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Review

Therapeutic potential and limitations of cholinergic anti-inflammatory pathway in sepsis

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ABSTRACT

Sepsis is one of the main causes of mortality in hospitalized patients. Despite the recent technical advances and the development of novel generation of antibiotics, severe sepsis remains a major clinical and scientific challenge in modern medicine. Unsuccessful efforts have been dedicated to the search of therapeutic options to treat the deleterious inflammatory components of sepsis. Recent findings on neuronal networks controlling immunity raised expectations for novel therapeutic strategies to promote the regulation of sterile inflammation, such as autoimmune diseases. Interesting studies have dissected the anatomical constituents of the so-called "cholinergic anti-inflammatory pathway", suggesting that electrical vagus nerve stimulation and pharmacological activation of beta-2 adrenergic and alpha-7 nicotinic receptors could be alternative strategies for improving inflammatory conditions. However, the literature on infectious diseases, such as sepsis, is still controversial and, therefore, the real therapeutic potential of this neuroimmune pathway is not well defined. In this review, we will discuss the beneficial and detrimental effects of neural manipulation in sepsis, which depend on the multiple variables of the immune system and the nature of the infection. These observations suggest future critical studies to validate the clinical implications of vagal parasympathetic signaling in sepsis treatment.

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1. Sepsis: definition and pathophysiology

1.1. Definition

The definition of sepsis was recently modified to include severe organ dysfunction caused by an uncontrolled inflammatory host response to the infection [1]. Moreover, septic shock is defined as a subset of sepsis in which underlying circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than sepsis alone [2].

The most prevalent sites of infection responsible to trigger sepsis in humans are the lungs, abdominal cavity, urinary tract and primary infections of the blood stream. A microbiological diagnosis is made in about half of the cases showing that Gram-negative bacteria account for about 60% and Gram-positive for the remainder cases [3,4]. Nevertheless, there is no available "gold standard" diagnostic test for sepsis, reflecting the complexity of this syndrome [5]. For this reason, clinicians have limited ability to distinguish sepsis from other inflammatory conditions or to predict outcome.

Sepsis onset is often insidious characterized by abnormal body temperature, mental confusion, hypotension, diminished urine output or thrombocytopenia. When the treatment of sepsis is unsuccessful, the patient may develop respiratory or renal failure, abnormalities of coagulation, and profound and unresponsive hypotension, characterizing septic shock, the leading causes of death in sepsis [1]. While a balanced inflammatory response is critical to fight the infection, an unregulated pro and anti-inflammatory response may induce organ damage in the host, being more destructive than the initial infection [6]. This imbalance is determined by several factors, such as pathogen virulence, bacterial load and patient-related factors (i.e. genetic background, age and comorbidities) [7].

1.2. Innate immune response and neutrophils

Toll-like receptors (TLR) are pivotal to trigger the defensive innate immune response against infections. TLRs identify pathogen signatures, such as the TLR4, which recognizes the lipopolysaccharide (LPS), a component present in the wall of Gram-negative bacteria [8]. Similarly, peptidoglycan (PGN), the principal component of Gram-positive bacteria, activates TLR2. The activation of TLR4 or TLR2 in immune cells triggers the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1β and IL-6, as well as chemokines, like CXCL1 and CXCL2. These cytokines contribute to the elimination of the pathogen by recruiting and activating neutrophils [9–11] (Fig. 1A).

Neutrophils are key players of the innate immunity to control bacterial growth. Their initial recruitment into the infection site associates with better survival outcome in sepsis [12]. The killing of pathogens is favored by the phagocytosis and also by the activity of powerful, but dangerous microbicidal molecules, such as reactive oxygen and nitrogen intermediates, as well as lytic enzymes (i.e. elastase, myeloperoxidase, lysozyme, cathepsin G, and serine proteases). In addition to the killing following phagocytosis, neutrophils are also capable of controlling the bacterial load by releasing neutrophil extracellular traps (NETs) [13].

