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CHAPTER 2

The vagus nerve as a modulator of intestinal inflammation

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ABSTRACT

The cholinergic nervous system attenuates the production of proinflammatory cytokines and inhibits inflammatory processes. Hence, in animal models of intestinal inflammation, such as post-operative ileus and DSS-induced colitis, vagus nerve stimulation ameliorates disease activity. On the other hand, in infectious models of microbial peritonitis, vagus nerve activation seemingly acts counteractive; it impairs bacterial clearance and increases mortality. It is originally indicated that the key mediator of the cholinergic anti-inflammatory pathway, acetylcholine, inhibits cytokine release directly via the $\alpha 7$ nicotinic acetylcholine receptor (nAChR) expressed on macrophages. However, more recent data also point towards the vagus nerve as an indirect modulator of innate inflammatory processes, exerting its anti-inflammatory effects via postganglionic modulation of immune cells in primary immune organs. This review discusses advances in the possible mechanisms by which the vagus nerve can mediate the immune response, as well as the role of nAChR activation and signaling on macrophages and other immune cells.

VAGUS MODULATION OF IMMUNE RESPONSES

The innate immune system is pivotal in the first response to invading pathogens or tissue trauma. When challenged, the host needs an adequate inflammatory reaction but also needs to prevent collateral damage to tissues due to excessive systemic spread of inflammation and release of inflammatory mediators. Hence, regulation of the acute inflammatory response is important, and regulatory mechanisms are required to accomplish this. Decades ago, the sympathetic nervous system was already identified as a ‘hard-wired’ counter-regulatory mechanism that can locally regulate immune responses¹. Besides the sympathetic nervous system, the parasympathetic nervous system, comprised by the vagus nerve (the largest nerve in the body) is increasingly recognized as a potent player in neuro-immune inflammation. The vagus nerve, in addition to its classically assigned function of controlling heart rate, hormone secretion, gastrointestinal peristalsis, and digestion, may also be involved in control of immune responses to commensal flora and dietary components. The vagus nerve is a mixed nerve composed of 90% afferent and 10% efferent fibers. The afferent vagus system is known to regulate the inflammatory response via activation of the hypothalamic pituitary adrenal axis. However, more recent evidence reveals that efferent vagus nerve cholinergic activity exerts quite potent immuno-modulatory potential as well².

The vagus nerve transmits signals by releasing acetylcholine (ACh), its principal neurotransmitter, at its peripheral nerve endings. ACh activates nicotinic acetylcholine receptors (nAChRs), ligand-gated ion channels on neuronal cells³. In this function, ACh is historically referred to as a neurotransmitter. Immune cells that have been shown to be especially sensitive to modulation by vagus nerve activity are macrophages (the main class of innate immune cells)². Macrophages express nAChRs and potently respond to ACh. This was corroborated by our observation of close anatomical association between cholinergic nerve fibers and enteric macrophages⁴. These data suggest that the classical neurotransmitter ACh also functions as neuro-immune cytokine, providing a molecular basis for the purported “neuro-immune axis” between the brain and immune system. The initial experiments to show the role of the parasympathetic nervous system in the regulation of the innate immune response were performed in a rat model of experimental sepsis². In these experiments it was shown that surgical dissection of the vagus nerve enhanced pro-inflammatory cytokine production and accelerated the development of septic shock, whereas electrical stimulation of the efferent vagus nerve prevented systemic inflammation and reduced lethality². Subsequently, in several studies it was demonstrated that activation of the cholinergic nervous system ameliorated disease in animal models of ischemia–reperfusion injury⁵, hemorrhagic shock⁶, and experimental arthritis⁷, pancreatitis⁸, peritonitis⁹ and DSS-colitis¹⁰. It is hypothesized that the vagus nerve exerts anti-inflammatory effects through the interaction of its principal

neurotransmitter ACh with acetylcholine receptors expressed on macrophages. The group of Tracey² have originally showed that *in vitro*, ACh inhibited the endotoxin-induced release of pro-inflammatory cytokines in human macrophages². Since ACh signals through nicotinic or muscarinic receptors, selective agonists and antagonists have been used to identify the receptors involved in the immunomodulatory effects of ACh. Nicotine was as efficient as ACh in inhibiting pro-inflammatory cytokine production in macrophages, indicating that the anti-inflammatory effects of ACh on immune cells are mediated through nicotinic receptors, rather than muscarinic receptors. nAChR are pentameric ligand-gated ion channels that can consist of a large number of different subunits (α 1- α 9, α 8, β 1- β 4, γ , δ and ϵ)¹¹ and it is reported that the nAChR α 7 subtype, which is expressed on immune cells, is essential in mediating the anti-inflammatory effect of ACh¹².

