**CD4⁺ T-Cell Modulation of Visceral Nociception in Mice**

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**Background & Aims:** Although inflammatory and immune cells are present in the gut in the absence of pathology, their presence does not result in sensitization of sensory nerves, implying the existence of a local antinociceptive influence. We hypothesized that a component of the immune system exerts an antinociceptive influence, thus enabling the gut to function in the absence of undue pain or discomfort. **Methods:** Visceromotor responses to colorectal distention were measured in mice with severe combined immune deficiency (SCID) and their wild-type controls. **Results:** SCID mice exhibited significantly lower pain thresholds. Transfer of CD4⁺ T, but not B lymphocytes, normalized visceral pain in these mice. The restoration of normal visceral nociception following T-cell reconstitution in SCID mice was blocked by naloxone methiodide. Using an enzyme immunoassay and immunohistochemistry for β-endorphin, we showed that in vitro stimulation of T lymphocytes induced the synthesis and release of β-endorphin and that transfer of T cells into SCID mice increased the expression of β-endorphin in the enteric nervous system. **Conclusions:** These findings indicate that the immune system is a critical determinant of visceral nociception and that T lymphocytes provide an important opioid-mediated antinociceptive influence in the gut.

The nervous system is involved in host defense and serves to provide early warning of imminent danger. The sensation of pain alerts the host to the threat of injury and results in the generation of avoidance or other protective responses. Although the induction of pain is a critical component of host defense, the persistence of pain is unhelpful in that it may interfere with wound healing and may become chronic and debilitating to the host.

The primary role of the immune system is to protect the host through the identification, containment, and elimination of noxious agents. Initially, the role of the immune system is to orchestrate an inflammatory response to contain and neutralize pathogens and other injurious agents. In the longer term, the immune response is critical for the successful repair of the tissue. Classically, the immune and nervous systems had been thought to work in isolation, but it is becoming increasingly evident that these systems work together in several biologically important contexts. For example, it has recently been shown that exposure to a bacterial toxin elicits a vagal reflex that down-regulates the inflammatory response. Conversely, the immune system signals the brain during intestinal inflammation to modify feeding behavior through the elaboration of cytokines in the peripheral and central nervous systems.

Products of the innate and adaptive immune responses contribute to the activation and sensitization of primary sensory neurons following tissue injury. The sensitization of sensory nerves in the periphery may result in changes in neural function in the spinal cord and brain to produce a persistence of pain. However, in most instances, pain does not become chronic, implying the existence of a system that is capable of efficiently down-regulating pain at the site of the original injury.

Studies in the field of somatic pain have identified an antinociceptive role for lymphocytes using a model of inflammation-induced analgesia in the rat hind paw. Inflammation induced by Freund’s complete adjuvant resulted in the release of opioids from lymphocytes and a subsequent increase in pain thresholds. It should be emphasized that this antinociceptive role for lymphocytes was identified in a model of local tissue injury and inflammation; a role for lymphocytes in maintaining normal somatic pain thresholds has not been identified.

The gastrointestinal tract is constantly exposed to a large number of bacteria and has evolved a sophisticated mucosal immune system that avoids or limits penetration of the commensal intestinal bacteria. As a result, there is a significant inflammatory and immune cell

**Abbreviations used in this paper:** CRD, colorectal distention; LMMP, longitudinal muscle myenteric plexus; NLXM, naloxone methiodide; SCID, severe combined immune deficiency; VMR, visceromotor response.

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presence in the gut in the absence of pathogenic bacteria or other overt inflammatory stimuli. Moreover, recent work has shown that subtle changes in resident inflammatory or immune cell activity in the gut alter visceral pain thresholds. Thus, although afferent nerves are readily receptive to the sensitizing effects of inflammatory or immune cell products, abdominal pain and discomfort only occur following overt injury in otherwise healthy subjects. This raises questions as to whether there is an antinociceptive influence in the gut that maintains visceral perception within an acceptable range in the face of a resident inflammatory and immune cell presence and whether the immune system itself is the source of this antinociceptive influence.

