Neural regulation of innate and adaptive immunity in the gut

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Cholinergic signalling in gut immunity

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ABSTRACT

The gut immune system shares many signalling molecules and receptors with the autonomic nervous system. A good example is the vagal neurotransmitter acetylcholine (ACh), for which many immune cell types express cholinergic receptors (AChR). In the last decade the vagal nerve has emerged as an integral part of an immune regulation network via its release of ACh; a system coined “the cholinergic anti-inflammatory reflex”. The perspective of cholinergic immune regulation in the gut mucosa has been widened by the recent discovery of populations of ACh producing immune cells in the spleen and other organs. As such, ACh, classically referred to as neurotransmitter, may serve a much broader function as bi-directional signalling molecule between neurons and non-neuronal cell types of the immune system.
INTRODUCTION

The concept of vagal immune modulation

In many physiological parameters the autonomic nervous system is known to initiate quick and precise responses to restore homeostasis. Recent advances have further broadened this spectrum to the immune system, and the autonomic nervous system is now an established component of the immune response. Where earlier reports have indicated the sympathetic nervous system as a ‘hard-wired’ system that can locally regulate immune responses (o.a. reviewed in (1)), more recently the vagus nerve (the parasympathetic branch of the ANS) was demonstrated to play a regulatory role in immune responses. Building on initial observations of ACh production in the spleen, the regulation of innate immune responses by vagal nerve derived ACh was initially shown in a rat model of experimental sepsis (2). In these experiments it was shown that surgical dissection of the vagus nerve enhanced splenic pro-inflammatory cytokine production and accelerated the development of septic shock, whereas electrical stimulation of the efferent vagus nerve prevented systemic inflammation, loss of blood pressure, and reduced lethality (2). Subsequently, several studies demonstrated the anti-inflammatory property of the vagus nerve in diverse mouse models such as ischemia–reperfusion injury (3), hemorrhagic shock (4), experimental arthritis (5), pancreatitis (6), peritonitis (7), DSS-induced colitis (8) and postoperative ileus (9,10). Vagal modulation of immune responses may have particular implications in the densely innervated gastrointestinal tract. It is known that the intestinal (colonic) lumen contains massive numbers of commensal gut microbes (estimated to amounts of $10^{11}$ to $10^{14}$ per gram stool in the colon) and the immune system must display a well-regulated and effective response to the presence of both beneficial and pathogenic microbes (11). In particular, macrophages were identified as the vagal targeted immune cells that orchestrate an anti-inflammatory effect through the alpha7 homopentameric nicotinic acetylcholine receptor (nAChR) in a murine model of septic shock (12). In addition, we and others have recently provided evidences indicating that ACh can affect various functionalities of macrophages such as phagocytosis, and killing through a different nAChR heteropentamer alpha4beta2 (13,14). This suggests that neuronal ACh production can potentially modulate macrophage activity at different cellular levels and through distinct nicotinic receptors. However, it has become clear that in addition to
macrophages a variety of non-neuronal cells express AChRs allowing these cells to respond to cholinergic activation. In this sense it should be noted that in the gut cholinergic release from pre-ganglionic neurons of the vagus has been shown to amplify vagal signalling through cholinergic enteric neuron excitability (15). The enteric neurons are not only an abundant source of ACh, but also of neuropeptides (co-transmitters of enteric cholinergic neurons such as vasoactive intestinal peptide (VIP)), which display immunomodulatory potential (extensively reviewed by o.a. (16)). Hence, cholinergic modulation of intestinal cells can be regarded as quite common in a gut context. Therefore it should be kept in mind that the neurotransmitter ACh has the potential to affect a multitude of cellular targets through differential receptor propagation that could be of cholinergic or non-cholinergic origin.