From the pioneering work of Tracey¹³ it is postulated that the cholinergic anti-inflammatory pathway may act as part of a anti-inflammatory reflex arch, in which the presence of proinflammatory cytokines in the periphery activates vagus afferents, resulting in a vagus efferent firing subsequently leading to an attenuation of cytokine release from macrophages via nAChR α 7. On the other hand, recent data indicate that the efferent arm of the cholinergic anti-inflammatory pathway may, at least in part be mediated via post-ganglionic events¹⁴. Here we review the recent reports on the possible pathways via which vagus nerve activity can exert its anti-inflammatory effects, with specific focus on intestinal disease. Moreover, the role of nAChR expressed on macrophages as well as other immune cells in intestinal and peritoneal tissue in the immunomodulatory effects of cholinergic signaling are highlighted.

VAGUS NERVE SIGNALING IN INTESTINAL INFLAMMATION

Neuronal tracing studies reveal that efferent vagus nerve fibers innervate the small intestine and proximal colon of the gastro-intestinal (GI) tract^{15,16}, leaving the possibility that cholinergic activity may modulate immune cells residing in, or recruited to, the densely innervated bowel wall. Therefore, vagus nerve stimulation, or the use of applied cholinergic agonists targeting distinct nAChR subtypes, has been studied extensively as a novel approach to treat intestinal inflammatory disease in several animal models.

One of the GI-disorders in which vagus nerve stimulation has been shown to ameliorate disease is post-operative ileus (POI)^{4,17}. POI is a commonly occurring post-operative complication that results from manipulation of the bowel during abdominal surgery, and is characterized by a transient hypomotility of the GI tract. With ~22 million surgical procedures being performed annually in the US (data derived from hospital discharge records between 1980 and 1993¹⁸), and with all of these procedures

causing some degree of POI, treatment strategies that can accelerate the recovery from POI represent an important unmet clinical need¹⁹. In rodent models of POI, intestinal manipulation leads to leukocyte influx into the muscularis externa, resulting in delayed gastric emptying²⁰ and impaired small intestinal transit^{21,22}. Electrical stimulation of the vagus nerve can reduce recruitment of neutrophils and restore gastric emptying in mice^{4,17}. The anti-inflammatory effect attained with electrical vagus nerve stimulation can be mimicked by AR-R17779, which specifically targets the nAChR $\alpha 7$ subunit¹⁷.

Ingestion of dietary fat stimulates the production of cholecystokinin (CCK), which is a characteristic hormone 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 hemorrhagic shock-induced TNF α and interleukin-6 release²³. 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 CCK²³. Along the same line, in mouse models of pancreatitis⁸, vagotomy exacerbates inflammation, and this effect is counteracted by pretreatment with nicotine or GTS-12, another selective nAChR $\alpha 7$ agonist⁸. These results demonstrate an important role for the nAChR $\alpha 7$ subunit in mediating the 'cholinergic anti-inflammatory effect'. Correspondingly, in experimental models of acute colitis, the vagus nerve seems to possess regulatory properties in inflammatory responses. Several studies show that nicotine administration attenuates disease in TNBS and DSS colitis models, although fairly high doses of nicotine are required^{24,25}. Ghia et al.¹⁰ demonstrate that acute colitis is more severe in vagotomized mice and in mice treated with the nAChR antagonist hexamethonium. Conversely, nicotine treatment resulted in reduction of the inflammatory response, independent of vagus nerve intactness, indicating that cholinergic signaling can be protective in animal models of experimental colitis. In addition, vagotomized nAChR $\alpha 7$ knock-out (KO) mice display more severe colitis than wild type (WT) mice, and nicotine pretreatment only attenuates disease activity in WT mice²⁶, pointing towards a role for the nAChR $\alpha 7$ in this process. However, in another study, experimental colitis is aggravated in nAChR $\alpha 5$ subunit-deficient mice²⁷, suggesting that not only the nAChR $\alpha 7$, but also other nAChR subunits can participate in the vagus modulation of colitis in mice. Finally, in IL10 KO mice, that develop colitis spontaneously, nicotine administration resulted in reduced colitis but enhanced jejunal inflammation²⁸. Overall, cholinergic activation can reduce inflammation and disease activity in various animal models of intestinal inflammation, likely via a mechanism involving activation of nAChR $\alpha 7$ subtype, although this receptor may not be the sole nAChR involved.

THE VAGUS NERVE AND CHOLINERGIC EFFECTS ON GUT EPITHELIUM

It is now well established that cholinergic enteric neurons participate in epithelial transport as well as mucosal immune defense. The intestinal epithelium is continuously exposed to a plethora of luminal antigens. The human gut harbors an estimate of 10^{14} microbes of 400 different species in the digestive tract²⁹, and the intestinal immune system has to fight invading pathogens while remaining tolerant to the beneficial flora and the many encountered food antigens. Under healthy conditions, specialized cells such as M-cells or CX₃CR positive dendritic cells protruding through the epithelial layer of normal mucosa or Peyer's patch, act as gatekeepers to the mucosal immune system³⁰. However, penetration of the mucosal barrier by luminal antigens does occur under pathological conditions, and regulatory mechanisms of epithelial permeability are a key factor in the balance between immunosurveillance and inflammation of the gut. For example during episodes of stress, inflammation or trauma, impairment of the epithelial barrier function is increasingly acknowledged as a key perpetuating factor in the pathogenesis of inflammatory bowel disease (IBD), food allergy and celiac disease³¹. Many hypotheses exist on the regulatory mechanisms behind these permeability changes³², but interestingly, several studies indicate that cholinergic nerve activity plays a significant role in gut permeability.

