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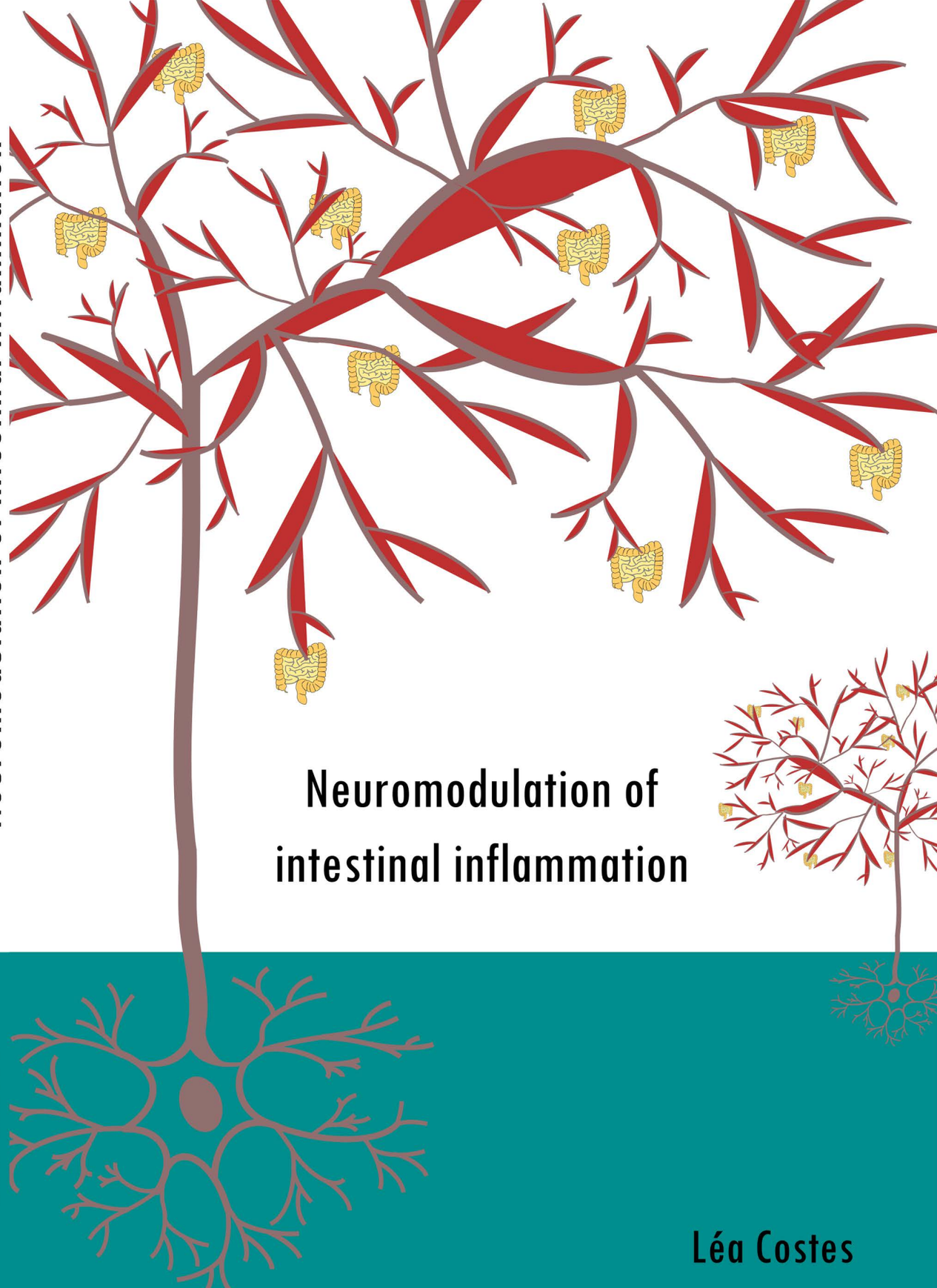

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Léa Marie Mathilde Costes

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PhD thesis of the University of Amsterdam

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Neuromodulation of intestinal inflammation

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General introduction

The gastrointestinal tract plays an essential role in the digestion of food, assimilation of nutrients and elimination of waste. As in other physiological systems, its key functions are governed by neural mechanisms but the innervation of the gastrointestinal tract however appears to be very unique. Indeed, as compared to other organs which are solely regulated by extrinsic parasympathetic and sympathetic fibers of the autonomic nervous system (ANS), the gastrointestinal tract is the only organ that possesses its own intrinsic nervous system or enteric nervous system (ENS) [1,2]. The ENS exerts an intrinsic neural control of the gastrointestinal tract, comprises of two plexuses, namely the myenteric and submucosal plexus and is able to regulate intestinal motility independently of the central nervous system (CNS) [3].

As a consequence of its digestive role, the intestine is constantly exposed to food proteins, commensals and pathogenic microbiota. In order to maintain the integrity of the organism by responding in an appropriate manner to threats while tolerating food proteins and commensals, a tightly regulated intestinal immune system has developed and a fine-tuned regulation of the immune response is required. The CNS, thanks to the proximity of its terminals to immune cells, plays a major role in this refined control of the immune system. Indeed, interactions between nervous and immune systems allow the modulation of the inflammatory response and offer new therapeutic targets to tackle pathophysiologies where a deregulation of the immune reaction constitutes the key element. Current data on the innervation of the intestine and its involvement in the modulation of the immune system in the gastrointestinal tract are reviewed in **Chapter 1**.

In particular, the vagus nerve was recently shown to be an essential regulator of the immune response. The ability of the immune system to activate the sensory arm of the vagus nerve is well established as vagal afferents have been shown to respond to pro-inflammatory stimuli. In the intestine, close anatomical contacts between vagal sensory fibers and mucosal granular cells resembling granulocytes [4] and mucosal mast cells [5] have been reported. These cells release inflammatory mediators such as IL-1β and prostaglandins which can in turn activate vagal afferents fibers [6]. The motor part of the vagus nerve, on the other hand, was only discovered as a modulator of the immune response ten years ago. Activation of the vagus nerve (by electrical stimulation) showed to suppress the release of the pro-inflammatory cytokine TNF-α by macrophages in a rat model of endotoxemia [7]. This effect mediated by the binding of acetylcholine

(ACh) to receptors located on immune cells was named 'cholinergic anti-inflammatory pathway' (CAIP) and led to the emergence of the concept of vagal inflammatory reflex [8]. This concept relies on the existence of close proximity of vagal fibers and immune cells. However, despite thorough study of the anatomical localization of nerve terminals [9,10], anatomical evidence of vagal neuro-immune interactions still remains a matter of debate. In **Chapter 2**, we aimed to bring further knowledge on the interactions between vagal nerve terminals and innate immune cells in the intestine. By using the high-definition dextran amine labeling anterograde tracer, we established that the vagus nerve does not directly make contact with resident macrophages in the intestine but contacts myenteric neurons whose nerve terminals are found in the close vicinity of those macrophages.

In the past decade, the vagal anti-inflammatory reflex triggered by VNS was shown to rely on the presence of the spleen in models of sepsis [11,12] and colitis [13]. However, the exact neural networks targeting the spleen involved in the inflammatory reflex still remain controversial as some studies provided evidence of a direct vagal innervation of the spleen [14] while others showed that nerve bundles innervating the spleen are solely sympathetic [15-17]. Finally, it was proposed that the vagal control on the spleen relied on vagal innervation of splenic postganglionic sympathetic neurons located in celiac ganglia and expressing the alpha 7 nicotinic acetylcholine receptor (α7nAChR) [11,12]. Vagal endings are indeed found in celiac ganglia [18], but anatomical evidence of synaptic contact between preganglionic vagal neurons and postganglionic sympathetic splenic neurons in those ganglia supporting this concept was however lacking. Therefore in **Chapter 2**, we investigated whether there existed a direct or indirect vagal innervation of the spleen.

One of the inflammatory disorders where the CAIP was shown to play a crucial role is postoperative ileus (POI) [19]. POI occurs in virtually all patients undergoing abdominal surgery and is characterized by a transient impairment of the gastrointestinal motility consequently to the manipulation of the intestine by the surgeon [20]. Its pathophysiology relies on an inflammatory reaction taking place in the gut muscularis with the activation of resident macrophages as well as the recruitment of leukocytes that will release pro-inflammatory cytokines which in turn activate neural inhibitory signals leading the paralysis of the gastrointestinal tract [21-24]. The infiltration of leukocytes to the gut wall represents a crucial event in the development of POI [25] but

little was known to date on the origin of these immune cells. Therefore in **Chapter 3** we investigated whether the spleen, a major secondary lymphoid organ known to act as a cell reservoir in several local and systemic inflammatory disorders [26,27], responded to the local intestinal inflammation and was involved in the inflammatory reaction underlying POI.

In POI, vagus nerve stimulation (VNS) applied prior to the intestinal manipulation prevents the inhibition of the gastrointestinal motility by suppressing the activation of resident intestinal macrophages via binding of ACh to α7nAChR [28], independently of the spleen innervation [29]. However, the existence and activation of a vagal reflex consequently to the inflammation under endogenous conditions had not been documented so far. In **Chapter 4 and 5,** we investigated whether an endogenous hard-wired neural circuitry was activated and led to a dampening of the inflammation in POI. To this end, we made use of the neuronal activation marker c-Fos to determine whether manipulation of the intestine triggered activation of sensory neurons leading to the integrated activation of motor vagal neurons (**Chapter 4)**. The results of this study allowed us to demonstrate that the intestinal inflammation triggered consequently to the intestinal manipulation led to the activation of vagal sensory neurons present in the nucleus tractus solitarius (NTS) as well as the dorsal motor nucleus of the vagus (DMV). In **Chapter 5**, we further investigated the involvement of this endogenous vagal reflex in the pathophysiology of POI. We hypothesized that, as observed for the anti-inflammatory effect of VNS, this endogenous vago-vagal reflex exerted an anti-inflammatory effect on manipulation-induced intestinal inflammation. To address this question, we selectively lesioned direct vagal inputs to the intestine and assessed the influence of the lack of those vagal inputs on the severity of the inflammation and ileus. Furthermore, to determine whether the splenic nerve played a role in the endogenous vago-vagal reflex, we lesioned the splenic nerve prior to intestinal manipulation and assessed whether the lack of splenic nerve impacted on the severity of POI.

The severity of POI varies considerably between patients. Accumulating evidence pointed towards a relationship between the intensity of the surgical trauma inflicted during manipulation of the intestine and the severity of the subsequent POI as increased surgical trauma was associated with systemic release of cytokines [20,22,30]. In the brain, specific areas such as the area postrema are devoid of blood-brain barrier and thus exposed to the systemic circulation. Interestingly, the presence of cytokines in

the systemic circulation was shown to trigger neuronal activation in the area postrema [31]. In **Chapter 6**, we hypothesized that the level of tissue damage induced during manipulation of the intestine was associated with enhanced brain activation and directly linked to the severity of POI.

Regulation of gastrointestinal inflammation by neural networks is not limited to the sole POI. Inflammatory Bowel Disease (IBD) is a chronic intestinal inflammatory disorder comprising two major forms, Crohn's Disease and Ulcerative Colitis (UC). A vagal anti-inflammatory role of the vagus nerve and its principal neurotransmitter ACh was demonstrated in a model of experimental colitis [32-34]. Subdiaphragmatic vagotomy in mice exposed to dextran sodium sulfate (DSS), an experimental model for UC, enhanced pro-inflammatory cytokine levels (i.e., TNF-α, IL-6 and IL-1β) hence worsening colonic inflammation [33]. In the same model, central activation of the vagus nerve showed to dampen the colonic inflammation, an effect relying on the integrity of the splenic nerve [13]. The approach used in these studies however relied on the general activation of the vagus nerve at a very high anatomical level and therefore did not allow to precisely determine which neural networks are involved in the anti-inflammatory effect. In **Chapter 7** we chose to use selective lesion of vagal inputs innervating the proximal colon, as well as lesion of the splenic nerve to unravel the exact involvement of these two neural pathways in the modulation of colonic inflammation in a DSS-induced model of colitis.

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Chapter 1

Neural networks in intestinal immunoregulation

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Abstract:

Key physiological functions of the intestine are governed by nerves and neurotransmitters. This complex control relies on two neuronal systems: an extrinsic innervation supplied by the two branches of the autonomic nervous system and an intrinsic innervation provided by the enteric nervous system. As a result of constant exposure to commensal and pathogenic microflora, the intestine developed a tightly regulated immune system. In this review, we cover the current knowledge on the interactions between the gut innervation and the intestinal immune system. The relations between extrinsic and intrinsic neuronal inputs are highlighted with regards to the intestinal immune response. Moreover, we discuss the latest findings on mechanisms underlying inflammatory neural reflexes and examine their relevance in the context of the intestinal inflammation.

Introduction

Key functions of the gastrointestinal tract such as motility, secretion and vasoregulation are regulated by neural mechanisms provided by the autonomic nervous system (ANS). The extrinsic control of the intestine is provided by two branches: the sympathetic and parasympathetic branches. Sympathetic preganglionic fibers arise from cholinergic neurons located in the thoracolumbar intermediolateral nucleus of the spinal cord and synapse with noradrenergic postganglionic neurons in para- and prevertebral ganglia. Parasympathetic preganglionic fibers originate from motor neurons of the dorsal motor nucleus of the vagus (DMV) and make synaptic contact with postganglionic neurons within the intestine (i.e., the myenteric plexus) (Fig. 1A). The density of the vagal innervation displays a proximodistal gradient along the intestine with the highest density observed in the duodenum and the lowest density observed in the distal part of the ileum [1]. The large intestine differs from the rest of the gastrointestinal tract as it receives a parasympathetic innervation from two distinct sources. The vagus nerve innervates the proximal colon whereas preganglionic neurons located in parasympathetic nuclei of sacral vertebrae (S2 to 4) contact post-ganglionic neurons in pelvic ganglia. These postganglionic neurons then give rise to rectal nerves providing vagal input to the distal colon. Of note, in some species (e.g. mouse and rabbit), direct vagal innervation of the distal colon is also provided by nerves arising from sacral parasympathetic nuclei [2].

In addition to the extrinsic neuronal control, the gastrointestinal tract possesses an intrinsic nervous system, the Enteric Nervous System (ENS), able to regulate intestinal motility independently of the presence of the central nervous system (CNS) [3]. The ENS is composed of 2 plexuses: the myenteric plexus located between the longitudinal and circular muscle layers of the muscularis externa and the submucosal plexus.

A tightly regulated immune system has developed in the intestine in response to a constant exposure to pathogenic and commensal microbiota. This system allows microbe sampling and surveillance and a quick response to microbial threat. The CNS, supported by the proximity of its terminals to immune cells, plays a major role in the regulation of this level of host defense. These interactions between nervous and immune system allow the modulation of the inflammatory response and offer new therapeutic targets to tackle inflammatory disorders where a deregulation of the immune reaction constitutes the key element.

In this review, we address the impact of neuro-immune interactions in the context of the intestine. The relevance of neural reflexes is discussed with regards to the regulation of intestinal inflammatory disorders.

Intestinal neuro-immune interactions

Sympathetic innervation of the gut and interactions with immune cells

The sympathetic regulation of the immune response relies on the binding of noradrenaline (NA) with α (subtypes α_1 and α_2) and β (subtypes β_1, β_2, β_3) adrenergic receptors. With a half-life of 1 to 2 minutes and a capacity to diffuse on a long distance (up to 1 µm) [4], a wide range of cell types can be affected by NA release without need of direct contact with neuronal fibers [5]. Sympathetic efferent fibers densely innervate the intestine (Fig. 1B). Terminals are found in the serosa [6] and the mucosa and an important network of sympathetic fibers contacts neurons of the myenteric plexus [7] [8]. Networks of fibers are also found in the circular muscularis [7] while some fibers pass through the longitudinal muscle layer [9,10]. Evidence of contacts between sympathetic terminals and non-vascular intestinal myocytes is however lacking. This innervation of the different intestinal compartments by noradrenergic fibers may therefore affect many immune cell types and thereby influence the innate and adaptive immune response. A good example of sympathetic immune regulation via such contacts is found in the lymphoid system of the gut.

The intestinal mucosa exhibits a dense network of immune cells particularly in organized lymphoid structures such as Peyer's patches (PPs). This component of the gut-associated lymphoid tissue (GALT), constituted by B and T-cell follicles and a subepithelial dome rich in dendritic cells (DCs), participates in the response to microbial threats. The presence of noradrenergic fibers was reported in PPs implying that the sympathetic nerve could play a role in the regulation of the immune response. Noradrenergic axonal fibers are found in close proximity to DCs [11], plasma cells and in T cell zones [12]. NA can affect DCs migration as well as their cytokine production (i.e., IL-12, IL-6 [13]) through the binding to adrenoceptors (β2 [13], α1 [14], α2 [15,16]). The potential of NA to dictate the cytokine profile secreted by DCs allows the sympathetic innervation to affect the skewing of naïve T cells and thereby indirectly influences the shaping of the adaptive immune response.

In vitro studies reported that NA can influence the skewing of naïve T cells expressing β-adrenoceptors and thereby their cytokine secretion (IFN-γ IL-12 for Th1 and IL-4 for Th2) (for a review see ref. [17]). B cell proliferation [18] and immunoglobulin secretion [19] are triggered by NA treatment through β2-adrenoceptors signaling. In vivo studies using 6-OHDA (a chemical sympathectomy) confirmed the effects of NA on T and B cells [20] and revealed the capacity of NA to modulate the migration and accumulation of lymphocytes in the GALT [21] during inflammation. Of note, the method used to deplete sympathetic inputs presents limitations as chemical sympathectomy not only depletes the noradrenergic input to the intestine but also affects the catecholamine content of immune cells [8]. Additional in vivo approaches are therefore required to determine the exact contribution of the intestinal sympathetic innervation in the modulation of the immune system.

The intestinal muscularis represents another compartment where neuro-immune interactions are observed. Recently, a direct contact between varicosities of sympathetic axons and resident intestinal macrophages was reported [22]. NA, through β-adrenoceptors signaling, can suppress TNF-α secretion [23] and phagocytosis of macrophages [24] thereby affecting their clearance capacity. Of note, sympathetic fibers synapsing with motor neurons of the enteric nervous system [25,26] could modulate immune functions via an indirect effect on enteric neurons.

Vagal innervation of the gut and interactions with immune cells

The vagus nerve modulates immune cells through the release of acetylcholine (ACh). In contrast to catecholamines, ACh exhibits a very short half-life of 1 to 2 milliseconds, due to the presence of ACh esterase which hydrolyzes ACh at a high rate. Close contact between cholinergic nerve terminals and cells expressing ACh receptors is therefore required for the cholinergic control of these cells.

Receptors for ACh are of two types: muscarinic receptors (mAChR) comprising of 5 subtypes (M1-M5) and nicotinic receptors (nAChR) constituted of homomeric or heteromeric combinations of 5 subunits (among 17 subunits). On a cellular level, ACh binding on one or the other type of receptors leads to significantly different mechanisms. Activation of muscarinic receptors, belonging to the G-protein-coupled receptors, leads to the activation of a cascade of secondary messengers only allowing a slow response

from the target cell [27]. On the contrary, nicotinic receptors belonging to the ligand-gated ion channel family allow a fast transmission of the cholinergic signal [28,29]. In immune cells however, these variations in transmission don't imply differences in the onset or length of the cholinergic signal as both nicotinic and muscarinic receptors trigger long-lasting effects.

The vast majority of immune cells express one or both types of receptors. Bone-marrow-derived dendritic cells, as well as PBMC-derived [30] and peritoneal macrophages [31] express both mAChR (M1-M5) and nAChR (α2, α5, α6, α7, α10, β2). T and B cells also exhibit receptors for ACh (nAChR α2, α5, α9, α10, β1, β2, β4 and M1, M3, M4, M5 mAChR for T cells; α4, α7, α2 nAChR and M2 mAChR for B cells) [31]. Interestingly, the expression of these different subunits on both innate and adaptive immune cells varies with their maturation and development stage. Intracellular signaling triggered by the binding of ACh to these receptors has been shown to modify greatly the function of these cells. In vitro studies demonstrated the capacity of ACh to modulate both the innate and adaptive compartments of the immune system. α7nAChR agonists suppress TNF-α secretion by peritoneal macrophages exposed to LPS. Moreover, treatment of freshly isolated murine T cells with both agonists and antagonists of nAChR and mAChR interferes with their skewing towards Th1, Th2 and Th17 profiles [32]. Finally, a suppressive effect of nicotine on B cell activation was reported and is mediated by α2, α4 and α2 subunits of nAChR [33,34]. Importantly, no contact between vagal terminals and immune cells in the intestine has so far been reported. However, preganglionic efferent terminals make synaptic contact with neurons of the myenteric ganglia [35-37] indicating that neural cholinergic influence on immune cells is solely provided by myenteric neurons (Fig. 1B). Importantly, T and B cells were recently shown as a crucial source of ACh able to modulate the immune response [38], [39].

The Enteric Nervous System and interactions with immune cells

Enteric neurons display a wide variety of neurotransmitters and neuropeptides determining the chemical coding of the ENS (for a review see refs. [40,41]). Enteric neurotransmitters and neuropeptides are able to bind and influence a variety of immune cells.

Cholinergic enteric neurons are targeted by both branches of the ANS [35,42,43].

Evidence of direct interactions between `basket-like' cholinergic endings and cholinergic myenteric neurons was recently reported [37]. This vagal innervation of cholinergic enteric neurons stimulates the production of ACh and may represent the neuronal circuitry by which the vagus nerve mediates its anti-inflammatory signal. Choline acetyltransferase (ChAT) positive fibers, most likely originating from the enteric neurons, are found in close proximity to resident intestinal macrophages [44]. Phillips and Powley recently brought evidence of direct interactions between cholinergic fibers (i.e., axons and dendrites) and macrophages in the gut muscularis [22]. This neuronal source of ACh can suppress macrophage activity through its binding to α7nAChR, and can thereby inhibit the release of pro-inflammatory cytokines [44]. On the contrary, sympathetic inputs to cholinergic enteric neurons inhibit ACh release through the binding of NA to α2 adrenoceptors [45,46]. This observation suggests that sympathetic inputs may counterbalance the cholinergic anti-inflammatory effect.

Of note, cholinergic enteric fibers are not restricted to the muscle layers. Some $ChAT^+$ fibers are found in PPs, suggesting a possible cholinergic modulation of the immune reaction [47]. However, no neuronal contact with immune cells has been reported so far in PPs. Determining what immune cells are contacted by cholinergic fibers in PPs is of crucial importance to better understand the interplay between cholinergic and immune system in the intestinal inflammatory response to microbial threats.

Vasoactive intestinal peptide (VIP), a well-established immunomodulator (for review see ref [39]), is expressed by enteric neurons. Sympathetic and vagal efferent fibers make contact with VIP^+ enteric neurons providing another neuronal mediator by which the brain can affect the gut immune response. In vitro and in vivo studies revealed the suppressive action of VIP on their activity as it inhibits their chemokine and cytokine production (e.g. MIP-1, MIP-2, KC and IL-12) during endotoxemia [4]. In addition, VIP treatment modulates DCs activity [48] in vitro and the expression of VPAC1 and VPAC2, the receptors for VIP, was observed on DCs isolated from PPs [49]. The modulatory effect of VIP on those DCs could potentially influence the IgA production by B cells, in addition to its direct effect already reported [50]. These results imply that VIP released by enteric neurons could interfere with both innate and adaptive immune systems.

In addition, both vagal [37] and sympathetic fibers [51] target nitrergic neurons in the ENS. Nitric oxide (NO) exerts both protective (e.g. T cell suppression [52]) and pro-

inflammatory effects (e.g. increase in IFN-γ production by natural killer cells [53]) and can greatly affect the inflammatory reaction (for review see ref [54]). This places NO as an additional neuromodulator of the innate immune system.

Serotoninergic enteric neurons receive inputs from both autonomic branches [25] [55,56]. Serotonin triggers the release of pro-inflammatory cytokines (i.e., IL-1β, IL-8) by DCs in vitro [57]. Interestingly, in postoperative ileus (POI) serotonin activates cholinergic enteric neurons leading to the dampening of intestinal inflammation [58]. Thus, despite a lack of evidence of contact between serotoninergic fibers and immune cells, serotonin can indirectly modulate the innate immune response. Of note, enterochromaffin cells are a major source of gastrointestinal serotonin [59] suggesting that its effect on the immune system could also be mediated through a non-neuronal release.

Extrinsic sympathetic inputs also target secretomotor neurons immunoreactive for substance P (SP) [60]. Receptors for substance P (i.e., NK) are found on a large variety of immune cells (e.g. mast cells, NK cells, macrophages, lymphocytes). SP induces the secretion of pro-inflammatory cytokines by macrophages [61] and increases T cell proliferation [62]. Importantly, mast cells are found in the close vicinity of SP positive fibers in the intestine [63] and SP activates peritoneal mast cells leading to their degranulation [64]. The degranulation and consequent release of inflammatory mediators can in turn activate sensory nerves, a phenomenon involved in the pathophysiology of irritable bowel syndrome (IBS) [65]. Moreover, the use of an inhibitor of Syk [67], a kinase involved in the downstream signaling of SP receptors, showed its efficacy in dampening the inflammation in POI in mice. Although the SP-mast cells interaction is a key event in IBS, the neuronal source of this neurotransmitter (i.e., enteric neurons or vagal sensory afferents) remains unclear [68].

In addition, a dramatic alteration in the expression of enteric neurotransmitters was observed in animal models of IBS and in IBD patients [69]. This phenomenon, most probably driven by the ongoing inflammation [70] is likely to influence the modulation of the intestinal immune system. Treatment with VIP in a TNBS-colitis model decreased the severity of the inflammation [71] suggesting a beneficial role of this neuropeptide on the colonic inflammation. In experimental colitis [72] and Crohn's disease (CD) patients [73], an increase in the number of VIP^+ neurons was measured and correlated

to an elevated colonic VIP content. However, contradictory results were found on VIP expression in ulcerative colitis and CD patients [74] leaving under debate the role of VIP in the colonic inflammation.

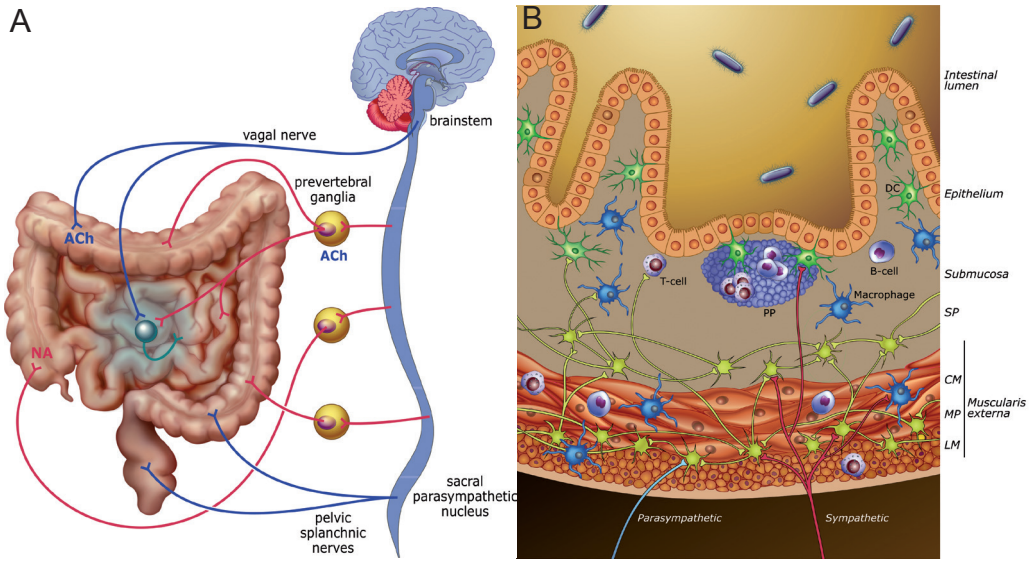

Figure 1. Intestinal innervation and interactions between nervous and immune system in the intestine. (A) Both branches of the Autonomic Nervous System innervate the small and large intestine. The parasympathetic innervation of the distal colon differs between species: direct innervation from sacral nuclei is observed in some species while postganglionic neurons located in pelvic ganglia provide inputs to the colon in others. (B) Vagal inputs (in blue) solely innervate myenteric neurons. Sympathetic inputs (in red) make synaptic contact with enteric neurons and immune cells in both smooth muscle layers and Peyer's patches (PPs). Enteric fibers (in green) also project to PPs and are found in the close vicinity of macrophages. CM: circular muscularis; LM: longitudinal muscularis; MP: myenteric plexus; SP: submucosal plexus

CNS control of the immune response: the inflammatory reflex

A fine tuning of the immune response is required to maintain the organism homeostasis. This refined control relies on interactions between the CNS and immune cells mediated through neural autonomic reflexes. The initiation of inflammatory neural reflexes is characterized by the activation of sensory nerve terminals by inflammatory mediators. This peripheral information is conveyed to the CNS where it triggers the activation of motor neurons which in turn provide signals to immune cells present in the target organ.

Sympathetic reflexes and involvement in intestinal inflammatory diseases

Sympathetic reflexes are involved in diverse intestinal pathophysiologies. In POI, sympathetic reflexes play a role in both stages of this inflammatory disorder: the early neurogenic and the late inflammatory phase [75]. In the early phase, the activation of spinal mechano-sensory afferent fibers [76] by handling of the intestine triggers NA release by sympathetic motor fibers. The inhibitory effect of NA on intestinal smooth muscle cells leads to an impaired gastrointestinal motility. In the late inflammatory phase, the activation of this sympathetic reflex is maintained through the release of NO and the induction of Cox-2 secretion by infiltrated leukocytes [77-79] thereby resulting in a prolonged intestinal paralysis. This sympathetic feedback loop is proposed to participate in the disseminative nature of POI [80].

In other intestinal inflammatory disorders, activation of sympathetic reflexes showed to play an important role in modulating the intestinal immune system. In intestinal parasitic infection (e.g. Trichinella spiralis) [81] and experimental colitis models (TNBS [82] or DSS [83]) motor sympathetic fibers are affected as shown by a decreased NA release in the gut. Importantly, observations of intestinal sympathetic innervation in CD patients showed a lack of TH^+ fibers in every intestinal layer [84]. This result however remains debatable as other studies showed an increased amount of TH^+ fibers in the myenteric plexus of CD patients [73]. Additionally, the α2-adrenoceptor antagonist RX821002 inhibited the colonic mRNA level of pro-inflammatory cytokines (TNF-α and IL-1β) in TNBS colitis [85]. In the same model, chemical sympathectomy showed to ameliorate colonic inflammation [86]. These findings underscore the pro-inflammatory role of NA and α2-adrenoceptors signaling in colitis. Interestingly, exposure to TNBS also led to an increase in the number of sympathetic `basket-like structures' in the dorsal root ganglia showing that colonic inflammation triggers sprouting of sympathetic fibers. These structures surround sensory neurons of the dorsal root ganglia, suggesting that sympathetic sprouting could influence the plasticity of these sensory neurons and be partly responsible for the visceral hypersensitivity in IBS and the chronic pain observed in colitis patients [87].

Vagal reflexes in intestinal inflammatory diseases

The ability of the immune system to activate the sensory arm of the vagus nerve is well

established. In the intestine, close anatomical contacts between vagal sensory fibers and mucosal granular cells resembling granulocytes [88] and mucosal mast cells [89] have been reported. These cells can release inflammatory mediators such as IL-1β and prostaglandins which in turn activate vagal afferents fibers [90]. Only ten years ago, the motor part of the vagus nerve was discovered as a modulator of the immune response. Indeed, activation of the vagus nerve (by electrical stimulation) showed to suppress the pro-inflammatory cytokine release by macrophages in a rat model of endotoxemia [91]. This effect mediated by the α7nAChR was named 'cholinergic anti-inflammatory pathway' (CAIP) and brought to light the concept of a vagal inflammatory reflex [92].

In POI, vagus nerve stimulation (VNS) applied prior to the intestinal manipulation prevents the inhibition of the gastrointestinal motility by suppressing the activation of resident intestinal macrophages via α7nAChR. In the same line, endogenous activation of the vagal reflex by administration of enteral lipid-rich nutrition [94] dampens the pro-inflammatory cytokine secretion by resident macrophages therefore preventing ileus. Altogether, these observations suggest two distinct functions of the vagus nerve in the modulation of the immune response: a preventive role when the vagus nerve is activated (by VNS) prior the immune challenge or insult; a role in the restoration of homeostasis when the vagal reflex is activated after the inflammation is settled.

The cholinergic anti-inflammatory pathway was also demonstrated in experimental colitis. Subdiaphragmatic vagotomy in DSS-exposed mice enhanced pro-inflammatory cytokine levels (i.e., TNF-α, IL-6 and IL-1β) worsening colonic inflammation [95]. Although the α7nAChR plays a key role in mediating the cholinergic suppressive effect on macrophage activity, another cholinergic receptor, α5nAChR, was shown to participate in the vagal anti-inflammatory mechanism during colitis [96]. With regards to the colon innervation, further investigations are required to determine the exact involvement of direct vagal innervation and sacral parasympathetic inputs in the cholinergic anti-inflammatory pathway regulating colitis.

Spleen innervation: involvement in the vagal reflex controlling immune responses

The spleen and its innervation were recently identified as key players in mediating the

vagal anti-inflammatory effect in sepsis [97,98]. Interestingly, no evidence of a direct vagal innervation to the spleen has been shown to date [99,100]. The vagal control on the spleen was consequently proposed to rely on vagal innervation of splenic postganglionic sympathetic neurons expressing α7nAChR located in celiac ganglia [97,98].

This vagal control of NA release by splenic sympathetic fibers was recently shown to target a specific memory T cell population producing ACh (as they express ChAT) [38]. This non-neuronal source of ACh is proposed to suppress pro-inflammatory cytokines (i.e., TNF-α) secretion by macrophages. However, some controversy exists as recent studies demonstrate the absence of neuronal contact between vagal and splenic nerve [101], suggesting that the anti-inflammatory effect observed may be driven by a spinal sympathetic reflex rather than a vagal reflex.

Nevertheless, the discovery of these cholinergic T cells may suggest a role of these cells in other inflammatory disorders where cholinergic regulation of the immune system is involved. The presence of this cell population may indeed not be restricted to the spleen but could also be found in other lymphoid structures such as PPs or mesenteric lymph nodes which present a comparable distribution of noradrenergic fibers. It would therefore be of interest to consider the contribution of this $ChAT^+$ T cell population in the cholinergic anti-inflammatory mechanisms regulating intestinal inflammatory diseases such as POI and IBD.

Conclusion and outreach

A large body of evidence points towards an integrated regulatory role of both extrinsic and intrinsic innervation in intestinal immunity. The importance of inflammatory reflexes in regulating acute and chronic intestinal inflammatory disorders is emerging and the ENS appears as a pivotal element linking sympathetic and more particularly vagal inputs to the immune system. Our understanding of the interactions between nervous and immune system in the context of the intestine has strongly increased during the past decade. However, the exact mechanisms underlying these neuro-immune interactions still remain partly unclear and numerous challenges need to be addressed to comprehend the functioning of this neuro-immune system. On the one hand, further anatomical evidence are required to determine precisely which neural

axis is able to affect the different components of the immune system in the intestine. On the other hand, the clear role of the nervous system in the pathophysiology of intestinal inflammatory disorders is not yet clarified in the current literature and further investigations are necessary to unravel the therapeutic relevance of this neural component. From a clinical point of view, the prevalence of intestinal inflammatory disorders such as IBD, IBS and POI and the limitations and cost of current treatments call for therapeutic alternatives. Regulating the immune system via the modulation of the nervous system may provide us with a powerful tool to resolve the intestinal inflammation underlying these disorders.

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Chapter 2

Neuroanatomical evidence indicating indirect modulation of macrophages by vagal efferents in the intestine but not in the spleen

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Abstract

Background: Electrical stimulation of the vagus nerve suppresses intestinal inflammation and normalizes gut motility in a mouse model of postoperative ileus. The exact anatomical interaction between the vagus nerve and the intestinal immune system remains however a matter of debate. In the present study, we provide additional evidence on the direct and indirect vagal innervation of the spleen and analyzed the anatomical evidence for neuro-immune modulation of macrophages by vagal preganglionic and enteric postganglionic nerve fibers within the intestine. Methods: Dextran conjugates were used to label vagal preganglionic (motor) fibers projecting to the small intestine and spleen. Moreover, identification of the neurochemical phenotype of the vagal efferent fibers and enteric neurons was performed by immunofluorescent labeling. F4/80 antibody was used to label resident macrophages. Results: Our anterograde tracing experiments did not reveal dextran-labeled vagal fibers or terminals in the mesenteric ganglion or spleen. Vagal efferent fibers were confined within the myenteric plexus region of the small intestine and mainly endings around nNOS, VIP and ChAT positive enteric neurons. nNOS, VIP and ChAT positive fibers were found in close proximity of intestinal resident macrophages carrying α7 nicotinic receptors. Of note, VIP receptors were found on resident macrophages located in close proximity of VIP positive nerve fibers. Conclusion: In the present study, we show that the vagus nerve does not directly interact with resident macrophages in the gut or spleen. Instead, the vagus nerve preferentially interacts with nNOS, VIP and ChAT enteric neurons located within the gut muscularis with nerve endings in close proximity of the resident macrophages.

Introduction

In the last decade it has become clear that the vagus nerve fulfills an important role in modulating the immune system [1,2]. Vagus nerve activation indeed has anti-inflammatory properties in a wide variety of disorders including systemic and local inflammation [3–8]. The first experiments leading to the introduction of this concept were performed in a rat model of sepsis [1], illustrating increased survival after vagus nerve stimulation. This effect is now believed to result from vagal activation of sympathetic neurons located in the mesenteric ganglion [9] rather than a direct effect of the vagus nerve in the spleen. These adrenergic nerve fibers release noradrenaline activating splenic T cells. These T cells subsequently release acetylcholine (ACh) that inhibits the release of pro-inflammatory cytokines from splenic macrophages through interaction with α7 (alpha7) nicotinic receptors [10,11].

Also in the gastrointestinal tract, vagus nerve stimulation dampens the inflammatory response in several immune-mediated disorders, including postoperative ileus (POI). In the latter, intestinal manipulation initiates an inflammatory cascade through the activation of muscularis resident macrophages that results in delayed gastrointestinal motility. Electrical stimulation of the vagus nerve (VNS) and systemic administration of selective nicotinic receptor agonists dampened pro-inflammatory cytokine production by macrophages resulting in reduced intestinal inflammation and shortened POI [12]. Recently, we showed that this subtle inflammatory response evoked by manipulation of the small intestine elicits neuronal activation in the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus [13]. This vagal output targeted mainly the inflamed zone (intestine) but also other organs such as the spleen.

Although the innervation of the intestinal myenteric plexus by vagal efferents is well described [14], its interaction with the immune cells residing in the intestine is poorly characterized. Similarly, the innervation of the spleen is still a matter of controversy with some studies providing evidence of cholinergic innervation whereas others propose that the spleen is only innervated by sympathetic neurons located in the mesenteric ganglion [9,13,15–17]. Hence, we aim to provide neuro-anatomical evidence on the interaction between vagal efferents and resident macrophages in the intestine and to bring more clarity on the vagal innervation of the spleen in mouse. To this end we labeled the vagal motor efferent fibers arising from the dorsal motor nucleus of the

vagus (DMV) by using the dextran amines anterograde tracer, recently reported to provide high-definition labeling of vagal motor fibers [18].

Material and Methods

Ethics Statement

All procedures were conducted in accordance with the Institutional guidelines and approved by the Animal Ethical Committee of the AMC/University of Amsterdam (reference protocol number 100096) and by the Ethical committee of the Catholic University of Leuven (Permit Number: 112/2011). All surgery was performed under anaesthesia (Hypnorm/Dormicum) and all effort was made to minimize suffering.

Animals

Mice (female BALB/c; Harlan Nederland, Horst, The Netherlands) were kept in 12h light/12h dark cycle (lights on at 8:00 AM to 8:00 PM) under constant conditions of temperature (20± 2°C) and humidity (55% humidity) with water and food *ad libitum*. Mice underwent surgical procedure at 11-13 weeks of age. Mice were anesthetized by FFM intraperitoneal injection, a mixture of fentanylcitrate/fluanisone (Hypnorm; Janssen, Beerse, Belgium) and midazolam (Dormicum; Roche, Mijdrecht, The Netherlands) in a ratio 1:1:2 (Hypnorm: Dormicum: H_20).

Tracer injection

Mice were anesthetized and mounted on the stereotaxic frame. Bilateral injections of the biotin or Texas red dextran amines (5% solution, D-B, D-1956 or D-TRD1863, Invitrogen) were performed at different rostro-caudal levels of the DMV: AP -7.8 mm, -7.9 mm and -8.0 mm. The stereotaxic coordinates used for lateral ventricle injection were AP -0.5mm, L -1.0mm and V -2.0/-1.50/-1.0mm. Injections (4 ms duration) were performed using a glass micropipette (25 µm). At the end of the injection procedure, the wound was closed by a suture with Mersilene, 6-0 silk.