**Cholinergic receptors and inflammation in the gut mucosa**

At a molecular level, most of the studies on the anti-inflammatory potential of the vagus nerve have been based on the effects of ACh. AChRs are prominently expressed in immune cells (lymphoid and myeloid cells). The functional implications have become clear after the finding that ACh controls the production of proinflammatory cytokines from macrophages (12). Since ACh signals through either muscarinic (G-protein coupled receptors) or nicotinic (ligand-gated ion channels) receptors, selective receptor agonists and antagonists were used to identify the receptors involved in the control of macrophage activation (17). Muscarine slightly inhibited macrophage activation at supraphysiological levels, but equimolar concentrations of nicotine were more efficient than ACh at inhibiting the production of Tumour-necrosis factor (TNF) in macrophages (12). These effects were specific for proinflammatory cytokines and neither ACh nor nicotine inhibited the production of anti-inflammatory mediators such as Transforming growth factor-beta (TGF-beta) or Interleukin-10 (IL-10). Hence, the anti-inflammatory effect of ACh on macrophages seems to be mediated mainly through nicotinic receptors. Most studies identified the alpha7 homopentamer nAChR as the mediator of the anti-inflammatory effect of the vagal signalling (by suppression of macrophage cytokine response). Furthermore, transcripts for nicotinic acetylcholine receptors (nAChR) subunit alpha7, beta2, as well as alpha4 have been detected in multiple inflammatory cell types, including macrophages derived from various tissues (2,13,14) (10). The finding of distinct nAChR subtypes expressed on immune cells suggests that nicotine
may differentially affect distinct inflammatory cells with its specificity based on receptor affinity for ACh, as is the case in neurons (18-20).

However, the concept of vagal immune modulation of intestinal inflammation rather needs to be considered in the context of vagal innervation of the gut. In appreciating a role for vagal immune modulation it remains to be elucidated if ACh released from vagus nerve termini actually reaches the immune cells, and if so, in what quantities. Given the turnover rate of ACh, cholinergic modulation of immune cell activation most likely requires close contact, i.e. via enteric neurons of cholinergic nature. Although macrophages are found in close anatomical apposition to cholinergic fibres in rat small intestinal wall (21), there is currently no evidence that parasympathetic fibres indeed innervate macrophages. Since pre-ganglionic neurons (from the dorsal motor nucleus of the vagus (DMV)) mainly innervate the myenteric plexus and sparsely the submucosal plexus (22), its plausible that the enteric nervous system (ENS), of which approx 26% of neurons of the ENS are cholinergic in nature, is the major neuronal network that conveys vagal signalling (23).

**The epithelium as a target of intestinal ACh**

In regarding the cellular targets of ACh produced in the intestine, epithelial cells express a variety of muscarinic receptors, may represent a class of players that are as yet underappreciated. The colonic epithelium forms a selective barrier, regulating the passage of nutrients, ions and antigens. Under normal conditions, enterocyte tight junction (TJ) proteins allow the gut epithelium to function as a barrier thus regulating access for luminal bacteria to mucosal phagocytes (24). Colonic epithelial cells extensively express muscarinic receptors (mAChR 1-3) (25,26). The group of McKay reported that mice deficient for M3 muscarinic receptor are more susceptible to DSS induced colitis. Colitis in these mice was restricted to the ileum, whereas colitis normally is restricted to the colon and ceacum (27) (Hirota and McKay, 2006). In line with these results, our preliminary in-vitro data imply that muscarinic receptor agonists restore cytokine induced paracellular permeability in differentiated epithelial cells (human colonic adenocarcinoma cell line Caco-2) (unpublished data).

