immune suppression. Human ghrelin has been administered into human subjects in clinical studies, showing excellent efficacy and safety profile (45). Ghrelin has 93% homology in its amino acid sequence between human and rat or human and mouse (46). In the current study, we used human ghrelin as a clinically relevant approach for implementing ghrelin as a novel therapeutic for elderly septic patients.

Communication between the nervous and immune systems is important for the regulation of immune function and inflammation (47–49). The vagus nerve has been shown to play a critical role in the inflammatory reflex and mediates rapid homeostatic response to protect against organ injury (47–49). The anti-inflammatory effect of ghrelin is mainly mediated through the vagus nerve as vagotomy diminishes the protective effect of ghrelin on septic animals (23). We previously showed that ghrelin alone can protect young animals with sepsis, but not aged septic animals (21). Aging is associated with the downregulation of the expression of ghrelin receptor in the brain and decrease in the neuronal activity in the nucleus that originates efferent vagus nerve, therefore compromising the responsiveness to ghrelin treatment (21, 22). Low dose of growth hormone has been proved to increase ghrelin receptor expression in the brain of aged rats and enhance the sensitivity of ghrelin receptor to ghrelin (21). The combination of ghrelin and GH significantly attenuates proinflammatory cytokines and organ injury after sepsis in aged rats (21, 22). We recently reported that ghrelin and GH in combination attenuated immunosuppression in aged septic rat (20). In the present study, we demonstrated the novel mechanism by which GG treatment reversed the immunosuppression in aged septic rats is through the downregulation of TGF-β production by utilizing the vagus nerve.

The afferent vagus nerve can be activated by peripheral inflammatory stimulation and the signal is transmitted to the efferent vagus nerve to suppress the inflammation (48, 50). The activation of the

vagus nerve during infection present a protective advantage to host and the defect on the vagus nerve anti-inflammatory pathway contributes to disease pathology (47, 51). In addition, the immunomodulatory effect of efferent vagal nerve in macrophages is mediated through the stimulation of cholinergic activity of the parasympathetic nerve system (47). Spleen is a primary organ of the cholinergic anti-inflammatory pathway (47, 49). The impaired cholinergic anti-inflammatory pathway in aged rats is responsible for the robust inflammatory response after onset of sepsis resulting in early morbidity and mortality in aged septic animals (21). On the other hand, survived septic aged animals showed an impaired immune response to LPS stimulation (20). It appears that these animals have inadequate ability to clean bacteria and defend secondary infection leading to high mortality rate after sepsis (21, 22). Despite the action of anti-inflammation through the activation of the vagus nerve, the vagus nerve has been showed to activate immune response in immune dysregulated condition (52). The vagus nerve stimulation increases immune activity by elevation of plasma inflammatory cytokines, such as TNF-α, IL-6 in human with depression (52). Depression has been characterized as dysregulation of immune function and associated with immunosuppression (53, 54).

In summary, we identified that GG maintained the immuneresponse in aged rats in sepsis through the vagus nerve that led to the inhibition of TGF-β. The decreased expression of TGF-β improved the immune response to infection in aged septic rats by maintaining lymphocytes counts and correcting monocytosis and basophilia that correlated with the decreased expression of cleaved caspase-3 and by reducing lymphocyte inhibitory receptor PD-1 and increasing HLA-DR expression.

Abbreviations

CLP	cecal ligation and puncture
GG	ghrelin and growth hormone in combination
GH	growth hormone
GHSR	growth hormone secretagogue receptor
PD-1	programmed death -1
TGF-β	transforming growth factor-β
Treg	T regulatory cells

Declarations

Author Contributions: Data curation, Mian Zhou and Mahendar Ochani; Formal analysis, Mian Zhou and Monowar Aziz; Funding acquisition, Ping Wang, Monowar Aziz; Investigation, Mian Zhou and Mahendar Ochani; Methodology, Mian Zhou and Mahendar Ochani; Project administration, Ping Wang; Resources,

Ping Wang; Supervision, Monowar Aziz and Ping Wang; Writing – original draft, Mian Zhou and Monowar Aziz; Writing – review & editing, Mian Zhou, Monowar Aziz and Ping Wang.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Figures