Tissue preparation

Nineteen days after injection of the tracer, anesthetized mice were sacrificed

by transcardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (pH 7.4, 4ºC). The spleen was removed prior the perfusion with fixative, quickly snap frozen and stored at −80°C. Then, brain, nodose and mesenteric ganglia and deep cervical lymph node were collected, post-fixed for 4h (4°C) and immersed in 30% sucrose/0.2M PBS (pH 7.4) overnight at 4°C. For the whole mount intestinal tissue preparation, samples were cut along the mesentery border, washed in cold saline and transferred to PFA for 4h and to 30% sucrose. Prior to the immunohistochemical procedures, the muscle layers were gently stripped out from the mucosa and sub-mucosa with fine-tip forceps. For intestinal coronal sections, tissue were frozen in OCT embedding compound (Neg 50, Thermo Scientific, Walldrof, Germany) and stored at −80°C.

In some cases, colchicine was used to enhance VIP immunoreactivity of cell bodies. Intestinal tissues were washed with PBS containing gentamicine (1:100 diluted) and incubated in Dulbecco's modified Eagle medium (Gibco, Life Technology) containing colchicine (0.01g/100ml) at 37ºC. Following incubation, the tissue was stretched and fixed with Zamboni fixative.

Immunohistochemical staining

Brainstem, nodose and mesenteric ganglia and deep cervical lymph node

Coronal sections of 30µm for brainstem, 16µm for nodose/mesenteric ganglia and deep cervical lymph nodes were collected. To reveal the biotin dextran amines, sections were pretreated first with a solution of Methanol (10%) and hydrogen peroxide H_2O_2 (0.1%) for 10min and were subsequently incubated for 1h with avidin-biotin complex (ABC, Vector Labs PK-4000). The reaction product was visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% H_2O_2 for 5min.

To reveal the Texas red dextran amines, section was incubated overnight with the anti-Texas red antibody (1/50; Invitrogen A6399) followed by an incubation overnight at 4ºC with Goat anti-rabbit$^{Poly\ AP}$ (1:50; BrightVision Immunologic B.V, DPVR55AP). The reaction product was visualized by incubation with Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Inc. SK-5300) for 20 minutes.

Intestinal whole mount preparation

ABC/DAB staining and the pan-neuronal marker cuprolinic blue were used to label

dextran-labeled vagal fibers and enteric neurons, respectively. Briefly, the whole mount preparation was pretreated (30 min) with a solution Methanol- H_2O_2 (4:1). The tissue was incubated for 4h at 37ºC with a solution of 0.5% cuprolinic blue (17052; Polyscience, Inc.) followed by incubation in buffer (0.05M NaAC, 1M $MgCl_2$, pH 4.9) for 30s. After thorough rinsing with distilled water and TBS, sections were incubated with ABC (1h) and with 1% DAB 0.05% nickel ammonium sulfate and 0.01% H_2O_2 for 8min. To visualize resident macrophages, preparations were exposed to the primary F4/80 antibody (1:500; Biolegend, biotinylated rat antibody; 1:200; Dako E0468) and were revealed by ABC/Nova Red (vector Labs, SK-4800).

Immunofluorescent labeling

Coronal section of intestinal tissue

Sections underwent a treatment with Biotin-Blocking System from DAKO (protocol provided by the manufacturer). An additional blocking step was performed by 2h incubation with 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) at room temperature (RT). Then, sections were treated with streptavidin conjugated with CY3 (1:400; Jackson ImmunoResearch, diluted in 1% BSA + 0.3% Triton X-100) for 1hr at RT. A counterstaining with DAPI to label nuclei was used to delineate the anatomical structure of the intestine wall.

Intestinal whole mount preparation

The preparations were subjected to Biotin-blocking system (DAKO) and 2h incubation with 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) at room temperature (RT). After blocking, the preparations were incubated with the primary antibodies (overnight, 4ºC) rabbit anti-PGP 9.5 (1:600,Chemicon), goat anti-ChAT (1:5000, Chemicon) and rat anti-F4/80 (1:200, Biolegend), rabbit anti-VIP (1:2000, kindly provided by Prof. Dries Kalsbeek from the Netherlands Institute for Neuroscience, Amsterdam), rabbit anti-nNOS (1:500, Santa Cruz), anti-PGP 9.5 (1:500, Chemicon) and goat anti-VPAC1 (1:500, Santa Cruz) diluted in PBS containing 1% BSA and 0.3% Triton X-100. Specificity of the primary antibodies used was confirmed by preincubation with the respective blocking peptide.

The next day, the tissues were incubated for 1h at room temperature with the following secondary antibodies at a concentration of 1:1000; goat anti-rabbit CY3

conjugated (Jackson ImmunoResearch), donkey anti-goat FITC conjugated (Jackson ImmunoResearch), donkey anti-rat CY5 conjugated (Jackson ImmunoResearch), donkey anti-rabbit Alexa 555 (Molecular probe) or goat anti-rabbit Alexa 555 (Molecular probe) and streptavidin conjugated with CY3 or CY5 (Jackson ImmunoResearch). The specificity of the secondary antibodies used or fluorescent streptovidin was confirmed by the lack of staining in the absence of preincubation with the primary antibody.
In case of nNOS/VIP, immunolabeling was performed sequentially including a step with citrate buffer prior to VIP labeling to avoid cross reactivity. In brief, the preparations were rinsed in citrate buffer (pH = 6) followed by 3 heating sessions (microwave 6min at 600W) and incubated in refresh citrate buffer for 20min.
For α7 nicotinic receptor labeling [19] and macrophage staining (F4/80), jejunum tissue was incubated with FITC-labelled α-bungarotoxin (Invitrogen) at 0.1µg/ml in RPMI 1640 medium (Lonza) at 4°C for 15 min. After thorough washes with PBS and post fixation with 4% of cold Paraformaldehyde (10 min), the mucosa and submucosa were gently removed from the muscle layers. The latter were subsequently processed for intestinal resident macrophages (F4/80) labeling.

Coronal section of spleen tissue

Eight µm sections were labeled with Tyrosine hydroxylase (1:100, T8700, Sigma) and anti-B220 (1:200; Clone 6B2 kindly provided by Dr Martijn Nolte, Sanquin, Amsterdam) antibodies. Before primary antibody incubation, sections were post-fixed with cold acetone (2min) and followed by pretreatment with Na Azide (0.1%) and H_2O_2 (0.3%) for 15 min. A 30 min blocking step with BSA 1% was performed prior the incubation with the primary antibodies (1hr). Anti-rat AF488 (1/400) and anti-rabbit AF546 (1/400) were incubated for 1 and 2h, respectively at room temperature.

Image acquisition

Preparations were examined by use of an Olympus BX4 epifluorescence microscope (Olympus America, Center Valley, PA). Immunohistochemical labelled tissues were visualized using a Zeiss LSM510 Meta confocal microscope (Cell Imaging Core, KU Leuven). The following lasers and emission filters were used to visualize the labeled structures and collect images: multiline Ar laser at 488 nm (used for the excitation of FITC); emission filter 535 ± 15 nm; 543 nm HeNe laser (used for Cy3); emission filter

575 ± 630 nm; and 633 nm HeNe laser (used for Cy5); emission filter 650 ± 700 nm. The confocal images were collected using the optimal pinhole size for the 63X oil objective or for the 20X air objective and confocal stacks were taken with *z*-axis step of 0.5 µm (63X objective) or 1 µm (20X objective).

Results

Tracer application

Injection of the tracer in the DMV (Fig. 1A) was achieved in 4 out 9 mice for the biotin-dextran and in 2 out 6 mice for the Texas red-dextran. Two extra mice were injected in the lateral ventricle (LV) to evaluate possible unspecific staining in the peripheral tissue (Fig. 1B). Leakage of the tracer to the cerebrospinal fluid is drained by the deep cervical lymph node (DCLN) and then released in the bloodstream [20]. In line we observed some trace of the dextran amine in the deep cervical lymph node in lateral ventricle injected mice and to a limited extent in some of the DMV injected mice, nineteen days after injection (Fig. 1 C and D).

Vagal innervation of the spleen

The spleen has been proposed to play a central role in the anti-inflammatory effect of the VNS in sepsis [9,21,22]. Although previous studies showed neuronal synapses between the vagal efferent fibers and sympathetic cells bodies of the mesenteric ganglia [15,23], our anterograde tracing experiments did not reveal any dextran positive vagal fibers or terminals in the mesenteric ganglion (Fig. 2A). Similarly, no labeled vagal efferent fibers or terminals were found in the spleen using Biotin-Dextran or Texas-red conjugated dextran (Fig. 2B). Only TH positive fibers were found throughout the spleen tissue along the blood vessel ending in the white pulp in close proximity to T cells (Fig. 3A). Occasionally, Texas-red dextran was found in the follicular dendritic cells in the B cell area (Fig. 3B) in both DMV and LV injected mice. This non-specific staining was most likely caused by the release of the tracer into the circulation as confirmed by the presence of the dextran in the deep cervical lymph node in both types of injection (Fig. 1 C and D).

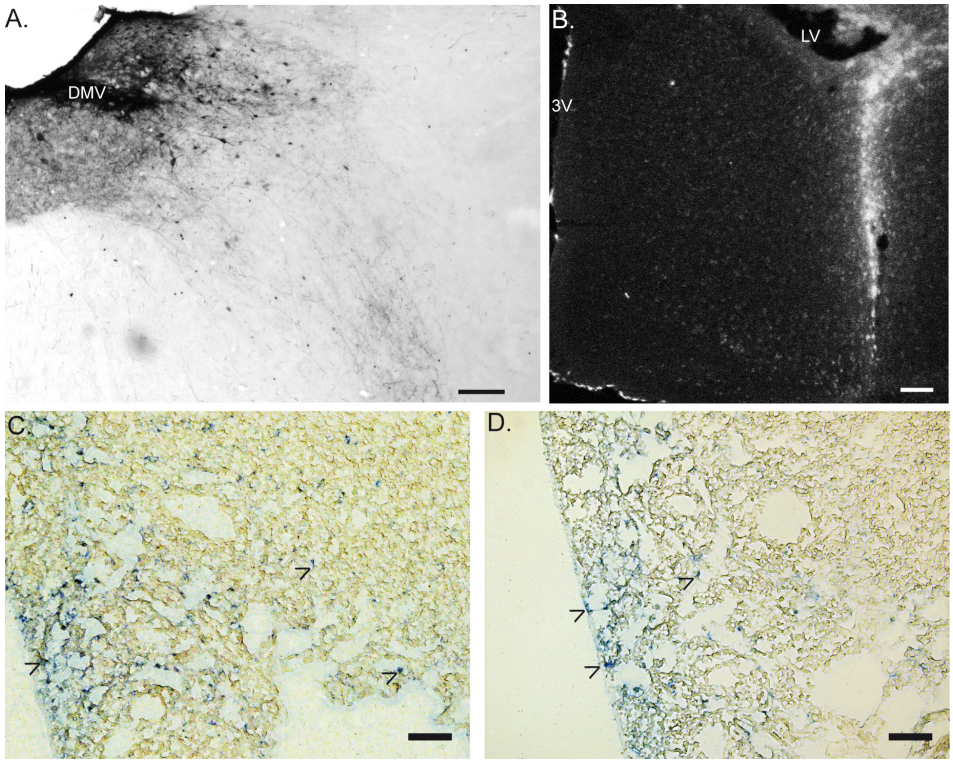

Figure 1. Injection sites of the neuronal tracer and deep cervical lymph nodes. Panel A shows the site of dextran amine injection at the level of the DMV, 19 days after injection. The tracer was revealed by DAB staining. B. Epifluorescent picture shows the distribution of Texas-red dextran amines after lateral ventricle injection. In the panels C and D, arrow heads show the presence of dextran amines (revealed by phosphatase alkaline staining to amplify the Texas red signal of the tracer) in the deep cervical lymph node. Of note, the presence of the tracer was found in all LV injected mice (D) and occasionally in DMV-injected mice (C). 3V: third ventricle. LV: lateral ventricle. DMV: dorsal motor nucleus of the vagus. Scale bar represents 0.1mm.

Vagal motor efferent fiber distribution in the intestine

Only DMV-injected mice exhibited dense dextran-labeled vagal fibers in the gut muscularis of the small intestine (Fig. 4). No dextran-labeled fibers were observed in LV injected mice. As previously reported by others [18], we did not find labeled cells bodies in the nodose ganglia 19 days post-injection, confirming the specificity of the labeled fibers to motor neurons arising from the DMV. Dextran amines injection in the DMV of mice provided a similar vagal distribution pattern in the gastrointestinal tract as previously reported in rats using the tracer DiI [14] and with a similar sensitivity and

specificity as reported by Walter *et al.* [18].

The distribution of the pre-ganglionic vagal efferent fibers (Fig. 4) was exclusively confined within the myenteric plexus located between the circular and longitudinal muscle layers of the intestine. No labeled fibers or terminal were observed within the submucosal plexus or lamina propria (Fig. 4A.1 and A.2). We did however notice a bright signal in the cells of the submucosal crypts (Fig. 4A.3). Based on the location (inside submucosal crypts) and the shape (round cells), the bright signal is most likely indicative of the presence of endogenous biotin expression in those cell types. In whole mount preparations, biotin dextran amine with nickel enhancement provided a clear distribution of the efferent vagal fibers/terminals connecting to enteric ganglia within the myenteric plexus region. The permanent staining with ABC and cuprolinic blue revealed the morphology of the terminals that synapse with neurons located in the myenteric ganglia (Fig. 4B). Labeling with a secondary fluorescent antibody combined with PGP 9.5 confirmed that vagal fibers and terminals in the small intestine were confined to the myenteric ganglia located at the level of the myenteric plexus (Fig. 4C and D).

Chemical coding of vagal efferent fibers in the small intestine

Immunoreactivity for various neurotransmitters and peptides confirmed that the vagal pre-ganglionic fibers were mainly positive for choline acetyltransferase (ChAT) (Fig. 5A). Dextran-labeled axons/terminals were negative for neuronal Nitric Oxide Synthase (nNOS), Tyrosine Hydroxylase (TH), Substance P (SP), Vasoactive Intestinal Peptide (VIP) and Calcitonin Gene-Related Peptide (CGRP) (Fig. 5B-F).

In rare occasions, we found that efferent vagus nerve terminals in the small intestine show VIP and CGRP immunoreactivity (at occasional points) at the level of the myenteric plexus region (Fig. 5 E and F, arrow head). However with the methodology used here, it is impossible to discriminate between VIP and CGRP stored in vagal nerve terminals or overlap of enteric nerve fibers with vagal efferents (Fig. 5E and F).

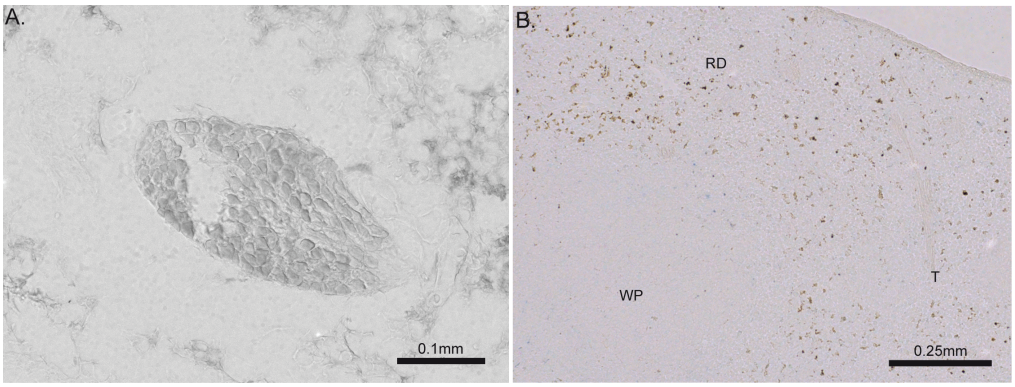

Figure 2. Distribution of dextran-labeled vagal fibers in the mesenteric ganglion and spleen coronal section. No biotin dextran-labeled fibers or terminals were found on coronal section of mesenteric ganglion (A) or spleen (B). Of note, similar observations were obtained with Texas-red dextran amine tracer. The brown spots on the spleen section were found in injected and non-injected mice, indicating of a strong endogenous biotin expression. WP: white pulp, RP: red pulp, T: trabeculae.

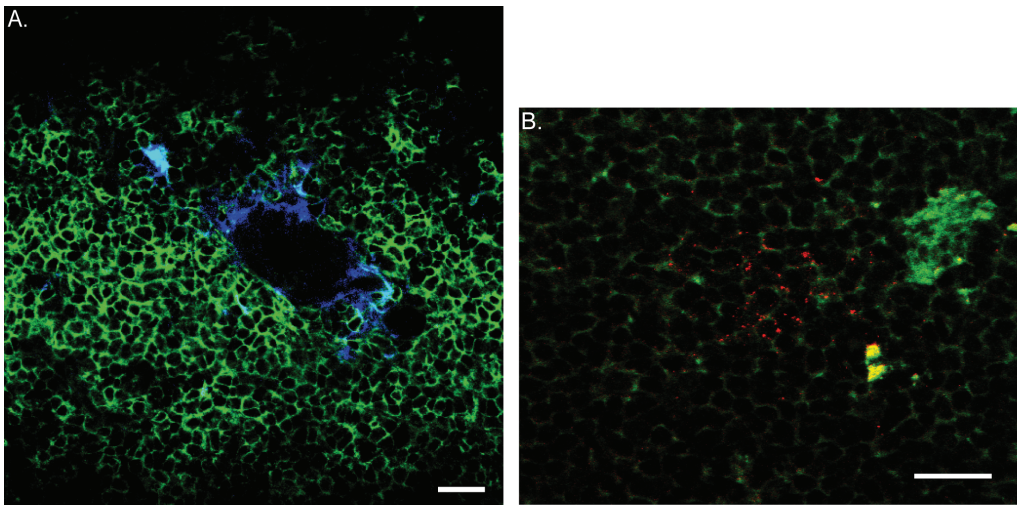

Figure 3. Sympathetic fibers and no vagal innervation of the spleen. A. Tyrosine hydroxylase (TH) staining was used to reveal the sympathetic innervation of the spleen. The central arteriole showed high TH positive fibers (blue) that are in close proximity of the T cells (green). B. Texas-red amine signal (red) was found occasionally in the B cells area (green). This dextran amine signal was found in mice that exhibit also dextran amine in deep cervical lymph node, indicating that the spleen signal is the result of tracer leakage into the cerebrospinal fluid. The scale bar represents 20µm.

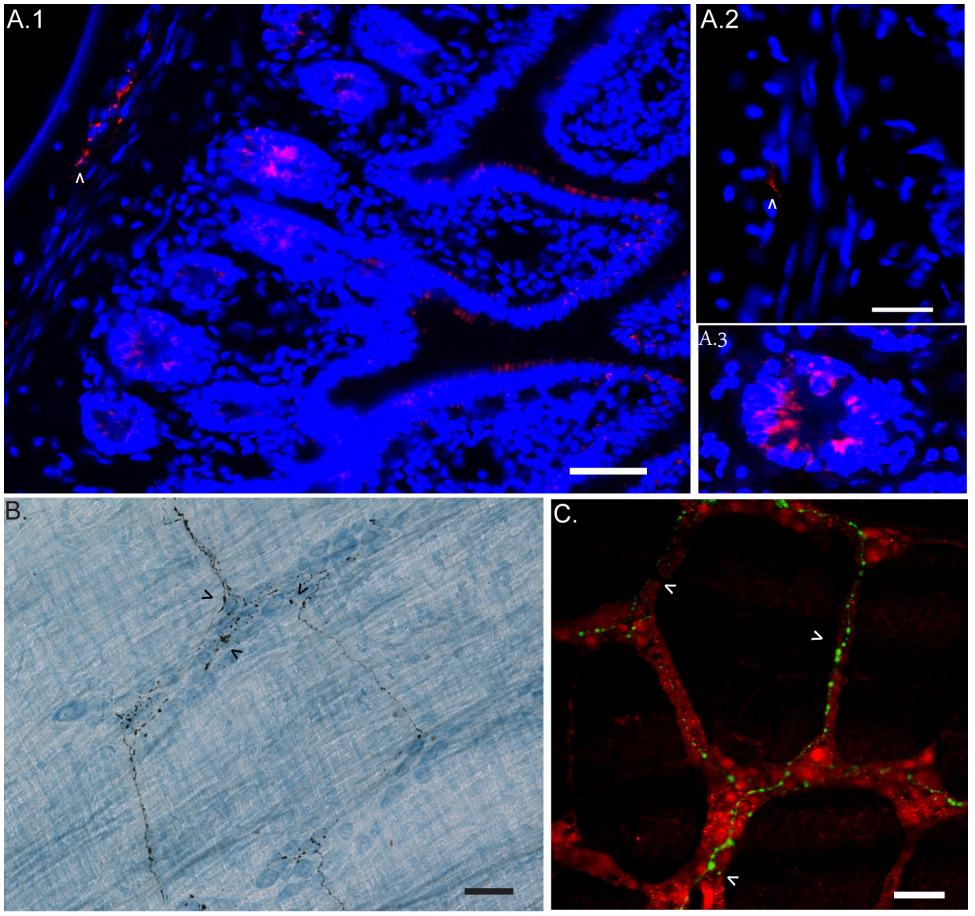

Figure 4. Vagus nerve efferent fibers and terminals reach myenteric plexus region in the intestine. A.1 Vagal motor efferent fibers (red; arrow head) were labeled using fluorescence conjugated streptavidin on 5µm thin coronal section of small bowel. Labeled vagus fibers were found between circular and longitudinal muscle layers at the level of the myenteric plexus. A.2. High power magnification field showing localization of vagal motor efferent fibers (red, arrow head) between nuclei (blue) of circular (CM) and longitudinal (LM) smooth muscle. A.3. The presence of intrinsic biotin was found in cells of the submucosal crypts. B. Labeled-fibers of the vagus nerve were revealed with ABC/DAB staining protocol while cuprolinic blue was used as panneuronal marker to visualize enteric neurons (blue). Labeled vagal efferent fibers and terminals with a basket-like shape terminals were found within myenteric ganglia (arrow head). C. Confocal image showing the presence of the dextran amine in the inter-ganglionic fibers (arrow). D. Epifluorescence image corresponding to vagal efferent fibers (green) densely found at the level of myenteric ganglion (PGP 9.5, red). The scale bar represents 50µm.

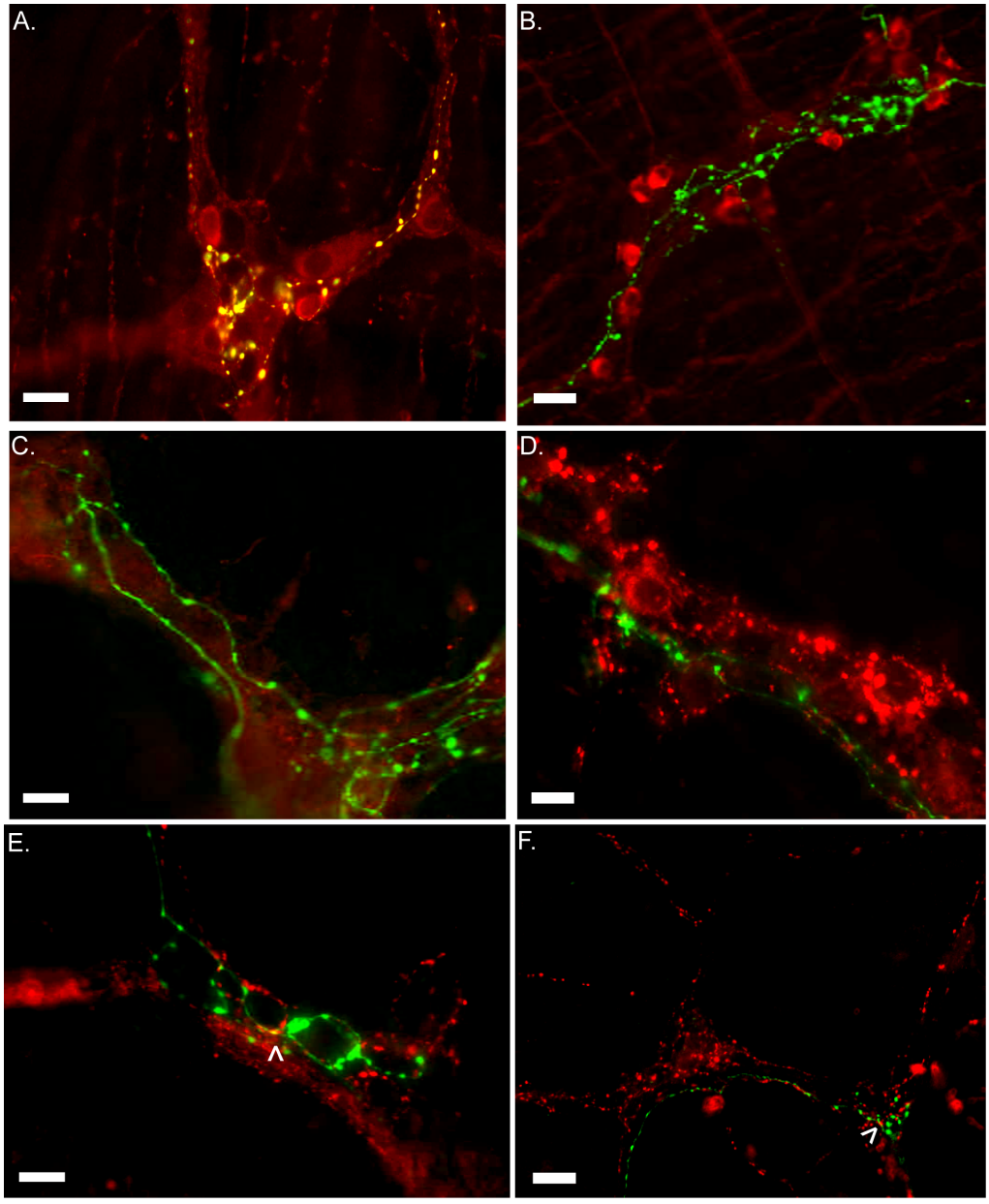

Figure 5. Vagus nerve efferent is fully cholinergic in nature. Epifluorescence images collected for the identification of the neurotransmitters (red) and dextran-labeled vagus efferent fibers and terminals (Green). Choline acetyltransferase (ChAT, A), neuronal nitric oxide synthase (nNOS, B), Tyrosine hydroxylase (TH, C), substance P (SP, D), vasoactive intestinal peptide (VIP, E) and Calcitonin gene related peptide (CGRP, F). Arrow heads point discrete co-localization between Vagus nerve and VIP or CGRP positive structures. Vagus nerve efferent fibers and terminals are only positive for ChAT and located in close proximity to ChAT and nNOS enteric neuronal bodies. Scale bars represent 20µm.

Contact between enteric neurons and macrophages

Intestinal handling is known to activate the resident macrophage network ($F4/80^{+}CD11b^{+}$) that resides in the gut muscularis. VNS applied before surgery implies that ACh released from the vagus nerve suppresses the activation of these resident macrophages ($F4/80^{+}CD11b^{+}$). Using immunohistochemistry and immunofluorescence techniques we observed a regular distribution of the resident macrophages ($F4/80^{+}CD11b^{+}$) located between the longitudinal and circular muscle layers of the small intestine (Fig. 6) with some of the macrophages located closely to the myenteric ganglia (Fig. 6A and D). The cholinergic dextran amine labeled terminals did not make contact with resident macrophages. We observed only `basket-like' endings around the cell bodies of the myenteric neurons (Fig. 6A). Fig. 6B.2 and C show the location of intestinal resident macrophages in relation to PGP 9.5 positive enteric neurons while figure 6.D shows a higher magnification of two intestinal resident macrophages in the proximity of one ChAT positive enteric ganglion.

Chemical coding of the myenteric neurons targeted by the vagus nerve

The vagal efferent terminals were found mainly close to nNOS and ChAT positive myenteric neurons (Fig. 7A and B). nNOS immunoreactive cells bodies showed an extensive co-localization with VIP (Fig. 7C) while only a few ChAT positive neurons exhibited VIP immunoreactivity (Fig. 7D). Double labeling procedures were performed to identify the neurochemical phenotype of the enteric fibers running close to the macrophages at point far from the myenteric ganglia. So VIP, ChAT and nNOS immunoreactive fibers were all found in close proximity to F4/80 positive macrophages (Fig. 8A-C). Although two commercial antibodies for α7nAChR have been successfully used to label immune cells in the rat gut or murine airway epithelium [24,25], we failed to observe specific labeling in whole mount preparations of the small intestine. The antibodies used provided the same signal in non primary controls specimens and also in tissue from $\alpha7nAChR^{-/-}$ mice (data not shown). In contrast, the fluorescent conjugate of the nicotinic receptor antagonist bungarotoxin specifically stained the resident macrophages present in the gut muscularis (Fig. 8 D). No specific staining was detected on muscle layers collected from $\alpha7nAChR^{-/-}$ mice (data not shown). Interestingly, these resident macrophages also showed immunoreactivity for the VIP receptor VPAC1 (Fig. 8E), suggesting that VIP could participate in the anti-inflammatory effect of vagus nerve stimulation.

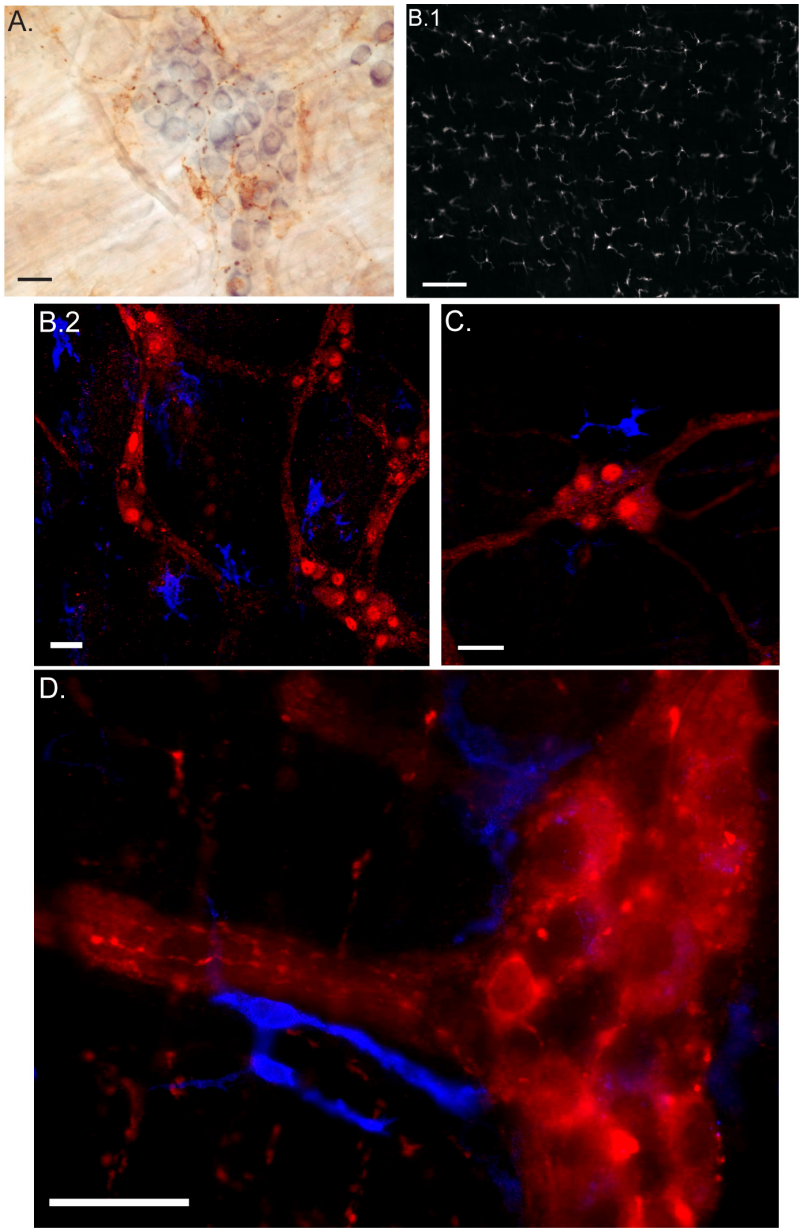

Figure 6. Intestinal resident macrophages are located in proximity to enteric neurons. A. Staining of F4/80 positive intestinal resident macrophages (brown) surrounding a myenteric ganglion (blue). Efferent vagus nerve fibers are shown in black. B.1 Regular distribution of resident macrophages (F4/80) in the muscularis of the murine small bowel. B.2 and C. Confocal image showing the distribution of the resident macrophage (F4/80, blue) close to enteric neurons (PGP9.5, red) in the muscle layers of the small intestine. D. Epifluorescence image showing the presence of resident macrophages (F4/80, blue) in close proximity to ChAT positive enteric ganglion (red). Scale bar represents 25µm, except for B.1 it represents 0.1mm.

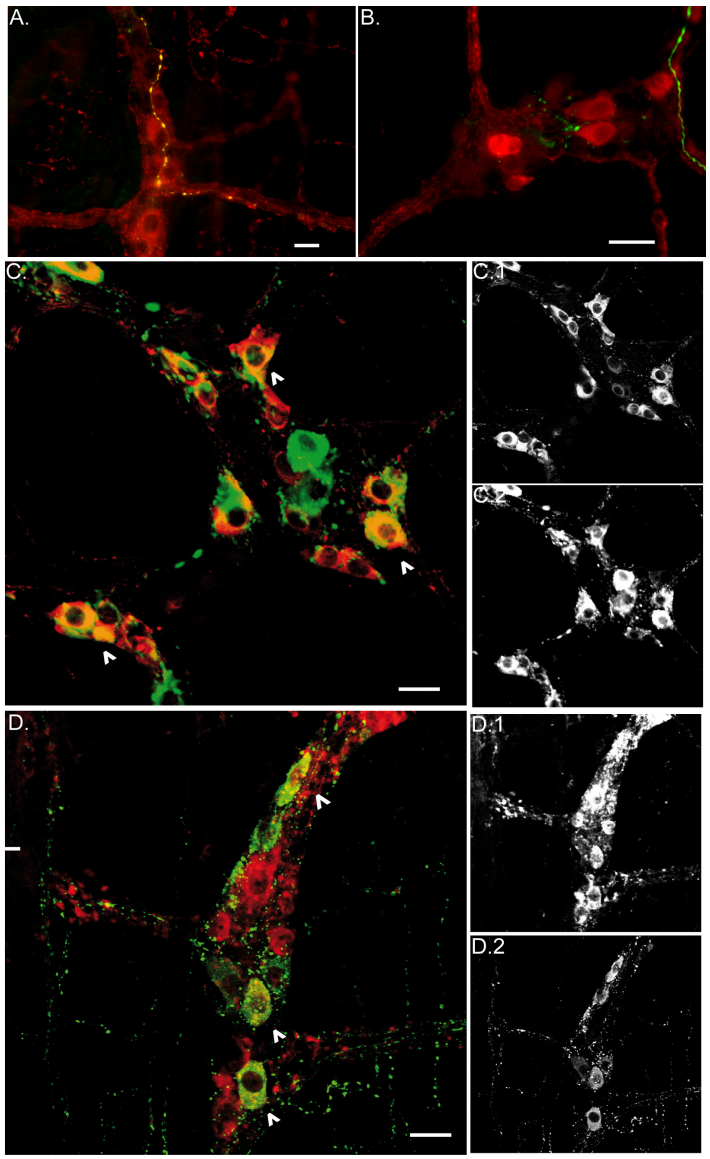

Figure 7. Vagus nerve efferent fibers and terminals are close to cholinergic and nitrergic enteric neurons. A. Epifluorescence image shows dextran-labeled vagal efferents (green) that co-localize with ChAT (yellow), and are in close contact with ChAT positive enteric neurons (red). B. Epifluorescence image shows labeled vagal efferent fibers (green) making contact with nNOS positive neurons (red). Of note, cholinergic neurons, and to lesser extent nitrergic neurons, are the main population targeted by the vagal efferent fibers. C. Confocal image of VIP (red) and nNOS (green) myenteric neurons. Most of the cells bodies exhibit co-localization of these two neurotransmitters (arrow head). D. Confocal image of VIP (red) and ChAT (green) myenteric neurons. Occasionally myenteric neurons showed immunoreactivity for both neurotransmitters (arrow head). C1 and C2 show the distribution for the nNOS and VIP positive cells bodies, respectively. D1 and D2 show the distribution for ChAT and VIP positive cells bodies. Scale bar represents 20μm.

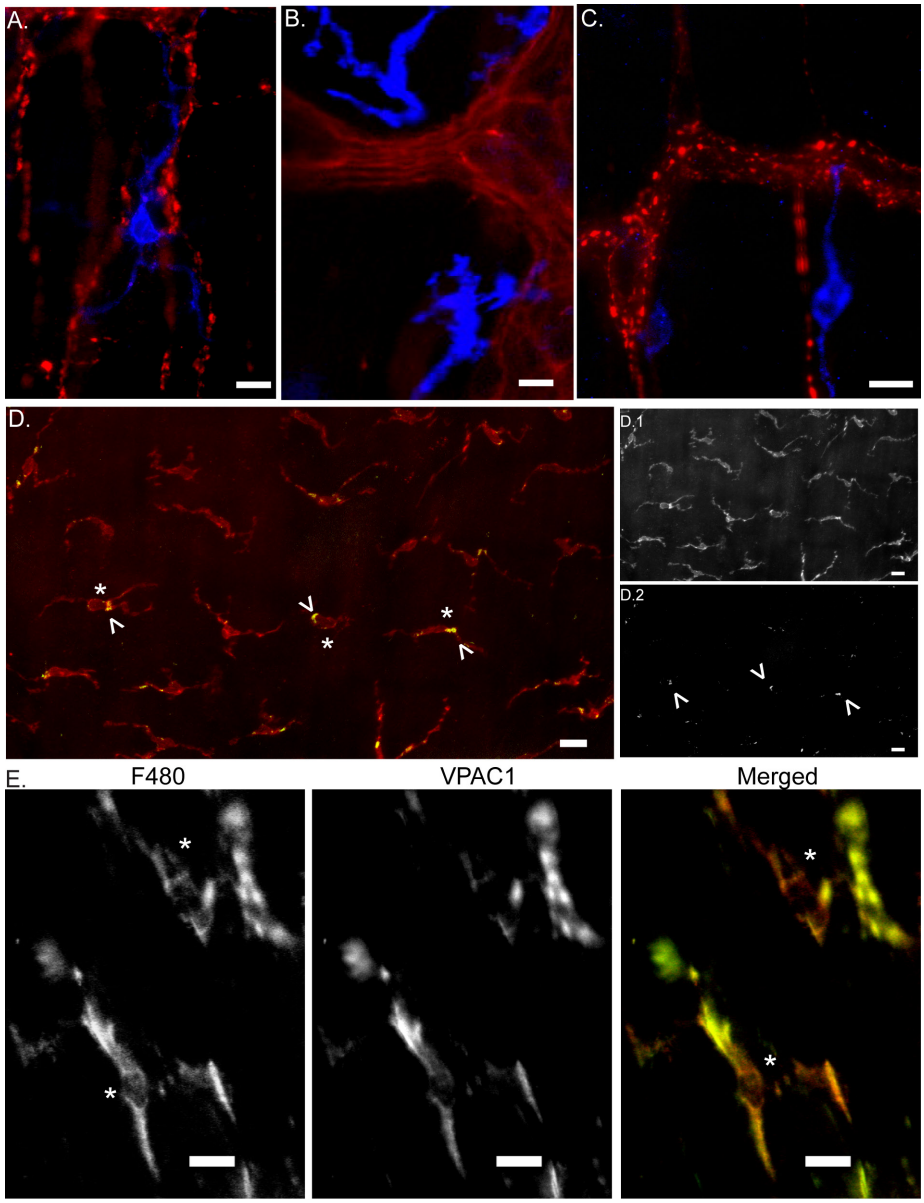

Figure 8. Neuronal fibers and intestinal resident macrophages: neurotransmitter and receptor expression. Epifluorescence images show F4/80 positive intestinal resident macrophages (blue) located in close proximity to inter-ganglionic enteric fibers positive for ChAT (A), nNOS (B) and VIP (C). D. Intestinal resident macrophages (F4/80, red, D.1) expressing α7 nicotinic receptor (green, arrow heads, D.2). E. Resident macrophages of the gut muscularis (F4/80, red) express VPAC1 receptors (green). Asterisk corresponds to intestinal resident macrophages located at the level of the myenteric plexus region. The other macrophage is located at the level of the submucosal plexus. Scale bar represents 10 µm from A-C and 20µm from D-E.

Discussion

In the present study, we show that the vagus nerve does not directly interact with resident macrophages in the intestine or spleen. In the intestine, vagal efferent fibers interact with nNOS and ChAT positive myenteric neurons, with nerve endings in close proximity to resident macrophages carrying α7nAChR. Of note, nNOS and some ChAT positive neurons co-expressed VIP, while VIP positive nerve fibers were identified in the vicinity of VPAC1 positive macrophages, suggesting that VIP could also be involved in the immuno-modulatory effect of VNS. In contrast, no evidence indicating vagal innervation, either direct or via the mesenteric ganglion was obtained for the spleen. Based on these data, we conclude that vagal modulation of the intestinal resident macrophages is indirect, most likely through cholinergic and nitrergic/VIPergic enteric neurons.

