Received: 23 August 2019 | Revised: 15 October 2019 | Accepted: 16 October 2019

DOI: 10.1111/nmo.13757

ORIGINAL ARTICLE

Neurogastroenterology & Motility NGM WILEY

CXCL1 is upregulated during the development of ileus resulting in decreased intestinal contractile activity

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Funding information
Karen Uray (all work except patient sample collection) was supported by institutional departmental funds from the Pediatric Surgery Department at the Univesity of Texas Health Science Center McGovern Medical School and the Hungarian National Research, Development, and Innovation Office (NKFI 120669 and Institutional Developments for Enhancing Intelligent Specialization Grant, EFOP-3.6.2-16-2017-0006); Charles Wade (collection of patient samples) was funded, in part, by the William Stamps Farish Fund and the Howell Family Foundation.

Abstract

Background: Although the development of ileus is widespread and negatively impacts patient outcomes, the mechanism by which ileus develops remains unclear. The purpose of our study was to examine the contribution of myogenic mechanisms to postoperative ileus development and the involvement of inflammation in mediating intestinal smooth muscle dysfunction.

Methods: Contractile activity and the effects of CXCL1 were studied in a gut manipulation model.

Key Results: Contraction amplitude in the ileum decreased significantly, while tone increased significantly in response to gut manipulation. Differences in contraction amplitude were affected by tetrodotoxin at earlier time points, but not at later time points. Agonist-induced contractions in the small intestine decreased significantly with ileus development. Intestinal transit slowed significantly after the induction of ileus. Myosin light chain phosphorylation was significantly decreased and edema increased significantly in the intestinal wall. Conditioned media from mechanically activated macrophages depressed intestinal contractile activity. CXCL1 (GroA) was significantly increased in the mechanically activated macrophages and intestinal smooth muscle within 1 hour after induction of ileus compared with control cells and sham animals, respectively. Treatment with CXCL1 significantly decreased contraction amplitude and agonist-induced contractile activity and increased tone in the small intestine. In the gut manipulation model, treatment with a CXCR2 antagonist prevented the decrease in agonist-induced contractile activity but not contraction amplitude.

Conclusions & Inferences: These data suggest that CXCL1, released from macrophages during intestinal wall stress, can suppress intestinal contractile activity. CXCL1 is a potential target for preventing or treating ileus in trauma patients.

KEYWORDS

CXCL1, gastrointestinal motility, inflammation, Intestine, mechanotransduction

1 | INTRODUCTION

Ileus, or depressed intestinal contractile activity, is manifested clinically as feeding intolerance, one of the most common postoperative complications. Grocott reported that 20% of orthopedic surgery patients, 92% of general surgery patients, and 51% of urology surgery patients experienced gastrointestinal postoperative complications.[1] Virtually, all abdominal surgery patients experience some degree of ileus. In general surgery patients, gastrointestinal complications were the leading reason for hospital readmission.[2] Ileus is also a common complication in trauma patients. Approximately 33% of moderate to severe trauma patients develop feeding intolerance, and 25% develop documented ileus.[3] Ileus significantly increases patient care costs by necessitating the treatment of resultant complications, feeding parenteral nutrition, and prolonging hospital stays.[4] More importantly, ileus causes significant patient discomfort and increases the risk of septic, pulmonary, and thromboembolic complications, and malnutrition.[5,6]

Despite the widespread and negative impact of ileus on patient outcomes, the mechanism by which ileus develops remains unclear. Ileus development is complex, with a number of contributing factors. Undoubtedly, opioid use contributes to ileus in surgical patients. However, in a trauma patient study, morphine equivalents were not significantly different between patients who developed feeding intolerance and those who did not,[3] indicating that other factors contribute to slowed gastrointestinal motility. Intestinal edema resulting from both excessive fluid use and/or inflammation also inhibits intestinal contractile activity,[7-10] and in recent years, inflammation has been shown to contribute to ileus development.[11-15] A combination of factors, including inflammation, edema development, and opioid use, is likely to contribute to the development of ileus; however, the mechanisms by which these factors induce ileus may be different. The effects of inflammation, in particular, are poorly understood. Inflammation affects the enteric nervous system (ENS) to suppress intestinal motility; however, studies suggest that intestinal smooth muscle is also affected by inflammation.[11,12] In abdominal surgery patients, intestinal manipulation is thought to activate resident macrophages in the intestinal muscle layers resulting in cytokine and chemokine release and recruitment of leukocytes. Inflammation results in contractile dysfunction, not only in the manipulated sections of the bowel, but in most of the small and large intestine. Systemic inflammation in trauma and general surgery patients may also contribute to the development of ileus.

