Beyond Neurotransmission: The Immunological Mechanism of ACh from The CAIP Perspective

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Abstract. Acetylcholine (ACh), traditionally recognized as a neurotransmitter involved in synaptic signaling, has emerged as a crucial player in the immune system, extending beyond its classical functions. Extensive scientific research has shed light on the intricate mechanisms underlying the cholinergic anti-inflammatory pathway (CAIP), which plays a pivotal role in regulating immune responses and preserving homeostasis. Primarily mediated by the vagus nerve, this pathway involves the interaction between the nervous and immunological systems. ACh, acting as a key signaling molecule, exerts anti-inflammatory effects by modulating immune cell polarization (encompassing both morphological and functional changes), cytokine production, and signaling pathways. T cells and macrophages, equipped with the cholinergic system, prominently contribute to this immunomodulatory process. Nevertheless, the precise mechanisms governing the CAIP and the specific contribution of ACh in immunological responses remain subjects of ongoing research and debate. This concise review explores the intricate neuro-immune interactions, with a particular focus on the CAIP. Additionally, we delve into the cholinergic system within immune cells, examining the influence of lymphocyte-derived ACh on immunological functioning, thereby illuminating its regulatory role in immune responses and homeostasis maintenance, providing new insights into the development of innovative therapeutic strategies to combat inflammation-related diseases.

1. Introduction

Acetylcholine (ACh), primarily acknowledged as a physiological neurotransmitter, represents an ancient signaling molecule that has persisted since the early stages in primitive lives including bacteria, algae, protozoa, sponges, and primitive plants and fungi, indicating its potential function independent of neurons. As evolution progressed, increasingly intricate organ systems such as the smooth and skeletal muscle, energy-generation, sexual reproductivity, immunity, and nerves underwent addition optimization of the cholinergic signaling machinery. Consequently, several shared interfaces developed, such as those linking the nerves and immunity.

Extensive researches conducted over the past two decades have elucidated numerous regulatory mechanisms mediated by the cholinergic system. T cells and macrophages both express a majority of the essential parts required for a well-functioning cholinergic system, including choline acetyltransferase (ChAT), acetylcholine receptors (mAChRs and nAChRs), as well as acetylcholinesterase (AChE). Also, the activation of immune system including splenic T cells triggered by neurons leads to an upregulation in cholinergic activity with increased expression of ChAT. Macrophages equipped with AChRs and ChAT are also engaged in the anti-inflammatory process. These leads to the emerging concept of cholinergic anti-inflammatory pathway (CAIP) and a revised understanding of the biological role of ACh, among other crucial functions, in safeguarding the organism against external and internal health threats by preventing inflammatory imbalances to maintain homeostasis.

Therefore, comprehending the intricate workings of the cholinergic system as well as the interactions within immune cells assumes paramount significance in order to devise efficacious drug regimens and therapeutic strategies for inflammatory disorders as well as cancer.

2. The CAIP: ACh's multifunctionality beyond neurotransmission

The intricate networks in the nervous system are responsible for integrating physiological processes and maintaining homeostasis, while the immune system is tasked with defending against infection and injury through immunological mechanisms. In recent years, research has revealed a multitude of interactions between these two disciplines that were previously overlooked due to a history of neglect. Traditionally, the regulation of inflammatory responses was believed to rely on humoral pathways, serving as intermediaries for neural and inflammatory signals to maintain a delicate balance between anti-inflammatory and pro-inflammatory responses. However, the concept of the "neuroendocrine-immune network," initially proposed by Besedovsky in

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1977 [1], emphasizes the involvement of neurotransmitters, hormones, and cytokines in preserving homeostasis among the nervous, endocrine, and immune systems. Furthermore, extensive research conducted over the past two decades has further elucidated the inflammatory reflex-an expeditious and efficient regulatory mechanism that facilitates direct communication linking the neurological and immunological systems by bypassing the time-consuming process of humoral transmission.

Previously believed to solely function in central and peripheral nervous systems, ACh acts as crucial part in the neuroimmune interaction, facilitating collaboration and communication between neurons and immune cells. This interaction is not limited to higher organisms but also extends to invertebrates, as evidenced by the specialized neurons in the simple nervous system of *Caenorhabditis elegans*, which control the unfolded protein response pathway necessary for innate immunity in this nematode.

In this section, we will provide a comprehensive overview of the structural basis and mechanisms underlying the CAIP within the context of the inflammatory reflex, particularly focusing on the role played by ACh as a key molecule in inflammation modulation. Furthermore, we will discuss the most recent discoveries and controversies surrounding this pathway, shedding light on its potential therapeutic implications in immune regulation.