Electrical stimulation of the vagus nerve, before and after intestinal manipulation, prevents the inflammatory response triggered by intestinal handling and consequently reducing postoperative ileus [12]. This effect results from the inhibition of the resident macrophages through acetylcholine-mediated activation of nicotinic receptors. To what extent the vagus nerve directly interacts with these resident macrophages however has not been studied. Using anterograde tracing, we aimed to detect the efferent nerve fibers innervating the intestine and spleen in a mouse model. Dextran amine was the most suitable tracer due to its low toxicity (water solubility) allowing multiple injections with minimal impact on the general condition of the mice. The duration of the transport of the tracer from the source (cell body) to the terminals relies on the type of neuron and its activity. In our case, the time period of 19 days was required for the motor axonal fibers to efficiently transport the tracer to the terminals. Following this time period, dextran amine selectively labeled the motor neurons axons. Indeed, no dextran-labeled cells bodies were found in the nodose ganglia while no labeled fibers were found in the gastrointestinal tract when the injections were outside the DMV (data not shown).

Using this technique, dextran-labeled vagal efferent fibers were densely found in the small intestine at the level of the myenteric plexus located between the longitudinal and circular smooth muscle layers, but not in the submucosal compartment. In the myenteric plexus region, the vagal efferents endings were predominantly found around ChAT immunoreactive cells bodies, but could not be detected in the vicinity of resident macrophages. Instead, resident macrophages were found in close proximity to

cholinergic fibers, i.e., mainly inter-ganglionic nerve fibers. Especially as we found no cholinergic vagal efferents in the vicinity of the macrophages, our data strongly suggest that mainly cholinergic enteric neurons rather than vagal nerve fibers directly interact with the resident macrophages. In addition to cholinergic neurons, we also observed close contacts between vagal efferents and nNOS positive enteric neurons. Similar to cholinergic nerve fibers, nNOS positive nerve fibers were found in close proximity to resident macrophages, suggesting a potential role modulating macrophage function. Taken together, our data indicate that the vagus nerve does not directly interact with the resident macrophages, but most likely modulates these immune cells through cholinergic and to a lesser extent nitrergic enteric neurons.

Vagus nerve stimulation potently suppresses the inflammatory response in sepsis and improves survival. This effect has been proposed to be mediated by vagal activation of sympathetic neurons in the coeliac ganglion innervating the spleen [9,21]. Although dextran amine is a sensitive anterograde tracer to label complex brain circuits [26,27], we were unable to detect this anterograde tracer in the mesenteric ganglion or in the spleen, indicating that no vagal fibers arising from the DMV are projecting to the spleen. The lack of anterograde tracer in the spleen coincides with data recently published [28]. Using transgenic GFP-ChAT mice to visualize pre and postganglionic cholinergic neurons, only a sparse ChaT positive innervation was shown in the spleen consisting of neuronal fibers of spinal origin (sympathetic) around arterioles and in lymphocyte-containing areas of the white pulp. The absence of labeled fibers in the celiac-superior mesenteric ganglia found in our current study did not correlate with previous studies using DiI anterograde tracer [15,16]. The labeling period (19 days) for dextran amines may not be sufficient to reveal the moderate density of the vagal fibers in the ganglia previously reported [18] even though it successfully labeled the vagal efferent fiber throughout the entire gastrointestinal tract.

In the intestine, the neurons contacted by the vagus nerve are predominantly cholinergic. In the spleen, acetylcholine released by T cells is proposed to suppress splenic macrophages [10,28], most likely through the activation of the α7 nicotinic acetylcholine (α7nAChR) receptors [1]. In the intestine, we collected evidence that this receptor is located on resident macrophages and mediates the anti-inflammatory effect of vagus nerve stimulation in a model of postoperative ileus ([29] in press). In the present study, we confirm these data by immunohistochemistry. The use of

the two commercially available antibodies for α7nAChR provides a similar staining pattern of α7nAChR on muscle layers as previously reported [30,31]. However, these antibodies exhibit similar results in α7 knock out mice, indicating that these antibodies lack specificity for α7nAChR. In contrast, using bungarotoxin staining, we indeed revealed the presence of α7nAChR (only) on resident macrophages. Interestingly, we also demonstrated close interaction between vagal efferents and nitrergic neurons co-expressing VIP. These NO/VIP positive neuronal fibers were found in close proximity to resident macrophages that express VPAC1 receptors suggesting that not only ACh, but also VIP and NO may modulate the function of resident intestinal macrophages [32,33].

In summary, no evidence supporting vagal or cholinergic innervation of the spleen could be provided. However, we collected neuro-anatomical evidence that the vagal modulation of intestinal resident macrophages is indirect and mainly involves cholinergic enteric neurons. Based on these data, we speculate that the cholinergic anti-inflammatory input to the intestine is mediated and thereby amplified by the enteric nervous system.

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Chapter 3

The spleen responds to intestinal manipulation but does not participate in the inflammatory response in a mouse model of postoperative ileus

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Abstract

Background: Postoperative ileus is characterized by a transient impairment of the gastrointestinal motility after abdominal surgery. The intestinal inflammation, triggered by handling of the intestine, is the main factor responsible for the prolonged dysmotility of the gastrointestinal tract. Secondary lymphoid organs of the intestine were identified as essential components in the dissemination of inflammation to the entire gastrointestinal tract also called field effect. The involvement of the spleen, however, remains unclear. Aim: In this study, we investigated whether the spleen responds to manipulation of the intestine and participates in the intestinal inflammation underlying postoperative ileus. Methods: Mice underwent Laparotomy (L) or Laparotomy followed by Intestinal Manipulation (IM). Twenty-four hours later, intestinal and colonic inflammation was assessed by QPCR and measurement of the intestinal transit was performed. Analysis of homeostatic chemokines in the spleen was performed by QPCR and splenic cell populations analyzed by Flow Cytometry. Blockade of the egress of cells from the spleen was performed by administration of the Sphingosine-1-phosphate receptor 1 ($S1P_1$) agonist CYM-5442 10h after L/IM. Results: A significant decrease in splenic weight and cellularity was observed in IM mice 24h post-surgery, a phenomenon associated with a decreased splenic expression level of the homeostatic chemokine CCL19. Splenic denervation restored the expression of CCL19 and partially prevented the reduction of splenocytes in IM mice. Treatment with CYM-5442 prevented the egress of splenocytes but did not ameliorate the intestinal inflammation underlying postoperative ileus. Conclusion: Intestinal manipulation results in two distinct phenomena: local intestinal inflammation and a decrease in splenic cellularity. The splenic response relies on an alteration of cell trafficking in the spleen and is partially regulated by the splenic nerve. The spleen however does not participate in the intestinal inflammation during POI.

Introduction

The vast majority of patients undergoing open abdominal surgery will develop postoperative ileus (POI). POI is characterized by a transient impairment of the gastrointestinal tract leading to pain and discomfort for the patient as well as increased hospitalization costs [1-3]. The pathophysiology of POI relies on an inflammatory process taking place in the gut muscularis in which the activation of resident macrophages [4,5] plays an important role. The release of pro-inflammatory cytokines such as IL-1β and IL-6 by these activated innate immune cells leads to the recruitment of leukocytes, namely neutrophils and monocytes to the gut muscularis. In turn, infiltrating leukocytes and activated resident macrophages secrete iNOS, Cox-2 and prostaglandins which are largely involved in the impairment of the gastrointestinal motility [6].

In POI, the paralysis of the gastrointestinal tract is not restricted to manipulated parts. Indeed, both the stomach and the colon are affected [7], a mechanism partially explained by the activation of neural inhibitory pathways by the local inflammation occurring in the small intestine [8]. A dissemination of the inflammation to unmanipulated parts of the gut was shown to also account for the generalized hypomotility, also referred to as “field-effect”. Enhanced pro-inflammatory cytokine and enzyme levels (i.e., IL-6, Cox2) as well as infiltration of leukocytes are observed in the colon after manipulation of the small intestine [9]. Recently, a crucial role for Th1 cells was unraveled in the dissemination of POI to the entire intestinal tract as intestinal manipulation leads to the activation of Th1 cells capable of migrating from the manipulated small intestine to the unmanipulated colon [10]. Secretion of IFNγ by these activated Th1 cells in turn triggers the activation of colonic macrophages, showing that both the adaptive and innate compartments are involved in the generalization of the ileus.

The origin of immune cells infiltrating the gut muscularis during POI remains largely unknown. However, gut associated secondary lymphoid organs were recently shown to play a role in the dissemination of the inflammation as the absence of mesenteric lymph nodes (MLN) and Peyer’s patches completely abolished colonic inflammation after manipulation of the small intestine [11]. Interestingly, in other acute inflammation models namely ischemic myocardial injury, stroke and peritonitis, the population of immune cells reaching the site of inflammation (i.e., monocytes, T cells, NK cells) was shown to be released from another secondary lymphoid organ, the spleen [12-

15]. In septic peritonitis, migration of $Ly6G^{+}CD11b^{+}$ splenic monocytes to the gut was associated with enhanced bacterial clearance and improved survival showing that the spleen can act as a cell reservoir during intestinal inflammation [15].

In light of the role of intestinal secondary lymphoid compartments in the local intestinal inflammatory process and the active role of the spleen reported during acute inflammation, we investigated whether the spleen responded to intestinal manipulation and was involved in modulating the intestinal muscular inflammation and in the pathogenesis of POI.

Material and methods

Ethical statement

All experiments were performed in accordance with the guidelines of the Laboratory Animal Use of the Netherlands and approved by the Ethical Animal Research Committee of the Academic Medical Center of Amsterdam (Protocol number: DMO 101319, DMO102498, DMO102688). All experiments were performed under fentanyl-fluanisone-midazolam (FFM) or ketamine-medetomidine-atropine (KMA) anesthesia and all efforts were made to minimize the suffering of the animals.

Mice

Ten to 12 week-old female Balb/c were purchased from Harlan Nederland (Horst, The Netherlands) and housed with a 12/12 light/dark with *ad libitum* food and water.

Surgical procedures and sample collection

Laparotomy and Intestinal Manipulation

Mice underwent Laparotomy (L) or Laparotomy followed by Intestinal Manipulation (IM) as described previously [16]. The peritoneum was opened by a midline abdominal incision and the small bowel was carefully removed from the peritoneal cavity and placed on a moist gauze pad. The entire small bowel was manipulated twice from the distal duodenum to the cecum with moist cotton applicators. Contact or stretch of the stomach or colon was avoided. Surgical procedures were performed under sterile conditions. Animals were sacrificed 24h after L/IM.

Treatment with CYM-5442 (0.7 mg/kg) (Sigma, St Louis, MO) or vehicle injection (2% DMSO, 2% Tween20 in water) was applied i.p. 10h after surgical procedure (Laparatomy or Laparotomy followed by Intestinal Manipulation). The injection time point was chosen according to our data showing that the loss of splenocytes begins 12h after IM.

Spleen denervation

Surgical removal of the splenic nerve (Sx) or Sham operation (Sham) was performed as previously described. Briefly, the abdominal cavity was opened through a midline incision. Blood vessels irrigating the spleen were exposed and nerve bundles running along those vessels [17] were removed just before and after the first branching point of the arterial supply to the spleen using micro-surgery instruments. Two weeks later, mice underwent L or IM. This led to 4 experimental groups: *Sham L* mice underwent first sham operation and 2 weeks later laparotomy; *Sham IM* mice first underwent sham operation and 2 weeks later laparotomy followed by intestinal manipulation; *Sx L* mice first underwent spleen denervation and 2 weeks later laparotomy; *Sx IM* mice first underwent spleen denervation and 2 weeks later laparotomy followed by intestinal manipulation. Tyrosine hydroxylase staining was performed to validate the completion of the denervation.

Sacrifice and sample collection

Mice were anesthetized and blood was collected by cardiac puncture. Spleens and mesenteric lymph nodes (MLN) were placed in cold RPMI1640 medium (Gibco, Bleiswijk, the Netherlands) for flow cytometry analysis or snap-frozen. The entire gastrointestinal tract was placed in ice-cold oxygenated PBS (Fresenius Kabi, the Netherlands). For transcript analysis, after stripping away both mucosal and submucosal layers, colonic and intestinal muscularis segments were snap-frozen in liquid nitrogen. For myeloperoxidase staining, 2 intestinal segments and the entire colon were placed in ice-cold absolute ethanol for 30 minutes and then stored in ice-cold 70% ethanol until further use.

Measurement of the gastrointestinal transit

The gastrointestinal transit was measured using the non-absorbable tracer 70 kDa

fluorescein isothiocyanate-labeled dextran (FD70) as previously described [18]. Briefly, mice were fed with 10 µL of FD70 in distilled water (6.25 mg/mL). Animals were sacrificed 90 minutes later and their entire gastrointestinal tract was placed in an oxygenated ice-cold PBS solution (Fresenius Kabi, the Netherlands) and divided into 15 segments (stomach, 10 segments of equal length for the intestine, cecum, 3 segments of equal length for the colon). FD70 concentration was assessed by fluorimetry in the supernatant of each segment. The distribution of FD70 was determined by calculation of the geometric center (GC) with GC=Σ(% of total fluorescent signal per segment x segment number)/100).

Myeloperoxidase quantification by Immunohistochemistry

Muscularis of intestinal and colonic segments were stained for myeloperoxidase (MPO) as a marker of leukocytic infiltration. Briefly, whole mount intestinal or colonic muscularis segments were stained with a 3-amino-9-ethyl carbazole (Sigma, St Louis, MO), 0.01% H_2O_2 in Sodium Acetate buffer (pH=5) for 20 min, as previously described [16]. Sections were analyzed using a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives) connected to a color-camera (JVC KY-F55 3CCD). Random counting of MPO positive cells was performed for each section, as previously described [16].

RNA isolation, cDNA synthesis and QPCR

Total mRNAs from intestinal or colonic muscularis and spleens were extracted after homogenization of the samples in TriPure isolation reagent according to the manufacturer's instructions (Roche Applied Science). cDNA synthesis was performed using the Revertaid first strand cDNA synthesis kit (Fermentas) and Real-time PCR was performed using a SYBR green master mix (Roche Applied Science) on a Lightcycler 480 (Roche Applied Science). The primers used (synthesized by Invitrogen, Bleiswijk, The Netherlands) are described in Table 1. Analysis was performed using the LinRegPCR program (AMC, Amsterdam, The Netherlands) [19]. The target gene expression was normalized over the expression of 2 reference genes. All data are expressed in AU and represent relative expression over the control group (i.e., L/Sham L).

Spleen and MLN digestion and quantification

Spleens and MLN were incubated in a solution of DNaseI and Liberase TL (Roche Applied Science) in RPMI 1640 medium (Gibco, Bleiswijk, The Netherlands) or PBS respectively at 37° C for 20 min and then mechanically homogenized on a 70 µm cell strainer to obtain a single-cell suspension. The spleen and MLN cell count was determined after lysing a fraction of the cell suspension and quantified using a Coulter counter. Erythrocytes, devoid of nucleus, were therefore excluded from the count.

FACS analysis

Spleen single-cell suspensions were incubated with a Fc-receptor blocking antibody 2.4G2 (kindly provided by Dr. Louis Boon, Bioceros BV, Utrecht, The Netherlands) and the percentage of spleen cell populations (macrophages, B cells, Dendritic cells, T cells, monocytes) were determined using the following antibodies: F4/80 (BM8) (Invitrogen, Bleiswijk, The Netherlands), CD45R (B220) (eBioscience, Vienna, Austria), CD11c (HL3) (BD Biosciences), CD4 (GK1.5) (eBioscience, Vienna, Austria), CD8α (53-6.7) (eBioscience, Vienna, Austria), CD3e (145-2C11) (eBioscience, Vienna, Austria), Lineage (Lin) (B220, NK1.1, CD90, CD49. Ly6G) (ebioscience, Vienna, Austria) CD45.1 (ebioscience, Vienna, Austria). Samples were analyzed with a LSR Fortessa II (Beckman Coulter) and the FlowJo software (Tree Star Inc., Ashland, The United States). The different spleen cell populations were defined as follow: DCs ($CD11c^{+}MHCII^{+}$), macrophages ($F4/80^{+}$), B cells ($CD45R^{+}MHCII^{+}$), $CD4^{+}$ ($CD3^{+}CD8^{-}CD4^{+}$), $CD8^{+}$T cells ($CD3^{+}CD8^{+}CD4^{-}$) and monocytes ($CD45^{+}Lin^{-}F4/80^{-}CD11c^{-}MHCII^{-}CD11b^{+}Ly\text{-}6C^{+}$). Percentages were reported to the total number of splenocytes of each mouse to calculate the number of cells per population (Fig. S2 and S3).

Cell death quantification

Apoptotic cell death was assessed using a TUNEL assay (In Situ Cell Death Detection Fluorescein kit; Roche Applied Science). Briefly, spleen sections were post-fixed in PBS PFA 4% and incubated with the Tdt enzyme diluted in digoxigenin-dUTP reaction buffer (TUNEL) for 1h at 37º C. Sections were analyzed with a Leica fluorescence microscope and positive cells were quantified using the ImageJ software. The density of positive cells was determined as the ratio of positive cells over the area of the section.

In addition, necrotic and apoptotic cell death was determined by FACS. Spleen cell

suspensions were stained with a fixable viablility Dye (eBioscience, Vienna, Austria) and Annexin V (eBioscience, Vienna, Austria) and analyzed using a LSR Fortessa II and the FlowJo software (Tree Star Inc., Ashland, The United States).

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc, Chicago, IL). Data are expressed as mean±SEM. Normal distribution was assessed using the Kolmogorov-Smirnov test. Square-root normalization was applied to non-normal data sets. Whenever two groups of data were compared (i.e., L vs IM), a Student t-test was performed. Whenever the influence of 2 independent variables (i.e., L/IM and denervation or L/IM and CYM-5442 treatment) was analyzed, a two-way ANOVA was performed to determine the interaction between denervation (Sham *vs* Sx) and treatment (L *vs* IM). When significance was observed (i.e., $p<0.05$) an unpaired Student t-test was performed to evaluate the significance between Sham *vs* Sx or L *vs* IM.

	Forward primer 5'-3	Reverse primer 5'-3
HRPT	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Cyclophilin	ACCCATCAAACCATTCCTTCTGTA	TGAGGAAAATATGGAACCCAAAGA
Ubiquitin	AGCCCAGTGTTACCACCAAG	ACCCAAGAACAAGCACAAGG
IL-1β	CTCCTGCTGTCGGACCCAT	TGCCGTCTTTCATTACACAGGA
IL-6	GAGTTGTGCAATGGCAATTCTG	TGGTAGCATCCATCATTTCTTTGT
TNF-α	TGGAACTGGCAGAAGAGGCACT	CCATAGAACTGATGAGAGGGAGGC
CCL19	ATGCGAAGACTGCTGCC	AGCGGAAGGCTTTCACGAT
CCL21	GCTGCAAGAGAACTGAACAGACA	CGTGAACCACCCAGCTTGA
CXCL13	CATAGATVGGATTCAAGTTACGCC	TCTTGGTCCAGACACAACTTCA

Table1. Primer sequences for analysis of mouse intestine and spleen samples

Results

Inflammation of the gastrointestinal tract 24h after intestinal manipulation

As previously described, intestinal manipulation increased the expression levels of pro-inflammatory cytokines such as IL-1β (L *vs* IM: 1.00±0.24 *vs* 11.12±1.90; $p<0.0001$), IL-6 (L *vs* IM: 1.00±0.16 *vs* 3.28±1.06) and TNF-α (L *vs* IM: 1.00±0.16 *vs* 6.18±1.31; $p=0.0002$) in the small intestine (Fig. S1A and 1). An increase in the number of MPO^+ cells in the muscularis of the small intestine (14.60±1.60 *vs* 81.20±13.7; $p=0.011$) (S1B) was also

observed in manipulated mice as well as a decrease in the GC values of the gastrointestinal transit (L *vs* IM: 10.43±0.55 *vs* 4.50±0.38; p<0.0001) (Fig. S1C). As previously reported [8,20], we did not observe any influx of MPO^+ cells in the colonic muscularis after IM (data not shown). Low transcript levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were measured in the colonic muscularis and no increase in their expression was seen 24h after IM (Fig. 1).

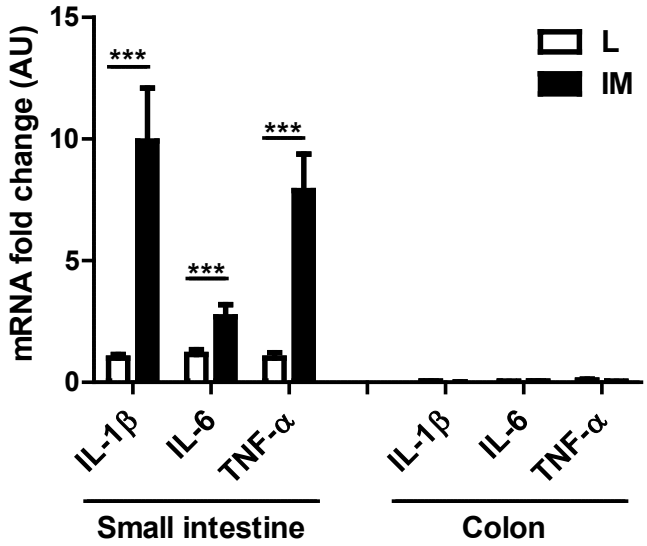

Figure 1. Intestinal manipulation triggers inflammation in the small intestinal muscularis but not in the colonic muscularis. Animals were sacrificed 24 after Laparotomy (L)/ Intestinal Manipulation (IM). Mice undergoing L or IM display very low levels of IL-1β, IL-6 and TNF-α in their colonic muscularis compared to the levels observed in the muscularis of the small intestine. IM leads to increased mRNA levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the small intestinal but not the colonic muscularis. Data shown are mean±SEM of 3 independent experiments (n=8-10 animals per group). *** p<0.001 (Student t-test).

Secondary lymphoid organs 24h after intestinal manipulation

Twenty four hours after surgery, no significant difference in the MLN weight and cellularity was observed between the L and IM groups (weight: 20.73±2.00 *vs* 22.00±1.98 for L and IM mice respectively; p=0.66; cell count: 19.72±2.62 *vs* 20.11±2.79 for L and IM mice respectively; p=0.92) (Fig. 2A). The spleen however displayed a decrease in weight in IM compared to L mice (L *vs* IM: 129.10±10.51 *vs* 94.54±4.77; p=0.003). This weight loss was associated with a 25% decrease in the number of splenocytes (L *vs* IM: 133.90±9.69 *vs* 100.40±6.72; p=0.008) (Fig. 2B).

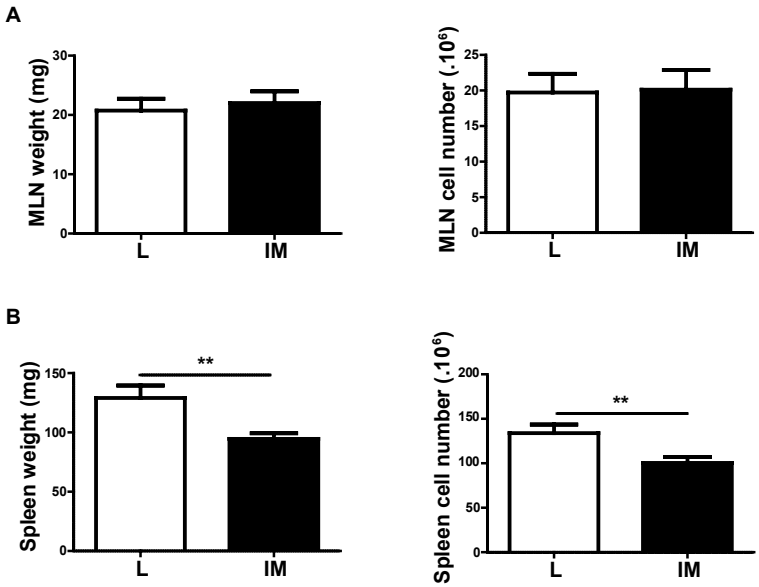

Figure 2. **The spleen but not the MLN responds to Intestinal manipulation 24h after surgery.** (A) IM leads to a significant decrease in spleen weight and splenocyte number (B) but doesn't affect the weight or cell number in MLN. Data shown are mean±SEM of 3 independent experiments (n=12 animals per group). ** p<0.01 (Student t-test).

Necrotic and apoptotic cell death are not increased in the spleen upon intestinal manipulation

Stress related to surgical intervention can trigger a loss of splenocytes due to cell death [21]. We therefore quantified splenic cell death 24h after surgery. No significant difference was observed in the density of cells in late apoptosis between L and IM mice (0.05±0.010.03±0.01; p=0.2) (Fig. 3A). Similarly, the percentage of necrotic and apoptotic cells did not significantly differ between the L and IM groups (Necrosis: 3.56±0.66 vs 3.12±0.73; Apoptosis: 11.74±0.98 *vs* 13.30±1.56) (Fig. 3B).

Cell trafficking is altered in the spleen in response to intestinal manipulation

To investigate further the mechanism underlying the reduction in splenocytes, we analyzed the expression levels of homeostatic chemokines involved in the retention/attraction of cells to the spleen. No significant difference in the expression levels of CXCL13 or CCL21 was observed between L and IM mice. CCL19 mRNA level was however significantly lower in IM mice compared to L mice (L *vs* IM: 1.00±0.13 *vs* 0.45±0.08; p=0.002) (Fig. 4A). To determine whether the decrease in splenocytes was due to an

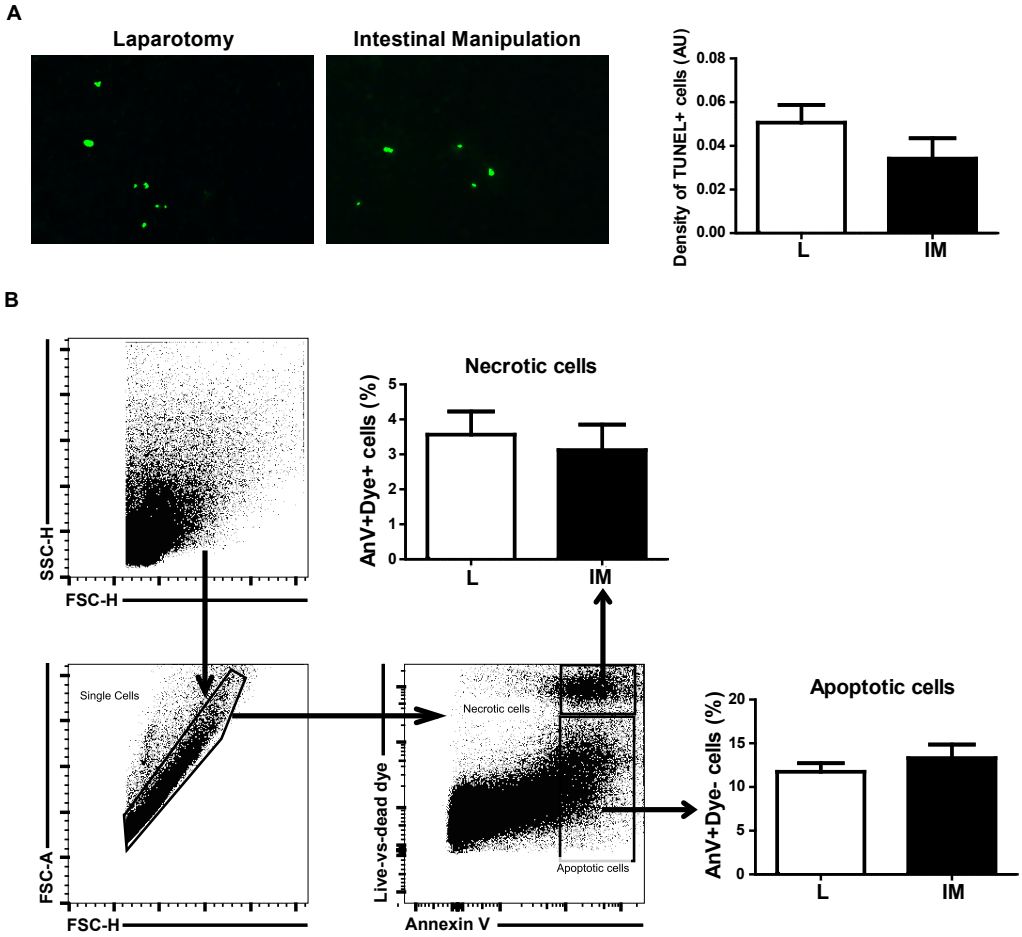

Figure 3. Intestinal manipulation does not increase splenocyte cell death. A. Late apoptosis was determined in the spleen by a TUNEL immunofluorescent assay 24h after IM. No difference was observed in the density of apoptotic cells (in green) between L and IM groups. B. Necrotic (AnnexinV$^+$ Live/dead marker (Dye)$^+$) and apoptotic cell death (AnnexinV$^+$ Live/dead marker (Dye)$^-$) in the spleen were quantified by FACS analysis 24h after IM. The percentage of necrotic and apoptotic cell death remains unchanged between L and IM mice. Data shown are mean±SEM of 2 independent experiments (n=4 animals per group) (Student t-test).

active departure of cells, the Sphingosine-1-phosphate receptor 1 ($S1P_1$) agonist CYM-5442 [22] was injected 10h after the surgical procedure. CYM-5442 treatment abolished the decrease in the number of splenocytes observed 24h after IM (Fig. 4B).

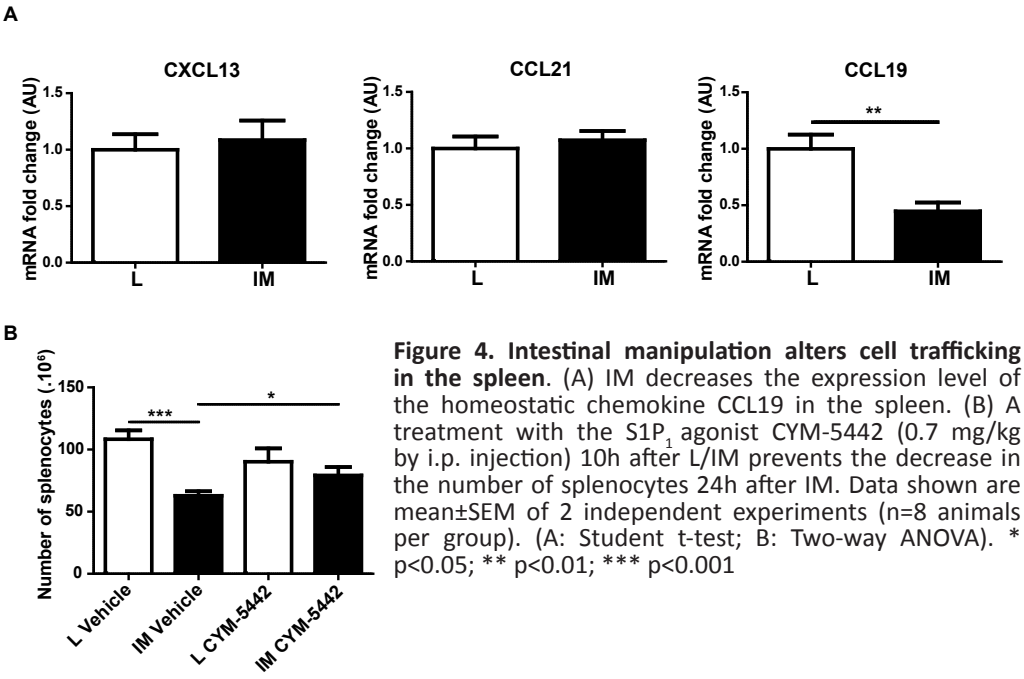

Figure 4. Intestinal manipulation alters cell trafficking in the spleen. (A) IM decreases the expression level of the homeostatic chemokine CCL19 in the spleen. (B) A treatment with the $S1P_1$ agonist CYM-5442 (0.7 mg/kg by i.p. injection) 10h after L/IM prevents the decrease in the number of splenocytes 24h after IM. Data shown are mean±SEM of 2 independent experiments (n=8 animals per group). (A: Student t-test; B: Two-way ANOVA). * p<0.05; ** p<0.01; *** p<0.001

Splenic innervation participates in the regulation of the egress of cells after IM

We next addressed the role of the splenic nerve in mediating the egress of splenocytes by surgically lesioning the splenic nerve. A 25% decrease in the number of splenocytes was again observed after IM (L *vs* IM: $164.90x10^6$±$13.94x10^6$ *vs* $120.60x10^6$±$10.10x10^6$; p=0.018) (Fig. 5A) but IM did not induce a significant decrease in the splenocyte number in mice lacking splenic innervation (Sx L *vs* Sx IM: $154.90x10^6$±$10.24x10^6$ *vs* $137.20x10^6$±$10.74x10^6$; p=0.25). Interestingly, removal of splenic innervation prevented the decrease of CCL19 expression observed in sham-operated mice after intestinal manipulation (Sham L vs Sham IM: 1.00±0.13 vs 0.46±0.06; p=0.003; Sx L vs Sx IM: 1.16±0.17 vs 0.89±0.11; p=0.21; Sham IM vs Sx IM: 0.46±0.06 vs 0.89±0.11; p=0.002) (Fig. 5B).

The number of $CD4^+$ T cells and monocytes in the spleen is decreased 24h after intestinal manipulation

We then aimed to identify which population of cells was released by the spleen after intestinal manipulation by analyzing the different spleen cell populations 24h after IM. A significant decrease in the number of $CD4^+$ T cells was observed 24h after IM

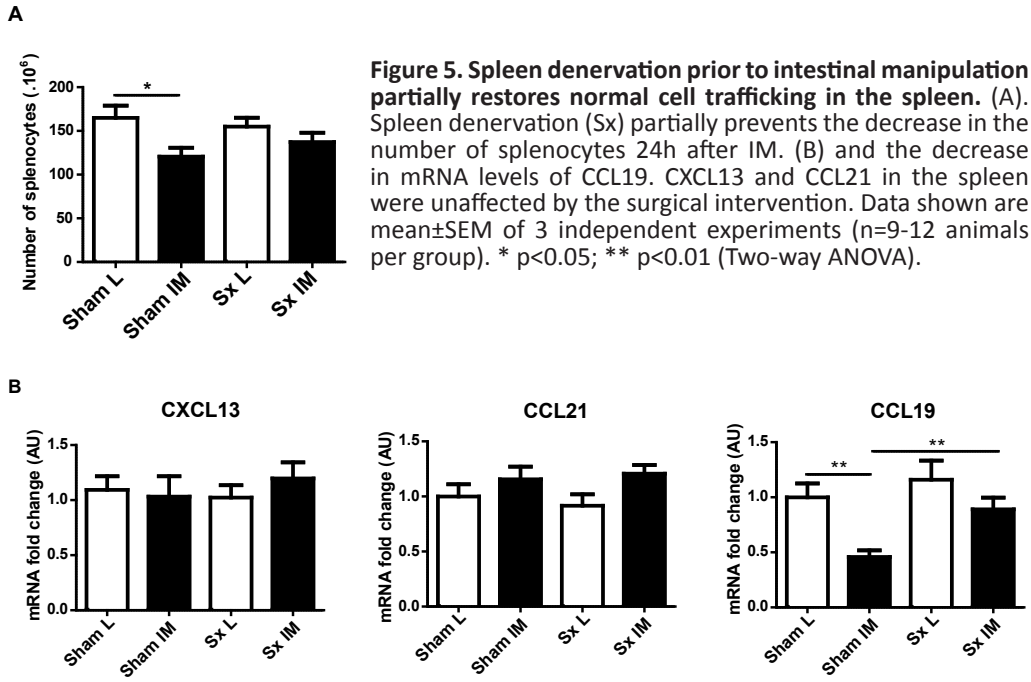

Figure 5. Spleen denervation prior to intestinal manipulation partially restores normal cell trafficking in the spleen. (A). Spleen denervation (Sx) partially prevents the decrease in the number of splenocytes 24h after IM. (B) and the decrease in mRNA levels of CCL19. CXCL13 and CCL21 in the spleen were unaffected by the surgical intervention. Data shown are mean±SEM of 3 independent experiments (n=9-12 animals per group). * p<0.05; ** p<0.01 (Two-way ANOVA).

(L *vs* IM: $20.54x10^6$±$1.96x10^6$ *vs* $15.23x10^6$±$1.24x10^6$; p=0.038) (Fig. 6A, S2 and S3). Similarly, the total number of monocytes was significantly decreased after IM (L *vs* IM: $5.24x10^6$±$0.49x10^6$ *vs* $3.38x10^6$±$0.59x10^6$; p=0.02). No significant decrease was observed in other splenic populations (i.e., DCs, macrophages, $CD8^+$T cells, B cells). No change in the percentage and number of cells of the different populations measured in the MLN was observed between L and IM mice (Fig. 6B, S2 and S3).

No influx of T cells in the intestinal or colonic muscularis 24h post-IM

Next, we analyzed the cell composition in the intestinal and colonic muscularis 24 after L/IM. As previously described, immune cells infiltrating the muscularis of the small intestine 24h after IM were mainly composed of neutrophils and monocytes (monocytes: $0.04x10^6$±$0.01x10^6$ *vs* $2.04x10^6$±$0.21x10^6$ for L and IM respectively; p<0.0001; neutrophils: $0.00x10^6$±$0.00x10^6$ *vs* $1.58x10^6$±$0.11x10^6$ for L and IM respectively; p<0.0001) (Fig. 7A). The number of monocytes and neutrophils found in the colonic muscularis 24h after IM were neglectable compared to the number of cells observed in the small intestinal muscularis (monocytes: $2.04x10^6$±$0.21x10^6$ *vs* $0.04x10^6$±$0.03x10^6$ for small intestine and colon respectively; neutrophils: $1.58x10^6$ *vs*

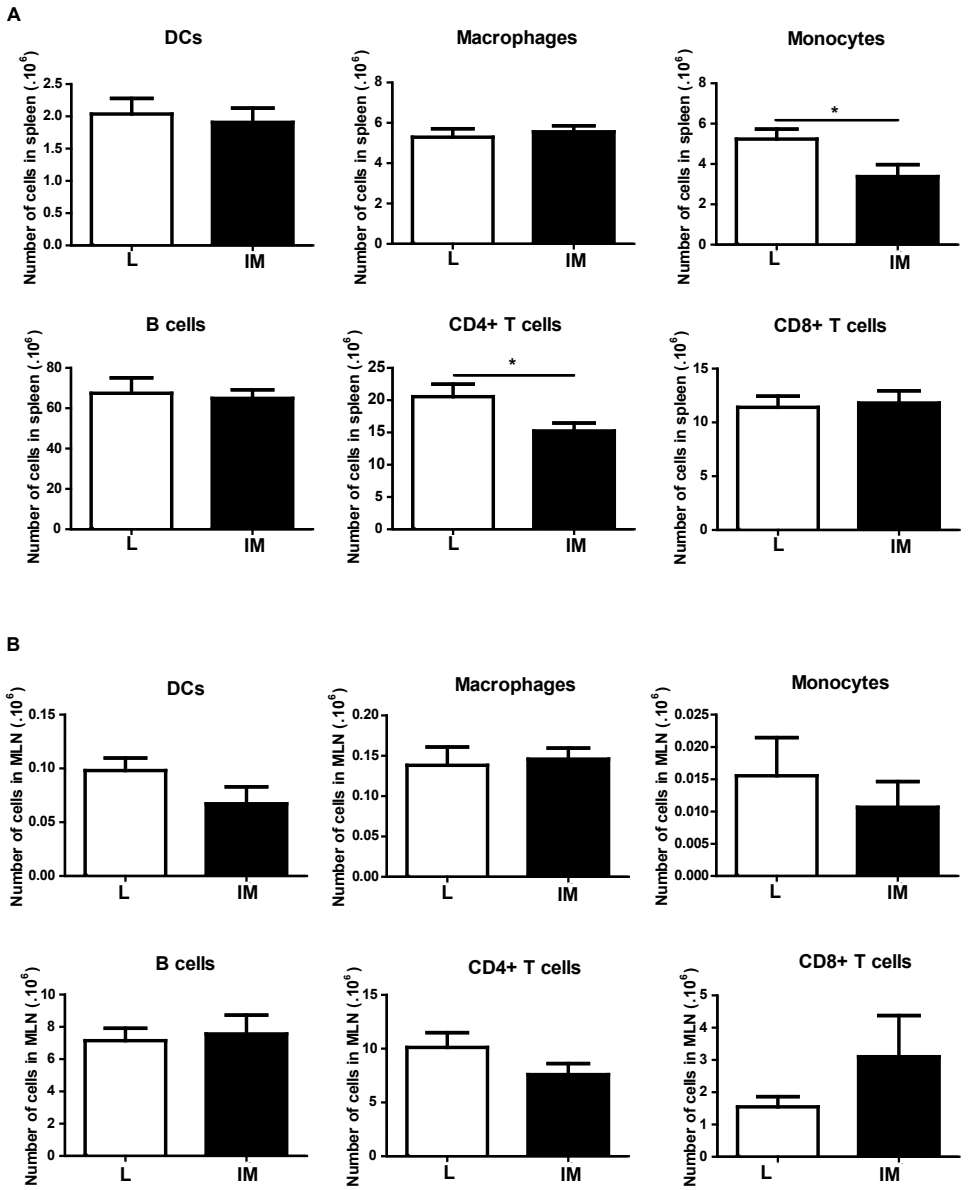

Figure 6. Intestinal Manipulation leads to decreased populations in the spleen but does not affect the MLN. (A). The number of DCs, macrophages, B cells and CD8 T cells is not significantly altered 24h after IM in the spleen whereas the number of CD4 T cells and monocytes is significantly decreased. (B). The number of cells of all population measured remains identical in the MLN after L and IM. Data shown are mean±SEM of 2 independent experiments (n=8-9 animals per group). * $p<0.05$ (Student t-test).

