Neuromodulation of intestinal inflammation
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Citation for published version (APA):
Chapter 7

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


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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.
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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
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*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 (H₂O₂) 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.

<table>
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<th>Forward primer 5’-3</th>
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<tr>
<td>Foxp3</td>
<td>TCCCCACGCTCGGTTTACAC</td>
<td>CCACATTGCAGACTCCATTTGC</td>
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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
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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.
References


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