While the effects of intestinal edema on the ENS are unclear, we have shown that intestinal edema alone, in the absence of inflammation, inhibits intestinal contractility by downregulating smooth muscle myosin light chain (MLC) phosphorylation.[10] Edema development results in increased intestinal wall stress.[16] Increased stretch of intestinal smooth muscle cells has been shown to down-regulate MLC phosphorylation.[17] Thus, edema-induced mechanotransduction in the intestinal smooth muscle layers is one mechanism by which edema causes smooth muscle dysfunction. Little is known about the interaction between mechanotransduction and inflammatory mediator signaling in smooth muscle. Furthermore, the effects of increasing intestinal wall stress during edema development on macrophage activation are unclear.

Key Points

- Spontaneous and agonist-induced intestinal contractile activities and intestinal transit are decreased after gut manipulation.
- Macrophages release CXCL1 in response to mechanical stress.
- CXCL1 also increased in intestinal smooth muscle after gut manipulation in a rodent model and in the circulation of trauma patients who develop ileus.
- CXCL1 decreased agonist-induced contractile activity.
- These data suggest that CXCL1, released from macrophages during intestinal wall stress, can suppress intestinal contractile activity.

While our investigations and others suggest that smooth muscle dysfunction contributes to the development of ileus, the drugs available to treat ileus target the ENS. We postulate that if intestinal smooth muscle is dysfunctional, drugs targeting the ENS will have limited effectiveness. Thus, the purpose of our study was to examine the contribution of myogenic mechanisms to the development of postoperative ileus, and the involvement of inflammation in mediating smooth muscle dysfunction.

2 | MATERIALS AND METHODS

2.1 | Animal model

A gut manipulation (GM) model of postoperative ileus, described by Bauer, was utilized.[18] Male Sprague Dawley rats weighing between 250 and 350 g were used for all experiments. All procedures were approved by the University of Texas Medical School Care and Use Committee and are consistent with the NIH "Guide for the Care and Use of Laboratory Animals". Intestines were exteriorized via an abdominal incision in anesthetized rats. The small intestine was gently compressed using a rolling motion between two sterile cotton applicators, in a proximal to distal direction. This procedure was repeated 3 times. After returning intestines to the abdominal cavity, the incision was closed. Sham animals underwent the same procedure with a laparotomy but no exteriorization or manipulation of the intestines. Naïve animals were not subjected to any procedures. Animals were sacrificed 0, 1, 2, 4, 12, and 24 hours after surgery, and the distal small intestine was collected for functional and biochemical analyses.

In a separate set of animals, rats were treated with the CXCR2 antagonist, SB265610 (3 mg/kg) or vehicle (3% DMSO) via intraperitoneal injection, immediately after gut manipulation or sham surgery. Animals were sacrificed 24 hours after surgery, and intestinal segments were collected.

2.2 | Intestinal contractile activity

Contractile activity was measured 0, 1, 2, 4, 12, and 24 hours after surgical manipulation in the distal small intestine, as described in previous publications.[7,10,17] After equilibration, 10 minutes of basal contractile activity data was recorded. A subset of intestinal strips was treated with tetrodotoxin (TTX, 0.3 μmol/L added to the bath), and another 10 minutes of contractile activity was recorded. Agonist-induced contractile activity was measured by adding increasing concentrations of carbachol to the organ bath in 5-minute intervals. Total contractile activity was calculated as the area under the curve. Basal tone was defined as the average minimum of the contraction cycle. Amplitude was calculated as average cycle height. All force development was normalized to tissue cross-sectional area. Measurements were performed on two separate intestinal strips and averaged.

For measurement of CXCL1 effects on contractile activity, intestinal sections were equilibrated for 30 minutes. After recording 5 minutes of baseline data, 200 ng/mL of CXCL1 (Biolegend, San Diego, CA) was added to the organ bath chamber and 10 minutes of data was recorded. After CXCL1 addition, a carbachol dose-response curve was generated as described above.

For determining the effects of conditioned media (see macrophage conditions below), animals were subjected to sham surgery only and 6 hours later tissue was collected for measurement of contractile activity. Equilibration and baseline measurements were as described above. Tissue was treated with media only, or media collected from macrophages after cyclical stretching. Media was added in a 1:10 V:V dilution. Measurements were normalized to baseline. An aliquot of conditioned media was pretreated for 5 minutes with 30 μmol/L oxyhemoglobin before adding to the organ bath chamber in a subset of experiments.