$0.09x10^6 \pm 0.06x10^6$ for small intestine and colon respectively). Moreover no significant difference in the number of these 2 populations was observed in the colon after IM (monocytes: $0.01x10^6 \pm 0.00x10^6$ *vs* $0.04x10^6 \pm 0.03x10^6$ for L and IM respectively; p=0.21; neutrophils: $0.00x10^6 \pm 0.00x10^6$ *vs* $0.09x10^6 \pm 0.06x10^6$ for L and IM respectively; p=0.17).

Finally, a very low number of $CD4^+$ T cells was observed in the intestinal and colonic muscularis in L animals (1297.00±431.80 *vs* 1211.00±234.20 for small intestine and colon respectively). No influx of $CD4^+$ T cells was observed 24h after IM either in the intestinal (1297.00±431.80 *vs* 2000.00±512.40 for L and IM respectively; p=0.32) or in the colonic muscularis (1211.00±234.20 *vs* 1243.00±263.40 for L and IM respectively; p=0.93) (Fig. 7B).

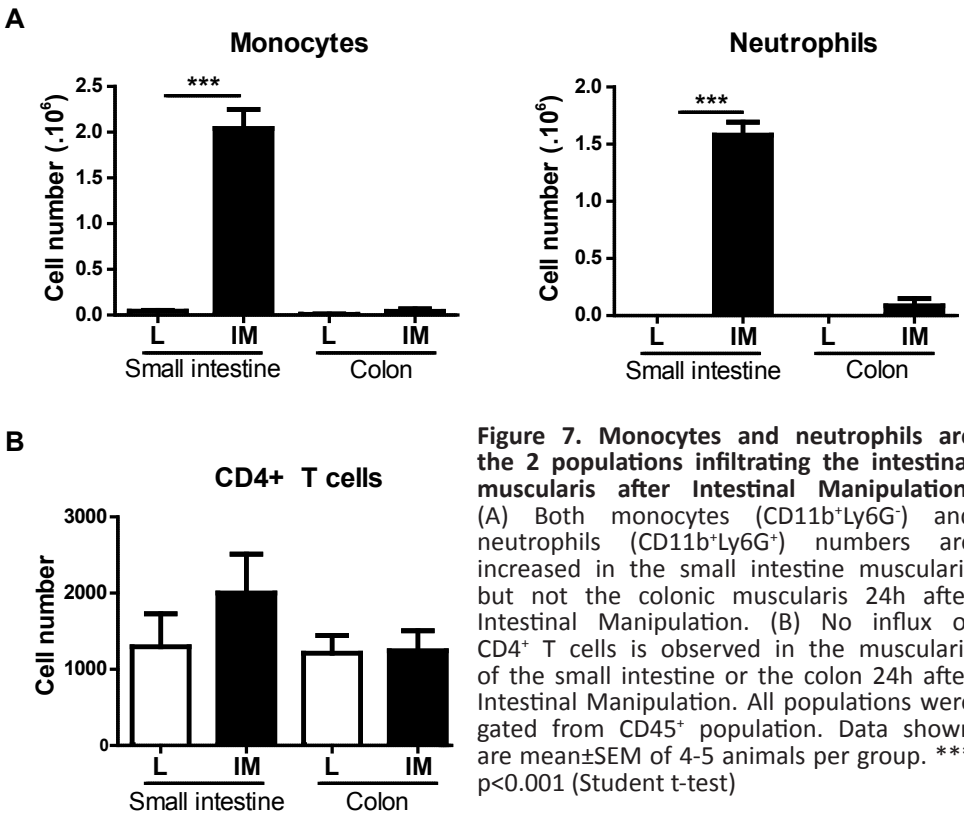

Figure 7. Monocytes and neutrophils are the 2 populations infiltrating the intestinal muscularis after Intestinal Manipulation. (A) Both monocytes ($CD11b^+Ly6G^-$) and neutrophils ($CD11b^+Ly6G^+$) numbers are increased in the small intestine muscularis but not the colonic muscularis 24h after Intestinal Manipulation. (B) No influx of $CD4^+$ T cells is observed in the muscularis of the small intestine or the colon 24h after Intestinal Manipulation. All populations were gated from $CD45^+$ population. Data shown are mean±SEM of 4-5 animals per group. *** p<0.001 (Student t-test)

The spleen does not participate in the intestinal immune response 24h post-IM

To assess whether the egressing splenocytes contributed to the intestinal immune response, we analyzed the inflammatory state of the intestinal muscularis in mice treated with CYM-5442. Blocking the egress of splenocytes did not affect the level of the pro-inflammatory cytokines IL-6 (21.07±13.32 *vs* 23.68±10.22 for vehicle IM and CYM-5442 IM respectively; p=0.88), IL-1β (18.51±7.96 *vs* 19.74±8.01 for vehicle IM and CYM-5442 IM respectively; p=0.92) or TNF-α (9.40±2.17 *vs* 9.56±3.84 for vehicle IM and CYM-5442 IM respectively; p=0.97) (Fig. 8A) in the intestinal muscularis. The influx of cells was not significantly different between vehicle- and CYM-5442-treated mice undergoing IM as shown by the MPO^+ cell count in the small intestinal muscularis (68.91±8.49 *vs* 70.81±7.61; p=0.96) (Fig. 8B). The delay in gastrointestinal transit observed 24h after IM was identical in both vehicle- and CYM-5442-treated mice (4.83±0.50 *vs* 4.39±0.18 for vehicle IM and CYM-5442 IM respectively; p=0.37) (Fig. 8C).

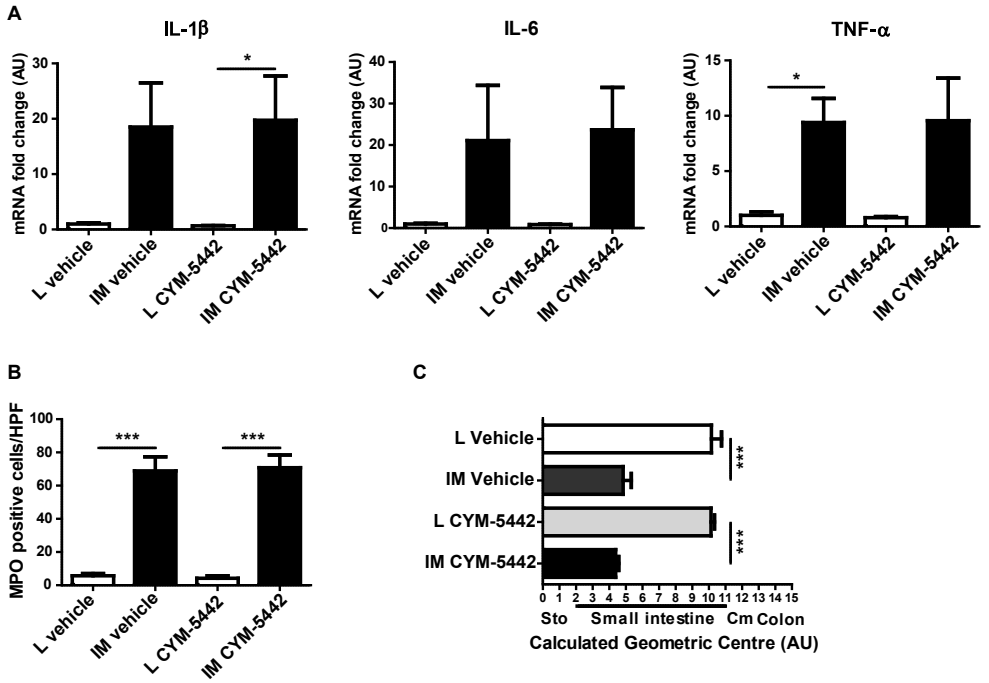

Figure 8. Blockade of S1P/$S1P_1$ signaling does not affect the inflammation or gastrointestinal transit 24h after Intestinal Manipulation. (A) CYM-5442 treatment administered i.p.10h after L/IM does not affect the mRNA levels of IL-1β, Il-6 and TNF-α in the small intestine. (B) The number of infiltrated MPO^+ cells in the intestinal muscularis 24h after is not significantly altered in CYM-5442-treated mice. (C) Delay in the gastrointestinal transit is identical between vehicle-treated or CYM-5442-treated mice. Data shown are mean±SEM of 2 independent experiments (n=5-6 animals per group). * p<0.05; *** p<0.001 (Two-way ANOVA). Sto=stomach, Cm=cecum.

Discussion

In the present study we demonstrate that intestinal manipulation leads to a response from the spleen as shown by the striking decrease in spleen weight and cellularity observed after IM. This mechanism is associated with an active egress of cells, namely $CD4^+$ T cells and monocytes. Blocking this active departure however does not affect the severity of the intestinal inflammation after IM showing that the spleen is not involved in the intestinal inflammation and pathogenesis of postoperative ileus.

The spleen constitutes an important immune cell reservoir which can be mobilized rapidly after a trauma or at the onset of an inflammatory process [12-15]. Here we report that intestinal manipulation also leads to a rapid egress of cells from the spleen. We indeed observe a decrease in the number of splenocytes following manipulation of the small intestine, a phenomenon that relies on an alteration of cell trafficking to the spleen with both retention/attraction and egress signals affected during POI. The analysis of the splenic expression of homeostatic chemokines revealed a prominent decrease of CCL19 expression during POI. CCL19 acts via its receptor CCR7 to provide a homing signal to lymphoid organs for several immune cell types such as DCs, T cells [23,24] and B cells [25]. It is therefore likely that its decreased expression observed after IM affects cell trafficking in the spleen and contributes to the decreased number of splenocytes. The sphingolipid S1P was shown to be a major modulator of cell trafficking in lymphoid organs [26]. Binding of S1P to its specific receptors expressed on immune cells triggers egress signals from lymphoid organs. Here, pharmacological blockade of this S1P/$S1P_1$ axis by the administration of an S1P agonist prevented the decrease of splenocytes observed after manipulation of the intestine. Our data therefore demonstrate that manipulation of the small intestine results in an active egress of cells from the spleen through the activation of the S1P/$S1P_1$ axis. We hypothesized that the egress of cells occurred consequently to intestinal inflammation rather than handling of the intestine itself. The administration of the S1P agonist was therefore performed 10h post-surgery, a time point where intestinal inflammation has already begun as shown by the elevated levels of pro-inflammatory cytokines such as IL-1β and IL-6 in the small intestinal muscularis [20,27].

Monocytes represent one of the major cell types infiltrating the intestinal muscularis during POI [28] but the origin of these infiltrating cells remains unclear. A recent study

provided evidence of the existence of a splenic reservoir of monocytes that can rapidly egress from the spleen in case of acute inflammation [12]. Here we report that a massive and rapid egress of monocytes and $CD4^+$ T cells from the spleen occurs during postoperative ileus but that contrarily to other acute inflammation models, these cells do not participate to the intestinal inflammation. Indeed, using flow cytometry, we failed to report any major influx of $CD4^+$ T cells to the intestinal muscularis. Similarly, preventing the egress of cells from the spleen did not ameliorate the degree of inflammation in the small intestine, influx of immune cells (neutrophils/monocytes) to the gut muscularis or delay in the gastrointestinal transit further confirming that immune cells infiltrating the gut muscularis upon intestinal manipulation do not originate from the spleen. In line, labeling of splenocytes by injection of CFSE in the spleen prior to manipulation of the intestine failed to reveal any CFSE positive cells on sections of the small intestinal muscularis 24h after IM (data not shown). However, FACS analysis of splenocytes from injected spleens revealed that labeling of the cells was very poor as only 15% of the cells displayed positivity for CFSE (data not shown). Moreover, as the spleen is a highly vascularized organ, CFSE leakage from the spleen to the circulation could occur and lead to labeling of cells that did not egress from the spleen in response to intestinal manipulation consequently distorting the results. These technical limitations therefore prevented us to further corroborate our findings using this approach.

Of note, the pool of splenic $CD4^+$ T cells decreased by about 5 million cells 24h after IM while the pool of splenic monocytes decreased by about 2 million cells. Altogether, these reductions, although spectacular, cannot by themselves account for the decrease of 30 million splenocytes triggered by IM. Comparable to previous studies [29,30], the percentage or absolute cell number of other cell types was not significantly modified after IM. Furthermore, the decrease in the number of splenocytes observed here is independent of necrotic or apoptotic cell death. This strongly suggests a general egress of splenic cell populations after IM, a phenomenon also observed during stroke [12,29]. As blocking the S1P/$S1P_1$ axis abolishes the decrease in the number of splenocytes, this implies that other cell populations bearing S1P receptors (i.e., DCs, B cells, macrophages, CD8+ T cells) are likely to be released upon handling of the intestine. Interestingly, our data demonstrate that the sympathetic nervous system is involved in the regulation of the egress of splenocytes as shown by the absence of a significant decrease in the number of splenocytes after IM in spleen-denervated animals. Previous studies on the involvement of the splenic innervation in the trafficking of cells have reported

ambivalent results. In a local inflammation model, i.e., the carrageenan air-pouch model, splenic denervation alone was not sufficient to prevent the egress of $CD11b^+$ leukocytes from the spleen. However, in this same model, vagus nerve stimulation was shown to abolish the leukocytic egress from the spleen only when the integrity of the splenic nerve was preserved, therefore showing that the splenic innervation played an essential role [31]. On the contrary, it was recently shown that chemical sympathectomy enhances the migration of monocytes from the spleen during peritonitis [15]. In POI, splenic innervation seems to promote the egress of splenocytes out of the spleen as no significant difference is observed after IM in the number of splenocytes in spleen-denervated and sham-operated animals. Noradrenergic fibers densely innervate the T-cell zone in the spleen [32,33], an area where stromal cells secreting homeostatic chemokines such as CCL19, CCL21 and CXCL13 are located. Neural control of the production of homeostatic chemokines by stromal cells has previously been reported in the intestine [34]. The effect of sympathetic innervation on the production of these homeostatic chemokines in the spleen is however poorly documented. Here, we demonstrate for the first time that this neural control is not restricted to the gut as splenic denervation prevents the decrease in CCL19 expression induced by IM. This suggests that sympathetic innervation of the spleen may act on stromal cells located in the T cell zone area and regulates the secretion of this homeostatic chemokine therefore playing a role in the attraction/retention of splenocytes. As noradrenaline is also known to trigger spleen contraction and atrophy [35,36], we therefore cannot rule out that sympathetic innervation of the spleen capsule triggers a contraction of the spleen leading to a massive expulsion of cells from the spleen explaining the loss of splenocytes after IM. However, as blockade of $S1P/S1P_1$ signaling abolishes the decrease in the number of splenocytes, it seems unlikely that a simple mechanical phenomenon can explain the decrease in the number of splenocytes. Importantly, no significant difference was observed in the number of splenocytes between sham-operated and spleen-denervated animals after IM showing that sympathetic splenic innervation is not the only mechanism driving this response and that other mechanisms, independent of the nervous system regulate this phenomenon.

Altogether our data suggest that the splenic response observed after IM constitutes an emergency response affecting a vast majority of splenic cell subpopulations and more strikingly $CD4^+$ T cells and monocytes. Since our data clearly demonstrate that those cells do not participate in the intestinal inflammation underlying postoperative

ileus, one may ask what the physiological relevance of this splenic response is. The influx of leukocytes to the small intestinal muscularis was previously reported to result from extravasation of cells from blood vessels to the gut wall [4]. The physiological relevance of mobilizing splenic cells rapidly and massively during POI may therefore reside in the need for the organism to maintain cellular homeostasis in the circulation by replenishing the blood cellular compartment after leukocytes are sent from the blood to the manipulated intestine. The fact that we failed to detect an effect of blockade of efflux of splenocytes on intestinal inflammation, influx of cells to the gut muscularis and delay in gastrointestinal transit further supports this concept.

In conclusion, our study demonstrates that the spleen responds to intestinal manipulation by releasing immune cells, a phenomenon associated with an alteration of cell trafficking that is partly regulated by splenic innervation. This mechanism is however not involved in the pathogenesis of postoperative ileus.

Supplementary material

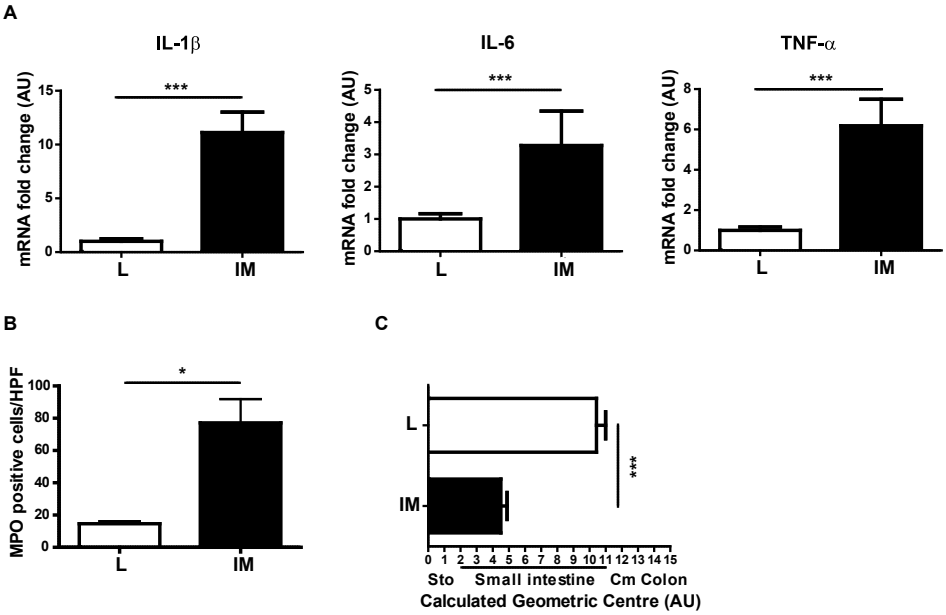

Figure S1. Effect of Intestinal Manipulation. Mice underwent Laparotomy (L) or Intestinal Manipulation (IM) and were sacrificed 24h after surgery. IM leads to enhanced pro-inflammatory cytokine levels (i.e., IL-1β, IL-6, TNF-α) in the small intestinal muscularis (A), influx of leukocytes (B) and delayed gastrointestinal transit (C). Data shown are mean±SEM of 3 independent experiments (n=8-10 animals per group). * p<0.05; *** p<0.001 (Student t-test). Sto=stomach; Cm=cecum

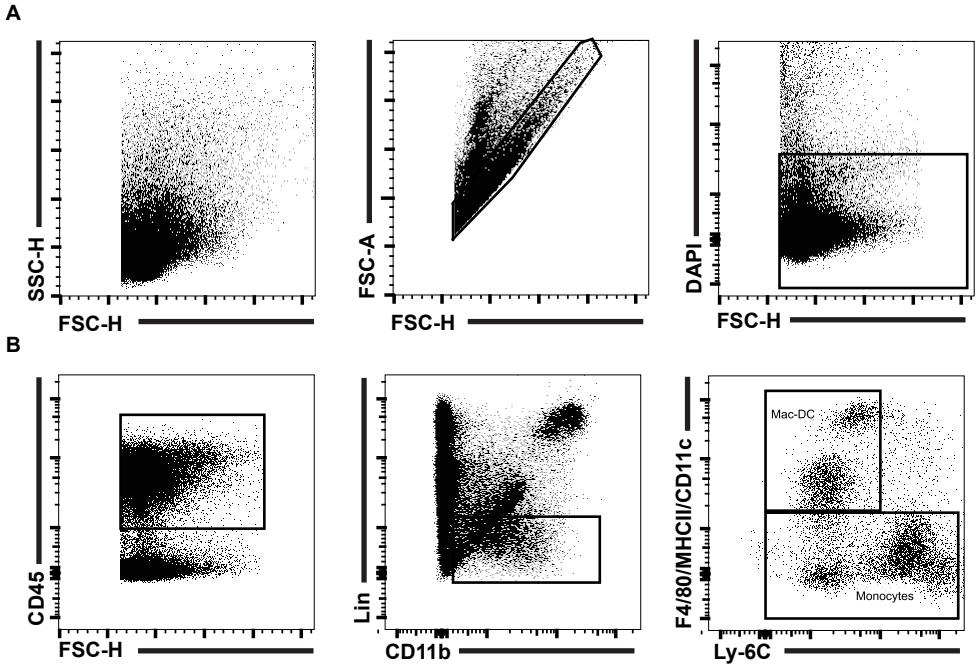

Figure S2. Gating strategies for Flow cytometry analysis. (A) Singlet cells were gated from the total spleen or MLN cell population. Gating on alive cells ($DAPI^-$) was performed on singlet cells population. All populations for both spleen and MLN were gated from singlet alive cells. (B) To measure the monocytic population, a Lin^-CD11b^+ population was gated from singlet alive $CD45^+$ cells. The monocytic population was determined as the $F4/80^-/MHCII^-/CD11c^-Ly6C^+$ fraction of the Lin^-CD11b^+ population.

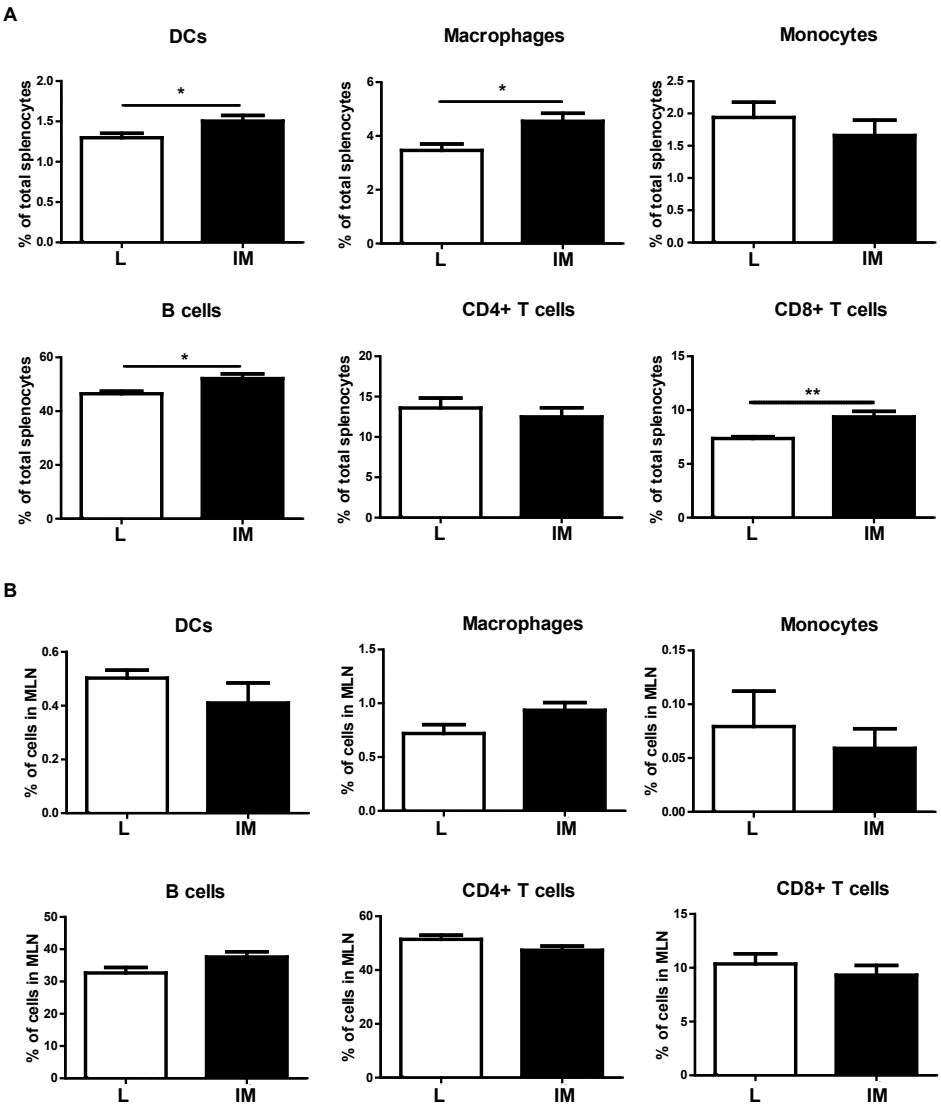

Figure S3. Percentages of populations in the spleen and the MLN. (A) Percentage of DCs ($CD11c^+MHCII^+$), macrophages ($F4/80^+$), B cells ($CD45R^+MHCII^+$) and CD8 T cells ($CD3^+CD4^-CD8^+$) increases in the spleen 24h after IM mice whereas the percentage of CD4 T cells ($CD3^+CD4^+CD8^-$) remains unchanged between L and IM mice. (B) Percentages of all populations measured in the MLN remain unchanged between mice undergoing L and mice undergoing IM.

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Chapter 4

Neuroanatomical evidence demonstrating the existence of the vagal anti-inflammatory reflex in the intestine

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Abstract

Background: The cholinergic anti-inflammatory pathway is proposed to be part of the so-called vago-vagal "inflammatory reflex". The aim of this study is to provide neuro-anatomical evidence to support the existence of a functional neuronal circuit and its activation in response to intestinal inflammation. Methods: The expression of c-Fos was evaluated at different levels of the neurocircuitry in the course of postoperative ileus (POI) in a mouse model. Specific activation of the motor neurons innervating the inflamed intestine and the spleen was monitored by retrograde tracing using cholera toxin-b. The role of the vagal afferent pathway nerve was evaluated by selective vagal denervation of the intestine. Results: Abdominal surgery resulted in subtle inflammation of the manipulated intestine at 24h (late phase), but not after 2 and 6h (early) after surgery. This local inflammation was associated with activation of neurons in the nucleus tractus solitarius and in the dorsal motor nucleus of the vagus. The vagal output mainly targeted the inflamed zone: 42% of motor neurons innervating the intestine expressed c-Fos immunoreactivity in contrast to 7% of those innervating the spleen. Vagal denervation of the intestine abolished c-Fos expression in the brain nuclei involved in the neuronal network activated by intestinal inflammation. Conclusion: Our data demonstrate that intestinal inflammation triggers a vagally mediated circuit leading mainly to activation of vagal motor neurons connected to the inflamed intestine. These findings for the first time provide neuro-anatomical evidence for the existence of the endogenous "inflammatory reflex" and its activation during inflammation.

Introduction

Vago-vagal reflexes coordinate gastric and intestinal digestive functions and motility [1-3]. These functions are controlled through functionally distinct pathways, called neurocircuitries, connecting the intestine with the brain stem nuclei, i.e., the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV). Mechanical (i.e., contraction, distention) and chemical (i.e., nutrients) signals are transmitted to the NTS via the vagal ascending fibers. After integration of the incoming information and through neuronal communication between the NTS and the DMV, vagal efferent output is triggered to adjust gastrointestinal (GI) functions such as secretion, absorption and motility.

Recently, it was suggested that also the immune system is modulated by the vagus nerve. Tracey and coworkers demonstrated that electrical stimulation of the vagus nerve prevents the development of endotoxin-induced shock by reduction of pro-inflammatory cytokine production, in particular TNF-α in the spleen [4,5]. This anti-inflammatory effect is mediated by activation of nicotinic receptors located on immune cells (in particular macrophages) in response to acetylcholine released by vagal efferent nerve terminals [6,7]. The discovery of the cholinergic anti-inflammatory vagal efferent pathway introduced the concept of the "inflammatory reflex" by which the central nervous system is capable of sensing inflammation and subsequently modulating the immune response [8,9]. To date, however no anatomical evidence has been reported supporting the existence of this neurocircuit.

Recently, we extended the anti-inflammatory role of the vagal efferent route in a confined/subtle model of inflammation, i.e., post-operative ileus (POI) [10]. POI is characterized by a generalized hypomotility of the GI tract, and occurs after almost every abdominal surgical procedure. The prolonged paralytic phase of POI is mediated by an inflammatory response in the muscular layer of the intestine triggered by activation of resident macrophages following intestinal handling [11,12]. Previously, we demonstrated that electrical stimulation of the vagus nerve inhibits the production of pro-inflammatory cytokines, reduces intestinal inflammation and shortens POI [10]. This effect was blocked by incubation of the intestine with the nicotinic receptor antagonist hexamethonium, indicating vagal modulation of the intestinal immune response within the intestinal wall. To what extent this anti-inflammatory mechanism is

also endogenously activated during inflammatory conditions, such as POI, and is part of a vago-vagal neurocircuitry remains however unclear.

The expression of the nucleoprotein Fos, a product of the c-Fos immediate-early gene, is widely considered a marker of neuronal activity and has been used repeatedly to map functional brainstem pathways in response to various stimuli [13-15]. The distribution of c-Fos expression induced by abdominal surgery enlightened specific neural circuits including brainstem nuclei (NTS, locus coeruleus, caudal ventral medulla and cuneate, lateral parabrachial nuclei) but also hypothalamic ones such as the paraventricular nucleus (PVN). In the current study, we focused on the expression of c-Fos at different levels of the neuronal pathway proposed to mediate the vagal anti-inflammatory mechanism. Finally, selective vagal denervation of the small intestine was performed to further evaluate the role of the vagus nerve in the modulation of peripheral inflammation.

Material and Methods

Animals

Mice (female BALB/c; Harlan Nederland, Horst, The Netherlands) were kept in 12h light/12h dark cycle (lights on at 8:00 AM to 8:00 PM) under constant conditions of temperature (20± 2°C) and humidity (55% humidity) with water and food *ad libitum*. Mice were studied at 10-12 weeks of age. All experiments were conducted in accordance with the guidelines of the Ethical Animal Research Committee of the Academic Medical Center.

CTb injection in peripheral organs: small intestine and spleen

The cholera toxin-B conjugated with Alexa Fluor 555 (1%; CTb-Alexa Fluor 555) (Molecular Probes, USA) was used to label the neuronal innervation of the small intestine and the spleen. 0.5µl of the retrograde tracer was injected (flow of 0.5µl/min) at different spots (5) along the ileum. 3µl of the tracer was injected in both ends of the spleen. The injection was performed with a fused silica tubing (40µm i.d., 105µm o.d.) (Aurora Borealis control, Schoonebeek, The Netherlands) protected at its end by a 30-gauge$^{1/2}$ needle. The probe was connected to an injection pump via a guide PEEK tube (PK005-02, Aurora Borealis control). The time period required for a retrograde tracer to reach

the brainstem was estimated to be 7 days. However, to avoid possible interference of inflammation triggered by tracer injection, we expanded the period of recovery to 15 days. To validate the specificity of the tracer injection within the small intestine, we performed injections of CTb-fluorophore 555 and 647 in the proximal and distal part of the ileum. Moreover injection of the same volume of the tracer was applied in the peritoneal cavity.
To further validate the specificity of the tracer protocol evaluating the vagal innervation of the spleen, we added an additional control in which tracer was applied on the top of the splenic hilum (i.e., the point of insertion of the splenic artery and vein). Finally, we included a group of mice that underwent vagal denervation of the spleen followed by CTb injection at the tip of the organ. Loss of CTb positive neurons in the DMV indicated successful (i.e., complete) vagal denervation of the spleen.

Experimental protocols

Protocol 1: Neuronal circuitry and c-Fos expression at different time points post-surgery

Control animals (no treatment) and animals that underwent laparotomy (L) or intestinal manipulation (IM) were examined at different time points after surgery: L and IM at 2, 6 and 24h (n=24) and controls at 2h (n=12). The controls mice were divided into two groups: one group underwent standard anesthesia (Control group) whereas the other was left untouched to estimate the baseline expression of c-Fos proteins (Baseline (BL) group). Mice were sacrificed by transcardiac perfusion with Phosphate buffered saline (PBS) followed by 4% paraformaldehyde PFA (pH 7.4). Brains and nodose ganglia were collected, post-fixed for 4h (4°C) and cryo-protected by immersion with 30% sucrose in 0.2M PBS (pH 7.4) at 4°C overnight. Intestinal tissue was collected prior to PFA perfusion. Intestinal tissue was cut along the mesentery border, washed in cold saline and directly fixed into 100% ethanol for 30min and then kept in 70% ethanol at 4°C until analysis.

Protocol 2: The spleen as target of the endogenous vagal anti-inflammatory pathway

Intestinal inflammation leads to a consistent c-Fos expression in the DMV that is not restricted to the small intestine (Fig. 3A, B). Recent data identified the spleen as an important player in the cholinergic anti-inflammatory mechanism [16]. Since a direct

vagal innervation of the spleen has been recently identified [17], we decided to investigate whether the vagal motor neurons that innervate directly the spleen express c-Fos immunoreactivity (IR) upon intestinal inflammation, i.e., 24h after IM. Mice (n=12) were injected with CTb in the spleen. Two weeks after recovery, mice underwent either laparotomy (n=5) or IM (n=7). 24h later, mice were sacrificed with transcardiac perfusion, brain and intestinal tissue was collected.

Protocol 3: Selective vagal denervation of the small intestine: analysis 24h post-surgery

To evaluate the role of the afferent vagal pathway in the activation of the anti-inflammatory efferent [18-20], the small intestine was selectively denervated (i.e., disruption of the sensory and motor nerves fibers of the vagus, IntX) and the effect on c-Fos expression in the brain stem nuclei involved in the "inflammatory reflex" was studied. Four groups were examined: L, IM, L+IntX and IM+IntX. In short, vagal denervation (n=24) or sham-operation (n=16) mice received intestinal CTb injection. Two weeks later, mice underwent IM or L and sacrificed 24h after surgery with transcardiac perfusion, as described above. Brain and intestinal tissue were collected.

Surgical procedures

Surgical experiments started at 8:30 am. Mice were operated at 11-13 weeks of age after one week of adaptation in the animal facilities. For all surgical procedure (tracer injection, denervation, intact, L or IM), mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of fentanylcitrate/fluanisone (Hypnorm; Janssen, Beerse, Belgium) and midazolam (Dormicum; Roche, Mijdrecht, The Netherlands). The anesthetic mixture was in a ratio of 1:1:2 of Hypnorm, Midazolam and water respectively. Subcutaneaous injection of Fynadine (0.03ml/100g, from a 10x diluted stock solution, 50mg/ml) was performed after the first surgery (i.e., denervation or injection of the tracer). The same length of the midline incision was performed in all surgical protocols (i.e., sham-operated *vs* denervated, L *vs* IM).

CTb injection

In protocols 1 and 3, mice were injected with the neuronal tracer in the small bowel.

In brief, the animals were anesthetized and the abdomen was opened by a midline incision. The small bowel was carefully eviscerated and placed on a moist gauze pad to allow CTb injections. In protocol 2, mice underwent CTb injection in the spleen.

Vagal intestinal denervation (IntX)

The right celiac branch of the vagus nerve supplies the jejunum, ileum and caecum and is embedded in fat/connective tissue that lies above the superior mesenteric artery. After removal of this fat/connective tissue, the nerve was cut. Vagal denervation was combined with CTb injection to evaluate the success of the denervation procedure. The absence of CTb labeled motor neurons in the DMV is considered indicative for complete vagal denervation of the bowel.

Intestinal manipulation (IM) or laparotomy (L)

Mice underwent L or L followed by IM, as previously described [10,21]. In brief, a midline abdominal incision was performed along the *linea alba* and the peritoneum was opened. The small bowel was carefully removed from the peritoneal cavity and positioned on a moist gauze pad. The entire small bowel was manipulated from the distal duodenum to the cecum with moist cotton applicators for 5 minutes. Contact or stretch on the stomach or colon was strictly avoided. The surgical procedures were performed under sterile conditions. At the end of the surgery, the abdomen was closed with Mersilene, 6-0 silk.

Histological techniques

Coronal sections of 30µm for brain/brainstem and 16µm for nodose ganglia were collected. After rinsing in 0.05M Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and/or rabbit anti-CTb (1:10 000; C3062, Sigma, USA, to recognize the CTb-conjugated alexa fluorophore 555) primary antibodies.
For c-Fos staining, sections were first incubated 1hr in biotinylated secondary antibody and then in avidin-biotin complex (ABC, Vector) for 1hr. The reaction product was visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide H_2O_2 for 5min. For c-Fos/CTb double staining,

sections underwent once more 1h incubation in secondary antibody followed by 1h incubation with ABC and ended with 7 min incubation in 1% DAB and 0.01% H_2O_2.
To count CTb and/or c-Fos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 color camera (Sony Corp., Tokyo, Japan). Brainstem sections, from bregma -7.20 to -7.76mm, were used for c-Fos and CTb counting in the NTS/DMV counted the brainstem section for the experiments. The counting of c-Fos in the PVN was performed on hypothalamic sections collected from Bregma -0.58 mm to -1.22 mm. The counting of c-Fos$^+$ cells was performed bilaterally for each nucleus. Data are represented as a mean of the relative density of c-Fos positive cells counts on 9-10 and 9-11 sections (non-adjacent section, at least separated 90μm) for the NTS/DMV and PVN, respectively.

Immunohistochemical staining for leukocyte infiltration of the small intestinal muscularis

Whole mounts of the small intestinal muscularis were used to determine the degree of inflammation. Myeloperoxidase (MPO) staining was performed with 3-amino-9-ethly carbazole (Sigma, St Louis, MO), 0.01% H2O2 in Sodium Acetate buffer (pH = 5) for 20 minutes, as previously described [21,22].

Random counting of whole mount sections

MPO$^+$ cells in the whole mount of the intestinal muscularis were counted using an image analysis system (ImagePro v4.5, Media Cybernetics, Silver Spring, USA) connected to a color camera (JVC KY-F55 3CCD) and a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives). For each section, an image covering the entire sample (2.5× objective) was loaded into the IBAS and displayed on the computer monitor. The region of interest, i.e., the muscularis devoid of Peyer's patch and damaged areas, was identified and a grid of rectangular areas (representing 20X magnification) was superimposed on the image. From this grid, 5% of the fields were selected randomly and from the selected images (20X), MPO$^+$ cells were counted.

Statistical analysis

Data from all experiments are presented as a mean±SEM Kolmogorov-Smirnov Test was

used to determine whether the data set followed a normal distribution (SPSS package v 16.0). Square root transformation was applied to the non normal data set. Two-way ANOVA analysis was performed to evaluate the two factors interaction: *time* (i.e., 2, 6 and 24h) and *treatment* (L *vs* IM) or *vagotomy* (i.e., intact *vs* denervation) *and treatment* (i.e., L *vs* IM). When the ANOVA analysis indicated difference, One-way ANOVA was performed to demonstrate a statistical significant difference among the different time point per treatment group (i.e., L *vs* IM) followed by post hoc (LSD) analysis. The student T test (un-paired) was applied to estimate a significant difference between L *vs* IM at a specific time point or between intact *vs* denervated. Repeated-measure analysis of variance (ANOVA) was conducted to test the effect of vagal denervation on the mice body weight during the recovery period. All statistical analysis was performed with a significance set at 0.05.

Results

Distribution of CTb labeled neurons after intestinal/splenic injection

Intestine

The retrograde labeling was limited to the circumscribed region of the DMV. CTb labeled neurons were localized in the lateral part of the DMV observed from -7.32 to -7.76mm Bregma, as expected from previous anatomical studies [23,24] (Fig. 1A) Given the distribution of the vagal sensory innervation of the gastrointestinal tract [18,25,26] and the distribution of the CTb positive neurons, brainstem sections from bregma -7.20 to -7.76mm were used for c-Fos and CTb counting in the NTS/DMV. Injections of CTb-fluorophore 555 and 647 in the proximal and distal part of the ileum respectively labeled 2 distinct set of neuronal population (Fig. 1B). No CTb^+ cells were observed in the DMV after injection of the same volume of tracer in the peritoneal cavity. This observation shows a restricted uptake of the neuronal tracer by the small intestine. Moreover the remarkable differentiation of innervation of the ileum by DMV neurons illustrates the specific uptake by defined sites of the ileum.

Spleen

The retrograde tracer labeled a restricted neuronal population within the DMV (bregma -7.32mm to -7.76mm; Fig. 1C), as previously reported [17,27]. Application of the tracer

on splenic vessels at the level of the hilum led to faint limited amount of labeled neurons (1 or 2) that were located in the DMV more rostrally of the brainstem from bregma -6.96mm to -7.08mm (Fig. 1B). These CTb positive neurons, however, were not found after injection of the CTb at the extremity of the spleen. Vagal denervation of the spleen followed by CTb injection at the tip of the organ led to a loss of CTb positive cells in the DMV (Fig. 1E) excluding the possibility that CTb positivity results from leakage of tracer into the peritoneal cavity. On the other hand, sympathetic post-ganglionic neurons exhibit CTb stained cell bodies in the mesenteric ganglion (Fig. 1F). We, therefore, used the brainstem sections from bregma -7.32mm to -7.76mm for c-Fos counting in NTS/DMV in the following experiments.

Early and late phase of postoperative ileus: role of inflammation

Intestinal inflammation was monitored by the degree of leukocyte infiltration at the different time points after surgery. MPO^+ cells were occasionally observed in the muscle layers of both L and IM mice early after surgery (2h L, 1±1 cells/mm^2 *vs* IM, 2±1 cells/mm^2; 6h L, 1±1 cells/mm^2 *vs* IM, 3±1cells/mm^2). In contrast, a clear infiltration of leukocytes was observed in the late phase of postoperative ileus (24h after surgery) (Fig. 2A-B) and was significantly higher in IM mice (276±64 cells/mm^2) compared to the L mice (1±1 cells/mm^2, p=0.007).