Although theoretically, stress is associated with a strong sympathetic nervous system response, studies in rodents have revealed that both acute and chronic exposure to stress can increase epithelial permeability³³ via cholinergic mechanisms. First of all, stress-susceptible rats have lower activity of cholinesterase in intestinal mucosa than less susceptible rats, leading to higher levels of mucosal ACh³⁴, which may account for altered epithelial barrier function in stress-susceptible rats³⁴. Second, the cholinergic muscarinic receptor antagonist atropine abolishes stress-induced epithelial barrier damage in rats, where nicotinic antagonists have no effect. This suggests that the cholinergic effects on epithelial barrier function are mediated via muscarinic, rather than nicotinic acetylcholine receptors. In stripped rat ileal epithelium mounted in Ussing chambers, cholinergic stimulation increases epithelial transport by disrupting tight junction integrity and induces the uptake of intact protein via endocytosis, which can be counteracted by atropine³⁵. In line, in jejunal mouse tissue, muscarinic receptor activation increases epithelial permeability to macromolecules via enhanced apical endocytosis³⁶. This is probably mediated via activation of muscarinic 3 receptors on epithelial cells and subsequent activation of phospholipase A2 and cyclooxygenase metabolites³⁶. In contrast to rat ileum³⁵, tight junction integrity is not affected by cholinergic signaling in mouse jejunum³⁶, which can probably be explained by species differences or variable pharmacological conditions. In rabbit jejunum, vagus nerve stimulation increases intestinal epithelial

permeability, resulting in the passage of serum proteins into the lumen, possibly by opening tight junctions and paracellular pathways³⁷.

On the other hand, other animal studies show that vagus nerve activity can be protective in maintaining gut barrier function under pathological conditions. Hemorrhagic shock results in gut barrier failure leading to translocation of endotoxin and bacteria. Luyer et al demonstrate that administration of high-fat nutrition, leading to the release of CCK inhibits bacterial translocation, reduces disruption of the tight junctions and attenuates TNF α and IL-6 production in hemorrhagic shock rat model³⁸. High fat nutrition failed to reduce the inflammatory response in VGX mice²³, indicating that this effect required CCK-induced activation of the vagus nerve. The observed maintenance of epithelial barrier integrity after vagus nerve stimulation may be a direct effect of cholinergic signaling, or indirect, via the reduced pro-inflammatory cytokine release³⁹.

Altogether, the epithelial barrier function is affected by cholinergic signaling, presumably via activation of muscarinic receptors expressed on epithelial cells. Differential effects of cholinergic signaling on tight junction integrity are reported. It is important to consider that altered barrier function may not necessarily be indicative of pathophysiology, but could also be a physiologically adaptive response to increase luminal antigen sampling.

THE VAGUS NERVE MODULATING THE RESPONSE TO PERITONEAL BACTERIAL INFECTION AND CLEARANCE

The function of vagus nerve firing has been well established in models of sterile inflammation, in which vagus nerve stimulation attenuates inflammation by dampening immune cell activation. However, in infectious diseases, such as bacterial peritonitis, or bacterial sepsis, the host defense is a delicate balance between pro-inflammatory pathways aimed at the rapid elimination of bacteria and anti-inflammatory responses to prevent systemic inflammation. Therefore, the role of cholinergic modulation of the immune response in microbial infection and bacterial clearance is an important topic of interest.

In mice that underwent cecal ligation and puncture (CLP), causing lethal peritonitis induced by a polymicrobial infection, nicotine administration attenuates clinical symptoms of sepsis and improves survival⁴⁰. These effects can be contributed to acute reduction of HMGB-1 and pro-inflammatory cytokines, rather than effects on bacterial outgrowth as mice received nicotine 24hrs after CLP. Moreover, to mimic the clinical scenario, these mice received antibiotics shortly after CLP, obscuring the potential effect of cholinergic signaling on bacterial outgrowth. In a septic peritonitis model induced by ip injection of *E.Coli*, *vagotomy* exaggerates, whereas nicotine reduces pro-inflammatory cytokine release, neutrophil influx and liver damage⁹. Interestingly,

nicotine treatment impairs bacterial clearance and significantly enhances mortality during this *E.Coli* induced peritonitis⁹. In line with this, *in vitro* studies indicate that nicotine can significantly impair antimicrobial activity of macrophages⁴¹. In contrast to nicotine, unilateral vagotomy does not affect bacterial outgrowth and survival in *E.Coli* induced peritonitis⁹. Bilateral vagotomy however, tested in a polymicrobial colon ascendens stent peritonitis model, has no effect on bacterial outgrowth, but does result in significantly increased mortality⁴².

