Loss of vagal anti-inflammatory effect: in vivo visualization and adoptive transfer

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The aims of this study were to quantify the impact of vagotomy in vivo by visualizing nuclear factor (NF)-κB activity and to determine if the proinflammatory impact of vagotomy could be transferred by lymphocytes. Real-time biophotonic imaging revealed that subdiaphragmatic vagotomy resulted in increased levels of NF-κB in vivo. NF-κB activation was further exaggerated in vivo following exposure to 4% DSS for 5 days. Vagotomized animals also exhibited higher disease activity scores and secreted more proinflammatory cytokines. Adoptive transfer of CD4+ T cells from vagotomized animals (but not CD4+ T cells from sham-operated controls) to naive dextran sulfate sodium (DSS)-treated recipients resulted in increased inflammatory scores. Further examination of the CD4+ T cells revealed that adoptive transfer of the CD25+ population alone from vagotomized donors (but not sham-operated donors) was sufficient to aggravate colitis in DSS-treated recipients. Increased DSS-induced inflammation was associated with reduced CD4+CD25+Foxp3+ regulatory T cell numbers in recipients. This study clearly demonstrates the ability of the vagus nerve to modulate activity of the proinflammatory transcription factor NF-κB in vivo. The proinflammatory effect of vagotomy is transferable using splenic T cells and highlights a previously unappreciated cellular mechanism for linking central parasympathetic processes with mucosal inflammation and immune homeostasis.

Vagus nerve; murine models of colitis; nuclear factor-κB; cytokines; regulatory T cells

The cholinergic anti-inflammatory pathway is a physiological mechanism whereby the vagus nerve modulates host inflammatory responses through the release of acetylcholine. In the absence of vagal signaling, vagotomized animals have increased sensitivity to endotoxin and produce more tumor necrosis factor (TNF)-α. Electrical stimulation of the vagus nerve activates the cholinergic pathway and is associated with inhibition of acute cytokine release. Acetylcholine release following stimulation leads to an interaction with the alpha-7 subunit of the nicotinic receptor on macrophages. This interaction inhibits macrophage activation and significantly contributes to the vagal anti-inflammatory effect (4, 39).

In addition to sepsis models, we and others (26, 40, 41, 13–15) have demonstrated that vagal signaling has a protective effect in experimental colitis. The vagus nerve plays an anti-inflammatory role protecting against acute relapses on a background of chronic inflammation induced by repeated exposure to dextran sulfate sodium (DSS) (14). A similar macrophage-dependent mechanism involving the nicotinic alpha-7 receptor, as already determined for protection against endotoxemia, has also been described for vagal attenuation of mucosal inflammation. However, other mechanisms are also likely to be important. For example, the length of recovery time after vagotomy may be relevant in the inflammatory reflex, since vagal protection has been shown to weaken and is compensated for over time with other anti-inflammatory mechanisms, e.g., induction of regulatory T cells and interleukin (IL)-10 secretion (15). In addition, vagotomy had no effect in the SCID T cell transfer model of colitis, suggesting that a direct interaction between the vagus and lymphocytes could also be relevant (41).

The purpose of the present study was: 1) to assess the significance in vivo of the vagal effect by quantifying activity of the proinflammatory transcription factor NF-κB using biophotonic imaging; and 2) to determine whether the impact of vagotomy is transferable. The loss of vagal anti-inflammatory signaling was of sufficient magnitude to be visualized in vivo and was transferable.

MATERIALS AND METHODS

Animals. BALB/c male mice were obtained from Harlan (Bicester, UK). Transgenic nuclear factor (NF)-κB reporter mice on a BALB/c background were obtained from Charles River Laboratories (Wilmington, NJ) and bred in-house. The reporter mice DNA construct contains three NF-κB binding sites from the Igκ light chain promoter coupled to the gene encoding firefly luciferase (3x-kB-luc) (6). The animal ethics committee at National University of Ireland approved all animal experiments, and experimental procedures were conducted under appropriate license from the Irish government or conducted in accordance with the requirements of the Animal Care Committee of McMaster University (Hamilton, Ontario, Canada). Mice were housed under barrier-maintained conditions within the biological services unit and were generally used at 6–8 wk of age.