2.3 | Intestinal transit

At the time of gut manipulation, a silastic catheter for transit measurements was introduced into the proximal duodenum of mice. The catheter was then tunneled through the musculature of the left abdominal wall and subcutaneous tissue and externalized behind the neck. At the time of sacrifice, a solution of 70k-dalton nonabsorbable fluorescein isothiocyanate (FITC) Dextran (150 μL) was injected into the duodenum via the catheter and 45 minutes later, animals were sacrificed. The entire small intestine was carefully removed and divided into 10 equal segments. The end of each segment was clamped at the time of separation. Each segment was flushed with 1 mL of 10% mmol/L Tris-buffer solution (TBS) into separate test tubes.[8,9] Spectrophotometry (490 nmol/L) was used to determine FITC-Dextran concentrations in each sample and expressed as absorbance units (AU). The geometric center was calculated based on a modified method published by Miller et al[19] using the following equation: geometric center = ∑ (fraction of FITC per segment × segment number).

2.4 | Measurement of MLC phosphorylation by Western blotting

The mucosa was removed immediately after collection of intestines, and smooth muscle tissue lysates were generated and subjected to Western blotting as described previously.[17] Antibodies used were MLC Ser19 (Cell Signaling) and total MLC. ImageJ [20,21] was used to quantify luminescence intensities. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to control for protein loading amounts.

2.5 | Intestinal tissue water

Wet to dry weight ratios were measured in the mid-section of the small intestine. Intestinal samples were weighed immediately after collection. After drying in a 65°C oven, samples were weighed again. Wet to dry weight ratio was calculated as [(wet weight) − (dry weight)]/dry weight.

2.6 | Cytokine array

Cytokine and chemokine levels in the rat ileal smooth muscle and conditioned medium were measured using a rat or human cytokine array (R&D Systems) following the manufacturer's directions. In the cytokine array kit, capture antibodies for the following 29 cytokines and chemokines are immobilized on nitrocellulose membrane: Cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-2α/β, CINC-3, ciliary neurotrophic factor, fractalkine, granulocyte macrophage colony-stimulating factor, soluble intercellular adhesion molecule (sICAM)-1, interferon-γ, interleukin (IL)-1α, IL-1β, IL-1 receptor agonist, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IL-17, interferon gamma–induced protein-10, lipopolysaccharide-induced CXC chemokine, L-selectin, monokine induced by interferon gamma, macrophage inflammatory protein, MIP-3α, regulated upon activation normal T-cell expressed, thymus chemokine, tissue inhibitor of metalloproteinase-1, tumor necrosis factor (TNF)α, and vascular endothelial growth factor. Antibody binding was quantified using ImageJ.[20,21] CXCL1 levels were confirmed by ELISA.

2.7 | Human primary cell isolation and culture

Disease-free small intestinal tissue was collected from organ donor patients with the generous consent of a family member and approval by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston. Human intestinal smooth muscle cells (hISMC) were isolated and characterized as previously described.[17]

2.8 | Cell stretching

THP-1 or hISMC were seeded onto 6-well BioFlex® culture plates with flexible silicone elastomer bottoms (Flexcell) coated with 1 mg/mL