In inflammatory bowel disease (IBD), the disturbed intestinal barrier function is recognized as a key event that perpetuates the disease course. Therefore, therapeutics aimed at protection or re-establishing epithelial barrier functions could be of interest. In
vitro and in vivo animal studies have demonstrated that intestinal permeability is regulated by multiple factors, including exogenous factors, cytokines, and immune cells. Cytokine-induced intestinal barrier dysfunction is thought to be critical in the predisposition to and exacerbation of numerous autoimmune and inflammatory conditions, including ulcerative colitis, food allergy, celiac disease, and diabetes. For example, IL-1, IFN-γ and TNF-α, which are central mediators of intestinal inflammatory diseases, have been clearly shown to reduce intestinal epithelial barrier function in cell systems (28). The mechanism of action of these cytokines appears to be primarily mediated through myosin light chain kinase–mediated phosphorylation of the myosin light chain, which promotes disruption of the TJ (29). Hence, cytokine release in the gut during active inflammation contributes to pathology by inducing changes in TJ integrity resulting in loss of intestinal barrier function. Not only inflammatory conditions but chronic stress or surgical trauma can alter expression of TJ and adherence junction (or zonula adherens) proteins and antimicrobial peptides allowing gut pathogens to adhere and cross the gut epithelium (24,30). Such proteins are composed of transmembrane proteins occludin, claudins and other junctional adherent molecules that are connected to the cytoskeleton via a complex of multiple proteins. Changes in epithelial barrier function and TJ expression also form the basis of defects in wound healing and/or increased electrolyte secretion that are often seen in IBD, thereby perpetuating disease progression (31). In this context, the role of the ENS in the regulation of barrier function and the gut immune response is emerging. Recent advances have highlighted the ENS as a key player in the control of barrier function and have indicated that alterations of the ENS could be directly associated with the development of IBD and its associated symptoms (32). Interestingly, ACh has been put forward as one of the neural-derived mediators that could be involved in this neuro-regulation of intestinal barrier function. One example thereof is that cholinergic activity has been shown to be involved in regulation of the mucosal barrier function, enterocyte endocytosis (33), and intestinal epithelial permeability (34). In addition, cholinergic signalling is implicated in intestinal permeability changes observed after chronic stress (24). The ENS may also exert regulation of intestinal barrier function via a network of enteric glia (35) that interact with epithelial TJs, possibly via release of TGF-beta or other mediators such as glial-derived neurotrophic factor (35).
Chapter 2
Cholinergic signalling in gut immunity

It is now well understood that the epithelium not only serves as a selective physical barrier but secretes mediators of host defence that protect against enteric bacterial pathogens. In the intestine this effect is mediated by secretory Paneth cells, located in the crypts of the small intestine. At the mucosal surface, Paneth cells secrete various anti-microbial peptides e.g. α-defensins, β-defensins, lysozyme, C-type lectins and phospholipases. Earlier studies suggest that aberrations in anti-microbial peptide (AMP) production by Paneth cells (36,37) and the resulting shift in the composition of luminal microbiota predispose individuals to infections and inflammatory diseases. Increasing evidences suggest that Paneth cells express mAChR3 on the mucosal surface and mAChR agonists may potentiate anti-microbial peptide secretion from Paneth cells (38,39). These results further suggest towards a role of homeostatic role of cholinergic signalling in the colon, under inflammatory conditions.

Non-neuronal sources of ACh in the gut
As already indicated by the widespread expression of AChR subtypes among intestinal non-neuronal cell types, cholinergic signalling is not restricted to neurons. In other organs such as the spleen, immune cells display a tightly regulated cholinergic activity that seems to be dictated by inflammatory activation (25). In another study, splenocytes were reported to down-regulate acetylcholine esterase (AChE) mRNA levels in vitro upon activation by Lipopolysaccharide (LPS), via a mechanism that involved miRNA 132 expression. This mechanism was also shown to regulate AChE expression in splenocytes in vivo, in a murine model of endotoxemia induced by intraperitoneal administration of LPS (40).

