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Cholinergic Regulatory Lymphocytes Re-Establish Neuromodulation of Innate Immune Responses in Sepsis

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Many anti-inflammatory strategies that are successful in treating sepsis in healthy animals fail in clinical trials, in part because sepsis normally involves immunocompromised patients, and massive lymphocyte apoptosis prevents immunomodulation. In this article, we report a new set of regulatory lymphocytes that are able to re-establish the cholinergic anti-inflammatory modulation and to provide therapeutic advantages in sepsis. The vagus nerve controls inflammation in healthy, but not in septic, mice. Likewise, vagus nerve and cholinergic agonists fail to control inflammation in splenectomized and nude animals. Unlike typical suppressor CD25+ cells, CD4+CD25− lymphocytes re-establish the anti-inflammatory potential of the vagus nerve and cholinergic agonists in immunocompromised and septic animals. These cholinergic lymphocytes re-establish splenic protection and the potential of cholinergic agonists to rescue immunocompromised animals from established sepsis. The study results revealed these new regulatory lymphocytes as, to our knowledge, the first known physiological target for neuromodulation of the innate immune responses and a potential therapeutic target for sepsis. The Journal of Immunology, 2011, 187: 718–725.

Regulatory anti-inflammatory mechanisms are essential for survival and are a critical target in many clinical disorders from trauma to infectious diseases. One of the most dramatic examples of the need to control inflammation is sepsis, the third leading cause of death in developed countries (1, 2). There are very few treatment options, and the mortality rate in sepsis remains extremely high, ranging from 30–70% depending on the underlying cause and the organs affected (3). A challenge in sepsis is that the pathology is associated with two factors: the infection and the inflammatory responses of the host. Sepsis is commonly caused by an infection, and new antibiotics are more effective at controlling infections (2). However, despite the advances in antibiotics and intensive care, sepsis remains the most common cause of death in hospitalized patients, killing >250,000 patients and accounting for 9.3% of the overall deaths in the United States annually (1, 2). In addition to the infection, sepsis is characterized by detrimental inflammatory responses produced by the host. Overzealous cytokine production can become more detrimental than the original infection and causes tissue damage, multiple organ failure (4–7), and mortality (7–9). Many studies showed the contribution of inflammatory cytokines to the pathogenesis of sepsis. Among others, inhibition of TNF, migration inhibitory factor, or HMGB1 provided promising results in experimental sepsis (7–9). Yet, none of these cytokines is specific for sepsis and, so far, the inhibition of single cytokines has produced modest effects in clinical trials for sepsis (10). A potential suggestion is that sepsis is not produced by a single cytokine and, hence, successful treatments may require the inhibition of several cytokines, rather than a single one. Thus, alternative strategies focus on the physiological and cellular mechanisms modulating the immune system and the potential alterations contributing to infectious or inflammatory disorders.

Physiological anti-inflammatory mechanisms represent efficient systems selected by evolution to control inflammation (11, 12). The vagus nerve is the most characteristic nerve connecting the immune system with the peripheral organs, because it orchestrates the immune responses according to the physiological needs (11, 12). Vagus nerve stimulation (VNS) can prevent systemic inflammation and protect against experimental sepsis (13, 14). The anti-inflammatory potential of the vagus nerve is mediated by the alpha7 nicotinic acetylcholine receptor (α7nR) (15–17). α7nR agonists, including acetylcholine (the principal neurotransmitter of the vagus nerve), nicotine, or choline, inhibit NF-kB and cytokine production in splenocytes (16). In vivo, treatment with cholinergic agonists attenuates systemic inflammation and protects mice from experimental sepsis (13–18). Different investigators confirmed that electrical or pharmacological stimulation of the vagus nerve restrains the production of inflammatory cytokines in experimental ischemia and reperfusion (19–21), hemorrhage and resuscitation (21), pancreatitis (22), colitis (23), endotoxemia (13, 24), and sepsis (16, 25). Despite their clinical implications, the cellular mechanisms mediating the anti-inflammatory potential of the vagus nerve remain unknown. Our studies indicated that the vagus nerve and nicotinic agonists prevent systemic inflammation through a mechanism mediated by the spleen. VNS and α7nR agonists failed to prevent systemic inflammation in splenectomized animals (24). From a clinical perspective, these results are particularly significant because experimental sepsis is normally studied in young, healthy animals; however, the clinical settings of sepsis typically involve immunocompromised patients. Indeed, most previous studies that
analyzed the vagus nerve focused on its potential to prevent TNF production when the stimulation was started before the endotoxic challenge. Little is known about the potential of the vagus nerve to modulate inflammation when the stimulation is started after the pathological onset, in animals that are already septic. In this study, we analyzed the anti-inflammatory potential of the vagus nerve to rescue animals from established sepsis. Our results indicated that the vagus nerve and selective ανR agonists’ control of systemic inflammation in sepsis is not mediated by typical suppressor CD4+CD25+ cells but occurs through a set of CD3+CD4+CD25− regulatory lymphocytes. Additionally, restitution of these regulatory lymphocytes re-establishes the anti-inflammatory potential of the vagus nerve and ανR agonists to rescue immunocompromised animals from established sepsis.