2.1. The vagus nerve as a crucial component in the neurological regulation of body homeostasis

The vagus nerve, also called pneumogastric nerve, is a major component of autonomic nervous network as a crucial component during the regulation of various physiological functions and body homeostasis.

Originating from the brain stem, which includes the pons, medulla oblongata, and midbrain, the vagus nerve extends from the medulla oblongata downwards, passing through the jugular foramen and traveling through the chest and abdomen. It is composed of afferent and efferent cholinergic fibers that project to peripheral visceral organs, such as the heart, liver, lungs, gastrointestinal tract, and pancreas. Within these organs, the vagus nerve forms synaptic connections with postganglionic neurons, allowing for the transmission of signals. The principal neuromodulator involved in this communication is ACh, which is released from the postganglionic fibers.

2.2. Inflammation and the anti-inflammatory role of ACh in neuroimmune

Inflammation is a response by the body's innate immune system when it detects tissue injury, invasion of pathogens, or danger signals. This response is triggered by the interaction between pattern-recognition receptors (PRRs, such as TLR4) and pathogen-associated molecular patterns (PAMPs), aiming to contain, neutralize, and eliminate these potentially harmful stimuli. Macrophages and other immune cells possess receptors such as TLRs and NLRs are functioning in the steps. These interactions promote further activation of intracellular pathways mediated by transcription factors. Additionally, they activate the inflammasome and increase the production of cytokines, chemokines, and other molecules involved in immune responses. Besides, additional inflammatory molecules like prostaglandins (PGE2) and leukotrienes are released, thereby facilitating the primary goal of the inflammatory response - neutralizing invading pathogens while promoting tissue repair. Moreover, macrophages and dendritic cells significantly contribute to adaptive immunity through antigen presentation to T and B cells, effectively containing the initial stimulus and establishing immunological memory.

The process of inflammation is an intricate event that involves several key events. These events include controlling the infiltration of leukocytes and promoting the phagocytosis of necrotic cells by neutrophils and macrophages, ultimately leading to the restoration of balance in the body. This restoration is achieved through the action of pro-resolving molecules like resolvins, protectins, lipoxins, and maresins. When there is a disruption in the signaling of these pro-resolving mediators, it can result in unresolved chronic inflammation which contributes to various disorders' pathologies.

It is increasingly acknowledged that, alongside the immunity, the nervous system provides valuable foundation to safeguard the function of wounding healing and anti-infection response. The convergence of neuroscience and immunological science has given rise to the emerging science of bioelectronic medicine, which focuses on elucidating underline mechanisms amenable to modulation through bioelectronic devices that selectively target neural circuits. Encouraging clinical trial results demonstrate that the bioelectrical activation of CAIP releasing ACh has exhibited significant anti-inflammatory efficacy in sepsis, rheumatoid arthritis, inflammatory bowel disease (IBD), diabetes, obesity-driven disorders, postoperative intestinal obstruction, stroke, brain injury, and other diseases characterized by aberrant inflammation by reducing inflammation and improving disease outcomes.

Thus, a comprehensive comprehension of the underlying mechanisms governing imbalances during inflammation holds great potential for the development of therapeutic interventions targeting the aforementioned inflammatory diseases.

2.3. Revealing the neuroimmune interaction mediated by ACh: the mechanisms underlying the functioning of the CAIP

Early evidence of immune-brain communication was discovered around 1995 [2], suggesting that the blockade of IL-1 could induce hyperthermia via subdiaphragmatic vagal transection, which provided a crucial clue leading to the discovery of neuroimmune regulation of

inflammation and the CAIP by Borovikova et al in 2000 [3]. Contributed an immunological perspective to the understanding of neurotransmitters, Borovikova's study discovered that the primary vagal neurotransmitter, ACh, effectively reduced the secretion of pro-inflammatory cytokines in human macrophage stimulated with lipopolysaccharide (LPS) in vitro, while leaving the inflammation inhibiting cytokine IL-10 unaffected. Additionally, vagus nerve stimulation in vivo with rats underwent lethal endotoxemia mitigates the LPS-induced serum and hepatic TNF surge, and ameliorates the event of endotoxic shock. Using various research approaches, including nerve transection and electrical stimulation, Tracey et al proposed the concept of the inflammatory reflex in 2002. Revised and extensively debated over the past two decades, this model continues to be a subject of controversy.