Mice that are deficient for the nAChR $\alpha 7$ subunit had enhanced neutrophil recruitment in early infection with *E.Coli* in comparison to WT mice⁴³. These results are in accordance with the anti-inflammatory properties of cholinergic nAChR $\alpha 7$ signaling¹². However, 20 hrs after infection, nAChR $\alpha 7$ KO mice displayed an accelerated bacterial clearance compared to WT mice⁴³. As a result of reduced bacterial loads, $\alpha 7$ nAChR KO mice had reduced numbers of infiltrating neutrophils and lower circulating cytokine levels at that time point⁴³. These data suggest that stimulation of cholinergic $\alpha 7$ nAChRs reduces early neutrophil migration to the site of infection, finally resulting in a reduction of bacterial clearance and decreased survival. Although vagus nerve stimulation can reduce excessive inflammation and acute reaction to sepsis, vagus nerve firing and nAChR $\alpha 7$ receptor activation can have a detrimental effect on host defense against bacteria.

HOW DOES VAGUS NERVE STIMULATION MODULATE THE IMMUNE RESPONSE *IN VIVO*?

Several lines of evidence indicate that vagus nerve stimulation can inhibit immune cell activation and modulate inflammation via its peripheral release of ACh. Many reports point towards the macrophage nAChR $\alpha 7$ as an essential player in mediating the anti-inflammatory effect of ACh^{4,8,12,17,44-47}. Specifically, nicotine exerts anti-inflammatory effects on human macrophages that can be counteracted by specific nAChR $\alpha 7$ antagonists or anti-sense oligonucleotides¹². In addition, nAChR $\alpha 7$ KO mice display enhanced TNF production compared to WT mice in an endotoxemia model, which cannot be counteracted by vagus nerve stimulation¹². In various animal models of inflammation, nAChR $\alpha 7$ agonists ARR17779 and GTS-21 ameliorate disease^{8,17,48}. These data point towards the nAChR $\alpha 7$ as a crucial player in cholinergic modulation of inflammation.

However, 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 short half-life of ACh, cholinergic modulation of immune cell activation most likely requires close contact. Although macrophages are found in close anatomical apposition to cholinergic fibers in rat small intestine⁴, there is currently no evidence that parasympathetic neurons indeed innervate macrophages. As the vagus nerve

mainly synapses with neurons of the enteric nervous system, it is very likely that the cholinergic terminals shown in proximity of macrophages may belong to enteric rather than vagus nerve fibers.

In this regard, recent data indicate that the spleen may play a role in effectuating the anti-inflammatory effects of vagus nerve activity, as electrical stimulation of the vagus nerve fails to attenuate serum TNF levels in splenectomized mice treated with endotoxin⁴⁹. This implies that the parasympathetic nervous system may regulate systemic inflammation by modulating immune cells in the spleen. Huston et al. show that vagus nerve stimulation fails to regulate splenic TNF production in $\alpha 7$ nAChR-deficient mice, and splenocytes from $\alpha 7$ KO mice do not respond to in vitro stimulation with ACh⁴⁹. In another study it was demonstrated that nerve endings are in close apposition to macrophages in the spleen, but interestingly, these nerve fibers, found in apposition to the TNF-secreting macrophages are catecholaminergic, not cholinergic¹⁴. The authors propose that ACh released by the vagus nerve does not reach the spleen directly, but acts on $\alpha 7$ nAChR at the level of the ganglia of the celiac-superior mesenteric ganglion to modulate splenic nerve function¹⁴. Hence, the vagus nerve via this ganglion, modulates adrenergic input to the spleen (via the *n. splenicus*), resulting in the release of catecholamines that stimulate adrenergic receptors on splenic macrophages and attenuate LPS-induced TNF production¹⁴

Whether or not the vagus nerve innervates the spleen directly, or indirectly, is currently under debate. Rosas-Ballinas et al.¹⁴ base their conclusion that the spleen does not receive direct vagus nerve input on the observation that ACh, choline acetyltransferase and the vesicular ACh transporter, are absent from splenic nerve terminals. Although this conclusion may seem justified, Buijs et al⁵⁰ show that the absence of the classically assigned vagus neurotransmitter ACh, or the ACh metabolizing enzymes in the spleen, does not directly imply the lack of direct input from the vagus. Indeed, they demonstrate that in rats, the spleen is directly innervated by the vagus nerve⁵⁰. Their results corroborate earlier observations regarding parasympathetic innervation of the liver. In parallel, ACh metabolizing enzymes are absent from the liver, although this organ has shown to be vagusly innervated⁵¹⁻⁵³.