Materials and Methods

Chemicals and reagents
Choline, nicotine, and LPS were purchased from Sigma-Aldrich (St. Louis, MO) and were freshly prepared in PBS (Life Technologies, Invitrogen, CA) for every experiment; LPS was dissolved in PBS as a stock of 5 mg/mL. Pan T cell and CD4+CD25+ Modulatory T Cell Isolation kits were obtained from Miltenyi Biotec (Auburn, CA). Mouse CD4+ Selection kit was obtained from Stemcell Technologies (Vancouver, BC, Canada). Anti-mouse CD3Ab (mCD3ε) was obtained from BioXcell (West Lebanon, NH) (26, 27).

Animal experiments
All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey-New Jersey Medical School. Male wild-type (WT) 6–8-wk-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), nude mice B6.Cg-Foxn1nu (The Jackson Laboratory), or mast cell-deficient WBB6F1-KitWsh/KitWsh mice (The Jackson Laboratory) were randomly grouped, and investigators were blinded to the experimental treatments. Endotoxemia and cell ligation and puncture (CLP) were performed as we described previously (16). Briefly, endotoxemia endotoxin (Escherichia coli) LPS 0111:B4 (Sigma-Aldrich) was dissolved in sterile, pyrogen-free PBS and sonicated for 30 min immediately before being used at the indicated concentration.

Cecal ligation and puncture
Animals were anesthetized with ketamine (75 mg/kg, i.m.; Fort Dodge, Fort Dodge, IA) and xylazine (20 mg/kg, i.m.; Boehringer Ingelheim, St. Joseph, MO) and subjected to CLP with an average 50% natural mortality, as described previously (16). Briefly, animals were subjected to abdominal incision and ligation of the cecum 5.0 mm from the cecal tip away from the ileocecal valve. The ligated cecal stump was punctured once with a 22-gauge needle, and stool was extruded (∼1 mm) to ascertain the patency of puncture. The abdominal wound was closed in two layers (peritoneum and fascia separately) to prevent leakage of fluid. All animals received antibiotic (0.9 mg/kg enrofloxacin, s.c.) dissolved in 0.9% normal saline (20 mL/kg, s.c.) immediately after surgery and every 12 h for 3 d. After collection, the organs were homogenized (Homogenizer; Omni International, Kennesaw, GA) in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 0.2 mM NaPO4, 25 µg/ml aprotinin, 25 µg/ml pepstatin A, 1 mM PMSF). The resulting suspension was centrifuged (10,000 × g for 25 min at 4˚C), and the supernatant was collected for analyses. The protein concentration was quantified by the Bradford method (Bio-Rad, Hercules, CA).

Vagus nerve stimulation
Cervical VNS was performed as we described previously (24). Briefly, a small incision was made to explore and identify the right cervical vagus nerve, and a platinum electrode was placed across it. The platinum electrode was attached to the Stimulation Device (STM 150) and controlled by AcqKnowledge software (Biopac Systems). Vagus stimulation was applied for 20 min at 5 V. Sham operation (cervical exploratory incision) was made on control animals with the exception of stimulation.