The present study investigated whether the immune system contributes to visceral nociception and specifically evaluated the role of lymphocytes. Our results provide the first demonstration that visceral nociception in the gut, in the absence of injury, is critically dependent on the integrity of the immune system and CD4+ T cells in particular. This is based on the demonstration of hyperalgesia in mice with severe combined immune deficiency (SCID) and demonstration that nociception is normalized following reconstitution of SCID mice with CD4+ T cells. Furthermore, our results identify opioids as the mediator of this immune-based peripheral antinociceptive mechanism.

Materials and Methods

Animal Housing and Handling

Male BALB/c and BALB/c SCID mice (6–8 weeks of age) were purchased from Harlan (Indianapolis, IN). Mice were kept under specific pathogen-free conditions at McMaster University Central Animal Care Facility (CAF). Upon arrival at our facility, mice were quarantined for 2 weeks before the start of experiments. Cages, bedding, and food were autoclaved as per standard procedure in McMaster University CAF. All experiments were approved by the McMaster University Animal Care Committee and the Canadian Council on Animal Care.

Lymphocyte Isolation and Mouse Reconstitution

Splenocytes from male BALB/c mice were isolated in Hank’s balanced salt solution (HBSS) + 10% fetal bovine serum (FBS) + 1% antibiotic/antimycotic (A/A). Purified cells were resuspended in phosphate-buffered saline (PBS), and each mouse received 1 × 10^6 cells/200 μL via intraperitoneal (IP) injection. Cells were 95% viable as determined by trypan blue exclusion.

B cells were isolated using B220-selective magnetic beads (Dynal Biotech, Lake Success, NY). Isolated cells were resuspended in PBS, and each mouse received 5 × 10^6 cells/200 μL IP. For CD4+ T-cell isolation, 1 × 10^6 splenocytes/mL were prepared in PBS + 2% FBS. EasySep negative selection mouse CD4+ T-cell enrichment cocktail with magnetic nanoparticles (Stem Cell Technologies, Vancouver, B.C., Canada) was used to isolate CD4+ T cells. The supernatant containing CD4+ T cells was collected, cells were resuspended in PBS, and each mouse received 5 × 10^6 cells/200 μL IP. Cell purity as determined by flow cytometry using anti-mouse CD4 (LT34) monoclonal antibody (BD Pharmingen, San Jose, CA) was approximately 90%.

Colorectal Distention

Electromyographic (EMG) electrodes were surgically implanted under sterile conditions in the anterior abdominal muscle wall of mice anesthetized with ketamine (Ketalean; Bimed-MTC, Cambridge, ON, Canada; 90 mg/kg) and xylazine (Rompun; Bayer, Toronto, ON, Canada; 20 mg/kg) IP, and a chronic fistula was exteriorized. Mice were then allowed to recover for a period of at least 7 days.

Mice were briefly anesthetized with enflurane (Enflurane USP, Abbott Laboratories, Saint-Laurent, QU, Canada) and a custom-made balloon catheter (20 × 10 mm) was gently inserted into the distal colon. A recording cable was connected to the chronic fistula, and mice were placed into Bollman restrainers. After connecting the catheters and cables to the barostat and EMG acquisition system, respectively, the mice were allowed a 5-minute rest. Colorectal distention (CRD) was then performed in a stepwise fashion. Each 10-second distention was followed by a 5-minute resting period. Each level of distention (30 and 60 mmHg) was repeated 3 times. EMG activity of the abdominal muscle was continuously recorded using customized software (Acquire 5.0; A. Bayatti). The area under the curve was calculated for 10 seconds before and after each distention period using customized software (GrafView 4.1; A. Bayatti). The median value for each distention level per mouse was then calculated; this value is described as the visceromotor response (VMR) and is plotted as area under the curve. Naloxone methiodide (Sigma-Aldrich, Oakville, ON, Canada) was administered (30 mg/kg IP) 30 minutes prior to CRD testing. This experiment was carried out 2 days after week 12 CRD testing.

Flow Cytometry

To confirm reconstitution, 1 × 10^6 isolated splenocytes were incubated with phycoerythrin-conjugated antibody to CD3 and fluorescein isothiocyanate-conjugated antibody to B220 (BD Pharmingen) was then analyzed by flow cytometry (FACScan, BD Pharmingen) using CellQuest software (BD Pharmingen).

