The vagus nerve as a modulator of intestinal inflammation
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Summary and conclusions
The goal of this thesis was to further unravel the working mechanism of the so-called 'cholinergic anti-inflammatory pathway', specifically in intestinal inflammation. The pioneering work of Tracey and colleagues has demonstrated that the vagus nerve, via its neurotransmitter acetylcholine (ACh), has an important role in limiting the inflammatory response\(^1\). Subsequently, several studies have demonstrated that cholinergic activation ameliorates disease in a range of animal models such as ischaemia-reperfusion injury\(^2\), hemorrhagic shock\(^3\), peritonitis\(^4\), DSS-colitis\(^5\) and rheumatoid arthritis\(^6\).

In this thesis, we focused on the role of cholinergic modulation in intestinal inflammation. The gastro-intestinal tract is under strict control of the vagus nerve\(^7,8\), rendering it susceptible to cholinergic immuno-modulation. Moreover, especially in the intestine, which homes our largest collection of microbes, tight regulation of immune responses to discriminate between self and non-self is crucial. An imbalance of this process has consequences and may lead to disease. An example of this is inflammatory bowel disease (IBD), including Crohn’s disease and ulcerative colitis (UC), which is caused by an inappropriate and exaggerated mucosal immune response to constituents of the gut flora in genetically predisposed individuals. Chapter 2 gives an overview of the current knowledge regarding cholinergic modulation of intestinal inflammation. This review discusses advances in the possible mechanisms via 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. Moreover, the clinical implications of the anti-inflammatory properties of vagus nerve signaling are discussed.

**JAK2-STAT3 signaling in cholinergic modulation of the immune response**

The ‘cholinergic anti-inflammatory pathway’ is characterized by a dose-dependent decrease in the production of proinflammatory mediators via activation of nicotinic acetylcholine receptors (nAChR) on macrophages\(^1\). Many reports point towards the macrophage nAChR α7 as an essential player in mediating the anti-inflammatory effect of ACh\(^9,10,12\). Wang et al showed that activation of the nAChR α7 inhibits transcriptional activity of the transcription factor NF-KBp65\(^9,13\), resulting in reduced cytokine production. In chapter 3, we evaluated the involvement of the transcription factor STAT3 in this process, since STAT3 is a potential negative regulator of inflammatory responses\(^14,15\). We demonstrated that nicotine exerts its anti-inflammatory effect on peritoneal macrophages via nAChR α7-Jak2 and STAT3 signaling in vitro. In vivo, we tested the involvement of STAT3 in the cholinergic anti-inflammatory pathway in an animal model of postoperative ileus (POI). POI is characterized by general hypomotility of the gastrointestinal tract and delayed gastric
emptying and is a pathological condition commonly noted after abdominal surgery\textsuperscript{16}. This condition is the result of inflammation of the intestinal muscularis layer due to activation of resident macrophages that are triggered by bowel manipulation\textsuperscript{17}. In an animal model, postoperative ileus was improved substantially by stimulation of the vagus nerve. Moreover, we found activation of STAT3 in intestinal macrophages in response to stimulation of the vagus nerve, which indicates activation of STAT3 induced by acetylcholine derived from vagal efferents. The finding that macrophage activation is under strict neuronal control may be substantiated by the observation that cholinergic nerve fibers are in close proximity to resident macrophages in intestinal myenteric plexus.

In conclusion, in chapter 3, we have shown that inhibition of macrophage activity via the cholinergic anti-inflammatory pathway is brought about via Jak2-STAT3 signaling. We speculated that nicotine repressed macrophage activity via direct interaction of dimerized STAT3 with the p65 subunit\textsuperscript{18}.

In chapter 4, the role of the JAK2-STAT3 pathway in the anti-inflammatory effect of nAChR activation was further analyzed. We studied the potential of STAT3 to modulate TNF responses using STAT silencing strategies, pharmacological blockade, and dominant negative STAT3 constructs. Two dominant negative STAT3 constructs were used: STAT3D, which prevents STAT3 binding to DNA, and STAT3F, which prevents STAT3 phosphorylation and subsequent dimerization\textsuperscript{19}.

Nicotine reduced TNF production in RAW cells transfected with STAT3F, but failed to do so in cells transfected with STAT3D. Hence, in contrast to our findings in chapter 3, we demonstrated that nicotinic inhibition of inflammation in macrophages is dependent on STAT3 DNA binding and STAT3 protein, rather than STAT3 phosphorylation. Hypothetically, unphosphorylated STAT3 (U-STAT) can be important in mediating the anti-inflammatory effect of nAChR activation, via binding to NF-κB\textsuperscript{20} and inhibition of NF-κB-activation of TNF transcription.