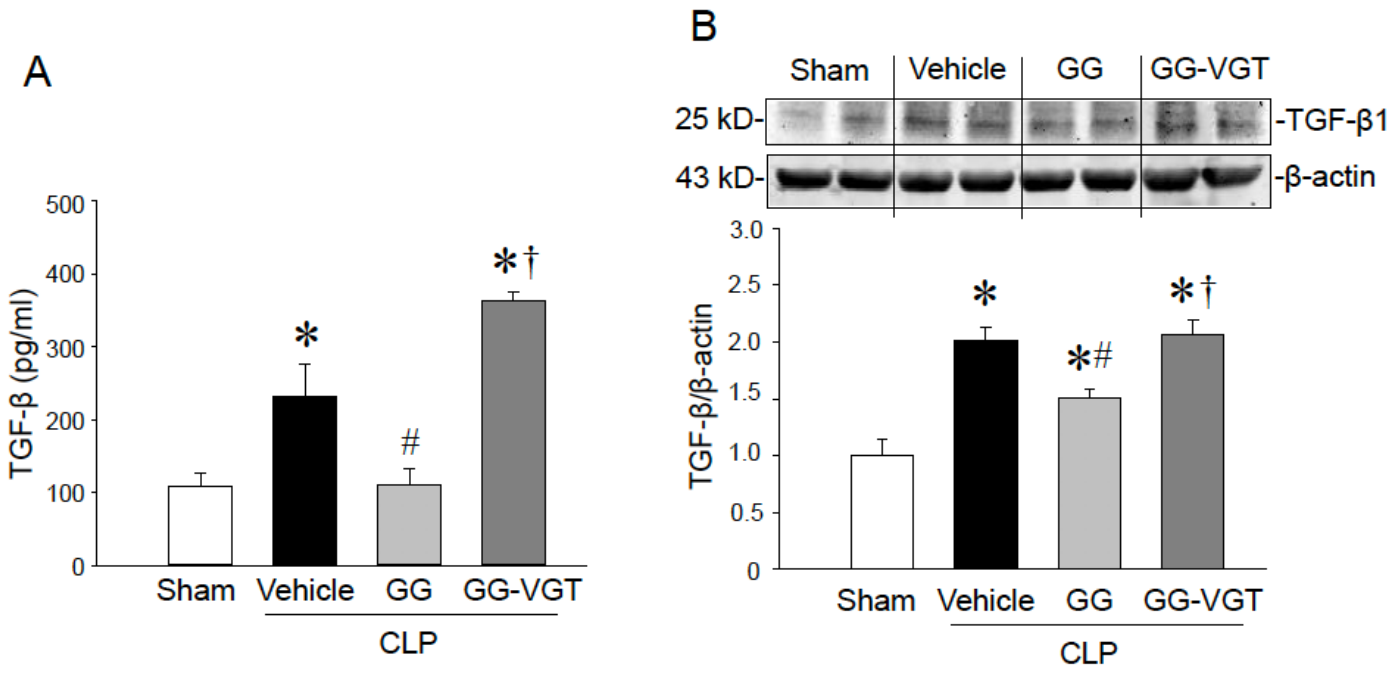

Figure 1

Ghrelin and GH in combination downregulates TGF-β in aged rats with sepsis. Rats were subjected to sham or CLP operation and treated with vehicle (normal saline) or GG (ghrelin 80 nmol/kg, GH 50 μg/kg) at 5 h after CLP. In an additional group of rats, vagotomy was performed at the time of CLP and treated with GG at 5 h after CLP. Serum and spleens were collected at 20 h after CLP. (A) The levels of TGF-β in the serum was measured by ELISA. Data are expressed as mean ± SEM (n = 4-7 rats/group). (B) The expression of TGF-β in the spleen were determined by western blotting. The representative western blot

images are shown. Sham was normalized as 1 in western blot analysis. Data are expressed as mean ± SEM (n = 4-5 rats/group). *P < 0.05 vs. sham; #P < 0.05 vs. CLP with vehicle treatment; +P < 0.05 vs. CLP with GG treatment. CLP, cecal ligation and puncture; GG, ghrelin and growth hormone in combination; VGT, vagotomy.

