Neural regulation of innate and adaptive immunity in the gut
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Discussion
The crosstalk between the nervous system and immune system has been well documented, and interest in the role of the gut-brain axis as a player in intestinal homeostasis has increased exponentially (1,2). In 2000, Tracey and colleagues demonstrated for the first time that vagus nerve stimulation suppresses cytokine production in a murine model of sepsis (3). This discovery also marked the beginning of a series of investigations into the so called ‘cholinergic anti inflammatory’ pathway. Since this ground-breaking study, us and several others, have reported an anti inflammatory role of vagus nerve stimulation in animal models of ischaemia-reperfusion injury (4), haemorrhagic shock (5), peritonitis (6), DSS-colitis (7) and rheumatoid arthritis (8). Chapter 1 gives an overview of current consensus on cholinergic anti-inflammatory pathway, and the key shortcomings in fulfilling the ‘gut-brain axis’ criterion. This review discusses the role of cholinergic receptor activation and signalling in macrophages and other non-immune cells in the intestine. It is widely believed that the efferent vagus nerve is the main regulator of immune responses in the intestine, via its peripheral release of acetylcholine (ACh) (9). However, unlike clear evidence of vagal-enteric nervous system synapse (10), neuroimmune synapses between the vagus nerve fibres and macrophage have not been reported (11). Therefore, in Chapter 1, we discuss the currently hypothesized mechanisms via which the vagus nerve is believed to mediate immune responses in the gut.

**Epithelial permeability and cholinergic signalling**

Altered barrier function is a hallmark of many chronic and acute inflammatory diseases of the intestine (e.g. IBD and POI) (12). Although vagal nerve stimulation has been shown to decrease intestinal barrier breakdown after injury (13,14), its effects are possibly relayed to the epithelium via the enteric nervous system (15). Furthermore, evidences show that enteric glial cells, a major constituent of the enteric nervous system, and outnumbering enteric neurons by a factor of 4 to 10 (16), play a major role in restoring epithelial barrier integrity under inflammatory conditions (17). Postoperative ileus (POI) is an almost inevitable phenomenon occurring after each abdominal surgical procedure that includes bowel manipulation, although POI may sometimes also
be associated with extra-abdominal operations (18). POI pathophysiology manifests two distinct stages; a neurogenic component in the early phase, and an inflammatory component in the late phase. In the inflammatory phase - characterized by enhanced intestinal permeability, the importance of mast cells was demonstrated in experiments using mast cell stabilizers, however, the role of mast cells in inducing permeability was poorly understood (19). In chapter 2 we show how mast cell activation in postoperative ileus (POI) contributes to intestinal permeability, and the subsequent migration of intestinal bacteria to intestinal lymph nodes, most likely mediated via CX3CR1 expressing macrophages. In this chapter we clearly show that intestinal manipulation (IM) elicits a mast cell dependent inflammatory response, leading to intestinal barrier dysfunction and bacterial translocation that may contribute to the pathogenesis of POI. In line with our observations, others have also reported that intestinal manipulation elicits a local immune response that involves an early production of mast cell mediators and IL1β initiates an inflammatory cascade following intestinal manipulation (20).

The intestinal epithelium forms a highly selective permeable barrier, limiting the passage of macromolecules from the lumen to the mucosa. At a molecular level, epithelial barrier consists of four principal intracellular junction complexes, the tight junctions (TJs), gap junction, adherens junction and desmosomes (21,22). TJs play a key role in maintaining the selective permeability between the epithelial cells, while adherens junctions, gap junctions and desmosomes are involved in cell-cell adhesion and intracellular communication (23). In inflammatory conditions, IL1β induces activation of myosin light chain kinase (MLCK) in colonic epithelial cells, which catalyzes the phosphorylation of myosin L chain (MLC); which in turn induces a contraction of peri-junctional actin-myosin filaments and opening of the TJ barrier (24). In clinical studies in IBD, an increased IL-1β secretion from colonic tissues is highly correlated with the disease severity, which underscores the central role of IL1β in acute and chronic intestinal diseases (25). On the basis of studies in macrophages by others and our group (26-28), in chapter 3 we hypothesized that ACh acting via muscarinic or nicotinic receptors, may physiologically antagonize IL1β induced NFκB activation, thereby restoring normal TJ function, through both inhibiting gene
transcription and direct dephosphorylation of MLC proteins (29). Indeed, by preincubating differentiated colonic epithelial cells with ACh and muscarinic receptor (mAChR) agonist we could antagonize IL1β induced paracellular permeability of 4kD dextran molecules. Since TJ proteins have also been shown to mediate other non-permeability associated activities, such as wound healing and cell proliferation (30,31). We performed a real time scratch assay and cell proliferation test in Caco2 and CMT93 cells, to confirm that AChR agonists did not affect cell proliferation or migratory capacity to counterbalance IL-1β induced permeability. IL1R activation either activates canonical (classical) or non-canonical (alternative) NF-κB pathways in enterocytes, ultimately leading to the transcription of NF-κB dependent genes, such as CXCL8 and MLCK (29). Although we observed a significant blockage of IL1β induced phosphorylation of MLC, neither ACh nor bethanechol affected IL1β induced phosphorylation of IKK-α/β, suggesting AChRs block the activation of non-canonical NF-κB pathway by IL1β in enterocytes. From a clinical standpoint, our observations are very interesting. While a success in blocking IL1 signalling might be anticipated in sterile inflammatory diseases (32), it’s therapeutic efficacy in IBD needs careful consideration given the role of IL1R in mucosal healing (33). However, downstream physiological antagonism (such as mAChR agonists) of IL1R signalling might be of therapeutic relevance in ameliorating intestinal permeability issues in IBD. Furthermore, we show the clinical relevance of cholinergic signalling in IBD. In line with previous studies, we observed a significant downregulation of ChAT expression in the epithelial fractions of UC and CD patients, compared to non-infamed controls. To summarize, in chapter 3 we provide clinical and molecular evidences that underpin the pivotal role of cholinergic signalling in maintenance of homoeostasis in the intestinal epithelium.