Not surprisingly, the over activation of TLRs has also been associated with an overwhelming inflammatory response during sepsis. One of the deleterious effects induced by TLR over-activation during sepsis is the failure of neutrophil recruitment to the infectious site and the accumulation of these cells in organs such as lungs, heart and kidneys (Fig. 1B). While the efficient neutrophil recruitment into the infection site is essential to control the spread of the pathogen, their unbalanced migration into organs away from the infection focus plays a deleterious and paradoxical role during sepsis. Thus, under a strong activation, neutrophils do not efficiently counteract their targets and are trapped into vital organs provoking unspecific tissue damage and organ dysfunction, which lead to multiple organ failure [14,15]. Mechanistically, it was demonstrated by our group that the chemokine receptor CCR2 mediates neutrophil infiltration into vital organs. CCR2 expression is induced on the neutrophil surface of mice and patients with sepsis after TLR activation. The blockade of CCR2 decreases organ damage and death in animals subjected to severe CLP. Moreover, CCR2 expression in septic human neutrophils is positively correlated with the severity of the disease, as measured using the Sepsis-related Organ Failure Assessment (SOFA) score. Accordingly, human neutrophils isolated from non-surviving septic patients show higher CCR2 expression than neutrophils from surviving patients [15]. This systemic entrapment of neutrophils is associated with a reduction in the recruitment of these cells in the infection site. Accordingly, mice lacking TLR2, TLR4 or TLR9-dependent pathways show higher numbers of neutrophils into the infection site than wild-type (WT). As a consequence, the absence of one of the TLR leads to lower bacterial loads and higher survival rates in septic mice [16–18]. It is noteworthy that in the absence of the signaling of one TLR, other TLRs are activated by distinct bacterial components during the polymicrobial sepsis, triggering the essential inflammatory response to control the infection.

1.3. Experimental models of sepsis

The complex interaction between pathogen-derived molecules and host immune cells during sepsis has been investigated using different models: endotoxemia, live bacteria administration and cecal ligation and puncture (CLP). Endotoxemia generated by bacterial LPS administration is a standard model widely used to investigate systemic inflammatory responses without the infectious component. In addition, the injection of live bacteria into the peritoneum or lung is used to simulate the human peritonitis and pneumonia, respectively. Finally, CLP is a gold standard model to mimic clinical sepsis with polymicrobial infection induced by exposure of cecal content into the peritoneal cavity and inflammation by both the infection and the necrotic tissue of the cecal ligation [19–22]. This polymicrobial peritonitis model can be performed with or without antibiotic administration. Anti-microbial treatment mirrors the clinical standards of sepsis treatment, but they are avoided in experimental models that intend to study the infection [19,20,23,24]. All these models reproduce some clinical manifestations that are observed in humans, such as systemic inflammation, hypotension, multi-organ dysfunctions and death. Basically, the key differences between these models are the kinetic/magnitude of cytokines production and the absence or presence of a microbial component. For example, LPS administration has a rapid and transient increase in systemic cytokines levels, while CLP has a less abrupt and continued cytokine production, better mirroring the clinical settings of sepsis. The main characteristics and differences between these classical septic models are shown in Table 1.

Although important findings contributed to better understand the pathophysiology of sepsis in the last years, the treatment of septic patients remains a major challenge. It is known that tight