Neuronal circuitry: c-Fos expression at the early phase (2 and 6h)

Two hours after surgery, all experimental groups (control, L and IM) exhibited a significant increase of c-Fos IR in the NTS and PVN compared to the baseline (Fig. 2). Even the control mice that only received anesthesia had a comparable number of $c\text{-}Fos^+$ cells as L or IM, suggesting NTS/PVN activation induced by anesthesia and/or peri-operative stress. In the same line, no difference was observed in c-Fos IR in nodose ganglia cells bodies (i.e., vagal afferent nerve) (Fig. 2D) between L and IM mice, 2h after surgery.
In animals receiving L only, the number of $c\text{-}Fos^+$ cells in the NTS decreased in time (one way ANOVA, p =0.002) and reached basal levels at 24h post-surgery (L 14.5±5.5 *vs* BL 15.44±2.7, p=0.689), suggesting a transient effect of the surgical intervention on c-Fos expression (Fig. 2C). In animals that underwent IM, the level of c-Fos expression remained high (one way ANOVA, p = 0,028) and was significantly different from L at 6h post-surgery.

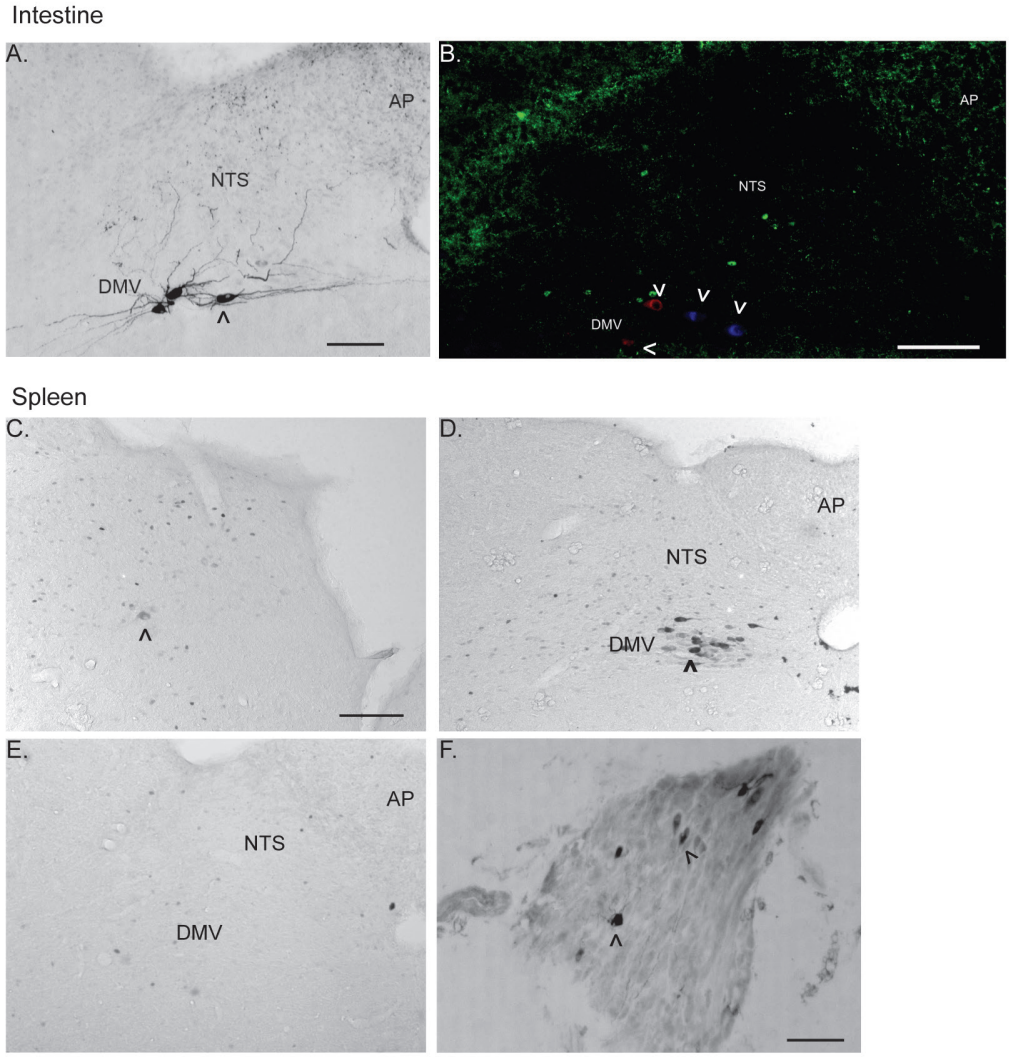

Figure 1. Distribution of CTb labeled cells in the brainstem nuclei after tracer injection in the intestine (A). Injection of Cholera toxin b (CTb) (fluorophore 555, red and 647, blue) into the proximal and distal part of the ileum labeled distinct motor neurons in the DMV (B). The distribution of the CTb^{+} cells in the brainstem nuclei after CTb application on splenic blood vessels differ from the CTb labeled cells (C) found in the DMV after tracer injection at the tip of the spleen (D). Vagal denervation of the spleen prevents CTb labeling of neurons in the DMV. Here we show an example of complete denervation in which no CTb positive cell body was found in the DMV (E). CTb labeled neuron population in the mesenteric ganglion (i.e., sympathetic post-ganglionic neurons) was found in denervated mice (F). The scale bars represent 100µm and 0.50mm for (A,B, F) and (C,D,E), respectively. Green: primary c-Fos antibody and streptavidin alexa-fluo 488. Sx: vagal denervation of the spleen

In the PVN (Fig. 2E), c-Fos IR in both L and IM groups significantly decreased with time after surgery (Two way ANOVA: *time* effect $p < 0.05$; *treatment* or *treatment*time* interaction, $p > 0.05$), suggesting a role of the PVN only at the early phase of the POI. The number of c-Fos IR motor neurons in the DMV was significantly increased in both L and IM compared to the control (anesthesia only) or basal level ($p < 0.05$; Fig. 3A) at 2 and 6h post-surgery, suggesting that additional sensory afferent activation triggered by skin incision and/or intestinal handling generates vagal output. In the L group, the number of c-Fos$^+$ neurons returned to baseline 24h after surgery.

c-Fos IR in CTb labeled motor neurons innervating the small intestine

To evaluate whether trauma/inflammation activates neurons in the DMV targeting the inflamed intestine, we counted the amount of c-Fos$^+$ cells in the CTb labeled neurons, i.e., motor neurons that specifically innervate the small intestine. The quantitative c-Fos analysis within the CTb labeled neuronal population showed that 2h and 6h after surgery a low percentage (below 10%) of CTb$^+$ neurons co-express c-Fos IR in both L and IM mice (Fig. 3C).

Neuronal circuitry: c-Fos expression at the late phase of POI (24h)

At the late phase of the POI (t=24h, inflammatory phase characterized by leukocyte infiltrates in the gut muscularis), activation of the NTS and nodose ganglia after IM was still significantly increased compared to L and baseline: IM 73±35 *vs* L 14± 4 or BL 15±3, $p = 0.005$) (Fig. 2C and D). However, no significant difference in c-Fos expression in the PVN was observed between L and IM (Fig. 2E). In contrast, the number of c-Fos$^+$ neurons in the DMV further increased after IM at 24h (IM 24.3±8 *vs* L 7.7±1.2, $p = 0.001$) while the number of c-Fos$^+$ neurons after L returned to basal level (Fig. 3A-B).

c-Fos IR in CTb labeled motor neurons innervating the small intestine

Nearly half of the CTb neurons that project to the manipulated intestine exhibited positive IR for c-Fos ($p < 0.05$), i.e., 42% in IM *vs* 4% in L (Fig. 3C-D). These data provide evidence that the late inflammatory phase is associated with c-Fos expression in NTS, DMV. This vagal neuronal circuit activation is based on a vagal output targeting specifically the inflamed zone, i.e., the intestine.

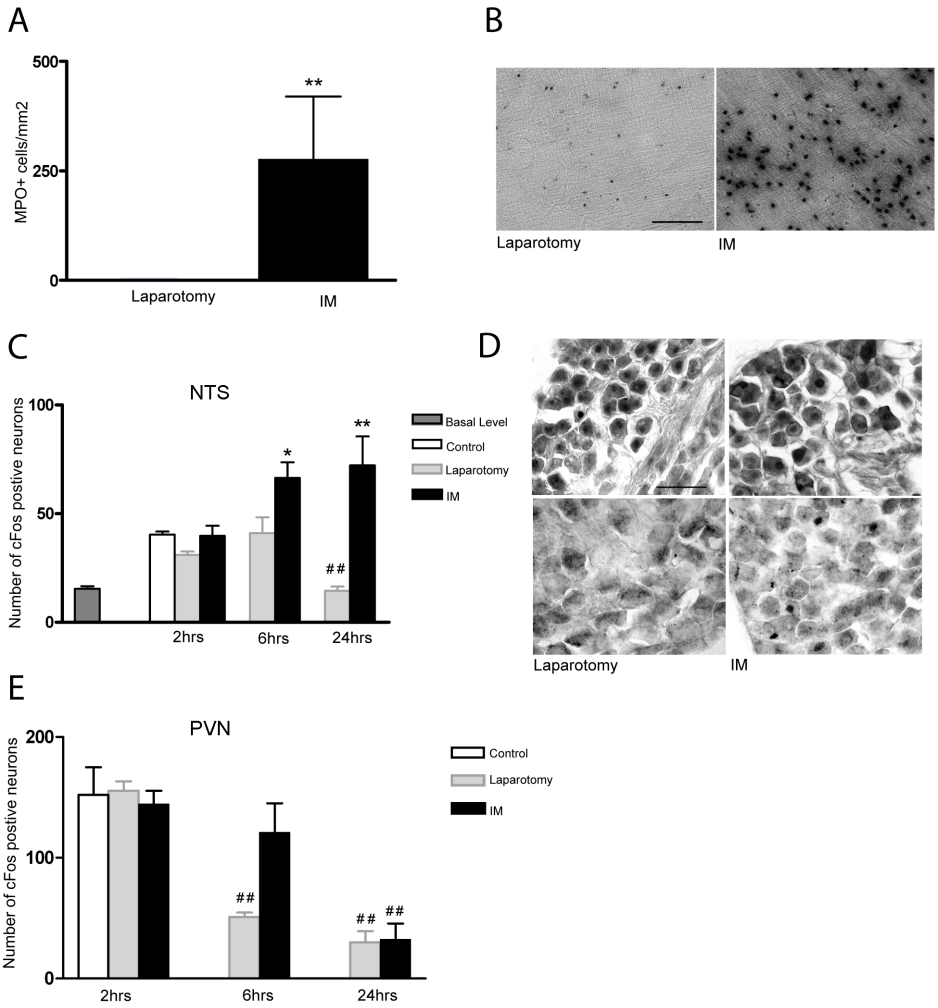

Figure 2. MPO⁺ cells in whole mount preparations at 24h post-surgery (A,B). c-Fos expression in the NTS (C), nodose ganglia (D) and PVN (E) at the early and late phase of POI. Data are expressed as mean±SEM for n=5-7 mice (A, C and E). The baseline expression of c-Fos expression in the PVN at 24h is similar in L or IM (data not shown**).** In the figure D, the upper and lower panel illustrated c-Fos expression in the nodose ganglia (after L or IM) at 2h and 24h, respectively. The scale bars represent 100µm and 50µm for (B) and (C), respectively. # indicates significant differences (#, $p < 0.05$; ##, $p < 0.01$) compared to t=2h. Asterisks indicate significant differences: *, $p < 0.05$; **, $p < 0.01$), L *vs* IM.

c-Fos IR in CTb labeled motor neurons innervating the spleen

Previous studies of Tracey et al., provided strong evidence that the spleen is involved in the vagal anti-inflammatory pathways [4,16]. Since the spleen is directly innervated by the vagus nerve [17,27], we investigated whether the vagal output triggered by intestinal inflammation also targeted the spleen. To this end, we retrogradely labeled the vagal motor neurons innervating the spleen prior to intestinal manipulation. As previously reported [17], injection of the CTb in both ends of the spleen labeled a neuronal population in the DMV (-7.20 to -7.76mm Bregma) (Fig. 3E). The average mean of c-Fos$^+$ cells counted in NTS and DMV from control mice (n=2; injection of CTb only) was not significantly different from the mice that underwent laparotomy and euthanized 24h after surgery. However, twenty four hours after surgery, IM triggered an increase of c-Fos expression in the NTS and DMV compared to L (NTS: 82±10 *vs* 5±1; DMV: 15±2 *vs* 1±0). Seven percent of the CTb$^+$ neurons that project to the spleen exhibited c-Fos IR ($p < 0.05$) in IM mice while no c-Fos$^+$ CTb$^+$ neurons were observed in L mice (Fig. 3C).

Selective vagal denervation: analysis 24h post-surgery

The neuro-anatomical proof of an endogenous vagal response triggered at the late inflammatory phase of POI and restricted to the inflamed intestine suggests the existence of a vago-vagal inflammatory reflex. To confirm our hypothesis, we selectively denervated the vagal innervation of the small bowel to prove that the afferent limb of the "reflex" is indeed of vagal origin. In the setup of the denervation procedure, we monitored the mice with sham-operation and denervation during their recovery period (sham-operated mice n=5, denervated mice n=7). Mice started with an average body weight of 22.7±0.2g prior surgery. At the end of the recovery period, we did not observe a difference between sham-operated and denervated mice body weight (23.7±1.5g and 23.9±0.96g, respectively). ANOVA with repeated measurement indicated a *time* effect (i.e., body weight measurement over days, $p < 0.05$) but no *interaction time*denervation* effect ($p = 0,740$) or *denervation* effect ($p = 0,432$). At the time of euthanasia, the control groups (laparotomy) did not exhibit distended stomach in sham-operated or denervated mice. Selective vagal denervation (IntX) completely abolished the presence of CTb$^+$ cells in the DMV indicating that the vagal denervation was successful. Among the 20 mice (out of 24) successfully denervated, 11 underwent IM and the 9 left underwent laparotomy.

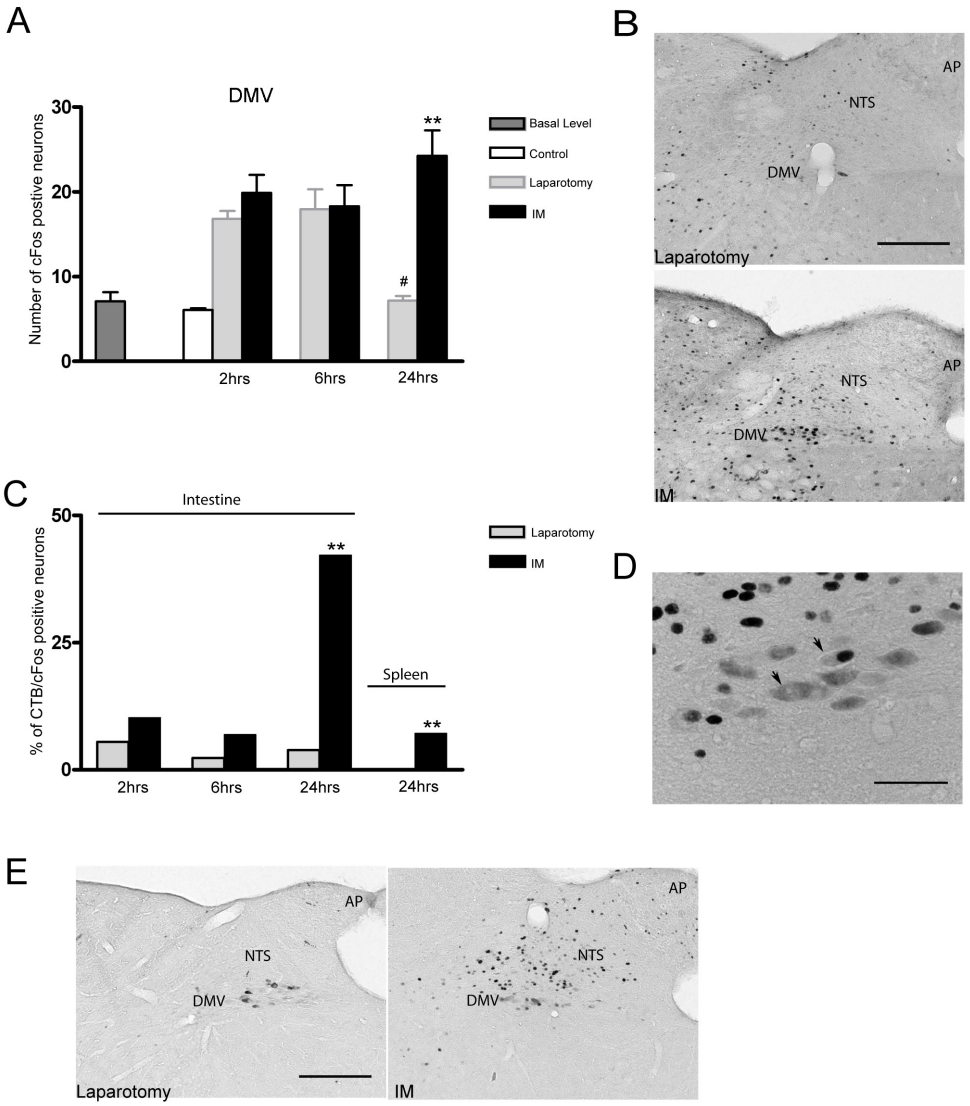

Figure 3. IM-induced c-Fos expression at different time points after surgery in the DMV (A, B) and in a subgroup of motor neurons innervating the small intestine (C). In panel D, part of the c-Fos^{+} neurons (black nucleus) is also stained with the retrograde tracer (cytoplasm), identifying vagal motor neurons innervating the intestine (black arrow). c-Fos IR was also detected in the motor neurons that innervate the spleen at t= 24h post-surgery (C, E). Data are expressed as mean±SEM for n= 5-7. The scale bars represent 200µm (B, E) and 50µm (D). # indicates significant differences (#, p <0.05; ##, p <0.01) compared to t=2h. Asterisks indicate significant differences: *, p < 0.05; **, p <0.01), L *vs* IM. AP, Area postrema; NTS, nucleus tractus solitarius; DMV, dorsal motor nucleus of the vagus; PVN, paraventricular nucleus of the hypothalamus; IM, intestinal manipulation.

In denervated mice, IM tended to increase the degree of intestinal inflammation compared to sham operated mice, but this difference did not reach statistical significance (336±79 *vs* 254±43, respectively; p = 0.188). In both IM groups, inflammation of the intestinal muscularis 24h after surgery (Fig. 4A) was significantly more pronounced compared to laparotomy mice (7±6 *vs* 9±4 for denervated and sham respectively). Even in the presence of inflammation, c-Fos expression in both the NTS and DMV was significantly reduced by selective vagal denervation (Fig. 4B, C and D). IM mice displayed the same levels of c-Fos expression as the control group (IM IntX *vs* L IntX; p = 0.289 for NTS and p = 0.043 for DMV). Selective vagal denervation did not affect c-Fos expression in laparotomy animals. These data indicate that the vagal sensory pathway indeed transmits the inflammatory signal to the brainstem activating the vagal output to modulate the inflammatory response.

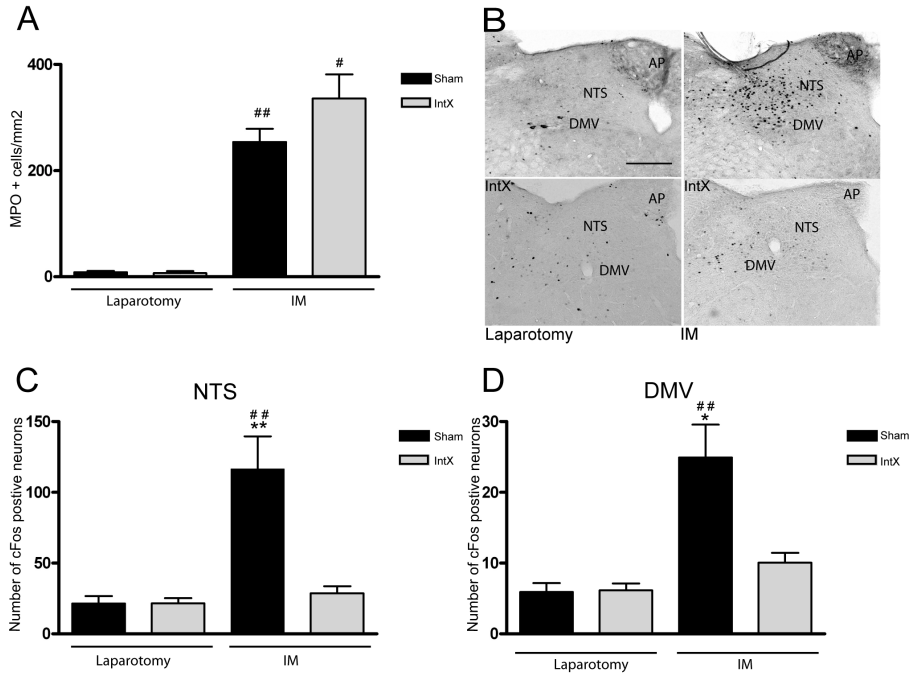

Figure 4. MPO⁺ cells in whole mount preparations at 24h post-surgery (A). c-Fos expression in NTS and DMV (B, C, D) in denervated and sham-operated mice 24h after L or IM. Data are expressed as mean±SEM for n= 5-7. # indicates significant differences (#, p < 0.05; ##, p <0.01), L *vs* IM. Asterisks indicate significant differences (*, p < 0.05; **, p <0.01), sham *vs* IntX. The scale bar represents 200µm. AP, Area postrema; NTS, nucleus tractus solitarius; DMV, dorsal motor nucleus of the vagus; PVN, paraventricular nucleus of the hypothalamus; IM, intestinal manipulation; IntX, vagal intestinal denervation.

Discussion

Recently, Tracey and coworkers introduced the concept of the vagal "inflammatory reflex" as a protective mechanism to restore immune homeostasis after an immunological challenge [28]. Although it is well established that pro-inflammatory cytokines and endotoxin [29] stimulate vagal afferents leading to brainstem activation, data supporting activation of motor neurons of the vagus nerve closing the anti-inflammatory loop are lacking. Here, we demonstrate that subtle intestinal inflammation leads to NTS and DMV activation that is abolished by selective intestinal vagotomy. Importantly, more than 40% of the activated DMV neurons targeted the inflamed intestine, supporting the existence of an endogenous vagal "inflammatory reflex" modulating intestinal inflammation.

During abdominal surgery, multi-synaptic neuronal pathways are activated involving mainly the NTS and the PVN. All experiments started early morning to avoid any circadian influence on c-Fos expression [30,31]. The effect of intestinal manipulation was compared to laparotomy group to avoid any influence of the circadian rhythm while intact mice control groups performed at ZT =2 were compared with 2 and 24h (ZT=2). The NTS is the brainstem nucleus receiving somato/visceral sensory information whereas the PVN is located in the hypothalamus known to regulate stress-related events and endocrine response [15,32]. The PVN, most specifically the parvocellular part, plays an essential role in mediating the immediate and early (up to 3h) hypomotility of the GI tract after abdominal surgery [14,15], most likely in response to surgery-induced stress and activation of visceral sensory afferents [33-35] by skin incision and surgery-induced noxious stimuli. In the present study, we confirmed activation of the PVN in the peri-operative phase (2h post-surgery). Interestingly, c-Fos expression was similar in animals that underwent anesthesia, laparotomy only or intestinal handling suggesting that factors such as stress induced by pain, anesthesia and others largely contribute to PVN activation and even appear to overrule the effect of intestinal handling. Alternatively, the PVN is maximally activated by anesthesia and peri-operative stress thereby obscuring the effect of adding intestinal handling to the surgical protocol. The fact that PVN activation returned to baseline levels at 6h in control and laparotomy animals suggests that peri-operative stress is indeed a major trigger in the early phase. In contrast, the degree of activation in NTS and PVN remained elevated in animals that underwent IM up to 6 hours after surgery. As the effect of peri-operative stress

and anesthesia has disappeared by then, as shown in the L mice, these data suggest that NTS and PVN activation at this stage is rather related to the procedure of surgery and intestinal manipulation. As shown in Figure 3, both L and IM activated the DMV, but this activation was not specifically directed towards the intestine as less than 10% these neurons were labeled with the retrograde tracer injected in the intestine. These data thus suggest that in the early phase of postoperative ileus, activation of the brainstem nuclei is triggered by a generalized stress response combined with activation of mechano/nociceptive pathways, most likely to control systemic homeostasis of the organism.

In the second inflammatory stage of postoperative ileus, however, different mechanisms come into play. It is well recognized that intestinal handling during surgery triggers the influx of inflammatory cells, mainly neutrophils and monocytes. This inflammatory response is observed from 6 hours post-surgery onwards and is well established by 24 hours [11,12]. Twenty four hours after surgery, infiltration of leukocytes within the muscle layers of the small intestine was observed in mice that underwent IM, associated with activation of the NTS and a subset of vagal motor neurons in the DMV targeting the inflamed zone. Indeed, we showed a significant increase of c-Fos IR in those neurons specifically innervating the intestine in IM mice (42%) compared to laparotomy mice (3.8%). Subsequently, we demonstrated that these motor neurons were activated by inflammation detected by vagal afferents. IM, but not laparotomy, resulted in c-Fos IR in the nodose ganglia. We are aware that the illustration of the presence of c-Fos$^+$ cells in IM in contrast to laparotomy is not sufficient to draw such statement. However the selective vagal denervation of the inflamed intestine abolished both NTS and DMV activation, confirming the importance of the vagal afferent activation in mediating the motor vagal neurons activation in response to IM. Although the latter could result from neuronal degeneration triggered by axotomy, studies demonstrate that neuronal degeneration is rather induced by vagotomy performed at the mid cervical level, i.e., close to the cell body of the neurons [36]. In our study, vagotomy was performed distally close to the nerve endings, reported to lead to neuronal regeneration rather than degeneration [37-39]. This makes the explanation of neuronal degeneration as explanation for the loss of c-Fos expression in the DMV/NTS after vagotomy less likely. Therefore, our data indicate that vagal afferents and not splanchnic afferent nerves (through the spino-solitary tract) triggered NTS activation, subsequently generating DMV activation and thereby closing the inflammatory reflex. Most likely, the vagal sensory limb of the inflammatory reflex

is triggered by the release of pro-inflammatory cytokines [11,12,40,41]. Vagal afferents indeed express IL-1 and PGE_2 receptors [42], two pro-inflammatory mediators known to be elevated 24h after intestinal manipulation [41,43]. However, further studies are needed to identify the exact underlying mechanisms. Nevertheless, our data clearly demonstrate that intestinal inflammation triggers vagal afferents resulting in the activation of a vagal efferent feedback loop targeting the inflamed area.

Although our data suggest a direct input of the vagal anti-inflammatory pathway to the gut, recent studies identified the spleen as a major player in the cholinergic anti-inflammatory effect in a model of sepsis [4] but also in a model of local inflammation such as the carrageenan air pouch model [16]. To evaluate whether intestinal inflammation would lead to increased vagal output to the spleen, CTb was injected in both ends of the spleen to retrogradely label the DMV neurons innervating the spleen [17]. Only seven percent of the labeled motor neurons expressed c-Fos IR after IM, indicating that the inflammatory reflex is mainly targeted to the inflamed area. Nevertheless, we cannot exclude that the spleen may contribute, albeit partially to the vagally mediated modulation of the inflammatory response.

In our previous studies, we demonstrated that perioperative electrical stimulation of the vagus nerve decreases intestinal inflammation most likely by inhibiting macrophage activation [10,22]. Here we provide evidence that this cholinergic inflammatory pathway (i.e., DMV activation) mainly occurs 24h after surgery, i.e., once leukocytes have infiltrated the gut muscularis, but not at the earlier stage where chemokines and cytokines are secreted by the resident immune cells [44]. This would indicate that the endogenous anti-inflammatory pathway is most likely involved in restoring homeostasis once the inflammatory response is established and has fulfilled its task of attacking micro-organisms and clearing tissue damage. Compatible with this hypothesis is the observation that vagal denervation of the small intestine did not significantly enhance the inflammatory response (nor prostaglandin E2 release, data not shown) 24h after surgery. Further studies are however required to confirm this assumption.

In summary, we provide the first neuro-anatomical evidence illustrating the existence of an "inflammatory reflex" triggered by inflammation. Subtle intestinal inflammation is detected by vagal afferents triggering NTS activation and generating a specific vagal outflow previously shown to modulate the inflammatory response. These data provide

solid evidence to accept the immune-modulatory role of the vagus nerve providing new opportunities to identify targets for the development of new anti-inflammatory strategies.

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Chapter 5

Endogenous vagal activation dampens intestinal inflammation independently of splenic innervation in postoperative ileus

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Abstract

Postoperative ileus is encountered by patients undergoing open abdominal surgery and is characterized by intestinal inflammation associated with impaired gastrointestinal motility. We recently showed that inflammation of the gut muscularis triggered activation of the vagal efferent pathway mainly targeting the inflamed zone. In the present study we investigate further the modulatory role of endogenous activation of the vagal motor pathway on the innate immune response. Intestinal or splenic denervation was performed two weeks prior to intestinal manipulation (IM) or laparotomy (L). Twenty-four hours post-surgery, the gastrointestinal transit, immune cell influx, and pro-inflammatory cytokine levels were measured in the gut muscularis. Manipulation of the small intestine led to a delay in intestinal transit, an influx of leukocytes and increased pro-inflammatory cytokine expression. Surgical lesion of the vagal branch that selectively innervates the small intestine did not further delay the intestinal transit but significantly enhanced the expression levels of the pro-inflammatory cytokines IL-1β and IL-6 in the gut muscularis. Splenic denervation did not affect intestinal inflammation or gastrointestinal transit after intestinal manipulation. Our study demonstrates that selective vagotomy, leaving the splenic innervation intact, increases surgery-induced intestinal inflammation. These data suggest that endogenous activation of the vagal efferent pathway by intestinal inflammation directly dampens the local immune response triggered by intestinal manipulation independently of the spleen.

Introduction

Postoperative Ileus (POI) is experienced by almost every patient undergoing open abdominal surgery. The pathophysiology of POI is characterized by a transient impairment of the gastro-intestinal tract leading to nausea, pain and discomfort for the patient. It is now well documented that the underlying mechanism relies on the recruitment of leukocytes as well as the production of pro-inflammatory cytokines (i.e., IL-β, IL-6, TNF-α) and induction of enzymes (i.e., Cox-2) in the gut muscularis after handling of the intestine [1,2].

During the last decade the vagus nerve was discovered as an essential player in the regulation of the immune response. Indeed, in diverse models of inflammation, increased vagal activity by electric stimulation of the vagus nerve was reported to dampen inflammation by suppressing the production of pro-inflammatory cytokines. The spleen was recently identified to play an essential role in mediating this anti-inflammatory effect of the vagus nerve in both endotoxemia [3,4], colitis [5,6] and local inflammation [7]. This phenomenon is thought to rely on the existence of synaptic connections between the vagus nerve and the sympathetic splenic nerve in celiac ganglia [8]. Activation of the vagus nerve leads to the release of noradrenaline by the splenic nerve which activates the production of acetylcholine (ACh) by splenic T cells. Binding of ACh to receptors present on macrophages was shown to inhibit their secretion of pro-inflammatory cytokines such as TNF-α, leading to dampening of the inflammatory response. Evidence of the existence of synaptic contacts between the vagus nerve and sympathetic post-ganglionic neurons in the celiac ganglion on which this model relies is however missing. Recent studies have on the contrary described the absence of such connections [9-11].

Several studies also demonstrated the anti-inflammatory property of the vagus nerve in POI. Indeed, vagus nerve stimulation (VNS) applied prior to the handling of the intestine down-regulates the secretion of pro-inflammatory cytokines by resident macrophages, inhibits the influx of immune cells to the gut muscularis and thereby prevents the delay in the gastrointestinal transit [12-14]. Besides, we provided substantial functional evidence that VNS directly suppresses pro-inflammatory cytokine secretion by macrophages in the gut muscularis independently of the spleen [14]. So far, most studies performed to unravel the cholinergic anti-inflammatory pathway regulating postoperative ileus

used electrical activation of the vagus nerve prior to intestinal manipulation and the subsequent intestinal inflammation. Data on the existence of an intrinsic manipulation-induced vagal reflex modulating the intestinal inflammation underlying postoperative ileus are however scarce. We recently demonstrated that intestinal inflammation triggered the activation of vagal motor neurons innervating the small intestine as well as the spleen [15]. The exact contribution of the vagal innervation targeting the intestine and splenic innervation in the modulation of the manipulation-induced inflammatory response underlying postoperative ileus has not yet been investigated.

In this study, we hypothesized that endogenous activation of vagal efferent fibers innervating the small intestine induced by intestinal manipulation, similar to vagus nerve stimulation, exerts a direct suppressive effect on pro-inflammatory cytokine release by immune cells during POI. We anticipate that this vagal anti-inflammatory effect is not mediated through the splenic nerve.

Material and methods

Mice

Ten to 12 week-old female Balb/c were purchased from Harlan Nederland (Horst, The Netherlands) and housed in a Specified Pathogen Free facility with a 12/12 light/dark cycle under constant conditions of temperature (20 +/- 2º C) and humidity (55%) and *ad libitum* food and water. Denervation procedures were performed under a mixture of fentanylcitrate/fluanisone (Hypnorm, Janssen, Belgium) and midazolam (Dormicum, Roche, The Netherlands) (5mg/mL). The anesthetic mixture was in a ratio of 1:1:2 of Hypnorm, Dormicum and water respectively and each mouse received 0.1mL/10g of that mixture by intraperitoneal injection. For the intestinal manipulation procedure, a mixture of ketamine (Ketalar 100 mg/kg) and xylazine (Rompun 10 mg/kg), known to have a short-lasting effect [16], was injected intraperitoneally. All efforts were made to minimize the suffering of the animals. All experiments were performed in accordance with the guidelines of the Laboratory Animal Use of the Netherlands and approved by the Ethical Animal Research Committee of the Academic Medical Center of Amsterdam.

Surgical procedures and sample collection

Selective vagal denervation of the intestine (Intx) and splenic denervation (Splx) were

performed as previously described [15]. Vagal intestinal denervation was achieved by cutting the right celiac branch of the vagus nerve supplying the jejunum, ileum and cecum. Splenic denervation was achieved by cutting noradrenergic fibers running along blood vessels supplying the spleen and by cutting nerve fibers present in the conjonctive tissue located at each tip of the spleen. Mice were monitored and weighed daily following the denervation procedure. Both sham-operated, Intx and Splx mice regained their initial weight and produced normal stool within 5 days after the surgical procedure. Two weeks after sham operation or denervation, mice underwent Laparotomy (L) or Laparotomy followed by Intestinal Manipulation (IM), as previously described [15]. After opening the abdominal cavity by a midline incision, the small intestine was carefully externalized from the peritoneal cavity and placed on a moist gauze pad. The small bowel was manipulated by compression with two moist cotton swabs: the two cotton swabs were placed on each side of the small intestine and manipulation was performed so that the luminal content of the small intestine was moved aborally. The entire small bowel was manipulated twice from the distal duodenum to the cecum. No contact or manipulation of the stomach or colon was applied. Sacrifice was performed 24h after surgery by transcardiac perfusion with PBS during anesthesia with pentobarbital (0.1mL of a 50mg/mL solution).

Tyrosine Hydroxylase staining

The completion of splenic nerve lesion was assessed by a Tyrosine Hydroxylase (TH) immunohistochemical staining on spleen sections. Eight µm sections of frozen spleens were air-dried, fixed in ice cold acetone for 2 min and air-dried again. Sections were blocked with 0.1% Triton (Sigma, St Louis, MO) 2% Normal Goat Serum (Dako Cytomation, Glostrup, Denmark) in TBS for 30 min. After a 2-hour incubation at RT with an anti-mouse TH antibody (Sigma, St Louis, MO), sections were incubated with a Brightvision Poly-AP-Anti Rabbit antibody (Immunologic, Duiven, The Netherlands) for 60 min at RT. The staining was revealed by incubating the sections with an alkaline-phosphatase solution AP kit III (Vector Laboratories, Burlingame, The United States) in Tris HCl with 5 mM Levamisole (Sigma, St Louis, MO) for 35 min. Sections were air-dried and mounted in gelatin-glycerol (Sigma, St Louis, MO). The absence of positive signal determined a complete splenic denervation. The success rate of the splenic denervation was 95%. One mouse was excluded from the analysis as its splenic denervation was not complete.

Measurement of the gastrointestinal transit

The gastrointestinal transit was measured using the non-absorbable tracer 70 kDa fluorescein isothiocyanate-labeled dextran (FD70) as previously described [14,17]. Briefly, 1.5 hour before sacrifice, mice were fed with 10 µL of a 6.25 mg/mL solution of FD70 in distilled water. The entire gastrointestinal tract (from stomach to colon) was divided into 15 segments (stomach, 10 segments of equal length for the intestine, cecum, and 3 segments of equal length for the colon). Each segment was flushed with PBS and FD70 concentration was assessed by fluorimetry in the supernatant of each segment. The distribution of FD70 was determined by calculation of the Geometric Center (GC) with GC=Σ(% of total fluorescent signal per segment x segment number)/100).

RNA isolation, RT-PCR and QPCR

The mucosa of segments 5, 6 and 7 (ileum) of the small intestine was stripped from the muscularis with 2 fine pincets. Intestinal muscularis of these 3 segments was snap-frozen. Total mRNAs of the frozen intestinal muscularis were extracted after homogenization of the samples in TriPure isolation reagent according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). cDNA synthesis was performed using the Revertaid first strand cDNA synthesis kit (Fermentas, Germany) and Real-time PCR was performed using a SYBR green master mix (Roche Applied Science, Indianapolis, IN) on a Lightcycler480 (Roche Applied Science, Indianapolis, IN). Primer sets were synthesized by Invitrogen (Bleiswijk, The Netherlands) and are described in Table 1. Raw data of the genes of interest were analyzed using the LinRegPCR program (AMC, The Netherlands) [18] and normalized with reference genes chosen after analysis with the Genorm software.

Cytokine analysis by ELISA

Segments 2, 3 and 4 (jejunum/ileum) of the small intestine were snap frozen and stored until further use. Small intestinal segments were homogenized in Greenberger Lysis Buffer. Analysis of murine IL-1β, IL-6 and TNF-α was performed using the commercially available ELISA kits according to the manufacturer's instructions (R&D systems, Abingdon, UK). Total protein content of the samples was measured using the BCA colorimetric assay (Thermoscientific, Breda, The Netherlands). The concentration of

the protein of interest was normalized on the total protein content of each sample.

Myeloperoxidase quantification by Immunohistochemistry

Segment 8 (ileum) of the small intestine was stored in 70% ethanol until further use. The mucosa was removed from the muscularis with 2 fine pincets and stained for Myeloperoxidase (MPO) as a marker of leukocytic infiltration. Briefly, the whole mount intestinal muscularis segment were stained with a 3-amino-9-ethyl carbazole (Sigma, St Louis, MO), 0.01% H_2O_2 in Sodium Acetate buffer (pH=5) for 20 min, as previously described [15]. Sections were analyzed using a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives) connected to a color-camera (JVC KY-F55 3CCD). Random counting of MPO positive cells was performed for each section, as previously described [15].

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc, Chicago, IL). The data are expressed as mean±SEM. Normal distribution was assessed using the Kolmogorov-Smirnov test. Square-root normalization was applied to non-normal data sets. A 2-way ANOVA was performed to determine the interaction between denervation (Sham *vs* Intx/Splx) and treatment (L *vs* IM). When significance was observed with the 2-way ANOVA, an unpaired Student t-test was performed to evaluate the significance between Sham *vs* Intx/Splx or L *vs* IM.

	Forward primer 5'-3	Reverse primer 5'-3
HRPT	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Cyclophilin	ACCCATCAAACCATTCCTTCTGTA	TGAGGAAAATATGGAACCCAAAGA
IL-1β	CTCCTGCTGTCGGACCCAT	TGCCGTCTTTCATTACACAGGA
IL-6	GAGTTGTGCAATGGCAATTCTG	TGGTAGCATCCATCATTTCTTTGT
TNF-α	TGGAACTGGCAGAAGAGGCACT	CCATAGAACTGATGAGAGGGAGGC
Cox-2	GGCCATGGAGTGGACTTAAATC	ACCTCTCCACCAATGACCTGAT
IL-10	GGACAACATACTGCTAACCG	GGGGCATCACTTCTACCAG
TGF-b	GCTGAACCAAGGAGACGGAATA	GGGCTGATCCCGTTGATTT
CD45	CCCCGGGATGAGACAGTTG	AAAGCCCGAGTGCCTTCCT
F4/80	TCATTCACTGTCTGCTCAACGGTC	GAAGTCTGGGAATGGGAGCTAAGG

Table 1. Primer sequences used for QPCR analysis

Results

Modulatory role of vagal motor efferent fibers in response to intestinal inflammation

Intestinal inflammation induced by handling of the small intestine delayed the gastrointestinal transit, as shown by the decrease in calculated geometric center (GC) (Fig. 1A) (11±1 *vs* 4±1 for Sham L *vs* Sham IM). We also reported a strong influx of immune cells to the gut muscularis, i.e., increased expression level of the general hematopoietic marker CD45 (L *vs* IM: 1±0 *vs* 5±1; p<0.001) and the macrophage marker F4/80 (L *vs* IM: 1±0 *vs* 3±0; p<0.001) and increased numbers of MPO^+ cells (neutrophils/monocytes) (Fig. 1B and C). Ablation of the vagal input to the small intestine did not affect intestinal transit in control mice (Laparotomy) and did not further delay the gastrointestinal transit in the inflamed intestine. In line, the immune cell influx was not affected by the denervation.