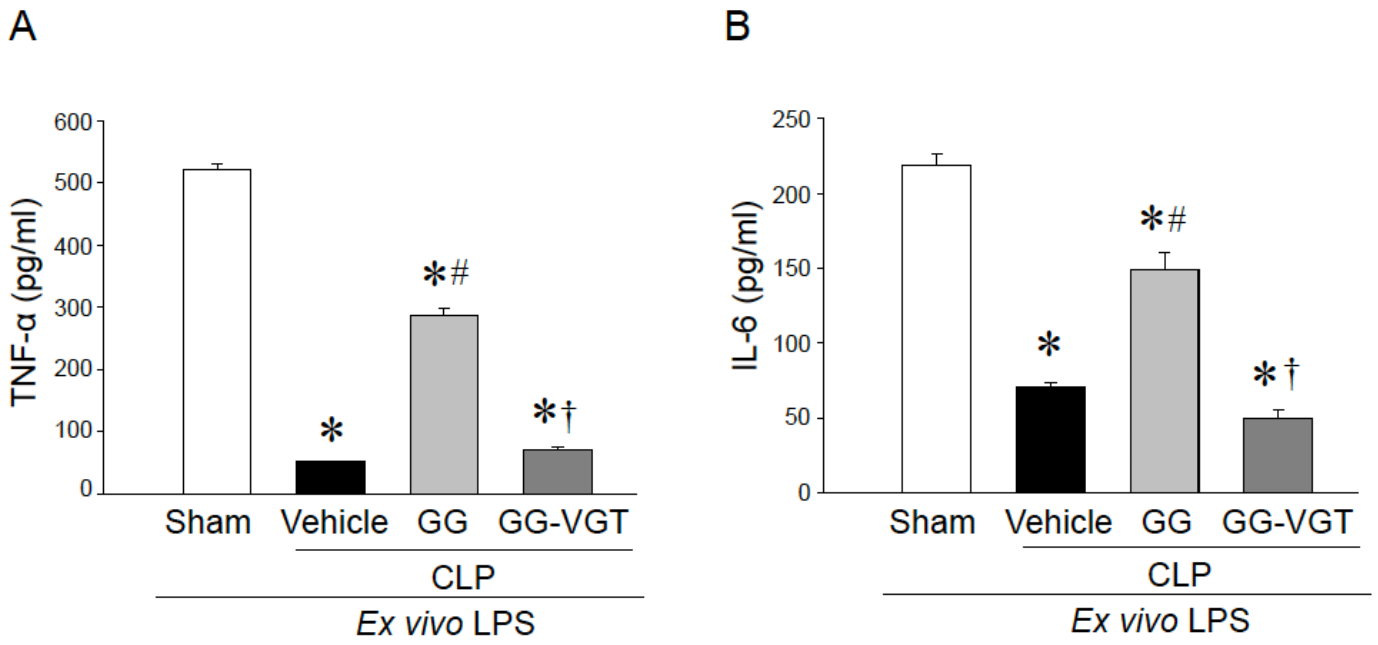

Figure 2

Ghrelin and GH in combination restores immune response in aged sepsis rats. Rats were subjected to sham or CLP operation and treated with vehicle (normal saline) or GG (ghrelin 80 nmol/kg, GH 50 μg/kg) at 5 h after CLP. In an additional group of rats, vagotomy was performed at the time of CLP and treated with GG at 5 h after CLP. Spleens were harvested at 20 h after CLP. Splenocytes were isolated and stimulated with LPS (100 ng/ml) for 5 h. The release of (A) TNF-α and (B) IL-6 in the medium were measured by ELISA. Data are expressed as mean ± SEM (n = 4-5 rats/group). *P < 0.05 vs. sham; #P < 0.05 vs. CLP with vehicle treatment; +P < 0.05 vs. CLP with GG treatment.