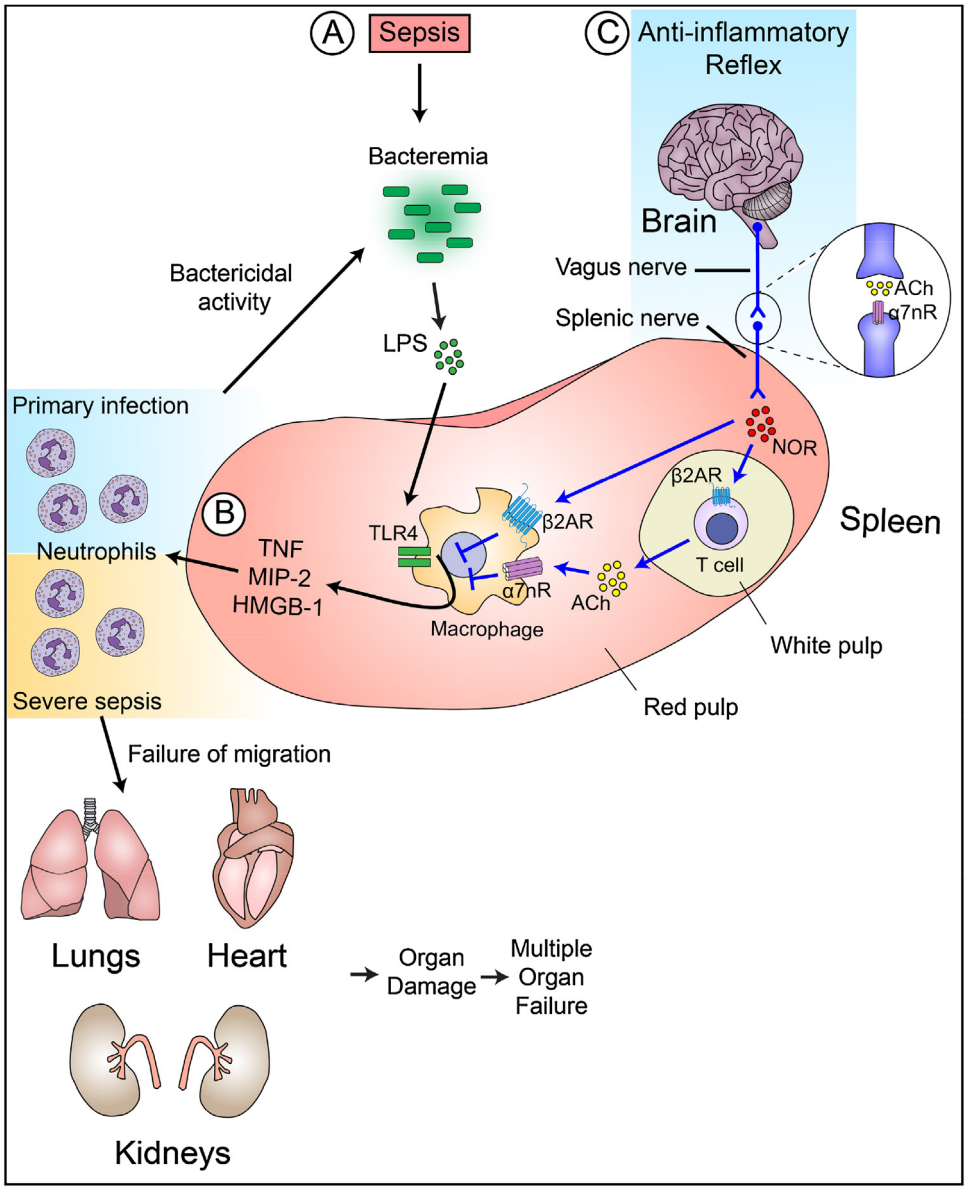

Fig. 1. Sepsis pathophysiology and cholinergic anti-inflammatory pathway description. (A) Bacteria or LPS (molecule isolated from Gram-negative bacteria) induce the release of pro-inflammatory mediators (cytokines, chemokines) by macrophages via activation of TLR4. (B) These mediators are involved in the neutrophil recruitment into the infection site. Indeed, neutrophils are key players in the elimination of bacteria, preventing the systemic bacterial dissemination. However, during the sepsis, the presence of excessive systemic inflammation is responsible for the failure of neutrophil migration into the infection focus. Subsequently, the circulating neutrophils infiltrate into vital organs (lung, heart and kidneys) provoking the multiple organ failure. (C) The efferent vagus nerve activation releases acetylcholine that stimulates the splenic nerve to liberate norepinephrine that, in turn, interacts with beta-2 adrenergic receptor expressed in T lymphocytes. These cells release acetylcholine, which activates alpha-7 nicotinic receptor expressed in macrophages and inhibits the release of inflammatory mediators.

Table 1
The characteristics and differences between experimental sepsis models.