However, a significant effect of vagal denervation was observed on the expression levels of pro-inflammatory cytokines in the gut muscularis. Indeed, Intx IM mice displayed a significant increase in the mRNA (Sham IM *vs* Intx IM: 8±1 *vs* 29±9; p=0.03) and protein level of IL-1β (Sham IM *vs* Intx IM: 8±1 *vs* 29±9; p=0.04). A significant increase in the mRNA level of IL-6 (Sham IM *vs* Intx IM: 6±2 *vs* 21±7; p=0.039) as well as a trend towards increased IL-6 protein level (Sham IM *vs* Intx IM: 13±4 *vs* 20±7; p=0.08) was also observed in Intx mice compared to Sham-operated animals 24h after IM. Of note, a non-significant trend was observed in the mRNA levels of TNF-α (Sham IM *vs* Intx IM: 8±2 *vs* 18±7; p=0.11) and Cox-2 (Sham IM *vs* Intx IM: 7±2 *vs* 10±3; p=0.34). As described previously, intestinal manipulation led to an increase in the expression of the anti-inflammatory cytokines IL-10 and TGFβ [17,19]. Vagal intestinal denervation however did not affect the expression level of these anti-inflammatory cytokines (Fig. 2A and B).

Splenic innervation does not participate in the vagal regulatory effect on the intestinal immune response

We previously demonstrated that the spleen is not involved in the anti-inflammatory effect of VNS during POI [14]. To determine whether the endogenous vagal anti-inflammatory effect triggered in response to manipulation of the intestine was also independent of splenic innervation, we investigated the effect of selective splenic denervation on IM-induced inflammation and delay in gastrointestinal transit.

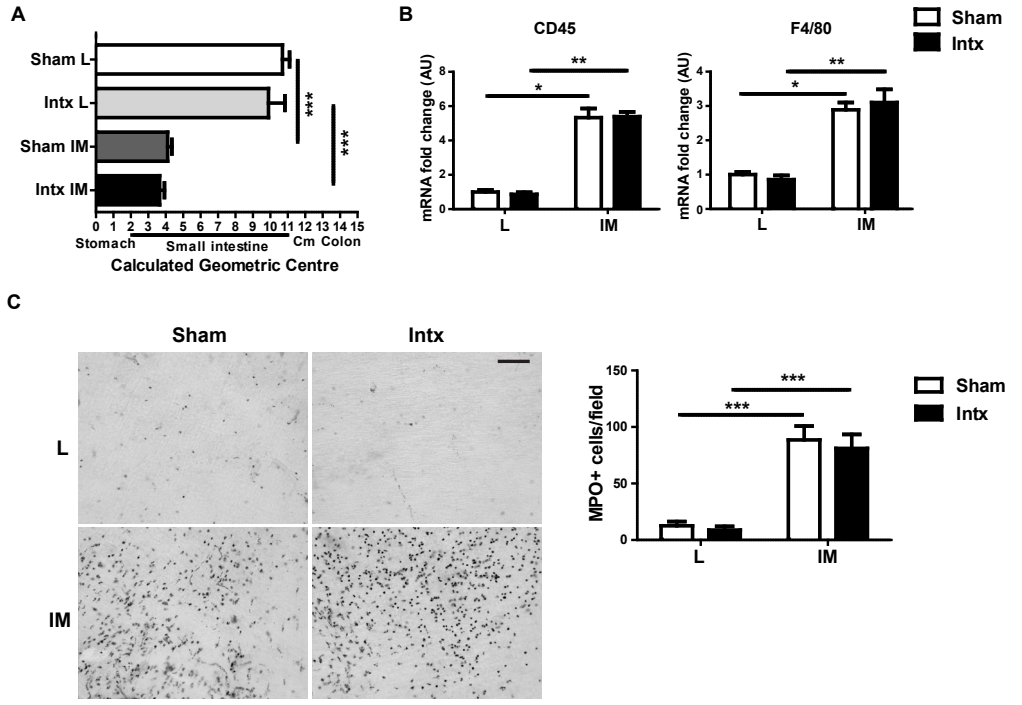

Figure 1. Intestinal vagal denervation does not influence the impairment of the gastrointestinal transit or the influx of immune cells to the small intestine. Lesion of the vagal fibers targeting the intestine was performed prior to Laparotomy (L)/Intestinal Manipulation (IM). (A) Intestinal manipulation (IM) triggers a delay in the gastrointestinal transit 24h post-surgery but intestinal denervation (Intx) does not affect the severity of this delay. Cm=cecum (B) IM triggers enhanced expression of the hematopoietic marker CD45 and the macrophage marker F4/80 in the gut muscularis 24h post-surgery. Intx doesn't affect the expression level of these markers. (C) IM triggers an enhanced number of myeloperoxidase positive (MPO^+) cells in the gut muscularis 24h after surgery. Intx does not affect the number of MPO^+ cells in the gut muscularis. Scale bar represents 50µm. Data are expressed as mean±SEM (n=6-7 animals per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Completion of splenic denervation was assessed by immunohistochemical staining for tyrosine hydroxylase, an enzyme present in noradrenergic fibers. The absence of positive signal determined a successful splenic denervation (Fig. 3A). The IM induced-delay in gastrointestinal transit was similar in sham-operated and splenic denervated mice (Fig. 3B). Similarly, no effect of the splenic denervation was observed in the number of immune cells infiltrating the gut muscularis (Fig. 3C), and the mRNA and protein levels of pro- (i.e., IL-1β, IL-6, TNF-α and Cox-2) or anti-inflammatory cytokine levels (i.e., IL-10 and TGFβ) (Fig. 4A and B) confirming that the spleen does not participate in the vagal anti-inflammatory pathway in POI.

Discussion

In the present study, we demonstrate that selective vagal denervation of the intestine aggravates pro-inflammatory cytokine production in the gut muscularis in response to intestinal manipulation but does not affect influx of immune cells or delay in the intestinal transit. This vagal immunomodulatory effect targets directly the gut muscularis and does not involve the splenic innervation.

We recently provided anatomical evidence of endogenous activation of a vagal reflex in response to intestinal inflammation [15]. Activation of vagal neurons was observed in both the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV) 24h after manipulation of the intestine demonstrating that the vagus nerve senses and responds to IM-induced inflammation. Here, we demonstrate that vagal innervation of the small intestine is essential to dampen the pro-inflammatory cytokines IL-1β and IL-6, known to increase early on after surgery. Interestingly the production of IL-1β and IL-6 in the gut wall is not restricted to the sole immune cells as enteric neurons are also able to produce these pro-inflammatory cytokines [20,21]. However, we recently demonstrated that VNS failed to dampen the inflammation in POI in wild-type irradiated mice reconstituted with α7nAChR$^{-/-}$ bone marrow.

This strongly demonstrates that the anti-inflammatory effect of the vagus nerve on the production of IL-1β and IL-6 relies on the presence of the α7nAChR cholinergic receptors on innate immune cells (i.e., macrophages and neutrophils) rather than enteric neurons [14]. Notably, especially IL-1β has recently been identified to play an essential role in the development of POI [21]. Given the crucial role of IL-1β in POI, a tight control of its production is of extreme importance. Our data demonstrating an increase in this cytokine following selective vagal denervation of the intestine indicate that IM-induced inflammation activates the vagal anti-inflammatory pathway reducing IL-1β production and contributing to restrict the inflammatory response.

Influx of mainly neutrophils and monocytes is considered to mediate the reduction in gastrointestinal motility in the postoperative period, mainly by release of nitric oxide and prostaglandins [1,2]. Previously, we showed that VNS significantly reduces this influx and shortens POI. Conversely, one might anticipate that selective vagal denervation of the intestine will increase cellular influx, an effect that we previously observed when cervical vagotomy was performed prior to manipulation of the intestine [12]. In the

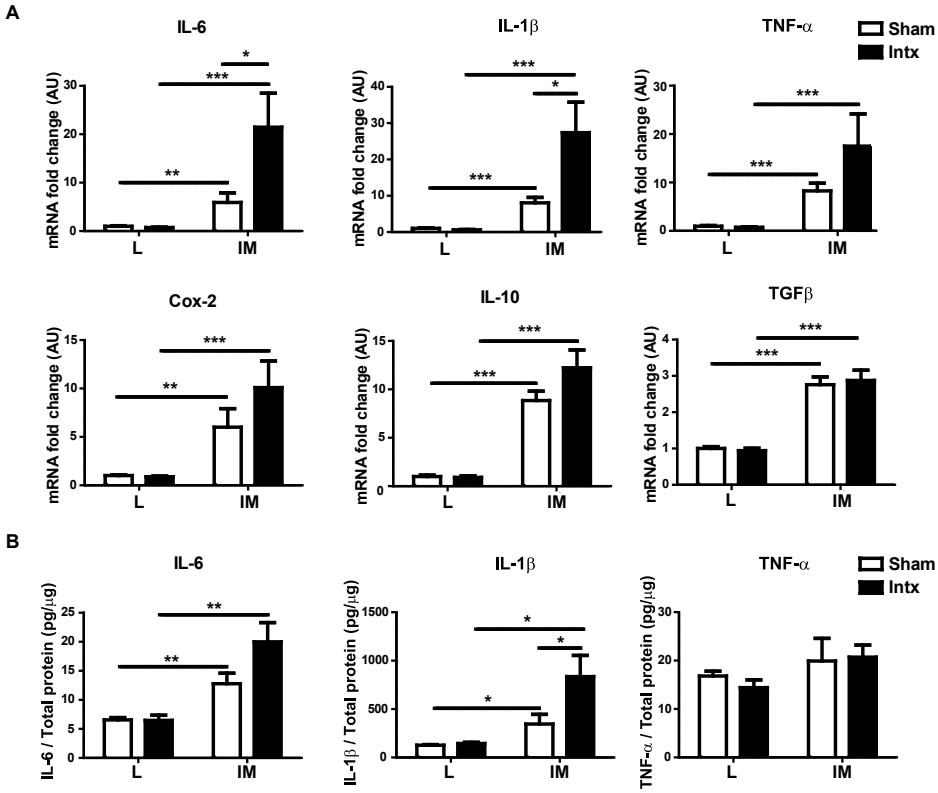

Figure 2. Lack of vagal intestinal innervation enhances the expression level of pro-inflammatory cytokines in the gut muscularis after IM. (A) Increased mRNA levels of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α are observed 24h after Intestinal manipulation (IM). Vagal intestinal denervation (Intx) increases the expression level of IL-6 and IL-1β after IM in comparison with sham-operated animals. (B) Cytokine levels as measured by ELISA 24h after IM. Intx leads to a significant increase in IL-1β and a trend towards increased IL-6 in the small intestine 24h after IM. Data are expressed as mean±SEM (n=12-14 animals per group). * p<0.05; *** p<0.001

present study, we fail to observe increased cellular influx to the gut muscularis in mice lacking vagal input to the intestine. Influx of cells to the gut muscularis represents a very early step in the pathophysiology of postoperative ileus whereas endogenous activation of the motor part of the vagus nerve occurs once the inflammation is settled [15]. This may explain why vagal denervation of the intestine does not alter the influx of cells after intestinal manipulation. Moreover, we previously showed that mild manipulation of the intestine leads to less production of pro-inflammatory cytokines in the intestine as compared to a severe manipulation of the intestine [22]. In this same study however, the influx of cells to the gut muscularis was as high in mice undergoing mild intestinal manipulation as in mice undergoing severe intestinal manipulation. This seems to indicate that the influx of cells after intestinal manipulation represents an on/off system that reaches its maximum intensity independently of the severity of the manipulation or the inflammation and may explain this absence of aggravation of cellular influx in intestinal-denervated mice. Moreover, the increase in the levels of pro-inflammatory cytokines observed after intestinal vagal denervation in the absence of a greater influx of cells implies that the vagus nerve exerts an anti-inflammatory role targeting immune cells residing in the gut muscularis and/or on infiltrating immune cells once they have reached the gut muscularis.

VNS was previously shown to prevent the delay in the gastrointestinal transit demonstrating the effectiveness of vagal signaling in ameliorating POI [13,14]. Although we observed increased inflammation 24h after IM in intestinal-denervated mice compared to sham-operated mice, we failed to report a further delay in the gastrointestinal transit. Importantly, VNS is applied pre-emptively to intestinal handling thereby preventing the release of pro-inflammatory cytokines by resident macrophages and consequently inhibiting the underlying cause leading to intestinal paralysis. On the other hand, endogenous activation of the vagal efferent pathway is only triggered once the intestinal inflammation is already settled and inflammatory signals such as pro-inflammatory cytokines and enzymes are released and detected by sensory vagal afferents. This endogenous pathway most likely contributes to resolution rather than prevention of the inflammatory response. This discrepancy might explain why, even though the endogenous vagal anti-inflammatory effect is able to dampen the production of pro-inflammatory cytokines by innate immune cells, it is not sufficient to prevent the impairment of the gastrointestinal motility. Of note, the delay in gastrointestinal transit observed after intestinal manipulation in our hands is very severe. It is therefore likely

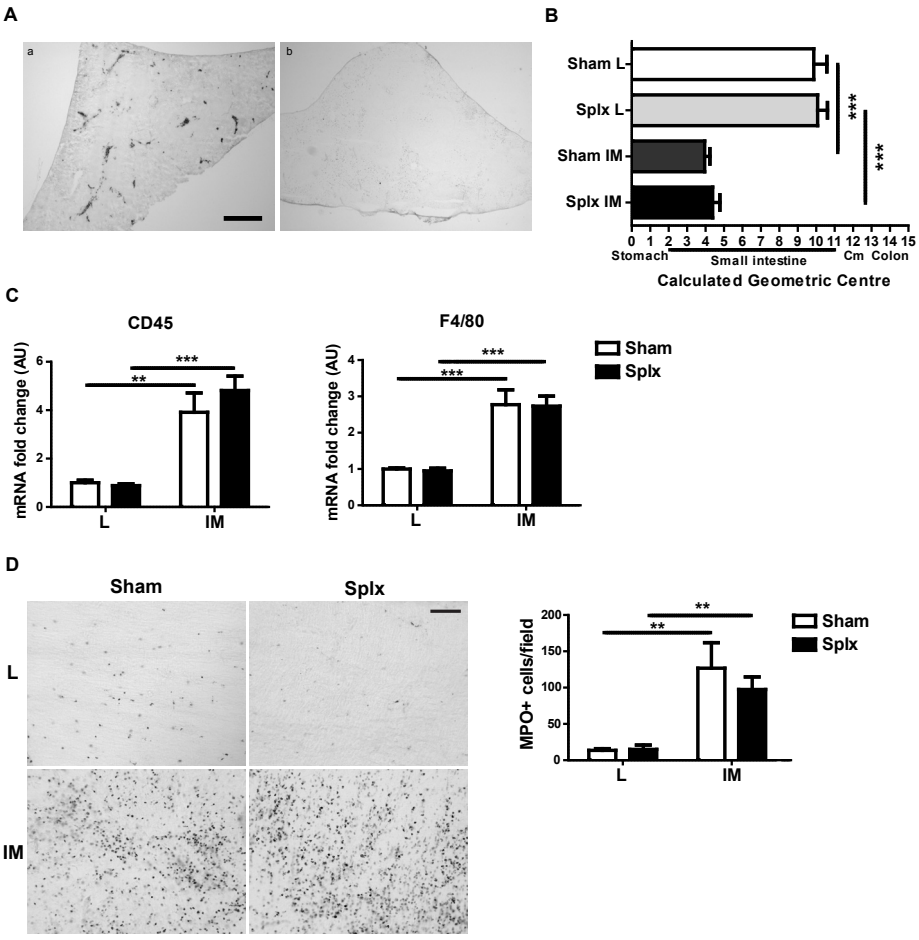

Figure 3. The vagal anti-inflammatory effect is independent of splenic innervation. Splenic denervation (Splx) was performed prior to Laparotomy (L)/Intestinal Manipulation (IM) (A) Immunohistochemical staining targeting Tyrosine Hydroxylase (TH) in sham animals (a) revealed the presence of noradrenergic fibers alongside blood vessels, trabeculae and in the white and red pulp of the spleen. The absence of TH^+ fibers in spleen sections of spleen-denervated (Splx) mice (b) was taken as a proof of completion of splenic denervation. (B) Splx does not affect the severity of the delay in the gastrointestinal transit or (C) the influx of cells to the gut muscularis 24h after IM. Scale bar represents 50µm. Data are expressed as mean±SEM (n=11-12 animals per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$

that, as for the cellular influx to the muscularis, this delay is already at its maximum and cannot be increased by denervation.

The action mode of the anti-inflammatory effect triggered by VNS was shown to rely on splenic innervation during sepsis and colitis [4,6-8]. In the context of POI however, VNS was shown to modulate the intestinal inflammation independently of the splenic innervation [14]. In these inflammatory models, little is known about the exact neural networks implicated in the inflammation-induced endogenous vagal anti-inflammatory effect. Here we demonstrate for the first time that also under endogenous conditions, splenic innervation is not implicated in the vagal neural network regulating postoperative ileus. On the contrary, we show that the vagal neural circuitry activated by IM-induced inflammation directly exerts its anti-inflammatory effect on the inflamed organ, i.e., the intestine. Furthermore, the approach we chose in the present study allowed us to provide evidence that the cholinergic anti-inflammatory pathway is also endogenously activated by intestinal inflammation. Notably, this results in a reduction of pro-inflammatory cytokines in the muscularis thereby contributing to resolution of the inflammatory response.

In conclusion, our study provides further insights in the cholinergic anti-inflammatory pathway regulating POI, showing that the vagal pathway activated endogenously during POI exerts an anti-inflammatory effect independently of splenic innervation.

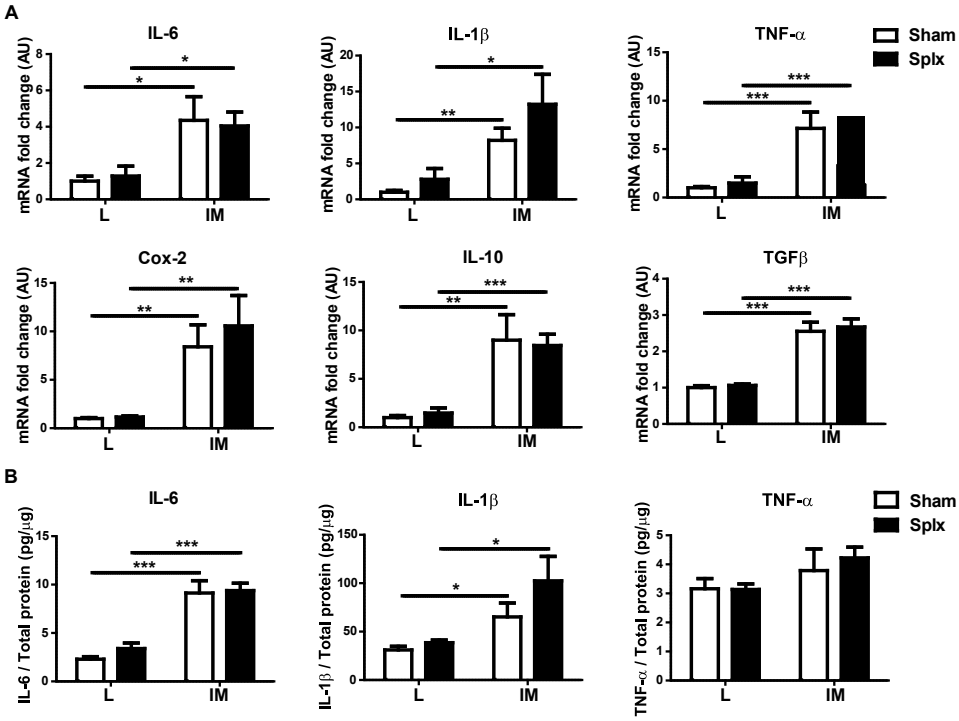

Figure 4. The vagal anti-inflammatory effect is independent of splenic innervation A. Splx mice display identical increased levels of IL-1β, IL-6 and TNF-α as sham-operated mice 24h after IM on mRNA level (A) as well as on a protein level (B). Data are expressed as mean±SEM (n=11-12 animals per group). * p<0.05; ** p<0.01; *** p<0.001

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Chapter 6

Systemic inflammation with enhanced brain activation contributes to more severe delay in postoperative ileus

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Abstract

Background: The severity of postoperative ileus (POI) has been reported to result from decreased contractility of the muscularis inversely related to the number of infiltrating leukocytes. However, we previously observed that the severity of POI is independent of the number of infiltrating leukocytes, indicating that different mechanisms must be involved. Here, we hypothesize that the degree of tissue damage in response to intestinal handling determines the upregulation of local cytokine production and correlates with the severity of POI. Methods: Intestinal transit, the inflammatory response, I-FABP (marker for tissue damage) levels and brain activation were determined after different intensities of intestinal handling. Key results: Intense handling induced a more pronounced ileus compared with gentle intestinal manipulation (IM). No difference in leukocytic infiltrates in the handled and non-handled parts of the gut was observed between the two intensities of intestinal handling. However, intense handling resulted in significantly more tissue damage and was accompanied by a systemic inflammation with increased plasma levels of pro-inflammatory cytokines. In addition, intense but not gentle handling triggered enhanced c-Fos expression in the nucleus tractus solitarius (NTS) and area postrema (AP). In patients, plasma levels of I-FABP and inflammatory cytokines were significantly higher after open compared with laparoscopic surgery, and were associated with more severe POI. Conclusions and inferences: Not the influx of leukocytes, rather the manipulation-induced damage and subsequent inflammatory response determine the severity of POI. The release of tissue damage mediators and pro-inflammatory cytokines into the systemic circulation most likely contribute to the impaired motility of non-manipulated intestine.

Introduction

Postoperative ileus (POI) is characterized by a transient inhibition of gastrointestinal (GI) motility following surgery. Patients experience significant discomfort, such as abdominal distention, nausea, and inability to pass stool or tolerate food. Especially prolonged ileus leads to an increased risk for wound dehiscence, pulmonary, and thromboembolic complications and a prolonged hospital stay and is associated with an enormous economic burden [1]. During the last decade, evidence has accumulated that intestinal inflammation evoked by handling of the intestine is a key mechanism underlying impaired GI motility following surgery, both in humans and in animal models. These studies demonstrated that infiltrating leukocytes inhibit the contractile activity of the manipulated intestine by local release of pro-inflammatory mediators such as nitric oxide and prostaglandins [2,3]. It is becoming increasingly clear that POI mainly results from intestinal handling of the intestine during surgery [4]. In rodents, Kalff et al. elegantly showed that manipulation of the intestine triggered the influx of leukocytes in the muscularis, starting from 3h onwards and further increasing up to 24h after surgery. Of note, the number of infiltrating leukocytes increased with the severity of intestinal manipulation (IM) with compression of the intestine yielding more influx than running along the intestine with cotton swabs [5]. These infiltrating leukocytes, mainly monocytes, subsequently release inflammatory mediators such as prostaglandins and nitric oxide impairing the contractility of smooth muscle strips of the intestine [6]. The latter has been proposed to underlie the delay in intestinal transit observed 24h after the abdominal surgical procedure. Recently, however, we observed that eventration of the small intestine and graded manipulation of the intestine up to 3.5 g did not delay GI transit, but was associated with influx of leukocytes to the same level as more intense manipulation that did lead to POI [7]. These data would imply that other mechanisms must be involved. Based on the above, we reasoned that the degree of tissue damage evoked by intestinal handling may be an important determinant of the severity of POI. Several clinical studies indeed have reported an increased postoperative inflammatory response related to increased operative trauma with systemic release of cytokines and systemic spread of the inflammatory response [5,8,9]. Tissue damage can trigger an innate immune response via the local release of damage associated molecular pattern molecules [10], evoking an inflammatory response involving macrophages and/or mast cells. The resulting enhanced local inflammation may result in a more systemic inflammatory response with increased serum levels of pro-inflammatory cytokines.

The latter will consequently affect distant regions of the gut and contribute to the generalized aspect of POI. In this study, therefore, we investigated the mechanism behind severe POI by studying the local and systemic inflammatory response, including brainstem activation after different intensities of intestinal handling.

Material and methods

Animals

Laboratory animals were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM with water and food *ad libitum*; 20–22 °C, 55% humidity). Ten to twelve weeks old C57NL/BL6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). Mice were maintained at the animal facility of the Academic Medical Centre in Amsterdam and were used at 12–14 weeks of age. Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands).

Patients

Patients undergoing elective segmental colectomy for colonic cancer were invited to participate. The protocol was approved by the Medical Ethics Review Board of the Academic Medical Center in Amsterdam (The Netherlands) and was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice guidelines.

Surgical procedures

Anesthesia was performed by an intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 $mg.kg^{-1}$) and Xylazine (Rompun 10 $mg.kg^{-1}$). Mice (five to eight per group) underwent a laparotomy (L) alone, or a L followed by small IM [11]. Surgery was performed as follows: a midline abdominal incision was made and the peritoneum was opened over the linea alba and the small bowel was carefully layered on a sterile moist gauze pad. The small intestine was manipulated from the distal duodenum to the cecum and back for a total of three times. Contact with or stretch on stomach or colon was strictly avoided. Gentle standardized bowel manipulation (gentle IM) was constructed

using a sterile moist cotton applicator attached to a device enabling the application of a constant pressure of 9 g to the intestine. The more intense manipulation (intense IM) was performed by compression of the small bowel using moist cotton applicators such that the luminal content was moved aborally as previously described [12].
After the surgical procedure, the abdomen was closed by a continuous 2-layer suture (Mersilene, 6–0 silk). After closure, mice were allowed to recover for 3h in a heated (32 °C) recovery cage.

Gastrointestinal transit measurements

Gastrointestinal function 24h postoperatively was determined in vivo by measurement of GI transit of liquid non-absorbable fluorescein isothiocyanate–dextran (FITC-dextran; 70 000 Da; Invitrogen, Paisley, UK). Ten microlitres of FITC-dextran dissolved in 0.9% saline (6.25 $mg.mL^{-1}$) was administered via oral gavage. Ninety minutes later, animals were sacrificed and the entire bowel from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), cecum, and colon (three segments of equal length) were collected and assayed in duplicate (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA; excitation wavelength: 485 nm, emission wavelength: 528 nm) for the quantification of fluorescent signal in each bowel segment. The distribution of the fluorescent label along the GI tract was determined by calculating the geometric center (GC): Σ (% of total fluorescent signal in each segment x the segment number)/100 for quantitative statistical comparison among experimental groups [13].

Colonic transit

Colon function was determined in vivo by the measurement of colon transit of a glass ball. One and a half hour before sacrifice, mice were briefly anesthetized with isoflurane (Abbott). Patency of the colon was carefully checked by inserting a polished metal rod 3 cm into the colon. The rod was pulled out and a 2.2 mm plastic ball was transanally inserted with blunt surgical forceps and pushed forward for 3 cm into the colon with a polished metal rod. The time from insertion until excretion of the plastic ball was considered as colonic transit time [14].

c-Fos expression in the brain

Twenty-four hours after surgery mice were sacrificed by transcardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA; pH 7.4). Brains were collected, postfixed for 4h (4 °C) and cryo-protected by immersion in 30% sucrose in 0.2 mol.L^{-1} PBS (pH 7.4) at 4 °C overnight. Coronal sections of 30 µm of the brainstem were collected. After rinsing in 0.05 mol.L^{-1} Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1 : 1500; SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) primary antibodies. However, sections were incubated 1h with biotinylated secondary antibody and after with avidin-biotin complex (ABC; Vector, Burlingame, CA, USA) for 1h. The reaction product was visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide H_2O_2 for 5 min to count the number of c-Fos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 color camera (Sony Corp., Tokyo, Japan). A minimum of seven sections was used for c-Fos counting in the NTS (from Bregma -7.20 to -7.76 mm) and Area Postrema (AP; Bregma -7.32 to -7.76 mm), and 9–11 sections for PVN (Bregma -0.58 to -1.22 mm).

Immunohistochemistry staining for leukocytic infiltration of the small intestinal muscularis.

To quantify the degree of inflammation in whole mounts of the intestinal muscularis, ileal segments were cut open and rinsed in ice-cold modified Krebs solution. The segments were fixed with 100% ethanol for 10 min, transferred to ice cold modified Krebs solution, and pinned flat in a glass-dish. Mucosa and submucosa were removed, and the remaining full-thickness sheets of muscularis externa were stained for polymorphonuclear neutrophils with Hanker Yates reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 min. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase (MPO) positive cells in 10 randomly chosen representative high-power fields (HPF; 668.4 x 891.2 µm) was counted and the average was calculated. Tissue sections were coded so that the observer was unaware of the surgical treatment of the specimens.

Blood analysis: tissue damage and plasma levels of inflammatory cytokines

Mice

tissue damage was assessed by determining plasma levels of intestinal fatty acid-binding protein (I-FABP) [15]. Levels of I-FABP in the plasma were determined using standard enzyme-linked immunosorbent assay (ELISA) for mouse I-FABP (Hycult Biotechnology (Hbt), Uden, The Netherlands). Interleukin (IL)-6, the murine IL-8 homologue KC, Monocyte Chemoattractant Protein-1 (MCP-1), Tumor Necrosis Factor (TNF-α) and IL-1β plasma levels of venous blood retrieved by cardiac puncture 1, 6 and 24h after surgery were determined using cytometric bead array kits (CBA) according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Biosciences). Cytometric bead assay results were analyzed using the FCAP ArrayTM software (BD Biosciences).

Patients

IL-6, IL-8, MCP-1, TNF-α and IL-1β plasma levels of venous blood retrieved 2h after surgery were determined using CBA kits for human IL-6, IL-8, MCP-1, TNF-α (for human TNF-α and IL-1β the enhanced sensitivity flex set kits were used) according to the manufacturer's instructions (BD Biosciences). Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Biosciences). Cytometric bead assay results were analyzed using the FCAP ArrayTM software (BD Biosciences). Before determination of I-FABP in the human samples, blood was centrifuged two times and the obtained plasma was concentrated with the use of Vivaspin 23 000 MW sample concentrators (Bio-Sciences AB, Uppsala, Sweden) by centrifuging at 3506 g at 4 °C for 2h and further processed according to the manufacturer's instructions. Levels of I-FABP in the concentrated plasma were determined using ELISA human I-FABP (Hbt).

RNA extraction and inflammatory gene expression

Total RNA was extracted from the intestinal muscularis externa of the distal stomach, jejunum, and distal colon at 6 and 24 after start surgery. The muscularis was microscopically dissected from the submucosa and immediately snap frozen in Tripure (Roche diagnostics, Mannheim, Germany) and stored at -80 °C. Tissue was homogenized by a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France).

RNA extraction was performed using RNeasy Mini Kit (Qiagen # 74104; Qiagen Benelux BV, Venlo, the Netherlands) according to manufacturer's instructions. Total of RNA were transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) according to manufacturer's instructions. Quantitative real-time transcription polymerase chain reactions (RT-PCR) were performed with the LightCycler 480 SYBR Green I Master (Roche) on the Light Cycler 480, Roche (Roche). Results were quantified using the $2^{\Delta\Delta Ct}$ method (PMID:11328886). The expression levels of the genes of interest were normalized to the expression levels of the reference gene (RPL32). PCR experiments were performed in triplicate, and standard deviations calculated and displayed as error bars. Primer sequences used are listed in Table S1.

Statistical analysis

The data on human plasma cytokine levels were not normally distributed. The Kruskal–Wallis test was performed to assess whether the cohort of data was statistically different. When variance of medians was statistically significant, the Mann–Whitney U test was used to identify the statistical differences within the cohort. For comparison of the time to recovery of GI function and plasma I-FABP levels between open and laparoscopy-treated patients, the Mann–Whitney test was used and results were shown as median with interquartile ranges (IQR). All other data were statistically analyzed by one-way ANOVA followed by Tukey's Multiple Comparison analysis and are presented as mean±SEM. A probability level of $P < 0.05$ was considered statistically significant. Graph Pad Prism version 5.01 software was used to perform statistical analysis and create graphs.

Results

GI transit and colonic transit

Twenty four hours after surgery, the intestinal transit was significantly delayed by IM compared with L (GC: 8.9±0.7). Notably, intense IM (GC: 3.8±0.2) induced a more severe delay in intestinal transit compared with gentle IM (GC: 6.3±0.8; Fig. 1A). Colonic transit did not differ significantly between L and gentle IM, but was significantly delayed after intense IM compared with L (Fig. 1B).

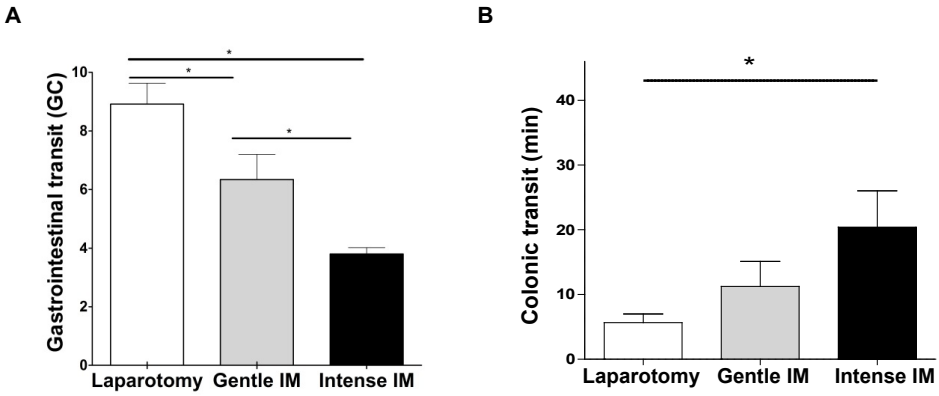

Figure 1. Intense manipulation leads to a more pronounced delay of intestinal transit compared to Gentle manipulation with similar leukocytic infiltrate in the manipulated small intestine. Mean geometrical center (GC) of orally administered FITC-dextran (A), or colonic transit time of a 2-mm plastic ball (B) after intestinal manipulation (IM) or sham operation (laparotomy) at 24 hours after surgery. Results are representative of three independent experiment in groups of 7-8 mice and data are mean±SEM. * $P < 0.05$, one-way ANOVA followed by Tukey's Multiple Comparison analysis

Intense handling leads to enhanced inflammation in the manipulated part of the intestinal tract

In the small intestine, IM but not L resulted in an influx of MPO-positive inflammatory cells in the muscle layer of the small intestine with similar leukocytic counts in the gentle and intense IM groups 24h after surgery (Fig. 2). In addition, IM resulted in the upregulation of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α at 6 and 24h compared with L. Importantly, intense manipulation induced significantly more upregulation of IL-6 and TNF-α as compared with gentle manipulation (Fig. 3). In the non-handled colon leukocyte infiltration was significantly increased after IM compared with L. However, no difference in leukocyte infiltration in the colon was observed after intense and gentle IM (Fig. 3). In line, IL-6, IL-1β and TNF-α mRNA levels were not significantly different after IM compared with L, indicating that there was no detectable inflammatory response (Fig. 3). However in the stomach, manipulation of the intestine did not result in an influx of MPO positive cells in the muscularis (data not shown), or an increase in mRNA levels of IL-6, IL-1β and TNF-α (Fig. 3).

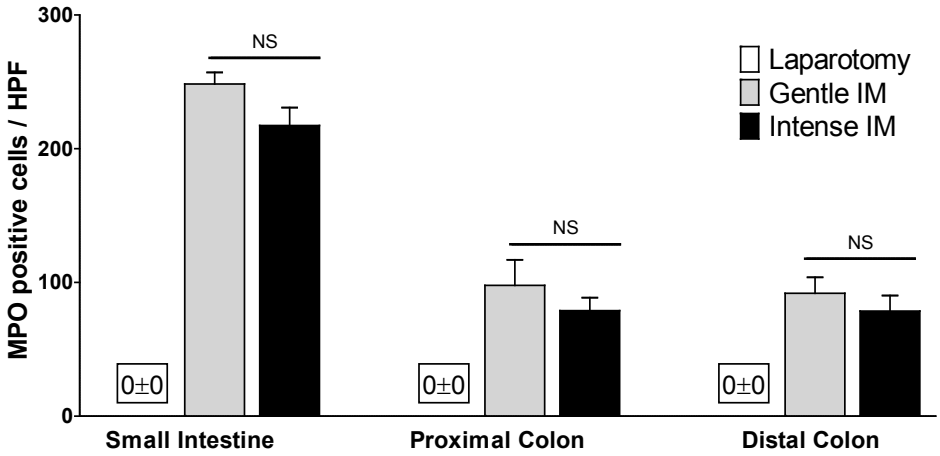

Figure 2. Gentle and Intense manipulation lead to a similar leukocytic infiltrate in the manipulated and non-handled parts of the intestinal tract. Leukocyte recruitment reflected as the number of myeloperoxidase (MPO) positive cells per high power field (HPF) in the muscularis externa in the different parts of the intestinal tract at 24 hours after surgery. However, the influx did not differ significantly (NS) between gentle IM (grey bars) and intense IM mice (black bars) in the small intestine or colon. Results are representative of three independent experiments in groups of 6-8 mice and data are mean±SEM * $P < 0.05$, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

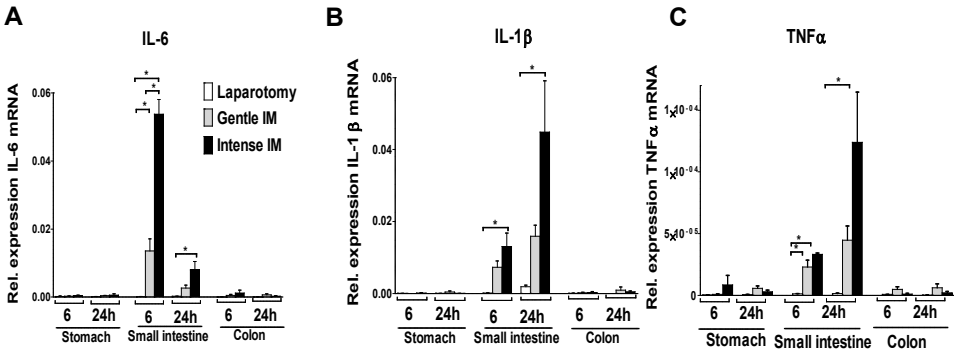

Figure 3. Expression of inflammatory cytokines in different parts of the gastrointestinal tract. Quantitative PCR for IL-6 (A), IL-1β (B) and TNF-α (C) in muscularis of the stomach, small intestine and colon at 6 and 24 hours after surgery. Results are representative of three independent experiments in groups of 4-7 mice and data are mean±SEM; * $P < 0.05$, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Intense IM results in tissue damage with release of inflammatory cytokines into the circulation

Next, we investigated whether increased levels of tissue damage could be associated with a more pronounced upregulation of pro-inflammatory cytokines reflected in increased cytokine plasma levels. One hour after surgery, plasma levels of I-FABP, a marker of intestinal tissue damage, were significantly elevated in mice subjected to more intense IM (Fig. 4A). After 24h, I-FABP was no longer detectable. To study whether intense IM results in a systemic inflammatory response, circulating pro-inflammatory cytokine levels were determined at 1, 6 and 24h following surgery. As shown in Figure 4, plasma levels of KC, MCP-1 and IL-6 were significantly increased following intense IM, but not after gentle IM or L, whereas manipulation of the intestine did not result in enhanced plasma levels of IL-1β and TNF-α (data not shown for TNF-α).