Myeloperoxidase Assay

Acute inflammation was assessed by myeloperoxidase (MPO) activity in distal colonic tissue. The assay was performed on frozen samples as previously described.
\(\beta\)-Endorphin Quantification

Isolated splenocytes were cultured (1 \(\times\) 10^6 cells/mL) in RPMI + 10% FBS + 1% A/A and stimulated with concanavalin A (con A) (5 \(\mu\)g/mL) (Sigma-Aldrich) for 24 hours. Supernatant was collected and assayed for \(\beta\)-endorphin using an enzyme immunoassay (Bachem, San Carlos, CA).

Colon tissue was collected and homogenized in glacial acetic acid (30%) containing phenylmethanesulfonyl fluoride (0.5 mg/mL) + bovine serum albumin (0.5 mg/mL). The homogenate was frozen and thawed 3 times, and the supernatant was lyophilized. The resulting product was resuspended in assay buffer, and \(\beta\)-endorphin was quantified using the above enzyme immunoassay. Peptide concentrations were normalized to tissue weight.

\(\beta\)-Endorphin Immunohistochemistry

Paraffin sections were incubated with rabbit anti-mouse \(\beta\)-endorphin primary antibody (Research Diagnostics Inc., Flanders, NJ) (18 hours at 4°C) following deparaffinization, peroxidase blocking, and protein blocking. Envision, horseradish peroxide (HRP)-coupled, anti-rabbit secondary reagent (DakoCytomation, Carpinteria, CA) was incubated with the sections for 30 minutes at room temperature. 3,3'-Diaminobenzidine (Sigma-Aldrich) was used for color development, and Mayer's hematoxylin was used to counterstain sections. Controls used included omission of the primary antibody and antibody preabsorption with \(\beta\)-endorphin peptide (Sigma-Aldrich).

Peptide staining was measured by immunostaining-based semi-quantification. Five positions of each section were photographed by a digital camera (Olympus Q-Color), and the stained areas were measured by ImageJ software (NIH, Bethesda, MD). The results are expressed as percentage of total area.

Immunofluorescence

Paraffin sections were incubated with rabbit anti-mouse \(\beta\)-endorphin primary antibody after deparaffinization and protein blocking. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) was added for 30 minutes at room temperature. After washing, guinea pig anti-mouse protein gene product 9.5 (PGP 9.5) (Chemicon International, Temecula, CA) was incubated with the sections for 1 hour at room temperature followed by Alexa Fluor 594-conjugated goat anti-guinea pig IgG secondary antibody (Molecular Probes).

CD3 Immunohistochemistry

BALB/c SCID mice were reconstituted with mixed splenocytes and were killed on 2 days and 1, 2, 6, and 12 weeks postreconstitution to monitor migration of CD3\(^+\) T cells in the colon. Paraffin sections of distal colon were incubated with rabbit anti-human CD3 antibody (1:250) (DakoCytomation) for 1 hour, following deparaffinization, peroxidase blocking, antigen retrieval, and protein blocking. Envision, HRP-coupled, anti-rabbit secondary reagent (DakoCytomation) was incubated with the sections for 30 minutes at room temperature. 3,3'-Diaminobenzidine (Sigma-Aldrich) was used for color development, and Mayer's hematoxylin was used to counterstain sections.

Longitudinal Muscle Myenteric Plexus Preparation and Culture

Colonic tissue from BALB/c SCID mice was mounted onto a glass rod, and the mesentery was removed. The longitudinal muscle myenteric plexus (LMMP) layer was carefully scraped off with a sterile cotton swab and cut into 2 pieces. One piece of LMMP was cultured in control medium (RPMI + 10% FBS + 1% A/A), and 1 piece was incubated with fresh supernatant of BALB/c-stimulated splenocytes for 18 hours. Supernatant was obtained from isolation of 1 \(\times\) 10^7 cells/mL from BALB/c spleens that were incubated with RPMI + 10% FBS + 1% A/A for 24 hours prior to LMMP culture. LMMP preparations were fixed after an 18-hour culture and prepared for \(\beta\)-endorphin immunohistochemistry.