		Sepsis Model		
		Endotoxemia	CLP	Live bacteria
Model Characteristics and Clinical Manifestations	Stimuli	LPS administration *in bolus*	Cecal material containing bacteria released continuously	Live bacteria administrated *in bolus* in the peritoneum (peritonitis) or lung (pneumonia)
	Severity	Depend on LPS dose	Depends on number of cecum holes, needle thickness and quantity of extruded cecal material	Depends on the number of colonies and characteristics of bacteria strain administrated
	Bacteremia	(−)	(+)	(+)
	Systemic Inflammation	(+)	(+)	(+)
	Hypotension	(+)	(+)	(+)
	Multi-organ dysfunction	(+)	(+)	(+)

(−) absence; (+) presence.

regulation of the innate immune system is essential for maintaining the balance between protective and detrimental inflammatory responses during sepsis. Moreover, despite the important role of the immune response during sepsis, the prognostic of septic patients depends also on other components such as hormonal, cardiovascular, metabolic and coagulation pathways [1]. More recently, the central nervous system has been shown to play a significant role on sepsis by regulating the immunity via the autonomic nervous system.

2. The inflammatory reflex

Several groups have demonstrated that the autonomic nervous system, which encompassed both the sympathetic and parasympathetic systems, control physiological homeostasis of the urogenital, cardiovascular and gastrointestinal activities, including the immunity [25,26]. Over the last fifteen years, the existence of a physiological crosstalk between the nervous system and the immune system has become evident [26,27]. The main example of integrative and complex pathway is called "inflammatory reflex" [28]. This intriguing bidirectional neuroimmune mechanism is depicted by both the afferent and efferent branches of the vagus nerve, which is the major parasympathetic nerve connecting the brain with the peripheral viscera. The afferent arm of vagus nerve is a crucial component for conducting peripheral immune signals to the brain. One illustration of the crosstalk between these systems is the influence of this nerve in the febrile response [29]. Fever is an adaptive physiological response generated by behavior and neural mechanisms, which alter the hypothalamic center of temperature leading to an increase in the heat production and conservation. This increased temperature improves different aspects of body's defense machinery, as phagocytosis, which favor the pathogen elimination [30]. Interestingly, subdiaphragmatic vagotomy on the hepatic branch prevented IL-1-induced fever [31]. It is also believed that microorganisms could activate the afferent vagus nerve directly at the level of nodose ganglion during an infection, because it expresses TLR4 [32].

Although the activation of the afferent vagal branch of the "inflammatory reflex" shows pro-inflammatory properties, the activation of the efferent branch exhibits a potent anti-inflammatory effect, suggesting that the neuroimmune route acts in an initial phase to eliminate the infectious agent, and in a posterior phase reestablishing the homeostasis. Historically, an initial study demonstrated that the electrical stimulation of the efferent vagus nerve prevented the increase in the serum levels of inflammatory cytokines after LPS administration [28,33] (Fig. 1C). Interestingly, the spleen has been described as the main source of TNF during endotoxemia [34]. Those results were quite surprising at the time because the spleen does not have vagal innervation. However, successive studies demonstrated that this neural pathway is highly complex because the vagus nerve is connected with the spleen via sympathetic splenic nerve [35,36]. Indeed, the stimulation of splenic nerve releases norepinephrine, which activates beta-adrenergic receptors expressed on a specific T-lymphocytes subpopulation found in the spleen white pulp [36–38]. Then, these cells release acetylcholine that bind to the alpha-7 nicotinic receptors (α7nAchR) expressed on macrophages localized in the red pulp, inhibiting the pro-inflammatory cytokines release [37,39] (Fig. 1C). The importance of the α7nAchR was also reported as a crucial neural component connecting the parasympathetic vagus nerve with the sympathetic splenic nerve at the mesenteric ganglion [36]. It is possible that the α7nAchR play critical roles regulating both the neuronal connection as the macrophage activation.

Moreover, the connection between the vagus and splenic nerves has been a matter of constant debate. For example, anatomical and physiological studies have demonstrated no connection between the vagus and splenic nerves [40]. We and other groups have suggested alternative central pathways involving the vagal anti-inflammatory signaling [36,41]. By contrast, both sympathetic and parasympathetic innervations have been recognized in the spleen [42]. Finally, an unconventional model has been proposed, suggesting a non-neural link from the vagus nerve to the spleen [43]. In this theoretical model, the vagus nerve stimulation activates T-cells that are not resident of the spleen to migrate in direction of this organ releasing subsequently acetylcholine. This neurotransmitter then binds in α7-containing nicotinic receptors expressed in peripheral terminals of splenic nerves (instead of the traditional cellular body) releasing norepinephrine.