Inflammatory mediators, such as cytokines, PGE2, nerve growth factor (NGF), serotonin, and histamine are released upon the activation of TLRs and NLRs in the periphery (e.g., Pathogens or tissue injury) by immune cells. Acting as the sensory members of the afferent arm in the inflammation reflex, vagus nerves detect these mediators mainly through PAMPs and PRRs.

These neurons send signals to the nucleus tractus solitarius (NTS), serving as a major relay station for vagal input to the central autonomic network (CAN). This can lead to a reduction in inflammatory molecules through two main negative feedback pathways: the traditional endocrine pathway, such as the hypothalamic-pituitary-adrenocortical axis that stimulates the production of anti-inflammatory glucocorticoids from adrenal glands, and a more recently discovered mechanism known as CAIP.

In the context of CAIP, communication with the NTS occurs through connections to three crucial nuclei: 1) the dorsal motor nucleus of the vagus (DMN), which houses neurons responsible for controlling the vagus nerve before it reaches its target; 2) the nucleus ambiguous (NA), containing both neurons involved in regulating the vagus nerve and those responsible for motor functions; and 3) the intermediolateral nucleus (IML), which is the lower central place of the sympathetic nerves of splanchnic nerve (SN). The initial formulation of this efferent cholinergic arm of the inflammation response was postulated that ACh secreted by the cholinergic vagal efferent neurons was directly exerted on macrophages based on evidence mainly using CNI-1493 (as a down-regulator of macrophage and TNF response) that macrophage cholinergic receptor activation down-regulates the synthesis of pro-inflammatory cytokines [4].

Within the context of endotoxemia and other inflammatory conditions, the spleen assumes a pivotal role as a primary source of TNF and other pro-inflammatory cytokines. Nevertheless, considering the evidence indicating an interplay between the efferent arm and splenic nerve that exerts inhibitory effects on proinflammatory cytokine production within the spleen, it is imperative to adapt this program. To date, substantial evidence supports the intricate model wherein the spleen exhibits limited or no direct innervation by cholinergic vagus fibers. Both the vagus nerve and visceral converge of nerves at the abdominal ganglion (CG), where output ACh binds to the α7-nAChR, facilitating neural transmission to the adrenergic splenic nerve (SpN) [5].

The recognition of the spleen's central role as a primary source of TNF and other cytokines promoting inflammation in conditions such as endotoxemia and inflammation has necessitated adjustments in our understanding. This is attributed to emerging evidence suggesting that the efferent arms collaborate with the splenic nerve to down-regulate the releasing of cytokines promoting inflammation within the spleen. Currently, existing evidence predominantly supports a complex model where direct innervation of the cholinergic vagus nerve on the spleen is minimal or absent. Instead, both the vagus nerve and splanchnic nerve converge at the coeliac ganglion, releasing ACh that binds to α7 subunit of α7-nAChR. This relayed neural information subsequently reaches the adrenergic SpN.

In the conventional depiction of CAIP, the β2-adrenergic receptor (β2-AR) found on $ChAT^{+}$ $CD4^{+}$ T-cells is targeted by the noradrenaline (NA) released at the termini of the splenic nerve which stimulates the releasing of ACh. The primary source of splenic ACh was identified by the splenic $CD4^{+}$ T cells in the mice, which exhibited expression of ChAT, which is responsible for catalyzing the production of ACh from choline and acetyl-CoA. When α7-nAChR is expressed, splenic resident macrophages, activated by exposure to LPS, TLR ligands, and other proinflammatory stimuli, are the key targets of locally produced ACh anti-inflammatory signal. The α7-nAChR can cause Ca^{2+} influx so that increases the Janus kinase /signal transducers and activator of transcription (JAK/STAT) and inhibits the nuclear factor κB (NF-κB) signal transduction pathway, reducing the level of TNF-α, IL-6, IL-1β, HMGB1, and other inflammatory factors in immune cells, and improving serum levels of anti-inflammatory factors like IL-10. Moreover, extracellular ATP-induced activation of immune cells triggers a prompt influx of ACh into the cytoplasm, which in turn mitigates the release of mitochondrial DNA through mitochondrial α7-nAChR and further inhibits inflammasome activation and cytokine secretion.