Given the recently purported role of the spleen in mediating the anti-inflammatory effect of vagus nerve activity, the question arises as to what is the role of macrophage nAChR $\alpha 7$ residing in the peritoneal or intestinal compartment is in this process. The interesting recent data on the role of the spleen in vagus nerve immune modulation put the role of nAChR $\alpha 7$ receptors on macrophages in a new perspective: although in vitro nicotine modulates macrophages function via nAChR $\alpha 7$, the in vivo effects of vagus nerve stimulation may rely on nAChR $\alpha 7$ on neurons rather than macrophages. Further research is required to assess the physiological mechanism by which the vagus nerve can modulate immune responses and whether this modulation is indeed the result of direct affects of ACh exposure to immune cells or whether vagus innervation of primary or secondary lymphoid organs plays a role (**Figure 1**).

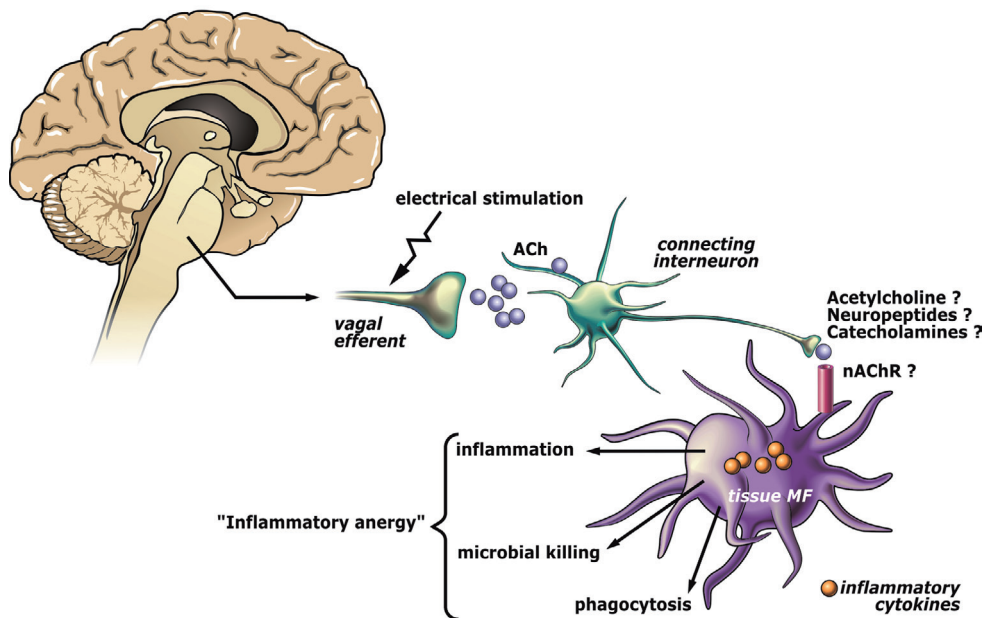


Figure 1. Hypothetical scheme of the mechanism via which vagus nerve activity can modulate immune cells *in vivo*. Vagal efferent fibres, that originate in the brainstem, release acetylcholine upon physiological or electrical stimulation. Vagus nerve stimulation may affect immune cells via direct release of acetylcholine, or via post-ganglionic mechanisms involving alternative neurotransmitters. This activates a connecting interneuron, resulting in the release of acetylcholine or other neurotransmitters, such as neuropeptides or catecholamines. Acetylcholine can act on nAChR which are broadly expressed on different types of immune cells, such as tissue macrophages. Possibly, neuropeptides and catecholamines acting on their specific receptors may be important as well. Receptor activation can lead to reduced pro-inflammatory cytokine production, abrogated microbial killing and enhanced phagocytosis. This skews immune cells to a more anergic phenotype, which can be beneficial in disorders that are characterized by an aberrant immune response.

WHICH nAChR IS INVOLVED IN THE VAGUS MODULATION OF IMMUNE CELLS?

The anti-inflammatory effects of nAChR activation on macrophages have previously been solely attributed to activation of the nAChR $\alpha 7$ ¹². However, some reports indicate that vagus activity may also convey its anti-inflammatory effect via distinct nAChRs expressed in macrophages. Mastunaga et al⁴¹ and others⁵⁴ have shown expression of $\alpha 4\beta 2$, but not $\alpha 7$ nAChR in alveolar macrophages. Likewise, we have failed to detect $\alpha 7$ nAChR transcripts in certain mouse macrophage types (unpublished 2008). Further analysis of potential $\alpha 7$ nAChR protein in these macrophages is hampered by the fact that commercially available $\alpha 7$ nAChR antisera seem not specific and stain an 57kD protein in brain homogenates from wildtype as well as $\alpha 7$ nAChR $-/-$ mice⁵⁵. Most previous studies, including our own⁴, have ascribed the effects of ACh and nicotine on peritoneal macrophage cytokine production solely to activation of

the $\alpha 7$ nAChR, although expression of alternative nAChR subtypes on macrophages has been described^{54,56}. Accordingly, we recently observed that $\alpha 7$ specific agonists are less effective in reducing pro-inflammatory cytokine production as compared to nicotine¹⁷. Surprisingly, $\alpha 7$ nAChR blockers are effective in counteracting nicotinic effects on pro-inflammatory cytokine production. These observations may question the selectivity of commonly used blockers α Bgt and MLA for $\alpha 7$ nAChR. In fact, both blockers have been shown to bear affinity for other nAChR subunits, including $\alpha 1$, $\alpha 6$, $\alpha 9$, $\alpha 10$ and $\beta 2$, as well⁵⁷.