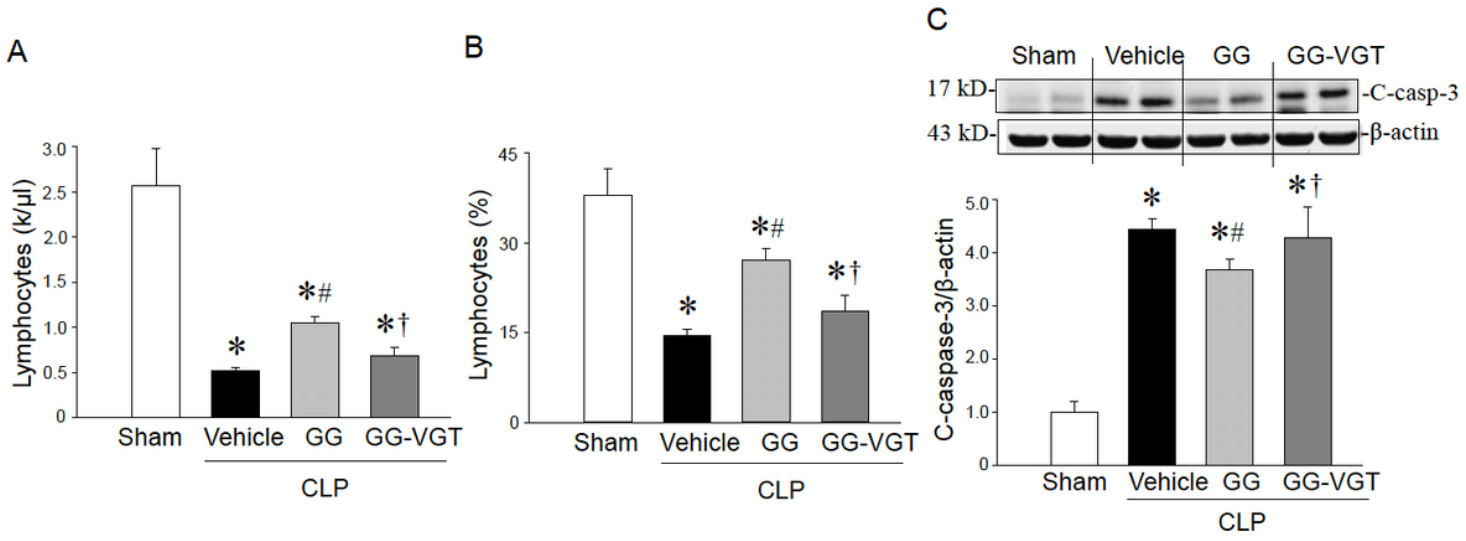

Figure 3

Ghrelin and GH in combination attenuates lymphopenia in aged rats with sepsis in a vagus nerve dependent manner. Rats were subjected to sham or CLP operation and treated with vehicle (normal saline) or GG (ghrelin 80 nmol/kg, GH 50 μg/kg) at 5 h after CLP. In an additional group of rats, vagotomy was performed at the time of CLP and treated with GG at 5 h after CLP. Blood and spleens were collected at 20 h after CLP. (A, B) Circulating lymphocytes were measured using a hematology analyzer. Data are expressed as mean ± SEM (n = 4-6 rats/group). (C) The levels of cleaved caspase-3 in the spleen were determined by western blotting. The representative western blot images are shown. Sham was normalized as 1 in western blot analysis. Data are expressed as mean ± SEM (n = 4-5 rats/group). *P < 0.05 vs. sham; #P < 0.05 vs. CLP with vehicle treatment; +P < 0.05 vs. CLP with GG treatment.