It is noteworthy that this model continues to be challenged. While the previously established impact of ACh (or other AChR agonists) on macrophage secretion of pro-inflammatory cytokines via α7-AChR remains unquestioned, a study conducted in 2023 by Simon et al revealed that the activated CAIP through stimulation of the vagus or nerves in the spleen doesn't necessarily rely on $CD4^{+}$ T cells [5]. Although nude mice require higher doses of LPS compared to other strains to trigger CAIP, stimulating the vagus nerve or splenic nerve in nude mice can still inhibit TNF production. Instead, it is attributed to the direct effect of NA released by SpN on spleen macrophage β2-AR. This finding appears to contradict the previous studies conducted by Rosas-Ballina, Tracey, and other researchers. Alternatively, it seems suggested that the effects of CAIP to fight against inflammation in the body may arise from a combination

of these distinct mechanisms (which may vary in their sufficiency and necessity).

Although the precise site of action and the underlying neuro-immune interaction mechanism of ACh in this pathway remain subjects of debate, the anti-inflammatory effect exerted by T cells and macrophages associated with this molecule is unequivocally supported by a substantial body of previous research. Consequently, we will now delve into an exploration at the molecular level, thus providing more possibilities for further elucidating the immune mechanisms underlying the cholinergic system.

3. ACh-related anti-inflammatory Mechanisms: Insights from T Cells and Macrophages in CAIP

3.1. Detection methods for the cholinergic system in immune cells

In 1996, Fujii employed radioimmunoassay (RIA) to ascertain the remarkably elevated ACh levels in three human leukemic T cell lines, in which both ChAT, and to a lower level of carnitine acetyltransferase (CarAT), are participated in the synthesis of ACh [6]. However, consistent with the correlation between ChAT activity and ACh content in T cell lines expressing ChAT mRNA, it has been observed that Daudi B cells lacking ChAT mRNA expression exhibit minimal levels of ACh despite their elevated CarAT activity, indicating that ChAT is accountable for ACh production in immune cells. Since then, anti-ChAT antibodies have been widely applied to identify cholinergic cells and terminals in peripheral tissues as cholinergic markers. However, these antibodies were not recommended while examining ChAT level in both peripheral neurons and non-neuronal cells. The challenges posed by technical limitations in accurately visualizing the cholinergic system through histochemical methods were successfully addressed with the utilization of transgenic ChAT-GFP reporter mice.

In 2006, $ChAT^{BAC}$-eGFP mice were developed [7]. ChAT expression regulated by transcriptional regulatory elements was faithfully detected in central and peripheral cholinergic neurons, also in immune cells. Rosas-Ballina et al then utilized flow cytometry and immunofluorescence analysis on these mice to effectively identify the clusters and microstructure distribution character of splenic ChAT-$EGFP^+$ T cells, greatly contributed the CAIP model [8]. Subsequently, ChAT-IRES-Cre transgenic mice were generated and characterized by Rossi et al in which a modified internal ribosome entry sequence (IRES) fused to Cre recombinase was inserted downstream of the stop codon of the ChAT gene [9]. The specification of labeling cholinergic cells by genetic tracer approach without tracer injection or immunohistochemical marker has been enhanced since ChAT-Cre-tdTomato mice was elegantly obtained by crossing ChAT-IRES-Cre mice with tdTomato reporter mice, which possess a loxP-flanked Stop cassette inhibiting the expression of CAG-driven tdTomato (a dimer with red fluorescence obtained from a mutant monomer of DsRed) expression [10]. In neurons expressing ChAT, the transcriptional termination sequence is removed, enabling the production of tdTomato.

In general, based on the established theory that ACh is mainly produced from choline and acetyl-CoA by ChAT in immune cells, currently ACh synthesis (as a symbol of cholinergic system) can be primarily detected by the following ways: 1) ACh, 2) ChAT mRNA , 3) ChAT enzyme activity, 4) immunohistochemical detection targeting ChAT, and 5) genetic tracer approach to detect ChAT-expressing in cell level which utilizes transgenic mice such as $ChAT^{BAC}$-eGFP and ChAT-Cre-tdTomato combining with histological section technique or flow cytometry analysis. In addition, since AChRs are essential components of cholinergic system, radiolabeled AChRs ligands or mRNA encoding any cholinergic components are also used for obtaining cholinergic clues.