Vagotomy procedure. Mice were anaesthetized using halothane (Inhalation Anesthetic; Merial, Harlow, UK), and the stomach and lower esophagus were visualized following an upper midline laparotomy. The skin and abdominal wall were incised along the ventral midline, and the intestines were moved aside to allow access to the left lateral lobe of the liver (LLL) and the stomach. The LLL was gently retracted, and a ligature was placed around the esophagus at its entrance to the stomach, which allowed access to the esophagus. The
stomach was gently pulled down beneath the diaphragm to clearly expose both vagal trunks, which were then transected. All neural and connective tissue surrounding the esophagus was removed to ensure transection of all small vagal branches.

To confirm completeness of vagotomy, a food intake analysis test was performed based on the satiety effect of cholecystokinin-octapeptide (CCK-8) (Sigma Aldrich, St. Louis, MO) (12, 18). Animals were deprived of food for 20 h and then received an intraperitoneal injection of 8 μg/kg CCK per mouse. Food intake over a 2-h time period was measured. Subdiaphragmatic vagotomy abolished the satiety effect of CCK-8 so the food intake in vagotomized mice was similar to saline-injected animals (vagotomy/saline 1.01 ± 0.15 g vs. vagotomy/CCK 1.09 ± 0.16 g food intake). Any vagotomized animal that decreased their food intake significantly was excluded from the study. A pyloroplasty was not deemed necessary with our mouse model. A recovery period of 2 wk was allowed after surgery before any further examinations.

Assessment of NF-κB signaling in vivo. In vivo imaging of NF-κBlux transgenic mice was performed by first anesthetizing the mice with isoflurane (Abbot Laboratories, Kent, UK). In Luciferin (120 mg/kg; Biothera, Handen, Sweden) was injected intraperitoneally (6) and immediately afterward the mice were placed in a ventral recumbent position in a light-sealed chamber in the In Vivo Imaging System (IVIS-100; Xenogen) and imaged continuously for 5 min. Organs were excised and placed in a culture dish and imaged with an integration time of 5 min. Organs examined were the spleen, liver, small intestine, and colon. A reference black and white image was taken in low light conditions while a sensitive cooled charged-coupled device camera collected the emitted photons. Photons were quantified using Living Image software (Xenogen), and the luciferase activity was quantified as the amount of light emitted per second per square centimeter from the tissue. The pseudocolored images represent light intensity (red is the strongest, and violet is the weakest).

Assessment of NF-κB signaling ex vivo. Activation of the NF-κB p65 subunit was determined ex vivo using an enzyme-linked immunosorbent assay (ELISA)-based transcription factor TransAM kit (Active Motif Europe, Rixensart, Belgium). Briefly, total T cell and dendritic cell populations were positively selected from the spleen using CD90.2 and CD11c MicroBeads as per the manufacturer’s instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Nuclear proteins were extracted from colonic homogenates, positive T cell, and dendritic cells isolates using the Nuclear Extraction kit according to the manufacturer’s protocol (Active Motif). Total protein concentration of the lysates was determined using the Pro-Stain assay (Active Motif). Activation of the NF-κB p65 subunit in 5 μg of nuclear extracts was determined.

Induction of acute DSS colitis. Colitis was induced by exposing mice to DSS (mol wt 36,000–50,000) (MP Biomedicals) in their drinking water at a final concentration of 4% (wt/vol). This dose of DSS was chosen from a range of doses tested as the optimal dose to ensure reproducible gastrointestinal inflammation within our animal unit (41). The acute model of colitis allowed mice free access to the drinking water at a final concentration of 4% (wt/vol). This dose of DSS solution for 5 days and tap water for two additional days. Unless otherwise stated, the DSS studies were completed within 21 days postvagotomy. Control mice were allowed to drink tap water.