The (para)sympathetic system in intestinal adaptive immunity

The cholinergic anti-inflammatory pathway has been described as a hard-wired system, and as key regulator of intestinal immune function it dampens inflammatory responses, as shown in various murine models (34). However, in appreciating the role of the vagus nerve as a modulator of intestinal immunity
(35), it remains unclear whether vagal ACh reaches intestinal cells, since there is currently no evidence that parasympathetic neurons indeed innervate macrophages or epithelial cells in the intestine (10, 36). On the other hand, sympathetic fibers have been shown in synapse with immune cells in Peyer’s patches and MLN’s (37). Besides the intrinsic innervation, studies, albeit conflicting, have shown that adrenergic fibres affect T cell migration (38), DC migration, antigen uptake and cytokine production (39-41). To understand the role of adrenergic and cholinergic receptor signalling on the modulation of DC function, in chapter 4 we compared the effect of the (para)sympathetic agonists ACh, nicotine, and epinephrine, on various DC functions such as endocytosis, maturation, cytokine production and assess the ability of these DC to induce/drive T helper (Th) cell differentiation. In monocytes β2-AR agonist salbutamol inhibits the production of IL-12 in a dose-dependent manner. The inhibition of IL-12 was specific, since salbutamol does not influence IL - 1α, IL - 1β, IL-6, or IL-10 production by LPS-stimulated monocytes (42). We observed that LPS-induced BMDCs, on pre-treatment with epinephrine or salbutamol, significantly increased IL-10 production, whilst IL-12p70 levels were almost completely blocked compared to vehicle. We next examined whether the potential of immature BMDCs to take up antigen was affected by pre-incubation with sympathetic or parasympathetic neurotransmitters. We observed that BMDCs pre-incubated with epinephrine, but neither by cholinergic agonists nor by salbutamol, exhibit a higher endocytic activity compared to control BMDCs, suggesting that epinephrine stimulates endocytosis in dendritic cells via α-AR, confirming earlier reports. In a previous report salbutamol enhanced IL-6 production following NOD2 activation of DCs, subsequently favouring Th17 cell development (39). However, in our study salbutamol pre-exposure to iBMDCs led to a significant induction of Th2 cells as determined by enhanced percentage cells having intracellular production of IL-4. A principal reason underlying this discrepancy might be explained by use of NOD2 ligand compared to TLR4 ligand – LPS used in our study. Although there is some documented evidence for a synergism between TLR2/4- and NOD2-mediated signalling in cytokine production, NOD2 is able to inhibit TLR2/4-mediated induction of inflammatory cytokine production and induce immune tolerance and homeostasis (43, 44). To
summarize, we provide a direct evidence of sympathetic control of DC function in the presence of LPS. However, we cannot exclude the possibility that ACh, or indeed, other neurotransmitters might have similar effects on APCs, when matured or activated with other TLR/PRR ligands.