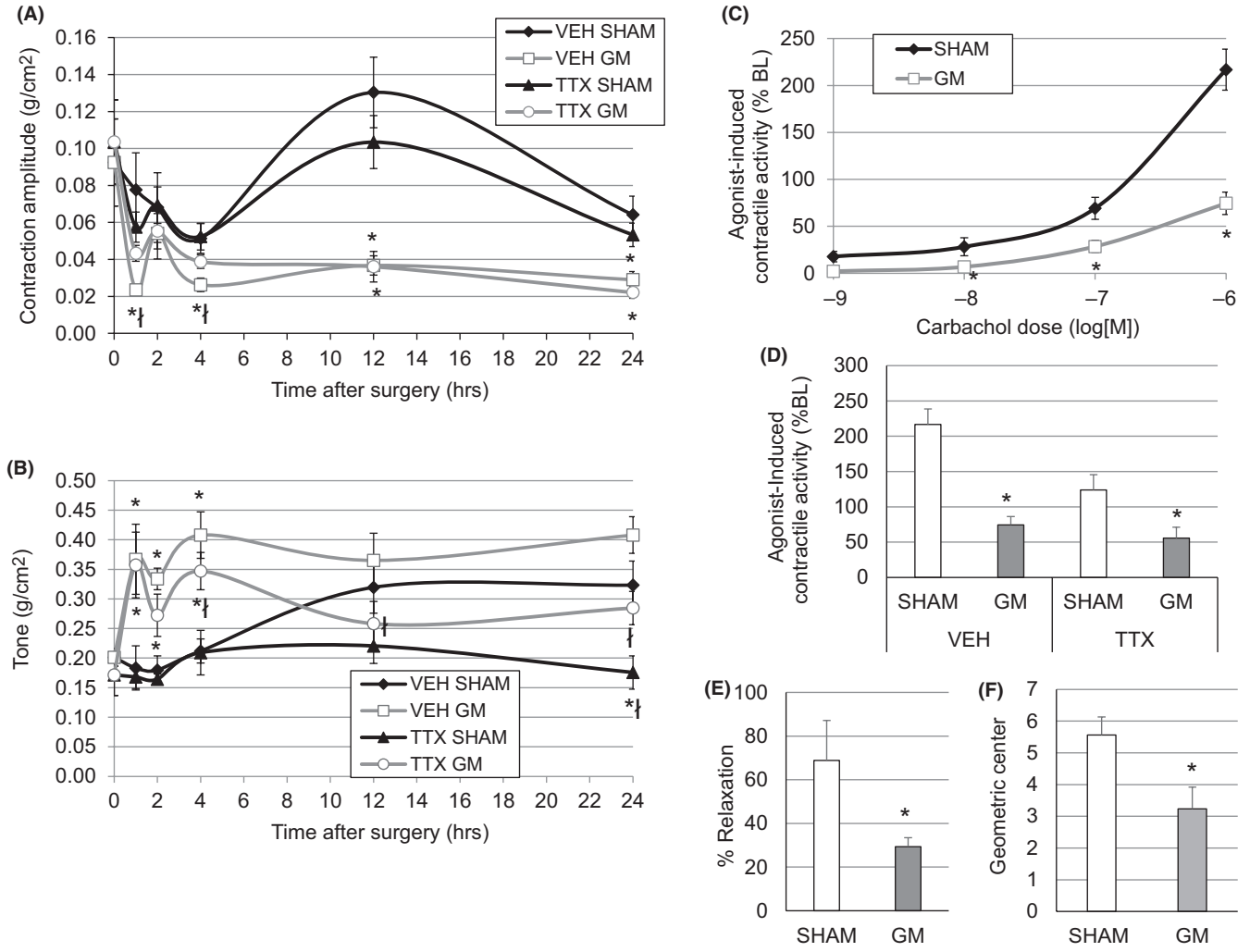

FIGURE 1 Contractile activity after gut manipulation. Contractile activity was measured in the ileum after gut manipulation to induce ileus or sham operation (laparotomy only). Intestinal sections were treated with tetrodotoxin (0.3 μmol/L) or vehicle. A, Contraction amplitude measured up to 24 h after surgery. B, Tone (average cycle minimum) measured up to 24 h after surgery. C, Carbachol dose-response curve to measure agonist-induced contractile activity 12 h after surgery. D, Agonist-induced contractile activity (10^{-6} M carbachol) measured 12 h after induction of ileus or sham surgery in the presence or absence of tetrodotoxin (0.3 μmol/L). E, Relaxation measured 4 h after induction of ileus or sham surgery. (Panels A-E: n = 6-10 per group; *, $P < .05$ vs Sham; ł, $P < .05$ vs TTX) F, Geometric Center, a measure of intestinal transit, measured 12 h after induction of ileus or sham surgery. (n = 4 per group; *, $P < .05$ vs Sham)

poly-D-lysine (Sigma). The FX-4000™ Tension System (Flexcell) was used to apply mechanical force to cells. The control cyclical stretch (CCS) and edema cyclical stretch (ECS) protocols were designed based on the spontaneous contraction amplitude and frequency measured in vivo in previous experiments.[16,17] For THP-1 cells, media was collected and used for subsequent cell and contractile activity experiments.

2.9 | Treatment of hISMC with conditioned THP-1 media

THP-1 cells are an immortalized human monocyte-like cell line. THP-1 cells (ATCC) were seeded onto Flexcell plates and 1 hour later activated with phorbol-12-myristate-13-acetate (PMA, 12.5 ng/mL). Twenty-four hours after PMA treatment, cells were subjected to either CCS or ECS protocols. Media, termed "conditioned media," was collected immediately after the 4 hours stretch protocol. Conditioned media was either used to treat intestinal strips, as described above, or added to hISMC, which were previously seeded onto Flexcell plates. Two hours after pretreatment with conditioned media, hISMC were subjected to either the CCS or ECS for 4 hours

2.10 | Patient blood sample collection

Blood samples were obtained from severely injured trauma patients 48 to 72 hours following their admission to the intensive care unit as part of an ongoing prospective study with prior approval obtained from the University of Texas Health Science Center Institutional Review Board (HSC-GEN-11-0213). We obtained consent from the patient or their legally authorized representative. Samples were centrifuged within 1 hour of collection, and plasma was stored at −80 degrees until assayed.