Assessment of colitis severity. Mice were monitored for colitis progression using a clinical scoring scale adapted from Okayasu et al. (28). This scale scored animals from zero (no disease) to seven (severe disease) and is shown in Table 1. In addition, colitis severity was evaluated by measuring colon weight and colon length, and results were expressed as milligrams per centimeter. Last, inflammation was assessed using myeloperoxidase (MPO) measurements, as previously described (21). Briefly, a full-thickness segment of the middle and distal colon was analyzed, and the supernatant was assayed spectrophotometrically for MPO activity and protein concentration. MPO activity was expressed as units per milligram of protein, where 1 unit (U) corresponded to the activity required to degrade 1 mmol of hydrogen peroxide in 1 min at room temperature. The protein concentration of the supernatant was determined using a Bradford assay kit (Bio-Rad Laboratories), and results were expressed as units of MPO per milligram of tissue protein.

Measurement of cytokine production. Single cell suspensions were generated from the spleens by mechanical means and filtered through a 70-μm cell strainer. Erythrocytes were lysed using the Mouse Erythrocyte Lysing Kit (R&D Systems, Abingdon, UK). Cells were seeded in 96-well tissue culture plates (Sarstedt) at 2 × 10^5 cells/well. Single cell suspensions were stimulated for 48 h with anti-CD3 (10 μg/ml) and anti-CD28 (2 mg/ml) antibodies (BD Biosciences, Oxford, UK) at 37°C and 5% CO2 humidified atmosphere. All supernatants were harvested for cytokine analysis. Aliquots were stored at −80°C for analysis of cytokine production. Cytokine levels were quantified using cytometric bead arrays (BD Biosciences). Levels were measured using a BD FacsCaliber flow cytometer, and analysis was carried out using the BD CellQuest software and BD CBA Software.

Cellular phenotypes. Single cell suspensions were obtained from the spleen as outlined above. Monoclonal antibodies to CD3, CD4, CD8 CD25 (BD Biosciences), CD90.2 and, CD11c (Miltenyi Biotech) were used to label cells for cell subset analysis. Antibodies to the transcription factor Foxp3 (eBioscience) were used to label permeabilized cells. Transcription factor analysis was performed on gated CD4+ cells. Cellular phenotypes were measured using a BD FacsCaliber flow cytometer, and analysis was carried out using the BD CellQuest software.

T cell adoptive transfer. Three weeks following vagotomy, or sham-vagotomy, spleens were removed, and a CD4+ T cell population was positively selected using CD4 MicroBeads as per the manufacturer’s instructions (Miltenyi Biotech). Adoptive transfer of 1 × 10^6 CD4+ T cells/ml in naive recipient mice was performed by intraperitoneal injection, and after 8 days recipient mice were administered DSS to induce colitis (as described above). In subsequent experiments, CD4+ T cells from vagotomized or sham control mice were further separated into CD25+ and CD25− subpopulations as per the manufacturer’s protocol (Miltenyi Biotech). Adoptive transfer of 1 × 10^6 CD4+CD25− T cells/ml in naive recipient mice was performed by intraperitoneal injection, and control mice were injected with PBS. Later (24 h and 8 days), the recipient mice were analyzed in vivo as described above using the Xenogen two-dimensional imaging system (IVIS-100; Xenogen). Briefly, a sensitive camera collected the emitted photons from the transferred cells in the recipient mice which were quantified using Living Image software. The pseudocolored images represent light intensity (blue is the strongest, and green is the weakest).