Role of cholinergic T cell in intestinal immunity

Despite the general acceptance of the vagal origins of cholinergic anti-inflammatory pathway, the model has been challenged, primarily due to the lack of neuro-immune synapses in the mucosa. It is plausible that the enteric nervous system, of which about 26% neurons are cholinergic in nature, is the source of ACh. However, in 2011 group of Tracey showed that VNS activates the splenic nerve, leading to the release of norepinephrine (NE) in the spleen (45). They further showed that NE stimulates a subpopulation of splenic memory T cell, which release ACh, thereby resulting in an anti-inflammatory effect of VNS. In chapter 5 we examined whether cholinergic lymphocytes might be the missing between postganglionic neuronal activity and the widely reported ‘cholinergic anti-inflammatory reflex’ in the intestine. Firstly, we observed that the majority of ChAT expressing T-cells in the human and mouse intestine have characteristics of Th17 cells, and co-express IL17A, IL22 and RORC. In line with other published studies, we found that ChAT expression in these cells is stimulated by adrenergic and IL1β receptor activation on dendritic cells. Interestingly, the relative number of ChAT+ T-cells identified in the intestinal Peyer’s patch was a factor 10 higher than reported previously in spleen. To be able to study the functional characteristics of ChAT+ T-cells in the gut, we generated CD4CreChATfl/fl mice (CD4ChAT-/-) in which the capacity to express ChAT was deleted in the CD4 compartment. Despite the lowered ChAT expressions in the small intestine of CD4ChAT-/- mice expression levels of IL17A and IL22 were unaffected, however a significant decrease in the expression of AMPs lysozyme, defensin A (DefA), and angiogenin 4 (Ang4) was observed. Cationic antimicrobial peptides (AMPs) form the first line of innate defense against pathogens and their deletion leads to increased bacterial colonization of the intestine and enhanced activation of intestinal adaptive immune responses (46). To validate whether T-cell derived ACh contributed directly to AMP
secretion in epithelia, we made use of crypt derived organoid cultures, wherein sorted spleen CD4+ChAT+ T-cells, and not CD4+ChATneg T-cells, added to the organoid culture, enhanced Reg3γ and DefA expression in a dose-response fashion.

The human gut hosts a complex ecosystem comprising of diverse microbial species, and although each individual harbouring a unique microbiome ‘fingerprint’, the relative abundance and distribution of bacteria in healthy individuals is similar. Although microbiome and our nervous system have rarely been linked to each other, it’s important to note that dysbiosis in functional gastrointestinal disorders is highly linked to mood disorders (47,48). Disruptions of the gut brain axis alongside an altered microbiome have been shown to play a major role in onset of irritable bowel syndrome (IBS) (49). The group of Bercik et al. showed that chronic colitis in mice was associated with increased anxiety-like behaviour, which was ameliorated by B. longum NCC3001 treatment (50). Therefore we investigated whether Intestinal ChAT T-cells could influence the microbial balance in the intestine. To this end, we analysed the effect of deletion of the T-cell derived ChAT expression on the composition of the intestinal microbiota. We used 16S rRNA sequencing and compared small intestinal, cecum, and colon lumen microbiota of ChATfl/fl and CD4ChAT-/- mice. Notably, deficiency of ChAT expression (and resulting reduced AMP expression) was associated with a significantly increased diversity and richness of microbiota, most pronounced in jejunum segments.

In conclusion, in this chapter we show that sympathetic fibres are a key driver of intestinal microbiome, via cholinergic T-cells. Through various functional experiments, we further showed that loss of ChAT expression in CD4+ T-cells leads to enhanced bacterial richness and diversity, demonstrating the ability of ChAT+ T-cells to modulate the epithelial host defence mechanisms.
Future perspectives

This thesis advances our understanding of neuroimmune interactions in the intestine. Our new observations identifying activation ChAT T-cells as a mechanism for intestinal AMP and barrier function regulation is a paradigm shift upon which the neuro-immune reflex is based. Based on the findings in this thesis we hypothesize that sympathetic fibres respond to disturbances in intestinal homeostasis that involves a triad of non-neuronal cholinergic T-cells, β-AR+ APCs (DC’s), and innate immune response, working in synchrony to orchestrate an anti-inflammatory response. On the other hand, the neuronal cholinergic (vagal) pathway is likely activated by the prolonged immune response in an attempt to restrain excessive pathological inflammation. In considering the clinical implications, this thesis opens up two avenues for further investigation – bioelectronics and diet.

The application of electronics technology to human biology is not new. In 1780, Luigi Galvani coined the term ‘animal electricity’ on discovering that electrical patterns made frog muscles twitch. We have come a long way since those days, patients suffering from IBD and Rheumatoid Arthritis are now offered an unconventional therapeutic option, vagal nerve stimulation (45,51), under the banner of ‘bioelectronics’ or ‘electroceuticals’ (52). However, basing clinical therapeutics on vagal anti-inflammatory pathway needs careful reconsideration. At a molecular level, our understanding of neuroimmune interactions is in its infancy, and the potential is unfathomable, should we get it right.

The second, hypothesis of dietary factors influencing gut inflammation may be explained through several biological mechanisms, including antigen presentation, change in prostaglandin balance, and alteration of the microflora (53). In this regard, supplements that support synthesis of ACh in the intestinal mucosa might be an effective non-invasive way of restoring homeostasis in the gut. One candidate to consider here is phosphatidylcholine (PC). Clinical trials results of enteric-delivered PC in UC have been very promising (54). Whether orally delivered PC modifies ACh bioavailability in the intestinal mucosa remains unclear, nevertheless, based on the clinical findings, it warrants further investigation.
REFERENCES