Statistical Analysis

All data are expressed as mean \pm standard deviation. Data were analyzed using 2 sample t tests (2-tailed) between unpaired groups and paired t tests between paired groups with \(P \leq .05\) considered as significant.

Results

Immunodeficiency and Visceral Hyperalgesia

To determine whether immunodeficiency was associated with visceral hyperalgesia, abdominal VMR during CRD was compared between BALB/c and BALB/c SCID mice. The response obtained at 0 mm Hg represented nociception with a deflated balloon inserted into the rectum. A distention of 30 mm Hg was considered as mildly increased.
painful, and 60 mm Hg was used as a painful stimulus. BALB/c SCID mice demonstrated significantly increased VMR to distention pressures of 30 mm Hg and 60 mm Hg compared with immunocompetent BALB/c mice (Figure 1). The response in SCID mice at 30 mm Hg suggests that these mice were hyperalgesic because they displayed an abnormally high response to a mildly painful distention.

**Reconstitution With Mixed Immune Cells Normalizes Hyperalgesia in SCID Mice**

To investigate whether the visceral hyperalgesia seen in SCID mice could be attenuated by replenishment of immune cells, we reconstituted SCID mice with mixed splenocytes isolated from age-matched wild-type mice and examined changes in VMR to CRD over time. At 8 weeks postreconstitution, SCID mice displayed a significantly reduced response to 0 mm Hg when compared with prereconstitution responses (Figure 2A). At 12 weeks, hyperalgesia was markedly attenuated because the VMR was significantly lower at all distention pressures when compared with prereconstitution responses (Figure 2A). Conversely, the responses in unreconstituted SCID mice did not vary significantly with time (Figure 2B). The normalization of hyperalgesia over time in reconstituted SCID mice indicated that the presence of lymphocytes was able to modulate visceral nociception to distention. The reduced response observed at 0 mm Hg, both at 8 and 12 weeks postreconstitution, suggests that immunodeficiency may also be associated with allodynia.

**Antinociceptive Effect of Lymphocytes Is Inhibited Using a Peripheral Opioid Receptor Antagonist in Reconstituted SCID Mice**

We next examined whether lymphocytes modulate pain through an opioid-dependent mechanism by using the opioid receptor antagonist naloxone methiodide (NLXM). As NLXM only acts in the periphery, any contribution from the central nervous system was excluded. NLXM administered 30 minutes before testing did not affect responses to CRD in unreconstituted SCID mice (Figure 3A), but it significantly increased the VMR at 0 mm Hg and 30 mm Hg of CRD in reconstituted SCID mice (Figure 3B). This finding demonstrates that the antinociceptive effect of reconstituting SCID mice is mediated via opioid receptors.
CD4+ T Cells Reduce Nociception in the Gut

To confirm the effectiveness of the reconstitution, flow cytometric analysis of isolated splenocytes was performed. Although the percentage of B cells (16.7% ± 3.8% vs 15.2% ± 2.5%, unreconstituted vs reconstituted, respectively) was similar between both groups of mice, a higher percentage of T cells (7.3% ± 3.1% vs 34.8% ± 10.7%, P = .01, unreconstituted vs reconstituted, respectively) was found in the reconstituted SCID group. The presence of some B and T cells in unreconstituted mice was not surprising because it is known that these mice can become "leaky" over time. As the proportion of B cells was similar in both groups, the increase in T-cell population in the reconstituted mice was most likely responsible for the changes in nociception observed in this group. To confirm this, we conducted CRD experiments in BALB/c SCID mice that were reconstituted with either CD4+ T cells or B cells only. CD4+ T-cell-reconstituted SCID mice displayed a significantly reduced VMR at 30 mm Hg 12 weeks postreconstitution compared with prereconstitution, whereas no significant changes in nociception were observed in B-cell-reconstituted SCID mice (Figure 4).