In an intestinal context, previous studies have implicated that a variety of non-neuronal cell types have the capability to produce bio available levels of ACh, albeit in low amounts, depending on cellular acetyl cholinesterase activity (23,41). ACh can be produced by a mechanism involving choline acetyltransferase (ChAT), which is expressed in both neuronal and non-neuronal tissues. Other than the epithelium (42), lymphocytes (43) have been extensively reported to express ChAT and produce measureable quantities of ACh. Kawashima et al., have previously reported that 60% of ACh in the blood is produced by mononuclear lymphocytes. Thus, ACh can no longer be considered simply as a neurotransmitter but rather as a ubiquitous intercellular messenger that is likely to be important in integrating many different aspects of
intestinal physiology in health and disease. For example, in the intestinal epithelia, non-neuronal ACh release was identified more than a decade ago (42), nonetheless its physiological relevance is still poorly defined. Further studies are awaited that may elucidate whether ChAT expressing non-neuronal cells play a regulating role in regulating actions of cells that express AChR’s such as epithelial cells and immune cell types. Importantly, it is shown that ChAT and high affinity transporters for the uptake of choline, are present in colonic epithelial cells. Only a fraction of epithelial cells express ChAT under healthy conditions, as demonstrated in transgenic ChAT-gGFP reporter mice (44), but irrespective, ACh release from the colonic epithelium fulfils many of the criteria to be considered as part of a non-neuronal cholinergic system (45) (See scheme in Fig. 1).

![Vagus nerve/enteric neurons (ACh)](image)

**Figure 1. The multifaceted role of vagally-derived ACh in gut immunity and barrier function.** Earlier data have indicated the vagal nerve as a potent immunomodulator via its release of ACh. More recent data point towards additional roles for non-neuronally produced ACh in the maintenance of epithelial barrier function and as a potential mechanism to restrain cytokine production.
In contrast to release of neuronal ACh at synaptic clefts, non neuronal ACh signalling may not necessarily require expression of ChAT or vesicular transporters (46). ACh in non neuronal cells can be produced by a mechanism involving carnitine O-acetyltransferase (CrAT), which is expressed in mammalian heart, retina, and urothelium (46). While ChAT exclusively relies on acetyl-CoA as a substrate for the synthesis of ACh, CrAT prefers short chain fatty acids for synthesis of ACh (47). This is interesting in the context of gut as short chain fatty acids such as butyrate or propionate are abundantly present in the lumen of the gut as bacterial fermentation products, and have been associated in the colitis disease course (48).

Although a direct evidence for the role of non neuronal cells producing ACh in the gut is lacking, recent studies on ACh producing immune cells in the spleen provide exciting insights into their potential role in inflammatory disease. Recently, the group of Tracey have isolated and characterised the role of ChAT-positive lymphocytes in a model of systemic inflammation (49). The authors identified a subpopulation CD4+ CD62L+ T cells as ChAT positive T cells that secrete ACh, express β-adrenergic receptors, and are located adjacent to adrenergic nerve endings in the spleen. These T cells were reported to be responsible for down regulating TNFα production following vagal nerve stimulation during endotoxic shock. Whether lymphocyte populations arriving in the gut during chronic inflammatory state are derived from the spleen remains debatable (50), but we can anticipate the circulating immune cell populations in gut-associated lymphoid tissue (GALT) to have a similar ChAT-positive subsets of immune cells as described in the spleen. As discussed in the previous section, GALT and in particular Peyer’s patches (PP’s) are extensively innervated by (peptidergic and sympathetic) nerve fibres (51), which under inflammatory conditions may be an important modulator of immune cell reactivity.