Based on the anatomical and physiological descriptions of "inflammatory reflex", several experimental studies provide novel potential strategies for treating sepsis.

2.1. Vagus nerve stimulation

As mentioned before, vagus nerve stimulation, the first form described to activate the cholinergic anti-inflammatory pathway, prevents the systemic release of pro-inflammatory cytokines and hypotension in endotoxemia [33]. In addition, experiments performed by our group confirmed these results [36,38,44]. Other beneficial effects from electrical vagal stimulation in endotoxemic animals include attenuation of the coagulant and fibrinolytic systems [45] and of disruption of tight junction in the intestinal epithelium [46]. To date, only two studies demonstrate the effect of vagus nerve stimulation on survival in polymicrobial peritonitis induced by CLP. In the first one, the protective effect on the survival rate was evaluated only for short periods (eight hours after the surgery) [47] as compared to the longer periods usually described in the literature (1–2 weeks). The second study demonstrated that the vagus nerve stimulation prevented systemic inflammation and mortality in polymicrobial peritonitis [48]. Importantly, the authors of this study used a transcutaneous carotid sinus massage in mice, instead of the conventional surgical approach of direct vagus nerve stimulation and treated the animals with antimicrobial drugs. Notable, the use of antibiotics reduces the bacterial load and could mask the effect of the stimulation of the vagus nerve in controlling the infection. Thus, although there are evidences in the literature that the electrical vagal stimulation prevents the systemic inflammation and mortality in endotoxemic and CLP models [33,44,48], other studies did not support these findings. Moreover, the electrical vagal stimulation in clinical studies is still under debate and warrant further investigation.

The vagal anti-inflammatory signaling is believed to be dependent on the nature of the sepsis. In fact, previous studies showed that the blockade of vagal transmission in vagotomized or α7nAchR-deficient animals under endotoxemic condition exacerbates cytokine production, accelerating the shock development [33,39]. However, although the vagotomy or α7nAchR-deficiency also increases the circulating cytokine levels in infectious conditions, the enhancement of the neutrophil migration toward the infectious focus improves the local control of microorganism growth, decreasing the bacteremia [49,50].

Further investigations on the role and clinical applicability of vagal stimulation are limited by the invasiveness of the surgical procedure required for direct nerve stimulation. The traditional surgical approach requires the induction of anesthesia, which limits the successive use of this technique during the progression of the disease and thus precluding the investigation of survival rates in both animals and humans. Moreover, anesthetic drugs interfere with the neuronal activation and they also showed anti-inflammatory properties. As consequence, these properties may not represent a real effect of nerve stimulation in a systemic inflam-

matory condition [51]. Another limitation of using the electrical vagus nerve stimulation in sepsis therapies involves the high rates of splenic lymphocytes apoptosis. After sepsis induction, splenic lymphocytes enter in apoptosis interrupting drastically the vagal transmission [44]. This interruption was circumvented in animals by the adoptive transfer of regulatory T lymphocytes [44]. Therefore, these results indicate that the anti-inflammatory potential of the vagus nerve may not be efficient in patients with severe lymphopenia or those that had splenectomy.

Finally, considering that neuroimmune pathways are reflexively activated to attenuate an inflammatory response, it is possible to claim that continuous vagus nerve activation could result in a sustained immunosuppressive status associated with an increased susceptibility to nosocomial/opportunistic infections.

2.2. Pharmacological approaches to mimic the vagal signaling

The limitations of conduct electrical vagus nerve stimulation in conscious septic animals for long periods raised alternative approaches using pharmacological tools mimicking the vagal anti-inflammatory signaling. For instance, the administration of nicotinic and adrenergic agonists has been shown to efficiently prevent the inflammatory responses and, as consequence, reduce organ damage and mortality in polymicrobial sepsis [38,52]. In this way, special attention will be given to the pharmacological aspects of the alpha-7 nicotinic and beta-2 adrenergic receptors activation.