We continued CRD testing in CD4+ T-cell-reconstituted mice to determine the duration of lymphocyte-induced antinociception. At 18 weeks postreconstitution, the VMR was significantly increased compared with week 12 and was similar to prereconstitution responses (Figure 4). As determined by flow cytometric analysis at the 18-week time point, the number of splenic CD4+ T cells in the reconstituted SCID mice was significantly lower compared with wild-type mice and was not different from unreconstituted SCID mice (2.45% ± 3.07% vs 12% ± 4.17%, P = .04, reconstituted SCID vs wild-type, respectively; 0.31% ± 0.1%, unreconstituted SCID), suggesting that the loss of antinociception in the reconstituted mice was due to a reduction in transferred cells (data not shown).

Inflammation Is Not Increased in SCID Mice Postreconstitution

We measured acute inflammation in unreconstituted and reconstituted SCID mice at 12 weeks postreconstitution. Both a microscopic assessment and an MPO measurement revealed no significant colonic inflammation in either group at this time point (data not shown).

β-Endorphin Is Increased in Reconstituted SCID Mice

We sought to elucidate the mechanism for lymphocyte-induced antinociception in our model and examined the contribution of β-endorphin. β-Endorphin release was measured from splenocytes isolated from unreconstituted and reconstituted SCID mice. The release of β-endorphin was significantly higher from cells from the reconstituted SCID mice compared with that from unreconstituted mice (Figure 5A). β-Endorphin content was also significantly elevated in colonic tissue from the reconstituted SCID group compared with unreconstituted mice (Figure 5B).

**Figure 4.** CD4+ T-cell reconstitution of SCID mice reduces VMR during CRD 12 weeks postreconstitution (n = 8). VMR is increased by 18 weeks postreconstitution. Reconstitution of B cells alone had no effect on VMR (n = 10). *P = .04 compared with Wk 0; #P = .01 compared with Wk 12.

**Figure 5.** β-Endorphin quantification. (A) β-Endorphin measured in the supernatant from isolated stimulated splenocytes of reconstituted SCID mice was higher than from splenocytes of unreconstituted SCID mice (n = 5). (B) β-Endorphin content was increased in colonic tissue of reconstituted SCID mice (n = 9) compared with unreconstituted mice (n = 6). *P = .01; **P = .04.
Immunohistochemical localization of β-endorphin in reconstituted mice revealed that the peptide was mainly present in the region of the myenteric plexus (between circular and longitudinal muscle). Unreconstituted SCID mouse colon showed minimal positive staining in the same region. Reconstituted SCID mouse colon labeled with the PGP 9.5 marker showed expression within the myenteric plexus region. Overlay of C and D verified β-endorphin to be localized within the neurons of the myenteric plexus. (Original magnification for A and B, ×630; for C–E, ×400).

**Figure 6.** β-Endorphin labeling in colon tissue. (A) Strong positive staining for β-endorphin (arrow) was seen in the colon of reconstituted SCID mice in the region of the myenteric plexus (between circular and longitudinal muscle). (B) Unreconstituted SCID mouse colon showed minimal positive staining in the same region. (C) Reconstituted SCID mouse colon labeled with the PGP 9.5 marker showed expression within the myenteric plexus region. (D) β-Endorphin labeling of the same section indicated peptide expression in a similar location to PGP 9.5. (E) Overlay of C and D verified β-endorphin to be localized within the neurons of the myenteric plexus. m, mucosa; cm, circular muscle; lm, longitudinal muscle.

Lymphocyte Mediators Increase Neural β-Endorphin Expression

To investigate further the lymphocyte-mediated antinociception, we considered both direct and indirect mechanisms of action. The possibility of a direct interaction of lymphocytes and neurons seemed unlikely because CD3+ T cells were not found in the myenteric plexus region at any time point observed postreconstitution (data not shown). Thus, we isolated LMMP preparations from SCID mouse colon for culture with supernatant obtained from BALB/c-stimulated splenocytes. LMMP tissue incubated with splenocyte supernatant showed a significant increase in β-endorphin expression in the myenteric plexus compared with preparations incubated in medium only (Figure 7B), suggesting that lymphocytes are able to indirectly induce neural changes.