CHOLINERGIC MODULATION OF IMMUNE CELLS IN THE GUT

Several immune cells express various nAChR subtypes⁴⁴, and other nAChR subtypes than nAChR $\alpha 7$ may play a more prominent role than originally assumed. Therefore, even though it is not clear how ACh released by the vagus nerve can directly interact with immune cells *in vivo*, it is plausible that different types of immune cells are sensitive to ACh. Here, we briefly describe effects of nAChR activation on macrophages, dendritic cells and mast cells.

Macrophages and monocytes

In response to inflammatory signals, monocytes can migrate from the bloodstream into the tissue and differentiate into macrophages. Macrophages play a fundamental role in early recognition of pathogens and the most important macrophage functions are ingestion of bacteria and debris, killing, and secretion of inflammatory mediators. Most research about cholinergic modulation of the immune response has focused on macrophages, and indeed macrophages are very responsive to ACh and nicotine. Macrophages and monocytes express the nAChR $\alpha 2$ - $\alpha 7$, $\alpha 9$, $\alpha 10$ and $\beta 2$ - $\beta 4$ ⁵⁴, although the expression pattern is dependent on the type of macrophage and the tissue where it resides. In addition, in human monocytes and monocyte-derived macrophages, several splice variants and two different isoforms of $\alpha 7$ nAChR are detected. Six mRNA splice variants of the $\alpha 7$ gene have been described in human brain as well as leukocytes⁵⁸⁻⁶¹, though it is uncertain whether any of these transcripts are processed to functional protein⁶¹. Interestingly, the human $\alpha 7$ -nAChR has been described to be partially duplicated on this chromosome. Exons 5 to 10 of the gene have been duplicated in a “tail-to-head” orientation and this partially duplicated gene is combined with four novel exons (A to D) to comprise a new gene, the “hybrid alpha7” or the “cholinergic receptor family with sequence similarity 7A” (CHRFAM7A)⁶¹. Although it is reported that this gene is transcribed as a 45 kD protein (i.e. in human leukocytes)⁶¹, it remains

unclear whether this hybrid transcript is appropriately translated and processed to form a functional receptor.

In macrophages and monocytes of various species, nicotine alters inflammatory properties. Several studies describe nicotinic inhibition of the secretion of pro-inflammatory mediators, such as TNF, IL-6, IL1 β , HMGB-1 and PGE₂^{2,4,40}. In monocytes, nicotine not only abrogates production of pro-inflammatory cytokines, but shifts the response to a IL-10 dominant anti-inflammatory profile⁶², while studies in macrophages report no difference in IL-10 production^{2,63}. Transcript levels of inflammatory mediators remain unchanged, suggesting a post-transcriptional effect of nicotine. Nicotine suppresses expression of CD14 and toll-like receptor 4 (TLR4) on monocytes, shifting the cells to a 'deactivated state' which can explain the nicotinic modulation of LPS-induced cytokine production⁶³. In contrast, one study reports that nicotine *increases* transcript levels and production of TNF, IL-1 β and iNOS⁶⁴, while another shows that nicotine stimulates iNOS expression and NO⁶⁵ production in mouse peritoneal macrophages, thus inducing inflammation.

Data on the effects of nicotine on macrophage-mediated functions, such as phagocytosis and bacterial killing, are limited. Matsunaga et al⁴¹ demonstrate that antimicrobial activity of alveolar macrophages to *Legionella pneumophila* infection is suppressed by nicotine. Some decades earlier, it was shown that nicotine partially inhibits endocytosis, pinocytosis, uptake and intracellular degradation and phagocytosis by macrophages⁶⁶⁻⁶⁸. This can partly be explained by the fact that nicotine accumulates in the lysosomes which impairs the digestive capacity⁶⁹. Nevertheless, we have strong evidence that nicotine augments phagocytosis by intestinal macrophages, while pro-inflammatory cytokine release and NF- κ B activation are decreased (van der Zanden et al, submitted). In conclusion, most reports show that nicotine attenuates pro-inflammatory cytokine release by macrophages and enhances phagocytosis, but inhibits antimicrobial killing (Figure 1).