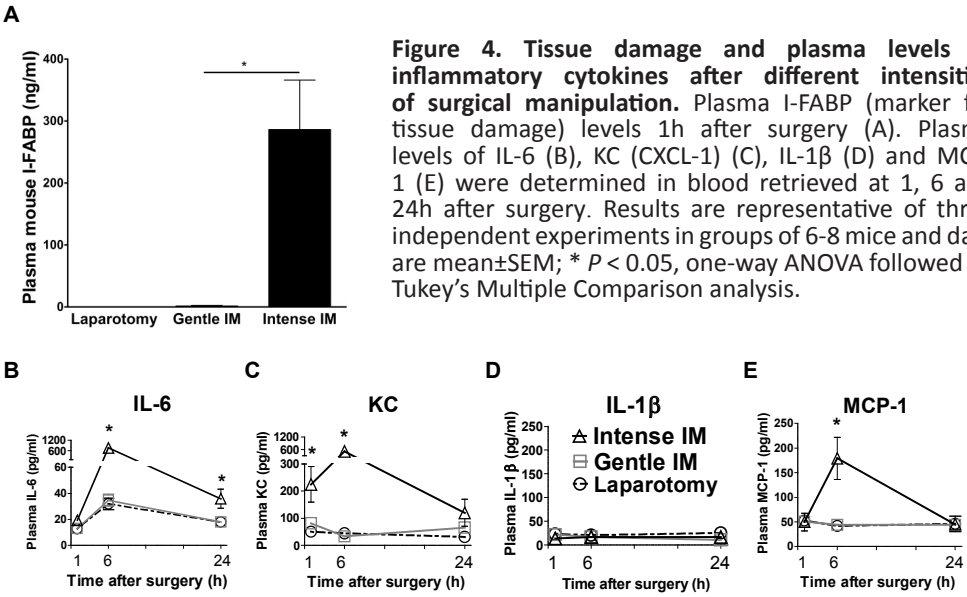

Figure 4. Tissue damage and plasma levels of inflammatory cytokines after different intensities of surgical manipulation. Plasma I-FABP (marker for tissue damage) levels 1h after surgery (A). Plasma levels of IL-6 (B), KC (CXCL-1) (C), IL-1β (D) and MCP-1 (E) were determined in blood retrieved at 1, 6 and 24h after surgery. Results are representative of three independent experiments in groups of 6-8 mice and data are mean±SEM; * $P < 0.05$, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Intense IM-induced tissue damage is associated with AP activation

Previous studies have reported activation of brain areas following abdominal surgery, a mechanism that was proposed to contribute to the development of POI [16]. To investigate whether brain activation contributes to the severity of POI or is associated

with increased plasma levels of cytokines, we investigated the expression of c-Fos to determine the amount of neuronal activation in the brainstem 24h after surgery. The neurons of the AP, which is exposed to systemic circulation, relay their signal to the nucleus tractus solitarius (NTS) and can thereby result in an enhanced activation of the NTS [17,18]. The number of c-Fos positive neurons in the AP and NTS was significantly higher after intense compared with gentle IM (Fig. 5). c-Fos expression was also significantly higher after intense IM at higher levels of the neurocircuitry, namely in the hypothalamic paraventricular nucleus (PVN; data not shown). In line, we observed a positive correlation of c-Fos expression in the AP with plasma I-FABP levels [Spearman's q correlation coefficient 0.65 (95% CI: 0.30–0.85; P = 0.0013)], suggesting that the degree of tissue damage is associated with activation of the AP (Fig. 5).

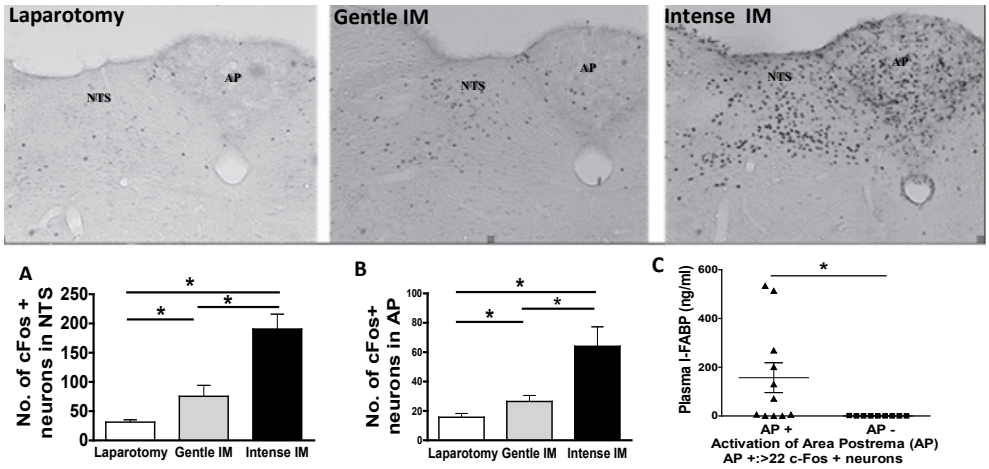

Figure 5. Intensity of IM and tissue damage are associated with enhanced brainstem activation. Representative images of IM-induced c-Fos expression in brainstem nuclei 24 hours after surgery (A). Panel B correspond to c-Fos expression in the nucleus tractus solitarius (NTS). Panel C shows c-Fos expression in the area postrema (AP). Data are expressed as mean±SEM for 6-8 mice per group. Activation of the AP is associated with tissue damage (plasma I-FABP levels 1 hour after surgery); Spearman's ρ correlation coefficient 0.65 (95% CI: 0.30 - 0.85; P = 0.0013 (D). * $P < 0.05$, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Recovery of GI function, tissue damage, and systemic inflammatory cytokines in humans after different intensities of surgical handling

In patients undergoing elective intestinal surgery baseline characteristics (age, sex, type of surgery, body mass index, and American Society of Anesthesiologists grade) did not differ significantly between open and laparoscopic surgery, except duration of surgery (median time: 139 min after open surgery *vs* 186 min after laparoscopy, P < 0.001). Plasma levels of I-FABP and the inflammatory cytokines IL-6, IL-1β, MCP-1, and IL-8 were significantly higher after open compared with laparoscopic intestinal surgery (Fig. 6B-C). This was associated with a longer duration of POI (median time until tolerance of solid food and passing defecation: 96h after open colonic surgery *vs* 72h after laparoscopic; Fig. 6A), confirming that more intense manipulation of the intestine leads to an increase in plasma levels of pro-inflammatory cytokines and more severe POI.

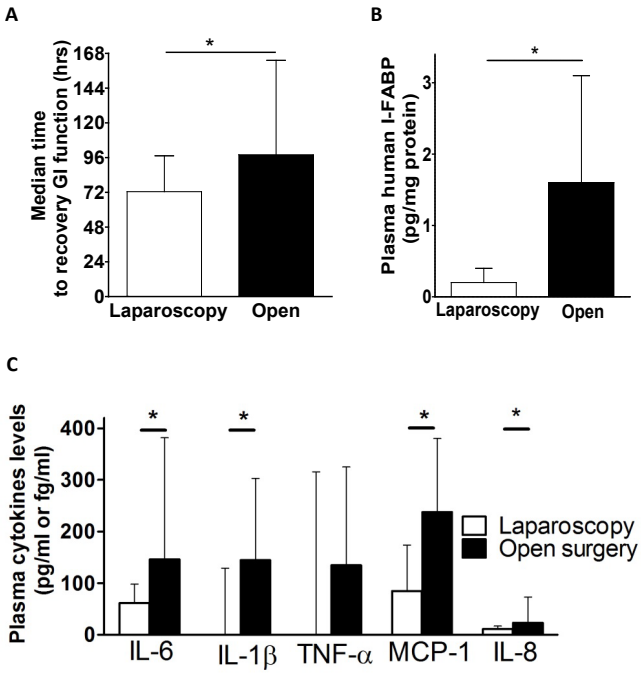

Figure 6. Tissue damage, systemic inflammation and duration of POI in patients after different intensities of surgical manipulation. (A) Recovery of gastrointestinal (GI) function: time until passing stool and tolerance of solid food after laparoscopic (white bars; n=26) and open (i.e., requiring more intense IM) colonic surgery (black bars; n=20). (B) Plasma levels of I-FABP (pg/mg protein) in concentrated plasma samples 2h after surgery (open (n=16); laparoscopy (n=19). (C) Plasma levels IL-1β and TNF-α (fg/ml), IL-6, MCP-1, IL-8 (pg/ml) 2h after surgery (open (n=15); laparoscopy (n=19). * *P* < 0.05; Median±IQR, Mann-Whitney U test.

Discussion

Inflammation of the intestinal muscularis is abundantly demonstrated to underlie POI. Here, we demonstrated that not the number of infiltrating leukocytes, but that rather tissue damage and the release of inflammatory cytokines into the circulation are important factors determining the severity of POI. Concomitantly, we found in humans that open abdominal surgery leads to more tissue damage and increased levels of circulating cytokines compared with minimally invasive laparoscopic surgery. Finally, increased tissue damage and plasma levels of cytokines lead to activation of the AP and PVN, possible contributing to the development of more severe POI. Taken together, our findings indicate that more severe upregulation of pro-inflammatory cytokines, in response to increased tissue damage, with `leakage' of pro-inflammatory cytokines into the systemic circulation significantly contribute to the severity of POI.

The pathophysiology of POI involves recruitment of leukocytes into the intestine impairing smooth muscle contractility [19]. Incremental degrees of manipulation of the small intestine cause a progressive increase in leukocyte infiltration [5]. These infiltrating leukocytes subsequently release inflammatory mediators such as prostaglandins and nitric oxide impairing the contractility of smooth muscle strips of the muscularis and have been proposed to underlie the delay in intestinal transit [20]. Up to date, there is still a scarcity of information on the influence of the severity and extent of surgery on the duration of POI [1]. Graber et al. subjected six monkeys to three operations varying in extent and site of dissection. In this cross-over study, the duration of postoperative dysmotility was independent of the extent, and site of the operative procedure [21]. However, years later Uemura et al. showed in rats that the magnitude of the abdominal incision does affect the duration of POI [22]. We previously demonstrated that only externalization of the intestine outside the abdominal cavity already induced a significant influx of leukocytes without resulting in POI [7]. However, in this study, no significant difference in leukocyte recruitment was observed in the small intestinal muscularis following intense IM compared with gentle IM. As we failed to demonstrate that increased influx of leukocytes is associated with prolonged POI, other mechanisms seem to determine the severity of POI.

It is reasonable to speculate that more severe handling of the intestine will result in more tissue damage. Veenhof et al. recently demonstrated a significant increase in

IL-6 in serum of patients undergoing open rectal procedures compared with patients undergoing a laparoscopic procedure [8]. In line, several studies have reported an increased postoperative inflammatory response related to increased operative trauma [6,8,9]. Damaged tissue releases pro-inflammatory mediators (also called pro-inflammatory damage-associated molecular patterns, or DAMPs), such as heat shock proteins, uric acid, HMGB-1, SAP130, DNA, and S100 proteins that are normally intracellular. Mast cells and macrophages, two cell types known to be involved in the pathogenesis of POI, may be activated by interaction with these DAMPs [10]. We reasoned that with increasing intensity of manipulation, the contribution of tissue damage in the pathogenesis of POI will increase, not only leading to more intense local inflammation reducing small intestinal motility but also leading to release of cytokines in the systemic circulation. The latter will affect neuromuscular function of unmanipulated segments of intestine, that is, the colon in our model. In this study, we indeed recorded higher levels of I-FABP both in mice and patients undergoing more severe intestinal handling. I-FABP is part of a family of nine different FABP types, each named after the tissue of its first detection, and is involved in the intracellular buffering and transport of long-chain fatty acids. I-FABP is currently used as a marker of intestinal tissue injury in experimental rodents and clinical studies [15,23,24]. Elevated plasma levels of I-FABP are detected in patients suffering from intestinal diseases. Hence, I-FABP shows to be a useful plasma marker for the detection of intestinal injury, especially in patients undergoing intestinal surgery [25]. In addition, we found a significant correlation between I-FABP plasma levels 1h after surgery and intestinal IL-6 production 24h postoperatively (Pearson correlation coefficient 0.7; $P = 0.0006$). Moreover, intense IM was associated with more pronounced upregulation of pro-inflammatory cytokines, associated with detection of these cytokines in the systemic circulation. Clearly, this increased inflammatory response in the handled intestine will impair smooth muscle function. A possible additional factor contributing to more severe ileus may result from the increased levels of plasma cytokines activating the hypothalamic-pituitary-adrenal axis. During tissue trauma, immune cells release the pro-inflammatory cytokines IL-1, IL-6, and TNF-α into the general circulation. These cytokines result in enhanced activation of the hypothalamus triggering hypothalamic-pituitary-adrenal activity [16,26-29]. This results in an enhanced sympathetic inhibition of intestinal motility through stimulation of α2-adrenergic receptors on monocytes leading to an increased release of nitric oxide [30]. Indeed, our preliminary brain histology data (unpublished) indicated enhanced activation of the hypothalamic PVN in the intense IM mice that still

had detectable systemic IL-6 levels 24h after surgery. Finally, the more enhanced delay in transit might result from direct activation of residential macrophages by circulating cytokines, DAMPs, and other tissue damage products, or even bacterial products. These muscularis-resident macrophages can induce nitric oxide synthase thereby further contributing to the postoperative impairment of GI motility [6,31].

As POI is characterized by impaired motility of the entire GI tract, including areas that have not been manipulated, other factors than local inflammation should be involved. Previously, evidence has been reported that the local inflammation, mainly via prostaglandins, activates afferent nerves triggering inhibitory neural pathways affecting motility of distant non inflamed areas [2,6,31-33]. More recently, Engel et al. showed that IM evokes local IL-12 production and thereby triggers TH1 memory cells to egress into the systemic circulation and migrate to non-manipulated areas of the intestine. There, these TH1 memory cells stimulate macrophages in the muscularis externa leading to dissemination of the inflammatory response [14]. In previous experiments, however, we were unable to demonstrate increased levels of IL-12 [34]. Moreover, we showed that $RAG1^{-/-}$ mice, devoid of T cells, developed POI to the same extent as wild type mice [35] suggesting that other mechanisms must be involved. In this study, we observed that IM of the small intestine resulted in an influx of leukocytes into the colon, but the degree of influx was not related to impaired motility. Notably, colonic transit was only delayed after intense IM but not following gentle manipulation although the influx of leukocytes was comparable. Similarly, the upregulation of inflammatory cytokine levels after intestinal handling, both after intense and gentle IM, did not differ from L mice (data not shown), indicating that reduction in colonic motility does not result from disseminated inflammation. Based on the observation that delayed colonic transit is rather associated with increased systemic levels of pro-inflammatory cytokines, we speculate that impaired colonic motility rather results from the known inhibitory effects of pro-inflammatory cytokines on smooth muscle function. Pro-inflammatory cytokines such as TNF-α, IL-1β, KC, and MCP-1 may affect directly enteric neural coordination of motility or intestinal muscle contractility [15-19]. In addition, we showed that increased plasma levels of inflammatory cytokines, only observed following intense IM, activate the AP and NTS [17,18]. This activation may subsequently trigger enteric inhibitory pathways, and thereby further contribute to more severe POI [2,6,31-33][36].

In conclusion, our findings indicate that more severe upregulation of pro-inflammatory

cytokines, in response to increased tissue damage, with `leakage' of proinflammatory cytokines into the systemic circulation significantly contribute to the severity of POI. Our observations may aid in the development of strategies to selectively block this response and reduce the severity of ileus. In addition, more insight into how tissue damage triggers the release of systemic cytokines may also lead to therapeutics to prevent this response.

Acknowledgements

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Supplementary material

Mouse

Gene	Forward	Reverse
rpl32	5’- AAGCGAAACTGGCGGAAAC -3’	5’- TAACCGATGTTGGGCATCAG -3’
IL-1β	5’-GACCTTCCAGGATGAGGACA -3’	5’-TCCATTGAGGTGGAGAGCTT-3’
IL-6	5’-CCATAGCTACCTGGAGTACATG-3’	5’-TGGAAATTGGGGTAGGAAGGAC-3’
TNF-*α*	5’-TCTTCTCATTCCTGCTTGTGG-3’	5’-CACTTGGTGGTTTGCTACGA-3’

Supplementary table 1. Primer sequences for qRT-PCR.

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Chapter 7

Splenic sympathetic innervation participates in the immunomodulation of DSS-induced colitis

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* authors contributed equally to the work

Manuscript in preparation

Abstract

In the last decade, the vagus nerve was discovered as an essential neuronal component by which the central nervous system can dampen peripheral inflammation. The anti-inflammatory property of the vagus nerve was shown to rely on its control over the sympathetic activity of the splenic nerve at the level of the celiac-superior mesenteric ganglion. In the present study, we aimed to unravel which neuronal circuitry is activated and participates in the vagal anti-inflammatory pathway modulating colonic inflammation in a dextran sodium sulfate (DSS)-induced acute colitis in mice. To this end, we selectively cut vagal innervation to the proximal colon or sympathetic innervation of the spleen prior to induction of colitis. We observed neuronal activation in the sensory nucleus tractus solitarius but surprisingly not in the dorsal motor nucleus of the vagus of mice exposed to DSS for 7 consecutive days which exhibited colonic inflammation. Selective vagal denervation did not significantly affect disease activity index (DAI) or production of the colonic pro-inflammatory cytokines IL-1β, IL-6 or TNF-α. In contrast, splenic denervation enhanced this pro-inflammatory cytokine production in the colon and was accompanied by an increased DAI and colonic Foxp3 expression during the recovery phase. Together, we demonstrated that the neuronal circuitry that dampens colonic inflammation during DSS-induced colitis in mice is not predominantly vagally mediated but rather relies on sympathetic activity of the splenic nerve.

Introduction

The importance of the central nervous system, and in particular the vagus nerve, in the regulation of immune responses has been extensively studied in the past decades. Vagus nerve stimulation (VNS) leading to the release of acetylcholine (ACh) was shown to dampen TNF-α production by macrophages thereby dampening the inflammatory response in sepsis. This discovery led to the emergence of the concept of vagal reflex with a sensing of the inflammation by vagal afferents leading to the activation of vagal sensory neurons in the nucleus tractus solitarius (NTS) and the subsequent activation of vagal motor neurons in the dorsal motor nucleus of the vagus (DMV).

Further studies however revealed a higher level of complexity in the neural networks implicated in this cholinergic anti-inflammatory pathway. In postoperative ileus, a surgery-induced local intestinal inflammation, the vagal control of inflammation is direct and solely targets the inflamed organ (i.e., the intestine). In other inflammatory models such as sepsis and colitis, the vagal anti-inflammatory effect is indirect as integrity of the splenic nerve is required for vagus nerve activation to exert an anti-inflammatory effect [1-3]. However, as evidence is lacking to support the direct innervation of the spleen by vagal inputs, this mechanism is thought to depend on the vagal control of splenic sympathetic neurons in celiac ganglia [4,5]. Vagal activation of the splenic nerve would lead to the release of noradrenaline able to bind splenic ACh-producing T cells [4]. However, evidence of the existence of neuronal contact between vagal and splenic nerve is lacking. Recent studies demonstrate an absence of contact between vagal and splenic nerve, suggesting that the anti-inflammatory role of the splenic nerve does not rely on its control by vagal outputs [6],[7]. Interestingly, an anti-inflammatory role of sympathetic inputs rather than vagal inputs has recently been described in sepsis [8]. Further investigations are therefore required to unravel the exact neural networks implicated in the vagal control of inflammation.

Here we aimed to clarify whether the vagus nerve exerts a direct or indirect anti-inflammatory effect on the colon during inflammation. To this aim, we made use of a dextran sodium sulfate (DSS)-induced mouse model of colitis which resembles the human inflammatory disease Ulcerative Colitis (UC) [9]. By using the early neuronal activation marker c-Fos, we first mapped the neuronal circuitry triggered by colonic inflammation. To determine whether the vagus nerve exerts a functional role in the immunomodulation of colonic inflammation, we then selectively removed direct vagal

inputs to the proximal colon and determined the influence of the absence of such inputs on the severity of the colitis. Finally, in light with the recent involvement of the sympathetic splenic nerve on inflammation, we assessed whether spleen denervation had an effect on the colonic inflammation.

Material and methods

Mice

Eight to 12 week old C57/Bl6 mice were purchased from Charles River (Maastricht, The Netherlands) and co-housed in a specified pathogen free facility with a 12/12 light/dark cycle under constant conditions of temperature (20 +/- 2º C) and humidity (55%) and *ad libitum* food and water. All experiments were performed under fentanyl-fluanisone (Hypnorm; Janssen, Beerse, Belgium)-midazolam (Hypnorm; Janssen, Beerse, Belgium) (FFM) anesthesia and all efforts were made to minimize the suffering of the animals. All experiments were performed in accordance with the guidelines of the Laboratory Animal Use of the Netherlands and approved by the Ethical Animal Research Committee of the Academic Medical Center of Amsterdam.

Surgical procedures and dextran sodium sulfate-induced colitis

Selective vagal denervation of the proximal colon

Selective denervation of the vagal innervation of the colon was performed by cutting the right celiac branch of the vagus nerve, as previously described [10], Olivier et al., not published.

Splenic denervation

Splenic denervation (Sx) was achieved by cutting noradrenergic fibers running along blood vessels supplying the spleen and by cutting nerve fibers present in the conjunctive tissue located at each tip of the spleen as previously described [10]. Completion of the denervation was assessed by Tyrosine Hydroxylase staining.

Acute DSS-induced colitis

Two weeks after Sham-operation or denervation (Sx or Vx), mice were given 2% DSS

ad libitum in drinking water for 7 consecutive days and then placed on normal drinking water for the remaining duration of the experiment. The DSS solution was replaced daily. Control mice were placed on normal drinking water throughout the whole experiment. Consistence of the stool, anal bleedings, general behavior and posture, and weight were assessed daily. Animals were sacrificed at day 7 or day 12 after the first day of DSS exposure.

Sacrifice and sample collection

Animals were anesthetized with pentobarbital (0.1mL of a 50mg/mL solution). Mice were sacrificed by transcardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA; pH 7.4). Colonic tissue was collected prior to PFA perfusion. Colon length, inflammatory score (i.e., severity of fibrosis) and diarrhea score were assessed as previously described [11] by a blinded observer. Entire colons were then snap-frozen for PCR analysis. Brains were collected after PFA perfusion, postfixed overnight (4ºC) and cryo-protected by immersion with 30% sucrose in 0.2 mol.L^{-1} PBS (pH 7.4) at 4ºC overnight and kept at 4ºC until analysis.

c-Fos staining

Coronal sections of 30 µm for brain/brainstem were collected. After rinsing in 0.05 mol.L^{-1} Tris buffered saline (TBS) (pH 7.4), sections were incubated overnight at 4ºC with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc.,Santa Cruz, CA, USA). For c-Fos staining, sections were first incubated 1h in biotinylated secondary antibody and then in avidin-biotin complex (ABC; Vector, Burlingame, CA, USA) for 1h. The reaction product was visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide (H_2O_2) for 5 min. To count c-Fos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 color camera (Sony Corp., Tokyo, Japan). Brainstem sections, from bregma -7.20 to -7.76 mm, were used for c-Fos counting in the NTS/DMV. The counting of c-Fos^+ cells was performed bilaterally for each nucleus. Data are represented as a mean of the relative density of c-Fos^+ cells counts on 9–10 sections (non-adjacent section, at least separated 90 µm) for the NTS/DMV.

RNA isolation, cDNA synthesis and QPCR

Total mRNAs from entire colon were extracted after homogenization of the samples in TriPure isolation reagent according to the manufacturer's instructions (Roche Applied Science). cDNA synthesis was performed using the Revertaid first strand cDNA synthesis kit (Fermentas) and Real-time PCR was performed using a SYBR green master mix (Roche Applied Science) on a Lightcycler 480 (Roche Applied Science). The primers used (synthesized by Invitrogen, Bleiswijk, The Netherlands) are described in Table 1. Analysis was performed using the LinRegPCR program (AMC, Amsterdam, The Netherlands) [12]. The target gene expression was normalized over the expression of 2 reference genes selected after analysis with the Genorm software. All data are expressed in AU and represent relative expression over the control group.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc, Chicago, IL). Data are expressed as mean±SEM. Normal distribution was assessed using the Kolmogorov-Smirnov test. Square-root normalization was applied to non-normal data sets. Whenever two groups of data were compared (i.e., Ctrl *vs* DSS), a Student t-test was performed. Whenever the influence of 2 independent variables (i.e., Ctrl/DSS and denervation) was analyzed, a two-way ANOVA was performed to determine the interaction between denervation (Sham *vs* Vx/Sx) and treatment (Ctrl *vs* DSS). When significance was observed (i.e., $p<0.05$) an unpaired Student t-test was performed to evaluate the significance between Sham *vs* Vx/Sx or Ctrl *vs* DSS.

	Forward primer 5'-3	Reverse primer 5'-3
HRPT	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Cyclophilin	ACCCATCAAACCATTCCTTCTGTA	TGAGGAAAATATGGAACCCAAAGA
IL-1β	CTCCTGCTGTCGGACCCAT	TGCCGTCTTTCATTACACAGGA
IL-6	GAGTTGTGCAATGGCAATTCTG	TGGTAGCATCCATCATTTCTTTGT
TNF-α	TGGAACTGGCAGAAGAGGCACT	CCATAGAACTGATGAGAGGGAGGC
Foxp3	TCCCACGCTCGGGTACAC	CCACTTGCAGACTCCATTTGC

Table 1. Primer sequences for QPCR

Results

Administration of DSS leads to colonic inflammation which activates sensory but not motor vagal neurons

As previously described [11], administration of DSS in drinking water for 7 consecutive days led to a significant reduction in body weight starting at day 6 (Fig. 1A), shortening of the colon at the time of sacrifice (Fig. 1B), and an increase of disease activity index (DAI) corresponding to the combination of diarrhea score, inflammatory score and presence of blood in the feces at the time of sacrifice (Fig. 1C). To determine the involvement of the vagus nerve in the regulation of this colonic inflammation, we first assessed whether colonic inflammation triggers the activation of an endogenous vagal reflex by quantifying c-Fos positive neurons in the nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV). The quantification of c-Fos positive neurons of the NTS revealed a large increase in DSS-exposed mice compared to control mice 7 days after the first exposure to DSS indicating that vagal sensory afferents sense the colonic inflammation. Interestingly, no c-Fos positive neurons were found in the DMV demonstrating the absence of reflex activation of vagal motor activity in response to the colonic inflammation (Fig. 1D and E).

Vagal input to the colon does not exert a prominent immunomodulatory effect on colonic inflammation

Electric vagus nerve stimulation was previously shown to dampen TNBS-induced colonic inflammation in rat models of colitis [13,14] while vagotomy at the subdiaphragmatic level aggravated colonic inflammation [15-17]. Here we failed to report evidence of neuronal activation in the DMV in response to colonic inflammation making a direct negative reflex loop involving the vagus nerve less likely. We concurrently reasoned that selective lesioning of the vagus nerve at the right celiac branch i.e., projecting to the proximal part of the colon would not affect the course of the disease. Indeed, selective vagal denervation (Vx) of the colon did not affect the body weight loss, shortening of the colon or DAI observed in DSS-mice (Fig. 2A, B and C). Analysis of the expression levels of pro-inflammatory cytokines in the entire colonic tissue revealed a trend however non-significant towards increased IL-1β and IL-6 mRNA levels in Vx DSS mice compared to sham DSS mice. TNF-α mRNA level was similar in Vx and non-denervated mice (Fig. 2D). As the vagus nerve only provides input to the proximal colon and not the distal colon, we next assessed the influence of selective Vx on the expression of

these pro-inflammatory cytokines in proximal colonic tissue. As observed for the entire colon, Vx did not influence the expression level of IL-1β, IL-6 or TNF-α in the proximal colon of mice exposed to DSS confirming that the vagus nerve does not modulate these cytokines during acute colitis (Fig. 2E).

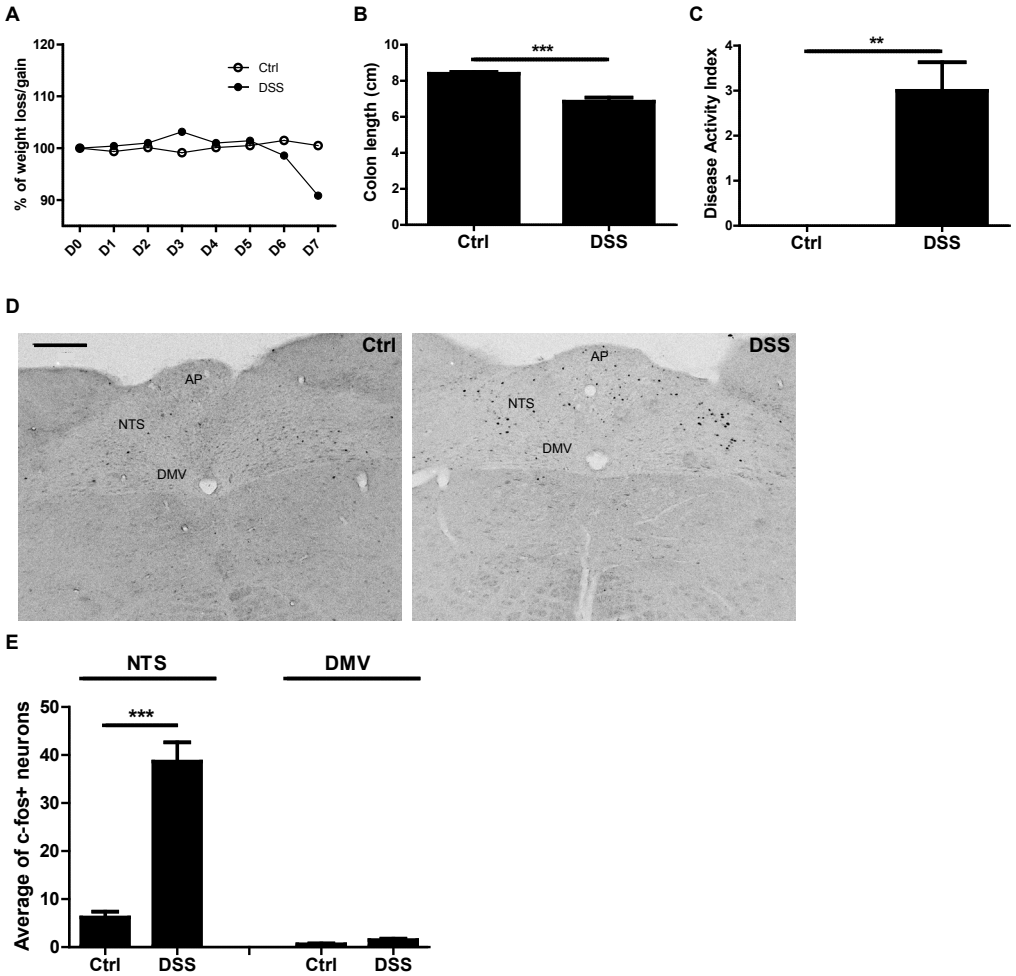

Figure 1. Acute DSS-induced colitis leads to activation of sensory but not motor vagal neurons. Administration of DSS leads to weight loss starting at D6 (A), shortening of the colon (B) and increased Disease Activity Index (C). D. c-Fos immunohistochemical staining of brainstem sections reveals an increase in activated vagal neurons in the nucleus tractus solitarius (NTS) but not in the dorsal motor nucleus of the vagus (DMV) after 7 days of DSS administration. The scale bar represents 100 µm. Data are expressed as mean±SEM (n=7-9 animals per group). ** p<0.01; *** p<0.001

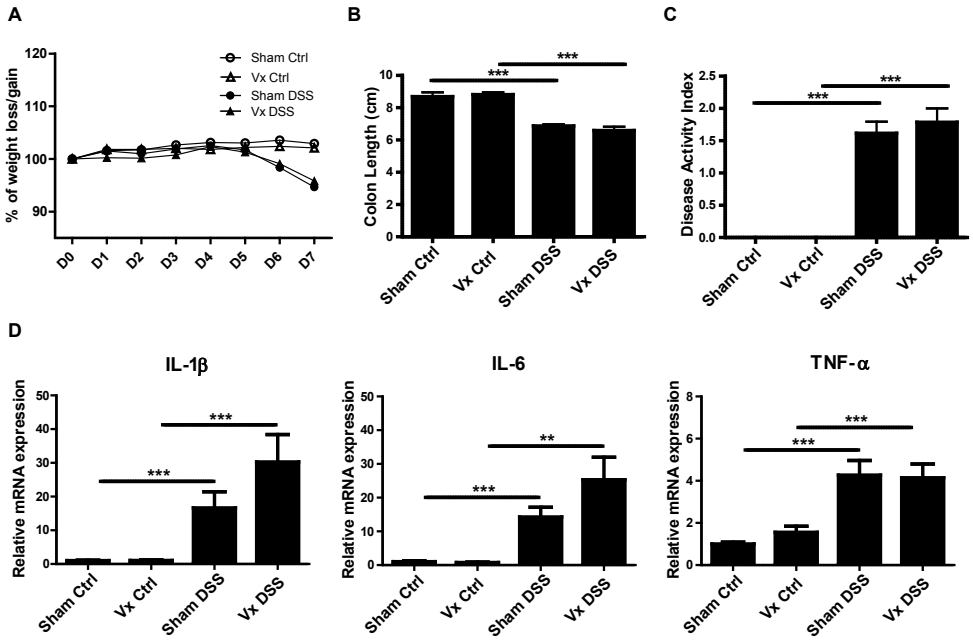

Figure 2. Colonic vagal innervation does not modulate colonic inflammation during acute DSS-induced colitis. Intestinal vagal denervation performed prior to DSS administration does not affect the weight loss (A), shortening of the colon (B) and Disease Activity Index (C). (D) DSS administration leads to increase in the expression levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α independently of vagal intestinal innervation. (E) Lesion of vagal innervation of the proximal colon does not affect the expression mRNA level of IL-1β, IL-6 and TNF-α. Data are expressed as mean±SEM (n=15-19 animals per group). ** p<0.01; *** p<0.001

Splenic denervation enhances pro-inflammatory inflammatory cytokine expression in the colon upon DSS exposure

We next sought to understand the nature of the innervation route involved in immune regulation in our colitis model. The splenic nerve was recently shown to play an essential role in the VNS-induced anti-inflammatory effect observed during colitis [3]. This role of the splenic nerve led us to postulate that splenic sympathetic inputs participate in the immune response observed in the acute DSS-induced colitis model. To investigate this, we lesioned the splenic nerve 2 weeks prior to DSS exposure. The absence of splenic innervation did not alter the body weight, DAI or colon length as was observed in sham-operated DSS-exposed mice (Fig. 3A, B and C). However, strikingly, splenic denervation did lead to an alteration in the expression levels of pro-inflammatory cytokines with a significant increase in the expression of IL-6, and TNF-α in the colon as well as a non-significant trend towards increased IL-1β (Fig. 3D) demonstrating that the sympathetic

activity of the splenic nerve exerts a modulatory effect on the expression of pro-inflammatory cytokines in DSS-induced colonic inflammation.

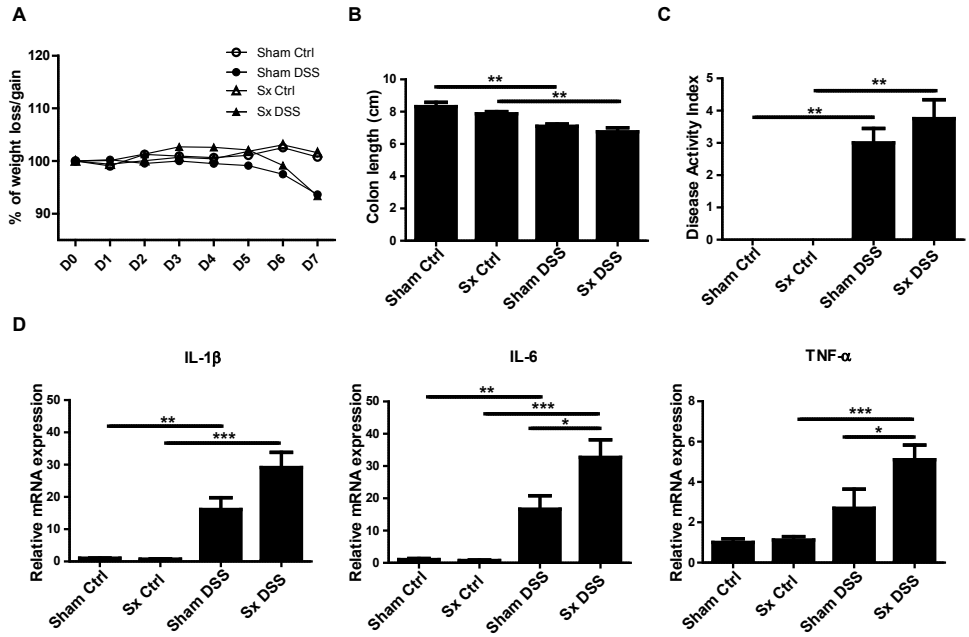

Figure 3. Sympathetic splenic innervation exerts an anti-inflammatory effect on the DSS-induced colonic inflammation. Spleen denervation performed prior to DSS administration does not affect the weight loss starting at D6 (A), shortening of the colon (B) or increase in the Disease Activity Index (C) induced by DSS administration. (D) Spleen denervation leads to a significant increase in the expression level of the pro-inflammatory cytokines IL-6 and TNF-α as well as a trend towards an increase in the expression level of IL-1β in mice exposed to DSS. Data are expressed as mean±SEM (n=11-15 animals per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Enhanced colonic inflammation induced by splenic denervation persists during the recovery phase following DSS exposure

Chronic colonic inflammation can lead to aberrant mucosal healing which has been shown to favor the development of colitis-associated colon cancer [18]. Enhanced inflammation, as observed here in spleen-denervated animals, can therefore have long term consequences. In order to determine if the pro-inflammatory effect of splenic denervation could influence the remission phase following DSS exposure where mucosal healing occurs, mice were exposed to DSS for 5 days and then placed back on normal drinking water. Mice were sacrificed 7 days after the last day of DSS exposure (i.e., day 12). As previously observed, both sham and spleen-denervated mice exposed

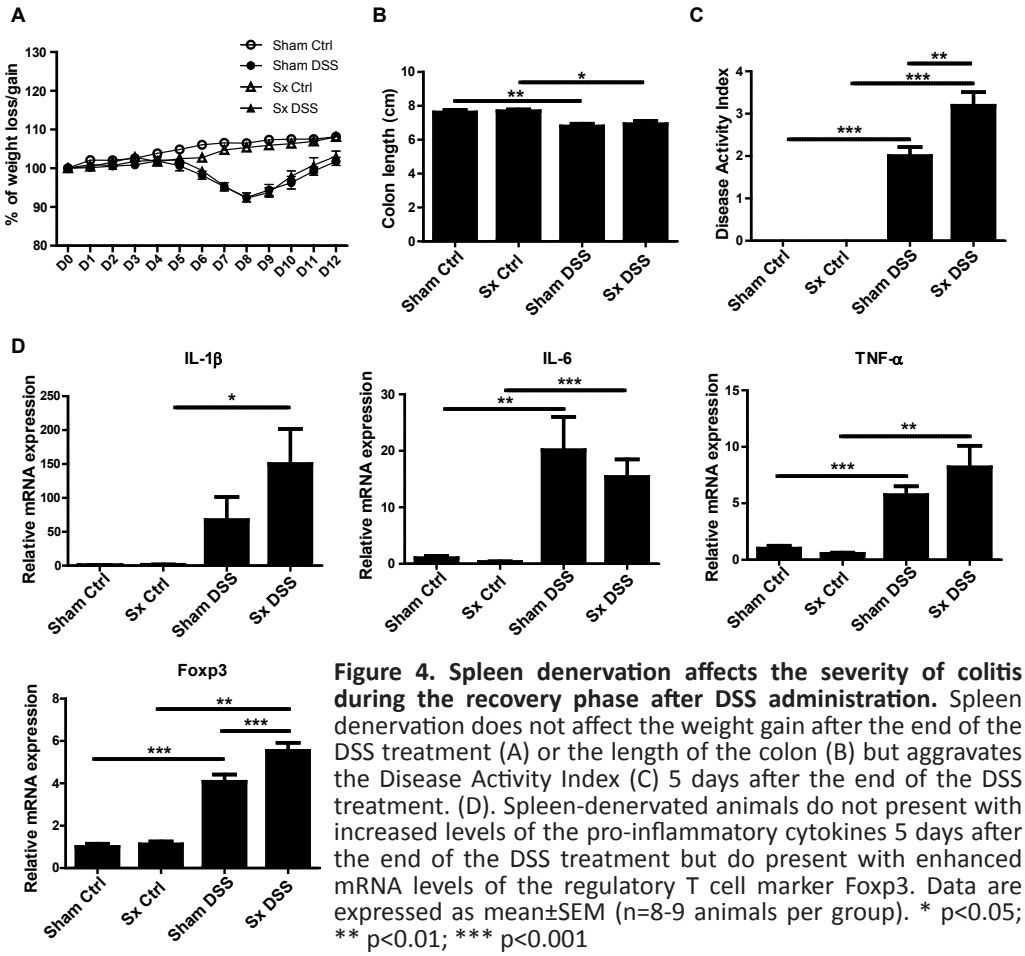

Figure 4. Spleen denervation affects the severity of colitis during the recovery phase after DSS administration. Spleen denervation does not affect the weight gain after the end of the DSS treatment (A) or the length of the colon (B) but aggravates the Disease Activity Index (C) 5 days after the end of the DSS treatment. (D). Spleen-denervated animals do not present with increased levels of the pro-inflammatory cytokines 5 days after the end of the DSS treatment but do present with enhanced mRNA levels of the regulatory T cell marker Foxp3. Data are expressed as mean±SEM (n=8-9 animals per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$

to DSS suffered from weight loss starting at day 6 after their first exposure to DSS. The weight loss persisted until 4 days after the last day of DSS exposure (i.e., day 9). DSS-exposed mice recovered their initial body weight 5 days after the last exposure to DSS independently of the presence of splenic innervation (Fig. 4A). The colon length at the time of sacrifice was shorter in mice exposed to DSS compare to control mice but did not differ between sham-operated and spleen-denervated mice (Fig. 4B). Remarkably however, mice lacking splenic innervation and exposed to DSS displayed a worsened DAI in comparison to non-denervated mice (Fig. 4C). The increased colonic expression of IL-1β, IL-6 and TNF-α was similar in spleen-denervated and sham-operated mice. $Foxp3^+$ T cells are known to arise in response to inflammation to limit the inflammatory reaction and induce the return to an immune homeostatic state [19]. We therefore determined

the Foxp3 mRNA levels in Sx and sham-operated mice exposed to DSS. Interestingly, mRNA levels of Foxp3, a marker for regulatory T cells (Tregs) was also elevated in spleen-denervated compared to Sham mice (Fig. 4D). Taken together these results seem to indicate that the immunomodulatory effect of the splenic nerve leading to enhanced inflammation may alter the mucosal healing following colonic inflammation.