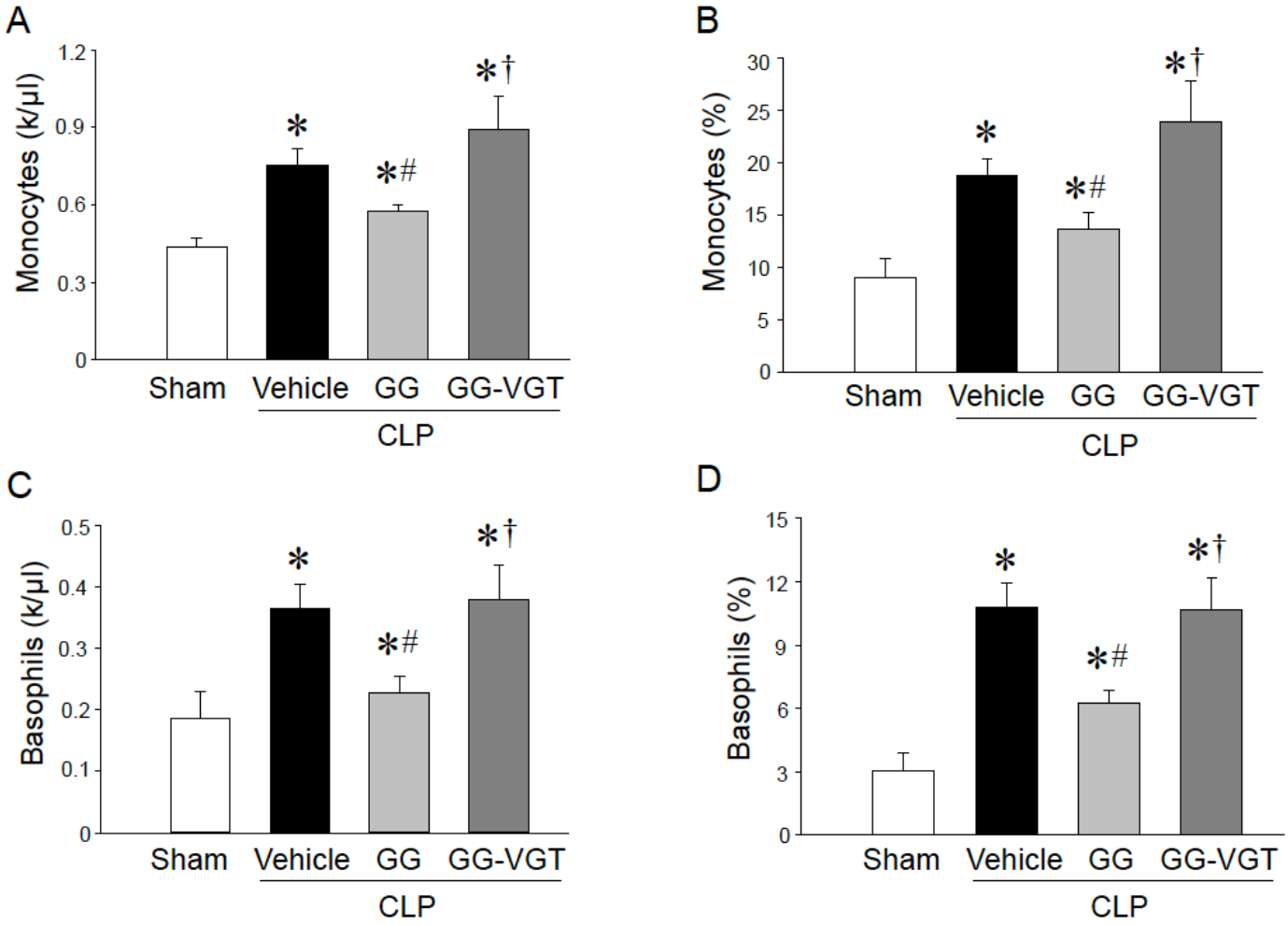

Figure 4

Ghrelin and GH in combination corrects the levels of circulating monocytes and basophils in aged rats with sepsis. Rats were subjected to sham or CLP operation and treated with vehicle (normal saline) or GG (ghrelin 80 nmol/kg, GH 50 μg/kg) at 5 h after CLP. In an additional group of rats, vagotomy was performed at the time of CLP and treated with GG at 5 h after CLP. Blood was collected at 20 h after CLP. Circulating (A, B) monocytes and (C, D) basophils were measured using a hematology analyzer. Data are expressed as mean ± SEM (n = 4-6 rats/group). *P < 0.05 vs. sham; #P < 0.05 vs. CLP with vehicle treatment; +P < 0.05 vs. CLP with GG treatment.