CD3+CD4+CD25− and CD3+CD4+CD25+ T cell isolation
Spleens were harvested from mice, and cell suspensions were prepared and filtered through a 70-µm cell strainer. RBCs were lysed with ammonium chloride solution for 3 min. Splenic cells were washed twice before suspension in culture medium. Total CD3+ T cells were purified using negative selection with the Pan T Cell Isolation Kit (Miltenyi Biotec). Next, CD4+ cells were isolated using Mouse CD4 positive selection kit (Stemcell Technologies), and CD25+ and CD25− cells were isolated using CD3+CD4+CD25+ modulatory T cell isolation kit (Miltenyi Biotec), according to the manufacturer’s instructions. Cell purity was assessed by staining purified cell populations with Cy5-labeled CD3 or -CD4 Ab and PE-labeled anti-CD25 Ab, followed by analysis using a FACSCalibur system (Becton Dickinson); results were processed using CellQuest Pro software. The T cells fractions were consistently >98% pure.

T cell transfer
CD4+CD25− and CD4+CD25+ cells populations were resuspended in 0.9% sodium chloride solution, and 1.5 × 106 cells were injected i.p., a similar route to that used for LPS administration. Cells were administered 24 h before the experimental challenge.

In vitro cytokine assay
Purified T cells populations (including CD3+CD4−, CD3+CD4+CD25−, and CD3+CD4+CD25+) depending on the specific experiment and as described in the figure legends) were cultured in 24-well polystyrene culture plates at 1 × 106 cells/well in 1 ml medium/well with serum-free Opti-MEM I medium for the experimental procedure. Nicotine or choline was added 30 min before LPS stimulation. TNF concentration in the culture cells was analyzed using ELISA kit from eBioscience (San Diego, CA) at 3 h after LPS stimulation using a Versamax plate reader and SoftMax Pro software (Molecular Devices). TNF levels in the serum were analyzed in vivo 90 min after LPS stimulation.

Neutralizing Ab
Anti-CD3–neutralizing mCD3ε Ab (BioXcell) was administered i.p. 3 d before VNS (20 mg/kg, i.p.), as previously described in the literature (26, 27).

Statistical analyses
All data are expressed as mean ± SD. Statistical analyses were performed using one-way ANOVA with multiple pair-wise comparisons, with the Bonferroni adjustment for multiple hypothesis testing. Normality and homogeneity of variance were analyzed. ANOVA was used to compare all treatments and specific pair-wise comparisons, as stated in the experiments. Statistical analyses of survival were determined using the log-rank test. Kaplan–Meier product-limit method was used for survival graphs. The p values < 0.05 were considered statistically significant.

Results
VNS prevents lethal endotoxemia
First, we analyzed whether VNS inhibited or delayed the onset of cytokine production in sepsis. VNS, started 20 min prior to the endotoxic challenge, inhibited serum TNF levels in endotoxemia by >70% (Fig. 1A). This mechanism induced a lasting inhibition of cytokine production and prevented mortality in lethal endotoxemia (Fig. 1B). Next, we analyzed whether VNS could rescue animals from established endotoxemia if the treatment was started after the induction of lethal endotoxemia. VNS started 6 h after the endotoxic challenge failed to rescue animals and to improve survival in established endotoxemia (Fig. 1C). VNS started 6 h after the endotoxic challenge also failed to attenuate serum TNF levels. Because serum TNF levels were very low at 6 h after the endotoxic challenge, we mimicked our original conditions with a secondary LPS challenge. All animals received an initial dose of LPS; 6 h later they underwent sham surgery or VNS and endotoxemia similar to the first experiments. A second LPS challenge induced serum TNF responses that were not inhibited by the vagus nerve, similar to that found in established endotoxemia (Fig. 1D). Because previous studies indicated that sepsis is characterized by a massive apoptosis of splenocytes (28–30), and the spleen contributes to vega modulation, we reasoned that the loss of splenocytes might abrogate vega modulation. Thus, surgical splenectomy may mimic the loss of splenocytes during endotoxemia, and splenectomized animals may resemble the failure of vega modulation in septic mice. VNS...
started 20 min prior to the endotoxic challenge improved survival in control, but not in splenectomized, animals (Fig. 1E). VNS also failed to attenuate serum TNF levels in splenectomized animals (Fig. 1F), similar to that described in endotoxic animals. Together, these results suggested that the loss of splenocytes may abrogate the anti-inflammatory potential of the vagus nerve.