2.2.1. Nicotinic acetylcholine receptors

The nicotinic acetylcholine receptors (nAchR) consist in cationic channels formed by five subunits delimiting a central aqueous pore, which allows cations influx [53]. The subtype α7nAchR expressed by immune cells is responsible for the modulatory effects of acetylcholine and nicotine in primary culture of macrophages. The molecular mechanisms of α7nAchR activation were observed in human monocytes after LPS stimulation. Acetylcholine binds to nAchR inhibiting the transcriptional activity of p38 mitogen-activated protein kinase (p38MAPK) and nuclear factor (NF)-kB (by suppression of I-kappa B phosphorylation) through inhibition of intracellular Ca2+ stores release. Furthermore, α7nAchR activation can also recruits janus kinase 2 (Jak2) to form a heterodimeric complex initiating an intracellular transduction mediated via signal transducer and activator of transcription 3 (STAT3) [54]. As result, this signaling culminates in the inhibition of the inflammatory components production such as cytokines and chemokines, CD14, TLR4, intercellular adhesion molecule 1, B7.1, and CD40 [54–56]. Moreover, the attenuation of cytokines release by macrophages stimulated with nicotine is not exclusively dependent on TLR4 signaling, since identical modulatory effects were also observed after selective TLR2, TLR3, TLR4, TLR9, and RAGE activation by specific ligands [57].

A preliminary study demonstrated the importance of α7nAchR activation on the anti-inflammatory potential of the vagus nerve in endotoxemia [39]. Nicotine, a prototypal non-selective nAchR agonist, prevented the high systemic TNF levels and subsequently death in endotoxemic animals [52]. Furthermore, the treatment with nicotine after the CLP induction reduced systemic inflammation and rescued mice from established sepsis [52]. Confirming these data, selective alpha-7 nicotinic agonists, such as choline [58] and GTS-21 (3-(2, 4 dimethoxybenzylidene)-anabaseine) [59], showed similar anti-inflammatory effects in endotoxemic mice. Due to the inhibitory effect on cytokines production, nicotinic agonists administration attenuated the infiltration of neutrophils in vital organs, such as heart, kidneys, lungs and liver, preventing the multiple organs failure in sepsis [60–64].

Moreover, an addictive beneficial effect of α7nAchR-agonists has been observed after the association with anti-TNF therapy during endotoxemia [65]. Therefore, the pharmacological α7nAchR activation of the cholinergic anti-inflammatory pathway characterizes an alternative approach to treating sepsis [66] and its importance has been supported by clinical studies. One of these studies describes that the *α7*nAchR gene expression levels in peripheral blood mononuclear cells of septic patients could be considered a clinical marker for sepsis prognostic, because higher α7nAchR expression is associated with a more efficient control of inflammation and subsequently lower disease severity [67].

Conversely, nicotine pretreatment impaired the host susceptibility after live *E. coli* administration due to an increase of bacteremia triggered by inefficient neutrophil recruitment to the infectious focus [49]. In another study, the nicotine also transiently impaired the innate host defense, increasing the bacterial loads in the lungs and blood in mice challenged by intranasal *Streptococcus pneumonia* [68]. It has also been shown that *in vitro* and *in vivo* nicotine exposition leads to a reduction of bacterial phagocytosis and killing on neutrophils and macrophages [69,70]. Finally, another study demonstrated that nicotine administration improved the survival rate in sterile (endotoxemia) but not in non-sterile sepsis (CLP) [71]. Considering that the treatment with antibiotics was not adopted in this study, it would be possible to suggest that the protective effect of nicotine in CLP-induced sepsis shown by the previous studies would be the result of combination of the nicotine and the antimicrobial drugs. However, data from other groups have confirmed that nicotine improves the outcome of polymicrobial peritonitis even in the absence of antibiotic therapy [72,73].

The apparent contradictory role of nicotinic agonists in sepsis could be explained by multiple variables, such as the time of the intervention, the effector mechanisms of innate immunity, the presence of infectious agent and the structural differences of bacteria. As a dynamic process, the systemic production of cytokines and the neutrophil migration to the infection focus do not occur at the same time. As a consequence, the activation of nicotinic pathway early in the pathogenesis of sepsis could block the production of cytokines, which would prevent the recruitment of neutrophils into the infection site. The failure of neutrophil migration could then culminate in high mortality rates. By contrast, the activation of the nicotinic pathway at a later time point could prevent the excessive production of the inflammatory cytokines as well as neutrophil recruitment in vital organs involved in the multiple organ failure. In this case, the initial response would induce the recruitment of neutrophils to control the bacterial infection, resulting in reduced mortality rates. Moreover, the sympathetic system can affect differently the Gram-negative or positive bacterial dissemination [74]. Altogether, these observations suggest that the use of nicotinic agonists remains to be more explored in order to validate their clinical potential in infectious disorders.