In conclusion, chapter 3 and 4 identify a novel molecular pathway involved in the vagal modulation of macrophage activity, and indicate that JAK2-STAT3 targeting may aid in further development of therapeutic strategies to modify the ‘cholinergic anti-inflammatory pathway’.

\textbf{Cholinergic modulation of phagocytosis}

Cholinergic inhibition of pro-inflammatory cytokine production by macrophages has been firmly established. However, especially in the intestinal compartment, macrophages may rather function as phagocytes that, along with dendritic cells, form critical effectors in the surveillance of luminal antigens. Therefore, it may be questioned if the anti-inflammatory effect of vagus nerve activity in intestinal inflammation exclusively rests on reduced macrophage cytokine production, or
Summary and conclusions

whether the vagus nerve also regulates other macrophage functions important in host defense. In chapter 5, we explored the effect of nAChR activation on more professional macrophage functions, such as endo- and phagocytosis by macrophages residing in the peritoneal and mucosal compartment. We demonstrated that nAChR activation enhanced endocytosis and phagocytosis in intestinal and peritoneal macrophages. This effect was mediated via enhanced recruitment of dynamin-221 to the phagocytic cup. The anti-inflammatory effects of nAChR activation on macrophages have previously been attributed to activation of the nAChRα79. Interestingly, we clearly showed that cholinergic agonists induced phagocytosis via nAChR α4β2, rather than the α7 nAChR. Despite enhanced phagocytosis, acetylcholine reduced NF-kB activation and pro-inflammatory cytokine production, while stimulating anti-inflammatory IL10 production. In vivo, vagus nerve stimulation enhanced luminal uptake by intestinal phagocytes. Moreover, nAChR activation in intestinal tissue induced a transiently enhanced mucosal passage of luminal bacteria. In line, stimulation of vagus activity enhanced mucosal uptake and drainage of luminal bacteria.

In conclusion, in chapter 5 we show that acetylcholine has a dual effect in macrophages, it stimulates phagocytosis via nAChR α4β2 activation, while reducing NF-κB activation and inflammatory cytokine production. Vagus nerve activity induces a transient increase in epithelial permeability and augments the uptake of luminal bacteria by mucosal macrophages. That way, vagus nerve activity assists in surveillance in the intestinal mucosa and peritoneal compartment.

Vagus nerve modulation of immune cells in vivo

In chapter 6, we tried to analyze how vagus nerve activity can modulate immune cells in vivo. We examined whether vagus activation restrains the immune response not only via the release of acetylcholine, but also via alternative neurotransmitters such as neuropeptides, released via post-ganglionic mechanisms. VIP and substance P are neuropeptides that are abundantly expressed in the gut and display important immunomodulatory functions. Vagus nerve stimulation altered expression levels of VIP and SP in mouse intestinal tissue. Co-administration of acetylcholine and VIP decreased LPS-induced production almost down to un-stimulated levels. In addition, substance P enhanced NF-kB transcriptional activity and TNF production in peritoneal macrophages, but when Substance P and acetylcholine were applied together, the pro-inflammatory actions of substance P were neutralized by acetylcholine. The immunomodulatory effects of ACh, nicotine, VIP and SP were independent of calcium signaling pathways.

In summary, VNS modulates VIP and SP expression in the intestine, and in vitro, cholinergic agonists can affect VIP and SP immuno-modulatory actions. These data
suggest that the vagus nerve anti-inflammatory effect may be amplified via modulation of co-released neuropeptides.

The finding that nicotine inhibits activation of immune cells, together with the observation that vagus nerve signaling attenuates disease in several inflammatory animal models, implies that therapeutic agents modifying cholinergic signalling might be beneficial in humans. However, clinical trials of nicotine treatment have shown variable outcomes. This could partly be explained by the finding that human immune cells display a variation in nAChR expression due to genetic or environmental factors. In chapter 7, we evaluated whether smoking or repeated nicotine