Dendritic cells

Mouse dendritic cells express nAChR α 2, α 5, α 6, α 7, β 2 and β 4 subunits⁵⁶. Immature DCs that mature in a nicotinic environment manifest lower endocytic and phagocytic activities. Mature DC's that are exposed to nicotine produce decreased levels of IL-12 and display reduced ability to induce T Cell responses⁷⁰⁻⁷². In contrast, other studies reveal that nicotine activates DCs and augment their capacity to perform endocytosis, stimulate T-cell proliferation, and produce IL-12^{73,74}. These different findings may be due to the exposure time and dose of nicotine used, or the maturation status of the dendritic cells at the time of assay.

These observations raise the possibility that some of the immunomodulatory effects of vagus nerve stimulation may be partly mediated by altered DC function.

Mast cells

Although best known for their role in allergy, mast cells play an important immune protective role as well, being intimately involved in wound healing and defense against pathogens. Mast cells express the nAChR $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$ and $\alpha 10$ ^{75,76} and appear to make intimate contact with afferent vagus fibers in the small intestinal mucosa⁷⁵. In human mucosal mast cells, ACh inhibits histamine release. However, this seems to be species specific, since in rats ACh stimulates mast cell degranulation^{77,78}. Host sensitization status may also affect the response of mast cells to ACh, as sensitization to a specific allergen makes rat mast cells more sensitive to ACh-induced histamine release⁷⁸.

In conclusion, cholinergic activation has broad effects on immune cell function. In animal models of intestinal inflammation, vagus nerve signaling may attenuate inflammation activity not exclusively via inhibition of macrophages but also other immune cells, such as dendritic cells and mast cells can be affected.

NICOTINIC ACETYLCHOLINE RECEPTOR SUBCELLULAR SIGNALING

The exact intracellular mechanism via which ACh exerts its effects on immune cells has been investigated in several studies. Most studies in immune cells focus on activation and subcellular signaling of the $\alpha 7$ nAChR. nAChR $\alpha 7$ receptor signaling on neuronal cells is mediated by ion channel fluxes due to ACh binding³, although the $\alpha 7$ nAChR can also activate alternative signaling pathways. In rat microglia, nicotine exposure elicits a transient increase in intracellular Ca^{2+} levels⁷⁹. This increase in intracellular Ca^{2+} may involve phosphoinositide 3-kinases (PI3K) and phospho-lipase C (PLC) activation and subsequent Ca^{2+} release from intracellular Ca^{2+} stores⁷⁹, which can finally lead to Ca^{2+} depletion and cell deactivation. However, unpublished data from our group indicate that no intracellular Ca^{2+} flux was observed after nicotine exposure in mouse peritoneal macrophages, although nAChR signaling was present (experiments performed at David Greaves' laboratory, Oxford University). Some studies link nAChR $\alpha 7$ to PI3K activation, possibly via phosphorylation of Akt^{62,80} and activation of Janus Kinase 2 (Jak2)⁸¹. In line with this observation, nAChR $\alpha 7$ activation in macrophages leads to recruitment and phosphorylation of Jak2 and subsequent activation of the Signal Transducer and Activator of Transcription 3 (STAT3) protein⁸². STAT3 is a negative regulator of inflammation, contributing to the anti-inflammatory effects of IL-10⁸². STAT3 does not inhibit transcription of pro-inflammatory cytokines directly⁸². Presumably, other signaling pathways are involved. Studies in macrophages indicate that nAChR $\alpha 7$ activation reduces NF-kB activation, resulting in decreased pro-inflammatory cytokine production⁴⁰. This

reduction of NF- κ B activation can be explained via induced STAT3 phosphorylation, as phosphorylated STAT3 has been shown to interact with NF- κ B p65⁸³ and thus inhibit p65 translocation to the nucleus, resulting in inhibition of NF- κ B transcriptional activity.

In monocytes and various other cell types, nicotine induces expression of COX-2 and the synthesis of one of its major products, prostaglandine E2 (PGE₂). PGE₂ is able to elicit an increase in cyclic AMP levels and protein kinase A (PKA) activity, leading to reduced adhesion molecule expression, cytokine production and lymphocyte proliferation⁸⁴. Other studies show modulation of mitogen activated protein kinases (MAPK), which are involved in various cellular activities, upon AChR activation⁶³.

In conclusion, nAChR α 7 activation triggers various signaling mechanisms that most likely interact with each other to achieve immunomodulatory effects. However, the precise mechanism requires further investigation.

CLINICAL IMPLICATIONS OF THE ANTI-INFLAMMATORY PROPERTIES OF VAGUS NERVE SIGNALING

The finding that nicotine inhibits activation of immune cells, together with the observation that vagus nerve signaling or specific nAChR α 7 agonists attenuate disease in several inflammatory animal models, implies that therapeutic agents modifying cholinergic signalling might be beneficial in humans.