2.2.2. β2 adrenergic receptors

Adrenergic receptors (AR) are a large family of seven transmembrane receptors responsive to catecholamines. The activation of β2AR can lead to both stimulatory and inhibitory responses of adenylate cyclase by coupling to Gs and Gi protein, respectively [75]. In addition, these receptors can activate the MAPK pathway through the G proteins coupling or β-arrestins recruitment [76]. Clinical evidences showed that the genetic variation of β2AR gene (ADRB2) renders the receptor less responsive to the anti-inflammatory properties of adrenergic agonists due to a higher desensitization. This effect aggravates the organ damage promoting mortality in septic patients [77].

We have shown that the vagus nerve stimulation depends on noradrenaline released from splenic sympathetic terminals followed by β2AR activation in T lymphocytes [38]. These results were later confirmed indicating that high concentrations of norepinephrine may activate lymphocytes to produce acetylcholine *in*

vitro [37]. These studies concur in suggesting that β2AR-activation could mimic the cholinergic anti-inflammatory signaling (Fig. 1C). The use of β2-adrenergic in the pharmacotherapy of sepsis is considered an alternative to the use of nicotine, which showed undesirable side effects in the central nervous system (e.g., addiction) and still has contradictory effects in sepsis.

Notably, salbutamol, a selective β2AR-agonist reduced systemic inflammation, organ damage and improved survival rate in both endotoxemia and polymicrobial peritonitis [38]. Corroborating this idea, protective effect of salbutamol was prevented in β2AR-deficient mice. Moreover, the importance of β2AR expressed in T lymphocytes was also confirmed with the adoptive transfer of WT lymphocytes into β2AR-deficient mice to restore the protective effects of salbutamol [38]. In addition, β2AR-activation can also act by additional mechanisms in the innate immune system that could prevent the progression of sepsis, such as directly inhibiting cytokine production or improving the bacteria phagocytosis in macrophages [78,79].

3. Conclusions

Sepsis still remains a worldwide challenge for the public health system in terms of treatment and cost of the intensive care units. It is clear that the unbalanced pro and anti-inflammatory response of the immune system during sepsis is one of the major issues of this pathophysiology. Thus, the search for new therapies that reduce inflammation in the initial phase of sepsis, without affecting the late phase or inducing immunosuppression, could be a promising therapeutic strategy. In this context, the present review describes the role of the efferent vagal transmission in controlling inflammation during sepsis and highlights the pharmacologic targets that can be activated without using the invasive technique of vagus stimulation. Moreover, the use of this vagal neuroimmune pathway is still controversial in specific situations such as sterile or pathogen-induced inflammation. The effect of the efferent vagal signaling at inhibiting exacerbated cytokine production and inappropriate neutrophil entrapment into vital organs could be beneficial to the host under systemic inflammatory conditions. By contrast, the cholinergic anti-inflammatory pathway can inhibit specific innate immune responses that are crucial to eliminate the bacteria (e.g. initial neutrophil migration), and subsequent increase of the mortality in sepsis.

4. The future of pharmacological and bioelectronic therapies based on the inflammatory reflex in sepsis treatment

Recent non-invasive techniques involving bioelectronic approaches (called “electroceuticals”), such as ultra-sound [80] and eletroacupuncture [81], have been studied as alternative tools in the treatment of systemic inflammatory responses, including sepsis. Moreover, several drugs derived from the molecular knowledge of vagal anti-inflammatory signaling have been proposed in pharmacological interventions of septic patients, including M1 muscarinic and D1 dopaminergic agonists [81,82]. Finally, ongoing clinical studies aim to investigate evaluating the effects of the cholinergic anti-inflammatory pathway have been suggested in systemic inflammatory conditions (e.g. https://clinicaltrials.gov/ct2/show/NCT02279147).

Conflict of interest

The authors declare no conflict of interest.

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