Statistical analysis. Statistical analysis was performed with GraphPad Prism software. A Student’s t-test was used for analysis of two groups. A one-way ANOVA with Bonferroni’s posttest was used where more than two groups were tested. Results were presented as

<table>
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<tr>
<th>Stool</th>
<th>Blood in Stool</th>
<th>Appearance</th>
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<tbody>
<tr>
<td>0. Well-formed pellets</td>
<td>0. No blood</td>
<td>Fur texture</td>
</tr>
<tr>
<td>2. Loose stool</td>
<td>2. Gross bleeding</td>
<td>Anal prolapse</td>
</tr>
<tr>
<td>3. Diarrhoea/no stool</td>
<td>3. Loose blood</td>
<td>0. Well-formed pellets</td>
</tr>
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Table 1. Scoring system for assessment of mucosal inflammation

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results are expressed as photons. C: vagotomy significantly increases DNA-binding activity of NF-κB p65 in colonic tissue as determined using an enzyme-linked immunosorbent assay (ELISA) system. Results are expressed as absorbance. All results are expressed as means ± SE (n = 3–4/group; *P < 0.05 and **P < 0.005).

Fig. 1. In vivo visualization of loss of vagal effect on nuclear factor (NF)-κB activation. A: representative whole body in vivo bioluminescent image showing NF-κB activation in sham-vagotomized and vagotomized mice. B: vagotomized animals display elevated whole body NF-κB activation in vivo compared with sham-vagotomized mice. Results are expressed as photons. C: vagotomy significantly increases DNA-binding activity of NF-κB p65 in colonic tissue as determined using an enzyme-linked immunosorbent assay (ELISA) system. Results are expressed as absorbance. All results are expressed as means ± SE (n = 3–4/group; *P < 0.05 and **P < 0.005).
Fig. 2. Visualization of vagotomy effect during dextran sulfate sodium (DSS)-induced colitis. A: representative biophotonic in vivo images showing that NF-κB activation in NF-κBα transgenic mice following DSS administration is higher in vagotomized animals (n = 3) compared with sham-operated controls (n = 4). B: vagotomy significantly increases NF-κB activation ex vivo 4-fold in the ileum and 3-fold in the colon, liver, and spleen. Data are presented as degree of increase. C: vagotomy significantly increases DNA-binding activity of NF-κB p65 in the colon after DSS administration (n = 5/group). D: NF-κB p65 expression in dendritic cells (CD11c⁺) and T cells (CD90.2⁺) is significantly higher following vagotomy and DSS administration (n = 5/group). All results are expressed as means ± SE.
Table 2. Proinflammatory effect of vagotomy on splenic cytokine production

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-6 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>MCP-1 (pg/ml)</th>
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<tbody>
<tr>
<td>Sham</td>
<td>75.5±30.5</td>
<td>165.3±67</td>
<td>8.6±5.4</td>
</tr>
<tr>
<td>Vagotomy</td>
<td>214.3±37.7*</td>
<td>666.7±3.7†</td>
<td>75.5±12.1†</td>
</tr>
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</table>

Data are expressed as means ± SE; n = 3–4 mice in each group. Units are pg/ml. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MCP, monocyte chemoattractant protein-1. Dextran sulfate sodium (DSS) cycle is defined as 4% DSS containing drinking water for 5 days followed by 2 days of tap water. On the final day of the study, mice were 21 days postvagotomy. Isolated splenocytes were stimulated with anti CD3/CD28 in vitro for 48 h. *P < 0.05 and †P < 0.001 vs. sham-vagotomized controls.

sham-operated controls (Fig. 4A). Moreover, disease activity scores were significantly elevated in DSS-treated mice that received CD4+ T cells from vagotomized donors compared with control recipients [5.9 ± 0.5 (n = 6) vs. 3.3 ± 0.9 (n = 8), P < 0.05]. Associated with higher disease scores, colonic MPO activity was also significantly increased (Fig. 4B) as was splenocyte release of TNF-α and MCP-1 from CD3/CD28-stimulated in vitro cultures (Fig. 4, C and D). No difference in IL-6 production was noted between recipients of CD4+ vagotomy-derived T cells or CD4+ sham-derived T cells (Fig. 4E).

Because vagotomy was shown above to be associated with decreased levels of T regulatory cells within the spleen, we also assessed the regulatory T cell population in the spleens of recipient animals. DSS-treated recipients of CD4+ T cells from vagotomized donors also exhibited a significant reduction in the percentage of CD4+ T cells that coexpressed CD25+ and Foxp3+ (Fig. 4F).