**Summarizing Figure**

Vagal efferent fibers, 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, such as VIP and Substance P. Acetylcholine (Ach) binds to nAChRs which are broadly expressed on immune cells, such as intestinal macrophages. Ach has a dual effect on macrophages: reduction of pro-inflammatory cytokines most probably partly via activation of nAChR α7, and stimulation of phagocytosis, via activation of the nAChR α4β2. The working mechanism of Ach induced reduction of TNF production ultimately involves modulation of JAK2-STAT3 pathways and prevention of NF-κB p65 transcriptional activity following nAChR activation. Our studies reveal that the anti-inflammatory effect of nAChR activation is dependent on STAT3 DNA binding and the presence of STAT3 protein, rather than STAT3 phosphorylation. The nAChR α4β2 dependent increase of phagocytosis is brought about via recruitment of dynamin-2 to the phagocytic cup. Altogether, this Summarizing figure shows how vagus nerve stimulation skews macrophages to a more anergic phenotype, which can be beneficial in disorders that are characterized by an aberrant immune response.
exposure could affect expression of nAChRα7, dupα7 or β2 on human monocytes. We demonstrated that repeated nicotine exposure up-regulated nAChRα7 expression in a human monocyte cell line. In conjunction, in a pilot study, nAChRα7 was only detectable in monocytes of smoking individuals. nAChR dupα7 was ubiquitously expressed on human monocytes, while nAChRβ2 was not detectable. However, the nicotine induced up-regulation of nAChR α7 in human monocytes did not render cells more susceptible to cholinergic immune-modulation, either via nicotine application or via administration of olive oil.

The **Summarizing Figure** is a model of the mechanism via which vagus nerve activity modulates intestinal macrophage function, according to the results obtained in this thesis.

**Therapeutic options and future perspectives**

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. *In vivo*, cholinergic activation can be accomplished in several ways. The best known, although not the most selective way, is by cigarette smoking.

In inflammatory bowel disease, cigarette smoking is an important environmental factor, but has differential effects in ulcerative colitis (UC) and Crohn’s disease (CD). While smoking increases the risk of developing CD, it appears to have a protective effect in the development of UC and reduces its severity. However, clinical trials using nicotine for the treatment of UC have showed no significant advantage for transdermal nicotine therapy compared to standard therapy, while nicotine did show more side effects. Therefore, the challenge is to define a specific nAChR agonist with highest anti-inflammatory potential and least side effects. Partial selective nAChR α7 and α4β2 agonists are already being tested in patients with neuronal disorders, since both receptor subtypes have shown to mediate improvement in attention, learning and working memory. The most characterized nAChR-agonist is GTS-21, a partial α7 nAChR agonist that also affects α4β2 nAChR, is well tolerated humans. *In vitro*, this agonist has shown to diminish production of pro-inflammatory mediators in mouse and human immune cells. In a recent study on the effects of GTS-21 on the innate response during human endotoxemia in 14 non-smoking individuals, there were no differences in the LPS-induced cytokine response between the GTS-21 and placebo-treated groups.

Targeting the nAChRs using specific agonists, requires exact knowledge of which nAChR is involved in the anti-inflammatory effects of cholinergic activation. These effects have previously been only attributed to activation of the nAChR α7. Nevertheless, nAChRα7 was not present on monocytes of non-smoking individuals (chapter 7), in conjunction we failed to detect α7 nAChR transcripts in certain mouse
macrophage types (chapter 5). Further analysis of potential α7 nAChR protein in these macrophages is hampered by the fact that commercially available α7 nAChR antisera seem not specific and stain an 57kD protein in brain homogenates from wildtype as well as α7 nAChR −/− mice31. In line, nicotine reduced TNF production in α7 WT, as well as in α7 KO mice (chapter 4). Accordingly, we observed that α7 specific agonists were less effective in reducing pro-inflammatory cytokine production as compared to nicotine (chapter 5). Altogether, these observations imply that the acetylcholine and nicotine effects on peritoneal macrophage cytokine production should not be exclusively attributed to nAChR α7 activation, but maybe also due to activation of alternative nAChR subtypes. Therefore, the use of selective agonists targeting other nAChRs than the α7 receptor could be successful, especially specific agonists of the nAChRα4β2, which is required for cholinergic activation of phagocytosis.

In addition to the use of specific cholinergic agonists, vagus nerve stimulation itself 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. Furthermore, high-fat enteral nutrition, sensed in the gastrointestinal tract, has shown to activate the parasympathetic nervous system. However, 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 models5, possibly clarified by the role of the spleen in exerting the anti-inflammatory effect of vagus nerve signaling32,33.

The finding that nicotine inhibits activation of immune cells, together with the observation that vagus nerve signaling or specific nAChR agonists attenuate disease in several inflammatory animal models, implies that therapeutic agents modifying cholinergic signalling might be beneficial in humans. However, one should keep in mind that targeting the cholinergic anti-inflammatory pathway in humans could be less straightforward than originally thought, as there might be an individual variation in response to future therapeutic agents modifying the cholinergic anti-inflammatory pathway in humans.

Overall, in this thesis, we further identified the working mechanism of the ‘cholinergic anti-inflammatory pathway’ to alleviate specific targeting of this pathway, in order to increase the translational potential.

Our data may aid in the development of therapeutic strategies to modify the cholinergic anti-inflammatory pathway, in order to treat various inflammatory conditions.
REFERENCE LIST