3.2. Cholinergic T cells (Lymphocyte)

As previously mentioned, the anti-inflammatory function of CAIP relies on ACh mainly derived from NA-activated $ChAT^+CD4^+$T cells. Firstly, Fujii et al conducted a study demonstrating that blood level of ACh is originated form lymphocytes. They discovered various ACh concentrations in 3 T cell lines [11]. T cells hold three times fold of ACh than B cells, with $CD4^+$ T cells having a larger concentration than $CD8^+$ T cells. Rosas-Ballina et al specifically discover that spleen ACh-synthesizing T cells show a memory T cell phenotype ($CD4^+$ $CD44^{high}$ $CD62L^{low}$ ChAT-$EGFP^+$) [8]. They did this by utilizing $ChAT^{BAC}$-eGFP mice. A modest population of splenic B-cells ($B220^+/CD19^+$) and T-cells ($CD3e^+/CD19^-$) were seen in ChAT-Cre-tdTomato mice. Adenylate cyclase activity is regulated, PKC activity is suppressed after TCR is activated with anti-CD11a, and cell adhesion molecules like LFA-1 are also involved in the modulation of cholinergic activity in T cells.

3.3. Cholinergic macrophages (myeloid cells)

After ACh and ChAT in human alveolar macrophages were firstly identified, research using $ChAT^{BAC}$-eGFP transgenic mice revealed reporter level of $CD11b^+$ $CD11c^-$ $F4/80^+$ macrophages in the spleen [8]. ChAT mRNA expression was not found in mouse peritoneal macrophages, either at rest or after stimulation with 3 g/mL Con A or 1 g/mL LPS, but the timing of the stimulus and the detection of the mRNA was unaffected [12]. No reporter expression in the macrophages was also seen in the lymphoid tissue linked with the gut or the spleen of ChAT-Cre-tdTomato animals [10].

Notably, at the molecular level, LPS stimulation induces the production of choline acetyltransferase (ChAT) in murine macrophages, leading to intracellular signal transduction mediated by MyD88 [13]. In a recent study conducted by Yingxu Ma et al, it was revealed that cold exposure leads to the activation of β2-ARs in cholinergic adipose macrophages (ChAMs), resulting in

an increase in ACh secretion, which, through α2-nAChRs, in turn stimulates beige fat thermogenesis [14]. These findings suggest that the macrophages ChAT expression may exhibit strain, stimuli, tissue, species, and/or immune status-dependent variations, and that ChAT expression levels in macrophages are likely to undergo delayed or dynamically altered changes following stimulation.

4. Conclusion

Considering the aforementioned relevant studies, the following aspects for discussion are proposed: At the cellular level, it is recommended to enhance the analysis of temporal and spatial expression dynamics in cells exposed to nerve stimulation or external stimuli, as immediate activation of ChAT expression and ACh anti-inflammatory effects may not be significantly induced upon stimulus exposure. Also, it is noteworthy that both non-neuronal and neuronal sources of ACh have been observed to play critical roles in various neuro-immune interfaces, thereby exerting distinct immune effects [15,16]. These observations indicate the existence of underlying classifications within the cholinergic pathways. Additionally, with flow cytometry, comprehensive investigations on the distribution pattern of ChAT-expressing cells and their alterations following stimulation remain insufficiently explored. At the level of biochemical and molecular mechanisms, it is imperative to comprehensively compare and investigate the diverse downstream effects of various stimuli (e.g., alterations in chat expression and activity) that have not been thoroughly examined. Although LPS has been extensively utilized in research, it remains uncertain whether this or other molecules can truly induce a rigorous activated cholinergic system in immunological cells. The inhibition of inflammatory progression through ChAT-mediated ACh production raises questions regarding potential mechanisms to inhibit ChAT or ACh production.

In conclusion, the expanding knowledge of the immunological mechanism of ACh from the CAIP perspective has revolutionized our understanding of the cholinergic system's role in immunological modulation. Beyond its classical neurotransmission functions, ACh exhibits multifunctionality in modulating immune cell activity, cytokine production, and signaling pathways. The CAIP, mediated by the vagus nerve, serves as a crucial interface between the neurological and immunological systems, maintaining immune homeostasis and preventing excessive inflammatory response. The identification of cholinergic T cells and macrophages further emphasizes the importance of ACh in immune regulation. Although some aspects of the CAIP are still debated, the accumulating evidence supports that targeting the cholinergic system for the development of novel therapeutic strategies for inflammatory-related neurological or immunologic derangement diseases, for example stroke, Alzheimer's disease (AD), multiple sclerosis (MS), arthritis, diabetes, and inflammatory bowel disease (IBD), is highly promising direction [17].

Future studies should focus on unraveling the precise mechanisms underlying the CAIP, exploring the heterogeneity of cholinergic immune cells, and investigating the therapeutic potential of modulating the cholinergic system in specific disease contexts. Overall, understanding the immunological mechanism of ACh from the CAIP perspective opens up exciting opportunities for advancing our knowledge of immune regulation and developing innovative therapeutic interventions.

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