Cigarette smoking is an important environmental factor in inflammatory bowel disease (IBD), but most strikingly smoking has differential effects in ulcerative colitis (UC) and Crohn's disease (CD). While smoking increases the risk of developing CD and worsens its course, epidemiological studies of smokers in UC point out that smoking appears to have a protective effect in the development of this disease and reduces its severity⁸⁵. The exact explanation for this discrepancy is far from clear, but it certainly adds to the current belief that UC and Crohn's disease are two different disease entities. About 90% of UC patients are non-smokers. Patients with a history of smoking acquire their disease after they have stopped smoking⁴⁴. Patients who smoke intermittently often experience improvement in their colitis symptoms during the periods when smoking^{86,87}. In ex-smokers, onset is nearly always after quitting smoking.

However, clinical trials using nicotine for the treatment of UC have provided different results. Transdermal nicotine appears to be superior to placebo for the induction of remission in patients with UC, but no significant advantage for transdermal nicotine therapy compared to standard medical therapy was found. Moreover, adverse events associated with transdermal nicotine are significant which will limit its use in patients⁸⁸. However, to avoid side effects caused by nicotine, more specific nAChR agonists are designed. Partial selective nAChR α 7 and α 4 β 2 agonists are already being

tested in patients with neuronal disorders, since both receptor subtypes have shown to mediate improvement in attention, learning and working memory⁸⁹. The use of specific $\alpha 7$ nicotinic agonists is expected to bear potential as a maintenance therapy for active UC. Such selective nicotinic agonists were originally designed to mimic the cognitive effects of nicotine in patients with neurological disorders while avoiding the toxicity of nicotine. The most characterized specific $\alpha 7$ nAChR-agonists are GTS21 (3-[(2,4-dimethoxy)benzylidene]-anabaseine), 4OHGTS (3-(4-hydroxy,2-methoxybenzylidene) anabaseine), ARR17779 ((-)-spiro[1-azabicyclo[2.2.2]octane-3,5'-oxazolidin-2'-one]), CAP55, Exo2 (exo-2-(2-pyridyl)-7-azabicyclo[2.2.1] heptane), and PNU-282987 ([N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride])^{90,91}. Among these, the most characterized is GTS-21, a partial $\alpha 7$ nAChR agonist that also affects $\alpha 4\beta 2$ nAChR⁹¹, is well tolerated in patients with schizophrenia, Alzheimer disease and in healthy volunteers⁴⁴. Unlike trials using nicotine, patients tolerated doses of up 450 mg/day of GTS21 well, and there were no clinically significant differences in adverse events between the treatment groups. Besides these selective agonists, recent evidence indicates that centrally acting cholinergic drugs used in the treatment of Alzheimer disease can modulate peripheral immune responses and would therefore be interesting to explore⁹².

Future studies are needed to perform larger clinical trials and determine whether the cognitive potential of nicotinic agonists are based on their binding to neuronal receptors or whether their anti-inflammatory potential in immune and glia may contribute to their therapeutic potential in neurological disorders. In addition to the use of specific cholinergic agonists, vagus nerve stimulation could be a potential therapeutic asset in the treatment of patients with inflammatory diseases. Interestingly, in patients with drug-resistant epilepsy and depression, vagus nerve stimulation is already in use as a new adjunctive therapy. A pulse generator transmits impulses to the left vagus nerve via an implantable electrode. Overall, vagus nerve stimulation has shown better control of seizures or depression, with marginal side effects⁹³. Moreover, it has been demonstrated that a noninvasive method of transcutaneous vagus stimulation, which has shown to improve survival in a mouse model of polymicrobial sepsis⁹⁴, is feasible in healthy young and elderly subjects⁹⁵. As the vagus nerve does not innervate the distal colon and rectum, the areas usually affected in IBD patients, vagus nerve stimulation may not be the first therapeutic choice in targeting IBD. Nevertheless, vagus nerve activity can regulate disease in animal models¹⁰, possibly clarified by the role of the spleen in exerting the anti-inflammatory effect of vagus nerve signaling or by changes in autonomic (para)sympathetic balance^{96,97}.

CONCLUDING REMARKS

The hypothesis that vagus nerve stimulation, via the release of ACh, ameliorates inflammation solely via down-regulation of tissue macrophage reactivity and cytokine release via nAChR $\alpha 7$ receptors, seems more complicated than originally thought. It is likely that *in vivo*, more complex mechanisms play a role, including a variety of different (immune) cell types, neurotransmitters and ACh receptors that converge in the cholinergic down-regulation of inflammatory responses (Figure 1). Irrespectively, it is firmly established that electrical stimulation of the vagus nerve can attenuate inflammation in several animal models.

In conclusion, results obtained in a wide range of *in vitro* and *in vivo* models of inflammation imply that therapeutic agents targeting the cholinergic anti-inflammatory pathway can be an important asset in the treatment of immune disorders in human. However, the challenge is to define a specific nAChR agonist with highest anti-inflammatory potential and least side effects. Future studies are needed to explore the protective effects of these methods in the treatment of inflammatory disorders in humans.

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