**Discussion**

Our results provide the first demonstration of an antinociceptive effect of lymphocytes in the gut and provide insight into mechanisms of visceral pain not explained by structural abnormalities. We have demonstrated that a direct consequence of immunodeficiency is visceral hyperalgesia. Reconstitution of SCID mice with
either mixed immune cells or CD4\textsuperscript{+} T cells was able to normalize hyperalgesia up to 3 months postreconstitution. The effect of immune cells was concentration dependent because transfer of 15 to 30 times fewer cells than the effective concentration of $15 \times 10^6$ cells per mouse conferred significantly less analgesia in SCID mice (data not shown). The fact that CD4\textsuperscript{+} T cells alone were able to reduce nociception highlights the importance of this cell population in the gut for normal pain sensation.

Our results differ somewhat from those obtained in the field of somatic pain in which the demonstrated ability of lymphocytes to produce \beta-endorphin was considered to be the basis for the observed antinociception.\textsuperscript{9,10} For instance, the somatic pain studies used an inflammation-based model of hyperalgesia, whereas we have studied immunodeficient mice in the absence of overt inflammation. SCID mice did not exhibit signs of acute inflammation postreconstitution of immune cells. In our model, we consider that a direct analgesic mechanism of lymphocytes plays only a minor role in pain reduction. We propose that lymphocyte-derived mediators induce changes in endogenous opioid expression in the enteric nervous system and that the latter is the principal contributor to the reduction in visceral nociception. This is supported by the demonstration of the striking up-regulation of \beta-endorphin in the myenteric plexus in SCID mice following reconstitution as well as its up-regulation in L MMP tissue upon culture with lymphocyte-derived supernatant.

The attenuation of pain in somatic studies was achieved only when inflammation was induced and a stressor was used to stimulate \beta-endorphin release at the site of inflammation.\textsuperscript{11} The subsequent analgesia was transient, lasting minutes. The long-lasting analgesic effect of lymphocyte reconstitution in the gut was achieved in the absence of both inflammation and stress. These results imply that a more complex and durable mechanism of lymphocyte-induced antinociception exists in the gut whereby immune cells engage in cross talk with the enteric nervous system to modulate pain. This concept further separates visceral pain mechanisms from those of somatic pain while still enforcing the significance of the immune system for normal pain sensation.

Although the nature of the signaling between immune cells and enteric nerves remains to be identified, the finding that a mere reduction of mucosal lymphocytes, in the absence of infection and inflammation, can result in increased visceral nociception illustrates the significance of these cells in the peripheral regulation of pain. This is further supported by the minimal effect of NLXM in unreconstituted SCID mice, suggesting that the opioid-mediated mechanism is almost absent in immunodeficient mice.

Although a role for the immune system in the development of acute and chronic pain is well-established, our understanding of the role of the immune system as a determinant of visceral nociception in the absence of pathology is in its infancy. A recent study has shown that small changes in resident inflammatory cells in the gut, following corticosteroid or antibiotic treatment, produces significant changes in visceral sensation in healthy mice.\textsuperscript{6} The current study extends our understanding of this field by demonstrating that CD4\textsuperscript{+} T lymphocytes are critical for the maintenance of visceral sensation within normal limits. Although we cannot fully disregard possible contributions of other T-cell subtypes to visceral sensation, we believe that our data strongly support a principal role for CD4\textsuperscript{+} T cells in visceral pain and corroborate evidence from somatic literature maintaining the antinociceptive properties of this particular cell type.\textsuperscript{5,10,12}

The demonstration of lymphocyte-mediated analgesia in the gut has clinical implications. The present study demonstrates that the absence of CD4\textsuperscript{+} T cells results in visceral hyperalgesia. If human lymphocytes also modu-
late visceral nociception, it is possible that a deficiency in CD4+ T cells might contribute to the abdominal pain seen so commonly in human immunodeficiency virus-positive patients.13,14 In addition, it is possible that chronic inflammation characterized by a predominance of CD4+ T cells could be associated with an increased threshold for pain. This speculation is supported by the demonstration that, although relapses of inflammatory bowel disease are associated with an acute inflammatory infiltrate and a reduction in visceral pain thresholds,15 studies in inflammatory bowel disease patients in the absence of relapse demonstrate an increase in visceral pain thresholds.16,17 This could be due, in part, to a relative predominance of lymphocytes conferring an opioid-mediated antinociceptive influence on sensory nerves.

References

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