**Vagus nerve immunomodulation requires splenic T lymphocytes**

The specific contribution of particular splenic cell types to the anti-inflammatory potential of the vagus nerve was analyzed using knockout mice. Because our previous studies indicated that mast cells contribute to sepsis (31) and they can be in the proximity of the vagus nerve (32), we analyzed their potential role by performing VNS in mast cell-knockout animals. Mast cell-deficient mice have normal phenotype, but they have higher mortality to sepsis (33) and limited bacterial clearance (34). VNS inhibited serum TNF responses in both control WT and mast cell-knockout animals (Fig. 2A). Because our results suggested the implication of other cell types, we analyzed apoptosis in splenic lymphocytes and macrophages during endotoxemia, which is characterized by a dramatic increase in apoptosis and a progressive loss of B and T lymphocytes but not of macrophages (Fig. 2B). Based on these results, we analyzed whether the loss of T lymphocytes abrogates the anti-inflammatory potential of the vagus nerve. T lymphocytes were inhibited using anti-CD3–neutralizing Ab, as previously described (26, 27). Inhibition of T lymphocytes did not affect endotoxin-induced TNF responses, but it specifically blunted the anti-inflammatory potential of the vagus nerve (Fig. 2D). We used T cell-deficient nude mice and their counterpart WT mice to confirm our studies. Bacterial endotoxin induced similar TNF responses in both WT and nude mice, but VNS failed to modulate serum TNF levels in the nude mice (Fig. 2E). Together, these results indicated that the vagus nerve requires T lymphocytes to inhibit the innate immune responses to bacterial endotoxin.

**CD3+CD4+CD25+ lymphocytes re-establish nicotinic modulation in culture of splenocytes**

Because acetylcholine, the principal neurotransmitter of the vagus nerve, inhibits cytokine production in splenocytes via α7nR (15, 16, 35), we analyzed whether α7nR agonists require T cells to control cytokine production in splenocytes. Primary cultures of splenocytes from nude or WT mice were activated with endotoxin and treated with choline, a well-characterized specific α7nR agonist (35, 36). Similar to the results described for the vagus nerve, cultures of splenocytes from nude mice had similar TNF responses to endotoxin as did those from WT mice. α7nR agonist inhibited TNF production in splenocytes from WT mice but not in those from nude mice (Fig. 3A). These results were further analyzed in specific splenic cell populations by fractioning splenocytes in CD4-\(^+\), CD3+CD4+CD25-\(^+\), or CD3+CD4+CD25-\(^-\) cells. The most significant production of TNF was found in the CD4-\(^+\) splenocytes (Fig. 3B). α7nR agonists (both choline and nicotine) failed to inhibit TNF production in any isolated population of splenocytes. The potential interaction between different cell types was analyzed by combining CD4-\(^+\) splenocytes with CD3+CD4+CD25- or CD3+CD4+CD25- splenocytes. α7nR agonists (both choline and nicotine) inhibited TNF production in cocultures of CD4-\(^+\) and