The CD4+CD25− subpopulation is sufficient to transfer the proinflammatory vagotomy effect. To investigate which subpopulation of splenic T cells was responsible for the proinflammatory effect, isolated CD4+ T cells were divided into CD25− and CD25+ T cells from sham-control donors did not impact on colitis severity in recipient mice. However, transfer of the CD25− subset derived from vagotomized donors resulted in a significant increase in colitis severity in DSS-treated recipients (Fig. 5A). Extending the observation showing Foxp3 regulatory T cell expression was downregulated in animals that were transferred CD4+ T cells from vagotomized donors, we also demonstrated that the CD25− T cell population was responsible for this effect (Fig. 5B). In addition, a significant increase in the splenic CD4+CD25+Foxp3− T cell phenotype was observed in mice that received the CD25− T cell subpopulation from vagotomized donors (Fig. 5C). In vivo fluorescent imaging of labeled CD25− cells illustrates that many of the transferred T cells traffic to the intestine of naive recipient mice (Fig. 5D).

DISCUSSION

In this report, we have extended our previous findings and those of others (13–15, 26, 39–41) highlighting the anti-inflammatory effect of vagal signaling. We have confirmed that...
The vagal effect is biologically relevant and sufficiently robust to visualize in vivo, not only in colitis but also under basal conditions. Furthermore, we demonstrate that the loss of vagal anti-inflammatory signaling is transferable and is associated with reduced numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T cells.

NF-κB is a master transcription factor controlling the expression of a wide range of proinflammatory genes. The vagus nerve has been previously shown to impact NF-κB activation as measured by in vitro Western blotting or mobility shift assays on homogenized tissue (36, 1). Our biophotonic imaging data confirm that the vagal influence on NF-κB activation is operational in vivo. Bioluminescent imaging of NF-κB activation is a powerful methodology that has been developed over the last decade that enables the study of ongoing biological processes in vivo (6). Increased systemic NF-κB activation is evident in vagotomized animals before induction of colitis by DSS. Indeed, direct quantification of NF-κBp65 activation shows overexpression ex vivo in the colon following vagotomy. After DSS administration, there is a significantly exaggerated NF-κB response within the mucosa of vagotomized animals that correlates with increased colitis disease scores. In vivo imaging also revealed a substantial, but not statistically significant, increase in splenic NF-κB activation while examination of isolated dendritic cells and T cells from the spleen revealed a statistically significant increase in NF-κB activation.

This highlights a limitation of the in vivo imaging technique whereby it is difficult to detect alterations in the activation state of individual cell types within a mixed population or intact organ. Nevertheless, these observations imply that the vagus nerve exerts an influence on the proinflammatory transcription factor NF-κB in specific splenic immune cell populations. This regulatory control may have a profound influence on T cell differentiation and DC activation states within the spleen. However, more extensive studies are required to further investigate these findings. Proinflammatory cytokine production by mononuclear cells isolated from the spleen was significantly increased in mice transferred with CD4⁺ T cells from vagotomized donors compared with those that received T cells from sham donors. Results are presented for each animal with the mean per group highlighted. The P value compares vagotomy-derived T cell recipients vs. all other groups.