Figure 5

Ghrelin and GH in combination inhibits PD-1 and increases HLA-DR expression in the spleen of aged rats with sepsis. Rats were subjected to sham or CLP operation and treated with vehicle (normal saline) or GG (ghrelin 80 nmol/kg, GH 50 μg/kg) at 5 h after CLP. In an additional group of rats, vagotomy was performed at the time of CLP and treated with GG at 5 h after CLP. Spleens were harvested at 20 h after CLP. The expression of PD-1 and HLA-DR in the spleen were evaluated by immunohistochemical staining. The representative images are shown. Original magnification of images is 200X.

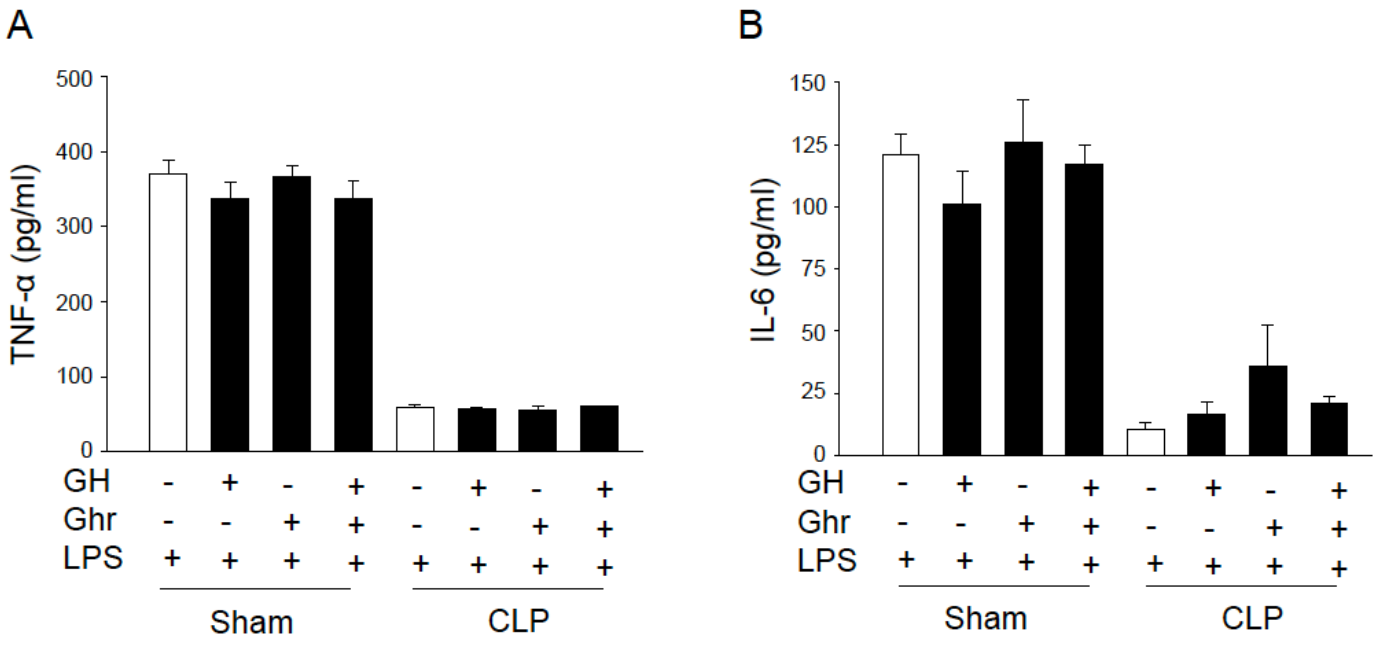

Figure 6

In vitro treatment of ghrelin and GH have no effect on the immune response of splenocytes of aged septic rats. Rats were subjected to sham or CLP operation. Spleens were harvested at 20 h after CLP. Splenocytes were isolated and treated with ghrelin (Ghr), GH or GG in vitro, followed by the stimulation with LPS (100 ng/ml) for 5 h. The release of (A) TNF-α and (B) IL-6 in the medium were measured by ELISA. Data are expressed as mean ± SEM (n = 3 rats/group).