**FIGURE 1. VNS lethal endotoxemia.** Mice underwent sham surgery (Control) or VNS started 20 min before LPS (6 mg/kg, i.p.). A, Serum TNF was analyzed by ELISA (n = 4) (one-way ANOVA with Bonferroni correction). B, Survival was analyzed for 2 wk, and no late deaths were observed (n = 15/group). \(p < 0.05\) versus control, log-rank survival test. C, Mice underwent sham surgery (Control) or VNS started 6 h after the LPS challenge. Survival was analyzed for 2 wk, and no late deaths were observed (n = 15/group). D, Mice underwent sham surgery (Control) or VNS beginning 6 h after the original LPS challenge. Then, animals received a second dose of LPS (2nd LPS), and serum TNF concentrations were measured 90 min later (n = 4) (one-way ANOVA with Bonferroni correction). E, Splenectomized (SPX) animals underwent sham surgery (Control) or VNS started 20 min prior to the endotoxic challenge improved survival in control, but not in splenectomized, animals (Fig. 1E). VNS also failed to attenuate serum TNF levels in splenectomized animals (Fig. 1F), similar to that described in endotoxic animals. Together, these results suggested that the loss of splenocytes may abrogate the anti-inflammatory potential of the vagus nerve. T lymphocytes but not of macrophages (Fig. 2B). Based on these results, we analyzed whether the loss of T lymphocytes abrogates the anti-inflammatory potential of the vagus nerve. T lymphocytes were inhibited using anti-CD3–neutralizing Ab, as previously described (26, 27). Inhibition of T lymphocytes did not affect endotoxin-induced TNF responses, but it specifically blunted the anti-inflammatory potential of the vagus nerve (Fig. 2D). We used T cell-deficient nude mice and their counterpart WT mice to confirm our studies. Bacterial endotoxin induced similar TNF responses in both WT and nude mice, but VNS failed to modulate serum TNF levels in the nude mice (Fig. 2E). Together, these results indicated that the vagus nerve requires T lymphocytes to inhibit the innate immune responses to bacterial endotoxin.
CD3+CD4+CD25− splenocytes but not in CD4− and CD3+CD4− CD25− splenocytes (Fig. 3C). Similar results were confirmed in the splenocytes from nude mice cocultured with particular populations of splenocytes from WT mice (Fig. 3D). Both choline and nicotine failed to attenuate TNF production in either splenocytes from nude mice or splenocytes from nude mice cocultured with CD3+CD4+CD25− splenocytes isolated from WT mice. However, the addition of WT CD3+CD4+CD25− splenocytes re-established cholinergic modulation in splenocytes isolated from nude mice. FACS studies confirmed that our CD3+CD4+CD25− lymphocytes were neither CD25+ nor Foxp3+ with 98% purity and did not have major production of IL-10. Together, these results suggested that TNF responses to endotoxin are mainly produced by CD4− splenocytes, but the anti-inflammatory potential of cholinergic agonists (to both nicotine and choline) specifically requires CD3+ CD4+CD25− splenocytes. Thus, T lymphocytes are required for

**FIGURE 3.** CD3+CD4+CD25− lymphocytes reestablish the anti-inflammatory potential of α7nR agonists in splenocytes. A, Primary cultures of splenocytes from WT or nude mice were treated with α7nR agonist choline (CHO) and/or LPS. B, Splenocytes from WT mice were fractionated to isolate CD4−, CD3+CD4−CD25−, or CD3+CD4+CD25− cells, stimulated with endotoxin, and treated with CHO (20 mM) or nicotine (NIC; 2 μM). C, Purified CD4− splenocytes were cocultured with CD3+CD4−CD25− or CD3+CD4+CD25− splenocytes and treated with CHO (20 mM) or NIC (2 μM). D, Splenocytes from nude mice were cocultured with CD3+CD4−CD25− or CD3+CD4+CD25− splenocytes from WT animals, stimulated with endotoxin, and treated with the indicated agonists. In all experiments, splenocytes were pretreated with α7nR agonists 30 min before LPS (100 ng/ml). TNF concentrations were analyzed by ELISA in the conditioned media 3 h after stimulation. Data represent mean ± SD from three experiments (n = 4). *p < 0.01, versus LPS group (one-way ANOVA with Bonferroni correction).
both the anti-inflammatory potential of the vagus nerve and cholinergic agonists in primary culture of splenocytes.