Discussion

In the present study, we demonstrated that DSS-induced colonic inflammation activates sensory vagal neurons located in the NTS but we failed to report endogenous activation of vagal efferent activity to the proximal colon. In addition, lesioning the vagus nerve at a level close to the proximal colon only led to a non-significant trend towards increased colonic pro-inflammatory cytokine expression while lesioning of the splenic innervation significantly increased the expression levels of those cytokines. Our results therefore suggest that in colitis, the splenic nerve seems to be at least of equal importance in the immunomodulation of colonic inflammation as direct vagal innervation of the proximal colon.

The concept of a cholinergic anti-inflammatory pathway emerged 15 years ago when vagus nerve stimulation was shown to dampen the production of the pro-inflammatory cytokine TNF-α by macrophages in a mouse model of endotoxemia [20]. Since then, the mechanisms underlying this vagal anti-inflammatory pathway have been studied, and its definition has evolved. The concept of a vagal reflex that emerged consequently to this discovery, comprises of sensing of the inflammation by vagal afferents with subsequent activation of vagal sensory neurons of the NTS, and a reflex activation of vagal motor neurons in the DMV leading to the release of ACh by efferent fibers [21]. We recently provided anatomical evidence of the activation of such a vagal reflex in a mouse model of postoperative ileus [10]. In this model, a subtle inflammatory response of the muscular layer and the peri-myenteric region, resulting from activation of resident macrophages, indeed was accompanied by activation of DMV motor neurons innervating the intestine. In the present study, however, acute DSS-induced colonic inflammation activates sensory vagal neurons, as shown by the increased c-Fos positivity in the NTS, but we failed to report c-Fos positivity in the DMV. These results were further confirmed by performing immunohistochemical stainings for Erk,

another early marker of neuronal activation, on brainstem sections (data not shown). Moreover, both c-Fos and Erk positivity were also assessed at different time points after the first exposure to DSS (i.e., day 1, day 3 and day 5, data not shown) but no positivity was observed at any of these time points in the DMV. These data show that the colonic inflammatory response is detected by the central nervous system but does not lead to increased c-Fos expression in the dorsal motor nucleus of the vagus. Although these findings cannot exclude subtle activation of the vagus nerve, our data at least suggest that there is no major contribution of direct vagal anti-inflammatory input towards the large intestine in case of submucosal inflammation.

Parasympathetic innervation of the colon is provided by two different sources: a direct innervation by the vagus nerve arising from the brainstem and targeting the proximal colon, and an indirect innervation arising from pelvic ganglia where parasympathetic preganglionic neurons contact postganglionic neurons which arise to form the rectal nerves targeting the distal colon [22]. Here we show that selective lesioning of vagal fibers arising from the brainstem and innervating the proximal colon does not significantly affect the production of colonic pro-inflammatory cytokines upon DSS administration either in tissue of the entire colon or in tissue of the proximal colon. Our data therefore indicate that direct vagal colonic innervation does not endogenously exert a strong immunomodulatory role during acute DSS-induced colitis. These results are in contrast with previous studies reporting that subdiaphragmatic vagotomy aggravates colonic inflammation in mouse models of colitis [16,17]. The discrepancy between those studies and ours is likely to be explained by the different denervation methods used. In previous studies conducted by others, vagotomy was performed at a subdiaphragmatic level thereby removing vagal innervation of numerous other visceral organs, including the spleen. Indeed, the splenic nerve is thought to be under the control of the vagus nerve [2,5] while several studies have recently indicated the importance of the spleen in the development and recovery of colitis in mouse models. Splenectomy performed prior to induction of colitis led to a delay in the recovery of mice. The beneficial role of the spleen is mediated through the accumulation of $Gr1^{+}$ cells in the spleen able to ameliorate inflammation when transplanted into colitic animals [23,24]. Furthermore, as observed in sepsis, splenectomy and more particularly ablation of the splenic nerve was sufficient to abrogate the anti-inflammatory effect of vagus nerve activation on the colonic inflammation demonstrating the crucial role of the splenic nerve in the modulation of colonic inflammation [3]. On the contrary, we chose to selectively

lesion vagal fibers targeting the proximal colon avoiding the effect of vagotomy on the spleen. As this approach failed to worsen colitis, our data suggest that direct vagal colonic innervation is not a main player in the modulation of colonic inflammation. It should be emphasized though that the distal part of the colon is not innervated by the vagus nerve, potentially explaining our negative findings. However, if we analyzed the proximal colon separately, similar results were obtained, i.e., no significant increase in inflammation could be detected. In contrast, the severity of colitis was aggravated, as shown by increased expression levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, by selective denervation of the spleen. These results are in line with previous findings establishing the splenic nerve as a crucial immunomodulator in colitis and other inflammatory disorders such as endotoxemia [2,3].

Of interest, taken together our data seem to suggest that the nature and/or localization (submucosal *vs* muscular) of the inflammatory response within the gastrointestinal tract determines the neural route activated to control the inflammation. In postoperative ileus, the inflammation is rather subtle and restricted to the small intestinal muscularis. Under these conditions, the vagus nerve provides local immunomodulation by targeting the resident macrophages, independent of the spleen [25]. In DSS-induced colitis, where the inflammation is located in the mucosal/submucosal compartment and is associated with a systemic component with circulating cytokines [9,26], more systemic neuromodulation is required with activation of neural pathways directed to the spleen. How the central nervous system determines to switch on the splenic pathway remains a matter of speculation. One potential mechanism could be through detection of circulating cytokines. Indeed, the presence of inflammatory mediators in the circulation, as seen during DSS-induced colitis can potentially be detected at the level of the circumventricular organs (CVOs) in the brain [27]. CVOs project towards various central structures among which nuclei of the hypothalamus [28]. Since infusion of neurotransmitters in the pre-optic hypothalamus has been shown to modulate the activity of the splenic nerve [29], one could indeed speculate that circulating inflammatory mediators detected by CVOs may account, at least partially for the activation of splenic adrenergic fibers. Taking this further, our data may suggest that subtle inflammatory response devoid of a systemic component may be modulated locally, whereas more severe inflammatory conditions with a systemic component will require (additional) modulation of the immune response through activation of the splenic anti-inflammatory pathway.

Inflammatory bowel disease is associated with episodes of inflammation followed by remissions and relapses. Repetitive inflammatory episodes have been shown to lead to dysbalanced mucosal healing which can ultimately favor the development of colonic polyps and cancer [18]. Data on the importance of neural modulation of inflammation on the remission phase of colitis are lacking. Here we show that enhanced colonic inflammation in mice lacking splenic innervation leads to enhanced DAI, namely on the degree of fibrosis of the colonic tissue. Moreover, our results show that Foxp3, a marker for regulatory T cells (Tregs), is also enhanced in those mice as compared to sham-operated mice. $Foxp3^+$ Tregs can arise in response to an inflammatory process to try and control inflammation and promote a return to homeostasis [19]. Thus, enhanced levels of Foxp3 in the colon of mice lacking splenic innervation during the recovery phase most likely reflect the enhanced inflammation observed during acute inflammation. These data seem to indicate that modulation of innate immune cell activity by the splenic nerve during colitis could potentially have an impact on the healing phase following inflammation but further investigation will be required to confirm the importance of neural modulation in colonic mucosal healing.

In conclusion, our study provides further understanding on the mechanisms underlying control of inflammation by the nervous system. We demonstrate that in the gastrointestinal tract, severe colonic inflammation triggers an anti-inflammatory response predominantly involving the splenic nerve rather than the direct gastrointestinal vagal innervation.

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Conclusions and discussion

Until recently, the central nervous system and the immune system were thought to constitute two independent entities. The discovery that the central nervous system could exert a tight and crucial control over inflammation through the release of neuropeptides [1] demonstrated the existence of interactions between these two physiological systems thereby opening a whole new field of research. Since then, the field of neuroimmunology has grown exponentially as these interactions have proven to be bi-directional, i.e., the central nervous system was shown to also perceive inflammation occurring at the periphery through the sensing of inflammatory mediators such as IL-1β and TNF-α [2]. In particular, the autonomic nervous system has shown to be largely involved in immuno-modulation. The vagus nerve was recently shown to exert a crucial anti-inflammatory effect in sepsis as vagus nerve stimulation applied prior to induction of sepsis in rodents decreased the production of pro-inflammatory cytokines thereby leading to improved survival [3]. From this observation the concept emerged of a vagal anti-inflammatory reflex defined as a sensing of the inflammation by vagal afferents leading to the activation of sensory vagal neurons in the NTS in turn leading to the activation of motor vagal neurons located in the DMV [4,5]. Vagal motor efferents subsequently release neurotransmitters such as ACh able to bind to nicotinic receptors present on macrophages and leading to a dampening of the production of pro-inflammatory cytokines such as TNF-α.

Cell-cell communication originating from the nervous system classically occurs via the release of various neurotransmitters by presynaptic terminals into synaptic connections and binding of these neurotransmitters on postsynaptic cells bearing the corresponding receptors for these neurotransmitters. Neurotransmitters released by the vagus nerve consist of a range of molecules with ACh representing the predominant one. Since cholinergic neurotransmitters and in particular ACh only have a limited range of action, close anatomical proximity between vagal nerve endings and immune cells is a prerequisite for the vagus nerve to be able to exert its modulatory action on the immune system. In the last decade, the vagus nerve has been shown to exert a crucial immunomodulatory role in intestinal inflammation. Despite thorough studies on the distribution of vagal nerve endings in the gastrointestinal tract, there still exists a debate on whether vagal nerve endings can be found in the close vicinity of immune cells. It therefore remains unclear if the vagus nerve acts directly on immune cells or whether preganglionic vagal nerve endings solely interact with postganglionic neurons of the enteric nervous system which in turn release neurotransmitters modulating immune

cells. Hence, **Chapter 2** focused on bringing further knowledge on the distribution of vagal efferents in the gastrointestinal system. Using injection of the anterograde tracer biotin dextran amine in the DMV, we confirmed that preganglionic vagal efferent fibers could only be found in the myenteric plexus and synapsed with post-ganglionic myenteric neurons [6]. We failed to detect cholinergic preganglionic efferent fibers in the close vicinity of immune cells. In contrast, nerve endings of enteric postganglionic neurons, mostly positive for ChAT (the enzyme responsible for the synthesis of Ach), were found in the close proximity of $F4/80^{+}$ macrophages. Of interest, vagal nerve fibers also contacted myenteric neurons positive for nNOS and VIP. In line, VIP receptors (i.e., VPAC) were found on $F4/80^{+}$ macrophages located in the close proximity of enteric postganglionic neurons suggesting that VIP may be also be involved in the vagal modulation of intestinal inflammation. Altogether our results demonstrate that the vagus nerve indirectly modulates the resident muscular macrophages in the intestine through interactions with the enteric nervous system.

The existence of a vagal regulation of inflammation was recently brought to light in postoperative ileus (POI). POI is characterized by a generalized paralysis of the gastrointestinal tract observed after abdominal surgery. It occurs as a consequence of a local inflammatory response in the intestinal muscularis triggered by handling of the intestine by the surgeon [7-10]. As seen in sepsis, vagus nerve stimulation applied prior to abdominal surgery was shown to lead to the dampening of the production pro-inflammatory cytokines by macrophages leading to a dampening of the inflammation and ameliorating the ileus [11]. Evidence of the existence of an endogenous closed neural circuitry in response to intestinal manipulation with, on the one hand, activation of sensory vagal fibers as a consequence of intestinal inflammation and subsequent activation of motor vagal efferents was however still lacking. By mapping the activation of vagal neurons located in the NTS and motor vagal neurons located in the DMV, we could demonstrate that intestinal manipulation leads to the activation of sensory and motor vagal neurons innervating the intestine 24h after surgery, an activation that was abolished by selective vagal denervation of the intestine (**Chapter 4**). Furthermore, activation of those vagal neurons was not observed in mice only undergoing laparotomy demonstrating that this neuronal reflex is not due to anesthesia or opening of the abdominal cavity but is specific to the manipulation of the intestine. This neuronal activation was long-lasting as still observed 24h after the manipulation of the intestine, at a time when inflammation is well established. It is therefore likely

that the intestinal inflammation rather than mechanical activation of the vagus nerve is the cause underlying this neuronal activation. Moreover, selective vagal denervation of the intestine increased the expression of pro-inflammatory cytokines IL-1β and IL-6 in the intestinal muscularis 24h after intestinal manipulation therefore showing that the endogenous vago-vagal reflex observed during postoperative ileus indeed exerts an anti-inflammatory effect (**Chapter 5**).

The severity and duration of POI has previously been shown to correlate with the severity of the surgical trauma [12]. Importantly, we showed in **Chapter 6** that a higher intensity of intestinal handling in a mouse model of postoperative ileus leads to higher degree of tissue damage associated with the systemic release of pro-inflammatory cytokines and activation of neurons located in the area postrema. It was previously reported that circulating pro-inflammatory cytokines can activate sympathetic inhibitory networks that were shown to be involved in the early neurologic phase of the ileus and participated in the inhibition of the gastrointestinal motility [13-15]. Furthermore, the area postrema is devoid of a blood-brain barrier allowing its direct contact with circulating blood and detection of systemic mediators such as circulating cytokines [16]. The activation of the area postrema in addition to the activation of the NTS and DMV usually observed after gentle intestinal manipulation, could enhance the activation of inhibitory enteric neural networks that could participate in the more severe ileus observed after intense intestinal manipulation. These results therefore underline the fact that different types of inflammation (i.e., local versus systemic) can lead to a differential activation of the autonomic nervous system that will ultimately affect the ongoing inflammation.

The vagal inflammatory reflex was recently shown to be more complex than first proposed. Indeed, splenectomy performed prior to vagus nerve stimulation showed to abolish its anti-inflammatory effect in models of endotoxemia identifying the spleen as a crucial player in the anti-inflammatory effect of the vagus nerve [17,18]. Further studies however demonstrated that removal of splenic sympathetic innervation was sufficient to prevent the anti-inflammatory effect triggered by vagus nerve stimulation [19]. Since evidence of vagal direct innervation of the spleen is still under debate [20,21], the cholinergic anti-inflammatory pathway is now thought to consist and rely on the vagal control of splenic sympathetic nerve fibers with synaptic connections between vagal preganglionic neurons and sympathetic postganglionic neurons occurring in celiac ganglia [22,23]. The subsequent release of noradrenaline by the splenic nerve

in turn acts on ChAT positive T cells able to produce and release ACh that can bind on cholinergic receptors present on innate immune cells. Importantly however, evidence of the existence of synaptic connections between the vagus nerve and the splenic nerve in celiac ganglia is still lacking. We (**Chapter 2**) and others indeed failed to report such connections suggesting that other neural connections may be responsible for the inflammation-induced activation of splenic sympathetic inputs [24,25].

In POI, we reported that even though the spleen responds to the intestinal manipulation by a dramatic decrease in the number of splenocytes, a phenomenon that is partly regulated by the splenic nerve, the spleen does not participate in the intestinal inflammatory response occurring after intestinal manipulation (**Chapter 3**). This strongly suggests that the activation of the endogenous vagal reflex observed after intestinal manipulation (**Chapter 4**) is independent of the splenic innervation. This result was further confirmed in the study performed in **Chapter 5** where we demonstrated that contrary to selective vagal denervation of the intestine, sympathetic denervation of the spleen prior to intestinal manipulation had no influence on the manipulation-induced inflammation. Similarly, splenic denervation prior to vagus nerve stimulation did not abolish its anti-inflammatory effect [26]. Altogether, the results of these studies demonstrate that in POI and unlike sepsis, the vagus nerve exerts an anti-inflammatory effect independent of the spleen and solely by targeting the inflamed area, i.e., the intestine.

The existence of a vagal anti-inflammatory neuromodulation was also demonstrated in another intestinal inflammatory disorder, i.e., Inflammatory Bowel Disease (IBD) consisting of Crohn's disease and Ulcerative Colitis. Both diseases are remitting and relapsing chronic intestinal inflammatory disorders. Alterations of the autonomic nervous system are observed in IBD patients and up to 35% present with a dysbalance in the autonomic nervous system [27,28]. In rodents, vagus nerve stimulation dampens colonic inflammation in models of colitis underlining the importance of neural control of inflammation in this disease [29,30]. Interestingly, a recent study provided evidence that, as in sepsis, this vagal anti-inflammatory effect did not directly target the inflamed intestine. Instead, vagally-mediated sympathetic inputs was shown to be responsible for this vagal anti-inflammatory effect [31]. The results of the study we performed in **Chapter 7** bring further evidence of the importance of the splenic innervation in the dampening of colonic inflammation. Indeed, we showed that splenic denervation

performed prior to induction of colitis dampened the production of pro-inflammatory cytokines. We however failed to report activation of vagal motor neurons by colonic inflammation. These data thus suggest that not vagal but other neural networks are activated during colitis and lead to the activation of the splenic nerve. This observation is in accordance with our study of the distribution of vagal fibers in the gastrointestinal tract where we failed to report synaptic connections between the vagus nerve and the splenic nerve (**Chapter 2**). Since the splenic nerve is under the control of sympathetic nervous system via the greater and lesser splanchnic nerves, it is tempting to hypothesize that the activation of said sympathetic inputs may be responsible for the activation of the anti-inflammatory effect of the splenic nerve during colitis. This hypothesis is in accordance with a recent study demonstrating a sympathetic rather than a vagal control of inflammation in sepsis [24]. Importantly, selective vagal denervation of the proximal colon showed a trend, however non-significant, towards increased secretion of pro-inflammatory cytokines in colitic animals. We can therefore not exclude the participation of direct vagal innervation of the proximal colon in the immunomodulation of colitis. Further studies are required to determine the extent of the participation of these vagal inputs in the regulation of colonic inflammation as well as the exact neural networks involved.

Altogether the results described here clearly underline the fact that the immunomodulatory effect of the autonomic nervous system is different in relation to the type and location of inflammation. In POI, where the inflammation is confined to the intestinal muscularis, we observed activation of a vagal reflex consisting in a closed circuit between the inflamed organ and the brainstem, independently of the splenic innervation. In contrast, in colitis, where the integrity of the intestinal barrier is compromised and where the inflammation becomes systemic, the spleen becomes a more prominent target for neuromodulation of the immune response. Altogether our results demonstrate that the modulation of the immune system occurs both on local and systemic level, most likely depending on the nature of the immune response triggered.

Perspectives and application

New technical developments to study neural networks

Despite numerous studies, the exact neural networks interacting with each other to exert a neuromodulatory effect on the immune system remain unclear and a matter of debate. This lack of clarity is largely due to the limitations of the techniques available to determine the distribution of nerve endings, their interactions with neuronal and immune cells and the neurotransmitters involved. In the past recent years however, new techniques have been developed allowing a more detailed insight in neural activities. These methods, including thermogenetics and optogenetics, use light to selectively control and monitor the activity of specific neurons [32]. By using these techniques one can for example determine the relevance of a set of neurons in the dampening of inflammation as well as the neurotransmitters involved. Such technical improvement will undoubtedly bring further understanding in the mechanisms underlying the anti-inflammatory neuromodulation in the coming years.

Clinical application of the vagal anti-inflammatory neuromodulation

Neuro-immune interactions have opened the way to new therapeutic strategies to modulate inflammation. Activation of the vagus nerve in particular has proven successful in several inflammatory disorders. Several approaches have been investigated to activate vagal inputs whose neurotransmitters could ultimately decrease the pro-inflammatory activity of immune cells. In postoperative ileus, gum chewing has been hypothesized has a potential safe and inexpensive mean to achieve activation of the vagus nerve that could ultimately lead to a dampening of the inflammation and ameliorate ileus. Meta-analysis studies have recently shown a beneficial effect of gum chewing on postoperative ileus [33] but whether this effect is actually due to activation of the vagus nerve remains and requires further investigation. Moreover, stimulation electrodes positioned around the cervical vagus nerve and stimulated by a subcutaneous pacemaker have proven to improve neurological disorders such as epilepsy [34]. In IBD, triggered by preclinical data in colitis experimental models, electrical stimulation of the vagus nerve is currently evaluated as a potential new strategy to obtain remission in patients. Both animal and clinical studies are currently being performed and the coming years will be decisive in determining whether activation of the vagus nerve represents an alternative to conventional anti-inflammatory treatments in IBD.

In conclusion, the results reported in this thesis bring further insight in the neuro-immune interactions existing in the gastrointestinal tract as well as in the mechanisms underlying the anti-inflammatory neuromodulation in the intestinal inflammatory disorders such as POI and colitis. Our results furthermore provide an important fundamental understanding necessary to further consider and use this anti-inflammatory neuromodulation as a new therapeutic strategy in the clinic.

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Summary, acknowledgements and appendix

Summary

Interactions between the central nervous system and the immune system have been shown to exert a crucial role in the tight regulation of the immune response in the intestine. In particular, the vagus nerve was recently unraveled as an important player in this neuromodulation of intestinal inflammation. Despite thorough investigations on the anatomical distribution of nerve endings of the vagus nerve in the small and large intestine during the last decades, there still exists a lack of knowledge on whether vagal nerve endings establish direct contact with immune cells to exert their neuromodulatory effect or whether this effect is indirect through the modulation of neurons found in the intrinsic nervous system of the intestine, i.e., the enteric nervous system. Moreover, activation of the vagus nerve was recently shown to be anti-inflammatory in intestinal inflammatory disorders such as postoperative ileus or experimental colitis. The exact neural networks involved in this vagal anti-inflammatory effect however still remain a matter of debate. Indeed, in some inflammatory models the vagus nerve has been shown to exert a direct effect solely targeting the inflamed organ whereas in others, this vagal anti-inflammatory pathway was shown to rely on the presence of the sympathetic splenic innervation. In this thesis, we aimed to bring further knowledge on the anatomical interactions between the vagus nerve and immune cells in the gastrointestinal tract and to unravel more precisely the role of neural networks, in particular of the vagus nerve, involved in the immunoregulation occurring during postoperative ileus and colitis.

In **Chapter 1**, we reviewed the current knowledge on the innervation of the small and large intestine and on the anti-inflammatory role of the vagus nerve in the intestine.

In **Chapter 2**, we studied the anatomical distribution of vagal fibers in the small intestine with a particular focus on synaptic contacts between preganglionic vagal neurons emerging from the brainstem and postganglionic neurons of the enteric nervous system. We provided evidence that preganglionic vagal neurons establish synaptic contacts with neurons from the myenteric plexus but we failed to report any direct contact between preganglionic vagal fibers and immune cells in the small intestine. In contrast, nerve endings emerging from postganglionic enteric neurons, in particular nerve endings containing acetylcholine, nitric oxide and vasoactive intestinal peptide were found in the close vicinity of macrophages strongly suggesting that the vagus nerve exerts its neuromodulatory effect indirectly through enteric neurons.

In previous studies, we demonstrated that postoperative ileus is caused by a subtle inflammation of the muscularis externa of the intestine caused by surgical manipulation, a phenomenon that can be prevented by preoperative vagus nerve stimulation. In **Chapter 3**, we investigated whether this subtle inflammatory response in the muscularis was capable to trigger endogenous activation of this vagal anti-inflammatory system. To map the neuronal activity of neurons in the sensory (nucleus tractus solitarius) and motor (dorsal motor nucleus of the vagus) neurons of the "vagal anti-inflammatory reflex", we used the early neuronal activation marker c-Fos. We reported that manipulation of the intestine, leading to postoperative ileus, triggers the activation of both brainstem nuclei. This neuronal activation was only observed 24h after intestinal manipulation but not after laparotomy alone. These results therefore clearly reveal anatomical evidence for the existence of a vagal reflex in postoperative ileus.

To further prove the anti-inflammatory role of endogenous activation of the vagal anti-inflammatory pathway, we selectively lesioned vagal inputs to the intestine prior to the manipulation of the intestine (**Chapter 4**). We reported that lack of vagal input to the intestine results in enhanced manipulation-induced intestinal inflammation. Recently, the vagal anti-inflammatoy effect was shown to rely on the presence of the splenic nerve in various inflammatory settings. Hence, we lesioned the splenic nerve prior to intestinal manipulation to evaluate the potential involvement of the splenic innervation in the anti-inflammatory effect observed during postoperative ileus. We however failed to report any change in the severity of the postoperative ileus in the absence of the splenic nerve therefore demonstrating that in postoperative ileus, the vagal anti-inflammatory pathway directly and solely targets the intestine.

The underlying mechanism of postoperative ileus has been well described and resides in an inflammatory process with recruitment of leukocytes and activation of resident macrophages in the gut muscularis triggered consequently to the handling of the intestine by the surgeon. The location from which these immune cells reaching the intestinal muscularis originate remains nevertheless unknown. In **Chapter 5**, we hypothesized that the spleen, a major secondary lymphoid organ that was recently shown to act as a cell reservoir able to rapidly release immune cells during acute inflammation, could be involved in the influx of leukocytes to the gut muscularis during postoperative ileus. We demonstrated that manipulation of the intestine triggers an important departure of cells, mainly monocytes and $CD4^+$ T cells from the spleen, a phenomenon that was partially regulated by the splenic nerve. We however failed

to report a role in the intestinal inflammatory process underlying postoperative ileus for these departing splenocytes. Our study therefore shows that the spleen responds to manipulation of the intestine but does not participate in the pathophysiology of postoperative ileus.

In postoperative ileus, the duration and degree of intestinal manipulation was recently shown to correlate with the severity of the ileus. In **Chapter 6**, we hypothesized that the degree of tissue damage in consequence of the manipulation of the intestine was closely associated with the severity of postoperative ileus. We observed that a harsher manipulation of the intestine associated with a higher degree of tissue damage, assessed by systemic levels of inducible fatty acid binding protein, correlated with enhanced brain activation in particular of the area postrema, a nucleus able to detect systemic inflammatory mediators, as well as with the severity of postoperative ileus. These results demonstrate that a more severe inflammation with a systemic component leads to the activation of additional neural networks than local intestinal inflammation.

The vagus nerve was shown to exert an immunomodulatory effect in murine models of chronic relapsing inflammatory diseases, such as inflammatory bowel disease. Recently, this vagal anti-inflammatory effect was shown to depend on the integrity of the splenic nerve as central pharmacological activation of the vagus nerve in mice lacking splenic innervation failed to dampen the colonic inflammation induced by oral administration of dextran sodium sulfate. However, since evidence of the existence of synaptic connections between the vagus and the splenic nerve is so far lacking, we wished to study the neural networks involved in the neuromodulation of colitis (**Chapter 7)**. Selective lesioning of vagal fibers targeting the proximal colon failed to affect the severity of colonic inflammation while lesioning of the splenic nerve led to a significant increase in the colonic level of pro-inflammatory cytokines. Although sensory neurons were activated once colonic inflammation was established, we failed to report activation of motor vagal neurons in colitic mice. Altogether our results suggest that the direct vagal innervation of the proximal colon is not the predominant player in the neural immunomodulation of colitis, a submucosal inflammation with a systemic component, but rather that the splenic nerve seems to modulate the colonic inflammation, strengthening the concept of a differential activation of neural networks regulating systemic and local inflammation.

In conclusion, the results described in this thesis provide further insights into both anatomical and physiological mechanisms underlying the neuromodulation of the

intestinal immune system by the vagus nerve. From a clinical point of view, the results presented here bring fundamental knowledge on neuromodulation of inflammation in the intestine, which represents a potent alternative to conventional anti-inflammatory treatments.

Samenvatting

Het is gebleken dat de interactie tussen het centrale zenuwstelsel en het immuunsysteem een cruciale rol speelt tijdens de strak gereguleerde immuun respons. Vooral van de vagale zenuw is recentelijk aangetoond dat deze een sleutelrol speelt tijdens de zogenoemde neuromodulatie van ontstekingen in de darm. Ondanks dat er de afgelopen decennia intensief onderzoek is gedaan naar de anatomische distributie van vagale zenuwuiteinden in zowel de dunne als de dikke darm, is het nog altijd niet bekend of de vagale zenuw zijn neuromodulaire effect via direct contact uitoefent of dat er sprake is van een indirect effect via de modulatie van neuronen die zich in het intrinsieke zenuwstelsel van de darm, het enterische zenuwstelsel, bevinden. Wel is gebleken dat de activatie van de vagale zenuw een voornamelijk remmende werking heeft op darmontstekingen in ziektebeelden als postoperatieve ileus en experimentele colitis. De neurale netwerken die bij dit ontstekingsremmende effect van belang zijn, blijven echter een bron van discussie. In sommige ontstekingsmodellen heeft de vagale zenuw een acuut effect welke direct gericht is op het ontstoken orgaan, terwijl in andere modellen de vagale ontstekingsremmende werking juist vertrouwt op de aanwezigheid van sympatische innervatie in de milt. Dit proefschrift heeft als doel om enerzijds een beter inzicht te geven in de anatomische interacties tussen de vagale zenuw en cellen van het immuunsysteem in het darmstelsel en anderzijds de rol van neurale netwerken, met de vagale zenuw in het bijzonder, die een rol spelen in de immunoregulatie rondom postoperatieve ileus en colitis te ontrafelen.

In **hoofdstuk 1** wordt de huidige kennis van de innervatie van de dunne en dikke darm alsmede de ontstekingsremmende rol van de vagale zenuw in de darmen besproken.

In **hoofdstuk 2** wordt de anatomische distributie van vagale zenuwuiteinden in de dunne darm onderzocht, met bijzondere aandacht voor synaptisch contact tussen preganglionaire vagale zenuwcellen afkomstig uit de hersenstam en postganglionaire zenuwcellen van het enterische zenuwstelsel. We leveren bewijs dat preganglionaire vagale neuronen synaptisch contact maken met neuronen van de myenterische plexus, maar hebben direct contact tussen preganglionaire vagale zenuwuiteinden en immuun cellen in de dunne darm niet kunnen aantonen. Daarentegen laten we wel zien dat zenuwuiteinden afkomstig van postganglionaire enterische neuronen, en vooral de zenuwuiteinden die acetylcholine, nitric oxide en vasoactive intestinal peptide bevatten,

in de nabijheid van macrofagen eindigen, wat sterk de suggestie wekt dat de vagale zenuw zijn neuromodulatie indirect uitoefent via enterische neuronen.

In voorgaande studies hebben we laten zien dat postoperatieve ileus ontstaat door een subtiele ontsteking van de muscularis externa van de darm, veroorzaakt door chirurgische manipulatie. Een fenomeen dat verhinderd kan worden door stimulatie van de vagale zenuw. In **hoofdstuk 3** hebben we onderzocht we of deze subtiele ontsteking in de muscularis in staat is om een endogene activatie van het vagale ontstekingsremmende systeem te bewerkstelligen. Om de neuronale activiteit in sensorische (nucleus tractus solitarius) en motorische (dorsale motor nucleus van de vagus) neuronen van de « vagale ontstekingsremmende reflex » in kaart te brengen, is gebruikt gemaakt van de vroege neuronale activatie marker c-Fos. We laten zien dat manipulatie van de darmen, wat leidt tot postoperatieve ileus, beide hersenkernen in de hersenstam activeert. Deze neuronale activatie is enkel 24 uur na de manipulatie van de darmen zichtbaar en is afwezig bij alleen een laparotomie. Deze resultaten geven daarmee duidelijk anatomisch bewijs voor het bestaan van een vagale reflex bij postoperatieve ileus.

Om de ontstekingsremmende rol van de endogene activatie van de vagale ontstekingsremmende reflex verder te onderbouwen hebben we, voordat de darmen gemanipuleerd worden, de vagale input naar de darm selectief weggenomen (**hoofdstuk 4**). Hiermee laten zien dat de afwezigheid van vagale input naar de darm resulteert in een versterkte ontstekingsreactie na manipulatie van de darmen. Recent is aangetoond dat het vagale ontstekingsremmende effect in verschillende ontstekingsprocessen afhankelijk is van de nervus splenicus. Om de potentiele betrokkenheid van de nervus splenicus bij het ontstekingsremmende effect tijdens postoperatieve ileus te evalueren, werd deze voorafgaand aan de manipulatie van de darmen doorgeknipt. Het is ons echter niet gelukt om enige verandering te laten zien in de ernst van de postoperatieve ileus tijdens afwezigheid van de nervus splenicus, met als conclusie dat het vagale ontstekingsremmende effect tijdens postoperatieve ileus, direct en uitsluitend gericht is op de darmen.

Het onderliggende mechanisme van postoperatieve ileus is uitgebreid beschreven en komt neer op een ontstekingsproces waarbij de werving van leukocyten en activatie van residerende macrofagen in de muscularis van de darm in gang wordt gezet door het

aanraken van de darm door de chirurg. De oorsprong van de immuun cellen die naar de muscularis van de darm migreren blijft echter onbekend. In **hoofdstuk 5** stellen we de hypothese dat de milt, een belangrijk secundair lymfoïde orgaan waarvan recent is aangetoond dat het als cel reservoir kan dienen voor onmiddellijke afgifte van immuun cellen tijdens acute ontstekingen, een rol kan spelen bij de influx van leukocyten naar de muscularis van de darm gedurende postoperatieve ileus. We laten zien dat manipulatie van de darmen een belangrijke efflux van voornamelijk monocyten en $CD4^+$ cellen vanuit de milt bewerkstelligt. Een fenomeen dat gedeeltelijk door de nervus splenicus wordt gereguleerd. Desalniettemin hebben we niet kunnen aantonen dat deze migrerende splenocyten een rol spelen in het ontstekingsproces in de darmen dat ten grondslag ligt aan postoperatieve ileus. Onze studie laat zien dat de milt reageert op de manipulatie van de darmen, maar niet participeert in de pathofysiologie van postoperatieve ileus. De duur en ernst van postoperatieve ileus is, zoals onlangs is aangetoond, gecorreleerd met de duur en hevigheid van de manipulatie.

In **hoofdstuk 6** testen we de hypothese dat de ernst van weefselschade als gevolg van de manipulatie van de darm nauw verbonden is met de hevigheid van postoperatieve ileus. We zagen dat een ruwere manipulatie van de darm niet alleen geassocieerd kan worden met een heftiger beeld van postoperatieve ileus, maar ook geassocieerd is met een hogere mate van weefselbeschadiging, gemeten in systemische niveaus van een induceerbaar vetzuurbindend eiwit welke weer correleert met verhoogde activatie in de hersenen, met name in en de area postrema, een nucleus in de hersenen die systemische ontstekingswaarden kan detecteren. Deze resultaten laten zien dat in tegenstelling tot een lokale darmontsteking, een hevigere ontsteking met een systemische component leidt tot de activatie van additionele neurale netwerken.

De vagale zenuw heeft een immuno regulerend effect heeft op muismodellen van chronische terugkerende ontstekingsziekten als inflammatoire darmziekten (IBD). In recent onderzoek is aangetoond dat dit vagale ontstekingsremmende effect afhankelijk is van een intacte nervus splenicus doordat centrale farmacologische activatie van de vagale zenuw in muizen zonder nervus splenicus niet in staat is om de ontstekingen in het colon, veroorzaakt door orale toediening van DSS (dextran sodium sulfate), te reduceren. Maar aangezien er tot nu toe geen bewijs is van synaptisch contact tussen de nervus vagus en splenicus, wilden wij de invloed van neurale netwerken op de neuromodulatie van colitis bestuderen (**hoofdstuk 7**). Na het selectief doorknippen

van vagale vezels die het proximale gedeelte van het colon innerveren kon geen verandering worden waargenomen in de hevigheid van de ontsteking in het colon, terwijl het doorknippen van de nervus splenicus wel degelijk tot een significante stijging van de niveaus van pro-inflammatoire cytokines leidde. Alhoewel sensorische neuronen wel degelijk geactiveerd waren op het moment dat de ontsteking in het colon zich had ontwikkeld, konden we geen activatie van de motorische vagale zenuw laten zien in muizen met colitis. Alles bij elkaar genomen suggereren onze resultaten dat directe vagale innervatie van het proximale colon niet de allesbepalende factor is bij de neuronale immuno-modulatie tijdens colitis, een submucosale ontsteking met een systemische component, maar dat de nervus splenicus de ontsteking in het colon moduleert. Dit onderbouwt het concept dat systemische en locale ontstekingen worden gereguleerd door gedifferentieerde activatie van neuronale netwerken.

Concluderend geven de resultaten, zoals besproken in dit proefschrift, een dieper inzicht in zowel de anatomische als de fysiologische mechanismen die ten grondslag liggen aan de neuromodulatie van het immuun systeem van de darmen door de nervus vagus. Vanuit een klinisch oogpunt geven deze resultaten een fundamentele kennis van de neuromodulatie tijdens ontstekingen van de darm, welke een krachtig alternatief geeft voor de conventionele ontstekingsremmende behandelingen.

List of abbreviations

ACh: acetylcholine

ANS: autonomic nervous system

AP: area postrema

CAIP: cholinergic anti-inflammatory pathway

CD: Crohn's disease

CGRP: calcitonin gene-related peptide

ChAT : choline acetyltransferase

CNS: central nervous system

CVOs: circumventricular organs

DAB: diaminobenzidine

DAI: disease activity index

DMV: dorsal motor nucleus of the vagus

DSS: dextran sodium sulfate

ENS: enteric nervous system

GALT: gut-associated lymphoid tissue

GC: geometric center

GI: gastrointestinal

IBD: inflammatory bowel disease

IBS: inflammatory bowel syndrome

IM: intestinal manipulation

IntX or Intx: vagal intestinal denervation

IR: immunoreactivity

L: laparotomy

mAChR: muscarinic acetylcholine receptor

MLN: mesenteric lymph node

MPO: myeloperoxidase

NA: noradrenaline

nAChR: nicotinic acetylcholine receptor

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NTS: nucleus tractus solitarius

PBS: phosphate buffered sodium

PFA: paraformaldehyde

POI: postoperative ileus

PP: Peyer's patch

PVN: paraventricular nucleus

S1P: sphingosin-1-phosphate

$S1P_1$: sphingosin-1-phosphate receptor 1

SP: substance P

Sx or Splx: spleen denervation

TH: tyrosine hydroxylase

TNBS: trinitrobenzene sulphonic acid

TNF-α: tumor necrosis factor alpha

Tregs: regulatory T cells

UC: ulcerative colitis

VIP: vasoactive intestinal peptide

VNS: vagus nerve stimulation

Vx: vagal denervation of the proximal colon

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Portfolio

	Year	Workload
Courses		
– International course for Laboratory Animal Science	2011	3
– Anatomy of the house mouse	2011	1.1
– Advanced immunology course	2011	2.9
Oral presentations		
– Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie (NVGE), Veeldhoven, The Netherlands	2012	0.5
– Voorjaarsvergadering NVGE, Veeldhoven, The Netherlands	2013	0.5
– Masterclass with Prof. Ruud Buijs, Amsterdam, The Netherlands	2013	0.5
– Masterclass with Dr Samuel Huber and Prof. Shigeo Koyasu, Rotterdam, The Netherlands	2013	0.5
Poster presentations		
– Dutch Society of Immunology (NVVI) Congress, Noordwijkerhout, The Netherlands	2011	0.5
– Digestive Disease Week, San Diego, USA	2012	0.5
– International Congress of Immunology, Milan, Italy	2013	0.5
(Inter)national conferences and symposiums		
– European Mucosal Immunology Group (EMIG) meeting, Amsterdam, The Netherlands	2011	1
– Dutch Society of Immunology (NVVI) Congress, Noordwijkerhout, The Netherlands	2011	0.5
– NVVI symposium, Lunteren, The Netherlands	2012	0.5
– Voorjaarscongres NVGE, Veeldhoven, The Netherlands	2012	0.5
– Digestive Disease Week, San Diego, USA	2012	1
– Voorjaarscongres NVGE, Veeldhoven, The Netherlands	2013	0.5
– International Congress of Immunology, Milan, Italy	2013	1.5
– 5th symposium on mucosal immunology, Rotterdam, The Netherlands	2013	0.2
Parameters of esteem		
– Travel grant from the NVGE to attend the Digestive Disease Week	2012	

Curriculum vitae

Léa was born on the 16th of August 1985 in Limoges, France. She obtained her Bachelor in Cellular Biology and Physiology in 2007 from the University of Limoges. She then specialized in Immunology and Neuroscience by performing a Masters degree at the University of Poitiers, France. During her Masters, she performed internships in the research team EA3842 under the direction of Pr. Dr. Marie-Odile Jauberteau and Pr. Dr. Jean-Claude Lecron. Upon her graduation, Léa engaged in the PhD project of this thesis, under the supervision of Dr. Cathy Cailotto and Pr. Dr. Guy Boeckxstaens. This project focused on studying the neural networks regulating intestinal inflammation by using murine models of postoperative ileus and inflammatory bowel disease. Léa is currently working as a post-doctoral fellow in the Laboratory of Pediatric Gastroenterology in Rotterdam, under the direction of Dr. Janneke Samsom on a project aiming to unravel the role of IL-21 in celiac disease and inflammatory bowel disease.

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List of publications

Costes LM, van der Vliet J, van Bree SH, Boeckxstaens GE, Cailotto C (2014a) Endogenous vagal activation dampens intestinal inflammation independently of splenic innervation in postoperative ileus. *Autonomic neuroscience : basic & clinical.*

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