**Dietary modulation of cholinergic immunomodulation**

As indicated above, the parasympathetic nervous system may also be involved in control of immune responses to commensal flora and dietary components. Recently, it was described that lipid-rich nutrition regulates the inflammatory response via activation of the autonomic nervous system (52). Subsequently nicotinic receptors on inflammatory cells are activated via the vagus nerve, leading to a reduction of cytokine release and organ damage. Activation of this anti-inflammatory pathway via administration of lipid-
Rich nutrition is an appealing and physiologic intervention to counteract excessive inflammation and organ damage in several diseases. Furthermore, this nutritional anti-inflammatory pathway might contribute to the largely unexplained unresponsiveness of the intestinal immune system to dietary and bacterial antigens. Ingestion of dietary fat stimulates the production of cholecystokinin (CCK), which is a characteristic neuropeptide released during ingestion to trigger several digestive functions including exocrine pancreas secretion, and activation of afferent vagus nerve signals to induce satiety. Interestingly, a recent study indicated that CCK, released as a result of high-fat enteral nutrition, inhibited haemorrhagic shock-induced TNF-α and IL-6 release (53,54). This anti-inflammatory effect of CCK release is mediated by the vagus nerve because surgical or chemical vagotomy abrogates the anti-inflammatory effect of high-fat diet and CCK (54). However, with respect to cholinergic anti-inflammatory effects of vagus nerve signalling in the GI-tract it should be kept in mind that vagal afferents are thought to be involved in maintenance of intestinal mucosal barrier function (55), plausibly via modulation of mast cell activity (56,57). Hence, the pro-inflammatory effect of vagotomy on intestinal and peritoneal inflammation could be indirect and involve other than direct cholinergic mechanisms.

Another aspect to consider is the dietary modulation of ACh synthesis by epithelial cells. As discussed above, the ACh productive capacity of intestinal epithelia is well documented. The challenge will be to develop new therapies to sustain the barrier function, via nutritional support of ACh production by non-neuronal cells, i.e. enterocytes. One candidate component to consider here is for instance choline, required for ACh synthesis. Choline is not only endogenously synthesized from the amino acid methionine, but also an essential nutrient required for ACh synthesis, found in a wide variety of foods. However, the dietary sources, specifically under pathological conditions, may become restricting (58). Of note is that there is a significant variation in the dietary requirement for choline that can be explained by common genetic polymorphisms. Current recommended intakes do not take into consideration these genetic variations as a modulator of dietary requirement. It is now clear that as much as 50% of the population may have genetic polymorphisms that leave them susceptible to choline deficiency. With respect to supplementation of choline in UC, a 70% reduction in phosphatidylcholine (PC) has been reported in UC, independent of the state of inflammation. PC is a substrate of ACh production and reduced levels may well be
limiting ACh metabolism and ACh production. Recently a number of delayed release formulation of oral PC was reported to be successful in UC (59). In a group of 60 patients with active disease and that were not on steroids, remission was achieved for 53% versus 10% in the placebo group. Such considerations may validate choline supplementation to clinical nutrition to enhance barrier function, a very attractive opportunity that deserves further examination in the near future.

Availability of substrates for ChAT, choline and acetylCoA, is of particular importance for regulating ACh synthesis. However, the mechanisms regulating ChAT and VAChT expression are partially understood. However, the organization of the ChAT and VAChT genes strongly suggests that they may share some transcriptional signals and that their expression may be regulated in a coordinated fashion by extracellular factors.

**DISCUSSION**

To summarise, homeostasis of the intestine relies on innate and adaptive immune responses initiated in the gut. Classically, the vagus nerve was proposed as the key player in executing ‘cholinergic anti-inflammatory reflex’ in a number of target organs. While the vagus nerve remains an important link between the CNS and the gut, mediating systemic and local immune responses, lack of neuroimmunological synapse either with immune cells or the epithelia in the intestine suggests that vagus derived ACh is unlikely to be the only mediator of this inflammatory reflex. Given the short half-life of ACh *in-vivo*, the juxtaposition of the nerve terminals and target cells is essential, and therefore it is well feasible that the biosynthesis of ACh by non-neuronal cell types is an important contributor of cholinergic immune modulation. In chronic inflammatory processes (e.g. IBD) all of these protective mechanisms are activated but may be inadequate. Many dietary factors can be absorbed, metabolised and subsequently be used as a substrate for ACh synthesis by neuronal and non-neuronal cells. In the light of recent advances on the immunomodulatory role of cholinergic signalling, it is important to consider the role of other factors, such as diet and intestinal flora, as other important factors that influence intestinal homeostasis and perhaps influence the outcome of such inflammatory processes.
REFERENCES