**CD3⁺CD4⁺CD25⁻ lymphocytes re-establish the anti-inflammatory mechanism of the vagus nerve in nude mice**

We also analyzed whether CD4⁺CD25⁻ splenocytes can re-establish cholinergic modulation in vivo. Similarly as described above for the VNS, the α7nR agonist, choline, failed to inhibit serum TNF levels in splenectomized animals. However, restitution of CD4⁺CD25⁻ splenocytes into splenectomized mice re-established the anti-inflammatory potential of the α7nR agonist (Fig. 4A). This mechanism is specifically mediated by the α7nR located in the lymphocytes, because α7nR-null CD3⁺CD4⁺CD25⁻ splenocytes from α7nR-knockout animals failed to re-establish the anti-inflammatory potential of choline (Fig. 4A). Because the anti-inflammatory potential of the vagus nerve is also mediated by α7nR, we also analyzed whether CD3⁺CD4⁺CD25⁻ splenocytes could re-establish the anti-inflammatory potential of the vagus nerve in splenectomized animals. VNS failed to inhibit serum TNF levels in control splenectomized animals. However, the transfer of CD4⁺CD25⁻ splenocytes into splenectomized mice re-established the anti-inflammatory potential of the vagus nerve (Fig. 4B). Based on our results, we reasoned that CD3⁺CD4⁺CD25⁻ lymphocytes could also re-establish the anti-inflammatory potential of VNS in nude mice. VNS failed to modulate serum TNF levels in nude mice. However, the transfer of CD4⁺CD25⁻ WT lymphocytes into nude mice re-established the anti-inflammatory potential of the vagus nerve (Fig. 4C). This effect was specific for CD3⁺CD4⁺CD25⁻ lymphocytes, because the transfer of typical regulatory CD25⁺ WT lymphocytes into nude mice did not re-establish vagal modulation. Together, these results indicated that CD3⁺CD4⁺CD25⁻ splenocytes re-established the anti-inflammatory potential of the vagus nerve and α7nR agonists in vivo.

**CD3⁺CD4⁺CD25⁻ lymphocytes re-establish splenic protection against sepsis**

Because VNS in splenectomized animals resembled that observed in endotoxemic mice, and CD3⁺CD4⁺CD25⁻ lymphocytes re-established vagal modulation in splenectomized and endotoxemic animals, we reasoned that CD3⁺CD4⁺CD25⁻ lymphocytes could re-establish vagal modulation in animals with established endotoxemia. VNS failed to modulate serum TNF levels in endotoxemic mice, when the treatment was started after the endotoxic challenge; however, restitution of CD3⁺CD4⁺CD25⁻ lymphocytes re-established the anti-inflammatory potential of VNS started at 6 h after the endotoxic challenge (Fig. 5A). Transfer of CD3⁺CD4⁺CD25⁻ lymphocytes could also re-establish vagal modulation of serum HMGB1, a late mediator of sepsis, even when the vagal stimulation was started at 24 h after the endotoxic challenge (Fig. 5B). Similar to VNS, nicotinic agonists also failed to control inflammation in splenectomized animals when the treatment was started after the onset of sepsis (24). Because VNS signals via α7nR, we reasoned that CD3⁺CD4⁺CD25⁻ lymphocytes could also re-establish the anti-inflammatory potential of nicotinic agonists in animals immunocompromised by splenectomy. From a clinical perspective, many pharmacological strategies modulate inflammation in healthy, but not in septic, patients. Likewise, nicotinic agonists modulate inflammation in healthy, but not in splenectomized, animals (24). The effects of α7nR agonist were analyzed in polymicrobial sepsis induced by CLP, a more clinically relevant model with polymicrobial peritonitis induced by the cecal puncture and the necrotic tissue induced by the cecal ligation. Treatment with α7nR agonist, started 24 h after CLP, failed to inhibit serum HMGB1 levels in splenectomized animals with polymicrobial peritonitis (24). Restitution of CD3⁺CD4⁺CD25⁻ lymphocytes re-established the splenic protection and the potential of choline to inhibit serum HMGB1 levels in splenectomized animals with established polymicrobial peritonitis (Fig. 5C). Restitution of CD3⁺CD4⁺CD25⁻ lymphocytes also re-established the potential of choline to rescue splenectomized animals from polymicrobial peritonitis, even when the treatment was started 24 h after CLP (Fig. 5D). Together, these results indicated that CD3⁺CD4⁺CD25⁻ lymphocytes re-established the splenic protection and the anti-inflammatory potential of cholinergic agonists to rescue animals from polymicrobial peritonitis.