Fig. 4. Vagotomy-associated proinflammatory activity can be adoptively transferred using splenic T cells. In A-F, the first bar represents control animals that did not receive transferred lymphocytes or DSS (n = 4). The second and third bars represent animals administered DSS 8 days after transfer of CD4⁺ T cells isolated from sham-vagotomized (n = 8) or vagotomized animals (n = 6), respectively. Recipients of CD4⁺ T cells from vagotomized mice have a higher disease activity score compared with recipients of CD4⁺ T cells from sham-operated animals as evaluated by colonic tissue weight (A) and colonic myeloperoxidase (MPO) activity (B). C: In vitro production of interleukin (IL)-6 was unchanged between recipient groups, whereas production of tumor necrosis factor (TNF)-α (D) and monocyte chemoattractant protein (MCP)-1 (E) was significantly elevated in mice transferred with CD4⁺ T cells from vagotomized donors. F: In addition, the percentage of splenic CD4⁺ T cells that coexpressed CD25 and Foxp3 was significantly less in animals that received CD4⁺ T cells from vagotomized donors compared with those that received T cells from sham donors. Results are presented for each animal with the mean per group highlighted. The P value compares vagotomy-derived T cell recipients vs. all other groups.
response to the superantigen favor the development of proinflammatory T cell subsets over 22, 43), and cytokine production following vagotomy may such as dendritic cells influence T cell lineage commitment (3, 20, 24, 25, 34, 37, 42). Although it is possible that the vagus may exert a direct influence on regulatory T cells, we hypothesize that other cellular intermediaries are terminally differentiated and no longer respond to 17, 33) and suppressors of autoreactive T cells (17, 33) and suppress additional regulatory cells. The transferred T cells maintain their proinflammatory activity and traffic to the intestinal tissues even in the context of intact vagal signaling in the recipient animals, suggesting that these T cells are not modulated directly by the vagus itself or they are terminally differentiated and no longer respond to vagal anti-inflammatory signals. It is tempting to speculate that these T cells are not modulated directly by the vagus itself or they are terminally differentiated and no longer respond to vagal anti-inflammatory signals. It is tempting to speculate that these T cells are not modulated directly by the vagus itself or they are terminally differentiated and no longer respond to vagal anti-inflammatory signals. It is tempting to speculate that these T cells are not modulated directly by the vagus itself or they are terminally differentiated and no longer respond to vagal anti-inflammatory signals. It is tempting to speculate that these T cells are not modulated directly by the vagus itself or they are terminally differentiated and no longer respond to vagal anti-inflammatory signals.

Transfer of CD4+ T cells from vagotomized donors in nonvagotomized colitic animals is sufficient to replicate the effect of vagotomy on splenic Foxp3+ regulatory T cells in recipient animals, and is associated with aggravated disease symptoms. Furthermore, transfer of the CD4+CD25- subpopulation alone, but not the CD4+CD25+ subpopulation, from vagotomized donors transferred the proinflammatory vagotom effect. Interestingly, transfer of CD4+CD25+ cells did not impact colitis severity. This may be attributed to the mouse model used in these studies, since previous published reports showing the protective effect of adoptively transferred regulatory T cells was performed in lymphopenic and knockout mouse models. In this report, we utilized immunocompetent recipient mice that already possess a regulatory T cell pool which might not be significantly influenced by the transfer of additional regulatory cells.

The transferred T cells maintain their proinflammatory activity and traffic to the intestinal tissues even in the context of intact vagal signaling in the recipient animals, suggesting that these T cells are not modulated directly by the vagus itself or they are terminally differentiated and no longer respond to vagal anti-inflammatory signals.
more severe inflammatory phenotype. This hypothesis could explain, in part, the lack of vagal influence in the SCID colitis model, since these animals lack T regulatory cells and therefore cannot be influenced by this mechanism. In addition, further support for this hypothesis is the observation that consumption of the anti-inflammatory bacterium *Bifidobacterium infantis* results in a greater protective effect in vagotomized animals compared with nonvagotomized colitis controls (41). We have recently demonstrated that this bacterium induces T regulatory cells in murine models (27) and therefore could plausibly be counteracting the negative impact of vagotomy on this lymphocyte subset.

Our data support the hypothesis that the vagus provides a direct or indirect tonic suppressive effect on lymphocyte activation. A better understanding of the molecular basis for this phenomenon will contribute to the pharmaceutical development of novel agonists of the vagal anti-inflammatory reflex (23, 29), providing alternatives for control of the inflammatory cascade in a range of inflammatory disorders not necessarily limited to the gastrointestinal tract.

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