**Discussion**

Immune homeostasis does not arise passively from an absence of inflammatory stimuli; rather, maintenance of health requires specific mechanisms to restrain reactions to inflammatory stimuli that
do not warrant a full response (9). In this sense, the regulation of inflammation seems to be proportional to the complexity of the immune system, and it can be considered in terms of checkpoints (9). Among these mechanisms, the nervous system is the main regulator of the immune system. We reported that the vagus nerve controls systemic inflammation, including both serum TNF and HMGB1 levels, by regulating cytokine production in the spleen during endotoxemia (24). However, the cellular mechanisms mediating vagal modulation and controlling inflammation in sepsis are unknown. Our current results now indicate that this mechanism involves two specific cellular responses, including an extrinsic cellular mechanism to control innate immune responses: CD4\(^+\) TNF-producing cells, representing splenic macrophages of the red pulp and the marginal zone, as previously described (37), and the cholinergic regulatory CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes, required by both the vagus nerve and cholinergic agonists to inhibit cytokine production in vitro (culture of splenocytes) and in vivo. It is important to note that our results mimick the histological organization of the spleen (38), where the nerve fibers are located mainly in the periarterial lymphoid sheath making synaptic-like connections with T lymphocytes located in the vicinity of macrophages (39–41). Previous studies described typical T suppressor lymphocytes characterized by the expression of IL-2R\(\alpha\) (CD25) and Foxp3\(^+\), our results are particularly significant, showing that neuro-immunomodulation requires a set of regulatory CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes that are neither CD25\(^+\) nor Foxp3\(^+\) and do not have major production of IL-10. The requirement of these cells seems to be specific, because the typical regulatory CD3\(^+\)CD4\(^+\)CD25\(^+\) lymphocytes failed to induce any significant effect on either the vagus nerve or cholinergic agonists in vitro or in vivo. Indeed, the transfer of CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes is sufficient to re-establish anti-inflammatory modulation in nude mice, even in the absence of the classic CD3\(^+\)CD4\(^-\)CD25\(^+\) T suppressor cells. Regulatory T cells are usually associated with modulation of a variety of in vitro immune responses of T cells to antigenic stimuli. Our studies suggested that regulatory CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes mediate vagal and cholinergic modulation of cytokine production in resident macrophages. Our studies included both cholinergic agonists and VNS to control TNF and HMGB1, as analyzed in vitro and in vivo. However, future studies analyzing other cytokines and the potential effects of these cells on activation, maturation, and proliferation of typical immune cells will be required to fully determine the regulatory potential of these cholinergic CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes. To our knowledge, these results revealed regulatory CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes as the first known physiological target for neuromodulation of the innate immune responses.

Pharmacological studies allowed the identification of specific cholinergic agonists to mimic the anti-inflammatory potential of the vagus nerve while avoiding the limitation of surgical nerve manipulation (17, 45). These studies focused on identifying the receptors mediating the anti-inflammatory potential of acetylcholine, the principal neurotransmitter of the vagus nerve. Cholinergic agonists (acetylcholine and nicotine) inhibit NF-\(\kappa\)B and cytokine production in splenocytes through \(\alpha\)7nR (15, 16, 24, 46). In vivo, both VNS and cholinergic agonists failed to modulate serum TNF levels in \(\alpha\)7nR-deficient animals (15, 47). From a pharmacologic perspective, \(\alpha\)7nR agonists mimic the anti-inflammatory potential of the vagus nerve, prevent organ damage, and improve survival in experimental sepsis (15, 16, 36, 48–50). However, both VNS and cholinergic agonists failed to control inflammation and improve survival in septic animals immunocompromised by surgical splenectomy (24). Indeed, treatment with nicotine decreased survival in splenectomized animals with sepsis (24). This is an important consideration because the clinical settings of sepsis typically involve immunocompromised patients, and overwhelming sepsis is the most typical risk of splenectomy and asplenia (51, 52). Our current results indicated that cholinergic agonists require regulatory CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes both in vitro and in vivo. In vitro, restitution of CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes re-established the anti-inflammatory potential of nicotine agonists in primary cultures of splenocytes from nude mice. In vivo, restitution of CD3\(^+\)CD4\(^+\)CD25\(^-\) lymphocytes re-established the anti-inflammatory potential of the vagus nerve and \(\alpha\)7nR agonists in nude mice. This effect was dependent
on α7nR, because the CD3^+CD4^+CD25^+ lymphocytes from the α7nR-knockout animals failed to re-establish cholinergic modulation. Moreover, these cholinergic regulatory lymphocytes re-established neuromodulation in splenectomized animals. This is a significant result considering the clinical implications of sepsis and asplenia (51, 52). These results also suggested that the cholinergic regulatory lymphocytes do not require the splenic structure; therefore, they can control inflammatory responses in addition to those in the spleen. Future studies are required to determine how these cholinergic regulatory T cells respond to the vagus nerve and re-establish vagal modulation in the absence of the spleen.

Severe sepsis remains a scientific challenge, with >30 unsuccessful clinical trials (3). Recombinant human activated protein C (Drotrecogin-α) is the only treatment approved by the U.S. Food and Drug Administration; however, because of the risk for hemorrhage, it is only approved for a small subset of patients with severe sepsis (53). A critical challenge is that many anti-inflammatory strategies that control inflammation in healthy animals fail to modulate inflammation in septic patients (42, 54, 55). One potential explanation is that many experimental strategies are effective for preventing systemic inflammation when the treatment is started before the pathogenesis but not when it is started after the pathological onset. Thus, these strategies fail to provide any therapeutic effects on animals or patients who are already sick. The immunological changes induced during sepsis might explain the failure of some of these strategies. Sepsis is characterized by a massive apoptosis of lymphocytes in the thymus and the spleen (29, 30, 56). Indeed, clinical studies indicated that leukocytes derived from septic patients exhibit T cell anergy (42, 57). Also, septic patients have shown a dramatic decrease in CD3^+CD4^+CD25^+ cells but a significant increase in CD3^+CD4^+CD25^- T cells that persisted only in nonsurviving patients (58, 59). Similar results were confirmed in trauma patients (60) and in mice after polymicrobial sepsis and stroke (61–63). The inhibition of lymphocyte apoptosis by caspase inhibitors can prevent mortality in experimental sepsis (28, 30). The mechanism by which lymphocyte apoptosis contributes to sepsis remains controversial. Caspase inhibitors can enhance immunity by preventing lymphocyte apoptosis, and lymphocytes act rapidly to control infection (28). In contrast, apoptotic cells can activate macrophages to release inflammatory cytokines, such as HMGB1 (30). Our results suggested that lymphocyte apoptosis can exacerbate inflammatory responses by preventing the anti-inflammatory potential of the vagus nerve and cholinergic regulatory lymphocytes. We confirmed that VNS failed to control serum TNF levels in splenectomized and nude mice, experimental models that mimic splenic apoptosis during sepsis. The restitution of cholinergic regulatory lymphocytes re-established the anti-inflammatory potential of the vagus nerve in splenectomized, nude, and septic mice. In addition to its physiological implications, the vagus nerve mediates the effect of multiple clinical treatments and anti-inflammatory compounds, including some nonsteroidal anti-inflammatory drugs, semapimod [CNI-1493; currently in clinical trials for Crohn’s disease (31)], melanocortin peptides (64), cholecystokinin (65, 66), and leptin (17, 45). Therefore, inhibition of the vagal immunomodulation renders these drugs inefficient in the control of inflammation. Among other examples, semapimod and adrenocorticotropic hormone (1–24) activate the efferent vagus nerve and limit circulating serum TNF levels during endotoxemia through a mechanism that requires a functional vagus nerve (64, 67). Inactivation of the vagus nerve abrogated the anti-inflammatory potential of these drugs (64, 67). Our study suggests a previously unrecognized role for splenic CD3^+CD4^+CD25^+ cholinergic lymphocytes in mediating the anti-inflammatory potential of the vagus nerve and clinical drugs (66). Thus, restitution of these regulatory lymphocytes might provide pharmacological advantages to re-establish the therapeutic potential of anti-inflammatory compounds in septic patients. One significant result is that the restitution of these cholinergic regulatory lymphocytes rescued the animals from established polymicrobial peritonitis, and it improved survival, even when the cells were transferred 24 h after CLP. The therapeutic window for the restitution of regulatory lymphocytes is significantly larger than that described for caspase inhibitors and typical inflammatory targets. Caspase inhibitors were used at 90 min after CLP (42, 57). Compared with other targets, administration of anti-TNF Abs increased mortality when administered after cecal perforation (68). Anti-macrophage migration inhibitory factor Abs (69) or lypoosphatidylcholine (70) are ineffective if administered >8 or 10 h after the induction of peritonitis (69). These results suggested the potential of harnessing regulatory lymphocytes for the treatment of sepsis. However, future studies are warranted to determine their potential clinical translation because, as opposed to the experimental models using healthy animals, the clinical settings of sepsis typically involve elderly patients with pre-existing disorders.

Disclosures

The authors have no financial conflicts of interest.

References