2.11 | Statistics

Analysis of Variance (ANOVA) and *t* tests were used to compare groups where appropriate. A *P* value <.05 was considered significant. When ANOVA was significant, Fishers LSD was used for post hoc analysis. Data are shown as the mean ± standard error of the mean.

3 | RESULTS

3.1 | Contractile dysfunction

Figure 1 shows the intestinal contractile activity measured 1 to 24 hours after gut manipulation. One hour after surgery, contraction amplitude decreased significantly in the GM group compared with the SHAM group (0.078 ± 0.020 vs 0.023 ± 0.002, Sham vs GM; $P = .038$); however, treatment with tetrodotoxin ameliorated the difference (0.057 ± 0.008 vs 0.043 ± 0.004, Sham vs GM; $P = .14$), as shown in Figure 1A. There were no differences in contraction amplitude 2 hours after surgery. Four hours after surgery, contraction amplitude decreased significantly in the GM group compared with the SHAM group (0.051 ± 0.008 vs 0.026 ± 0.004, Sham vs GM; $P = .013$) and treatment with tetrodotoxin ameliorated the difference, similar to 1 hour after surgery (0.052 ± 0.007 vs 0.039 ± 0.004, Sham vs GM; $P = .12$). At 1 and 4 hours after surgery, tetrodotoxin induced a significant increase in contraction amplitude, but had no significant effects at other time points. At 12 and 24 hours after surgery, contraction amplitude was significantly decreased in the GM group compared with the SHAM group (12 hours: 0.130 ± 0.019 vs 0.037 ± 0.005, $P = .0003$; 24 hours: 0.064 ± 0.010 vs 0.029 ± 0.004, $P = .006$; sham vs GM). In contrast to the earlier time points, tetrodotoxin did not ameliorate the differences in contraction amplitude at 12 and 24 hours after surgery. Contraction amplitude was significantly lower in the GM group compared with the Sham group after tetrodotoxin treatment (12 hours: 0.103 ± 0.014 vs 0.036 ± 0.008, $P = .001$; 24 hours: 0.053 ± 0.006 vs 0.022 ± 0.003, $P = .0004$; sham vs GM).

Figure 1B shows changes in intestinal tone after gut manipulation compared with sham surgery. From 1 to 4 hours after surgery, tone increased significantly in the GM group compared with the Sham group, even when tissue was pretreated with tetrodotoxin (Vehicle: $P = .026$, 0.00009, 0.001, at 1, 2, and 4 hours; tetrodotoxin: $P = .009$, 0.016, 0.014, at 1, 2, and 4 hours). At 12 hours after surgery, there were no differences in intestinal tone in the GM group compared with Sham either with or without tetrodotoxin ($P = .48$ and 0.45, respectively). At 24 hours after surgery, the tone was, again, significantly higher in the GM group compared with the Sham group, but only after tetrodotoxin treatment (0.32 ± 0.04 vs 0.41 ± 0.03, Sham vs GM, VEH, $P = .13$; 0.18 ± 0.03 vs 0.28 ± 0.03, Sham vs GM, TTX, $P = .016$). Tetrodotoxin significantly decreased tone in the GM group from 4-24 hours after surgery, and at the 24 hours time point in the Sham group.

The carbachol dose-response curve at 12 hours after surgery is shown in Figure 1C. Agonist-induced contractile activity was significantly reduced in the GM group compared with the SHAM group, from 10^{-8} M to 10^{-6} M carbachol concentrations ($P = .05$, 0.007, and 2×10^{-5} for 10^{-8}, $10^{-7,}$ and 10^{-6} M carbachol). The carbachol dose-response curve 24 hours after surgery looked similar to the 12-hour curve (data not shown). Carbachol-induced contractions were not different at the 1, 2 and 4 hours time points (data not shown). The effects of tetrodotoxin were measured at the highest carbachol dose (10^{-6} M) 12 hours after surgery, as shown in Figure 1D. Tetrodotoxin reduced agonist-induced contractile activity in the Sham group; however, agonist-induced contractile activity was significantly reduced in the GM group compared with the Sham group with or without tetrodotoxin ($P = 2 \times 10^{-5}$ and 0.02 for GM vs SHAM in VEH and TTX treated groups; $P = 9 \times 10^{-4}$, SHAM TTX vs VEH). Relaxation after agonist-induced contraction was measured 4 hours after induction of ileus (at the point of maximal differences in tone). Relaxation was significantly suppressed in the GM group compared with the Sham group, as shown in Figure 1E.

Intestinal transit, measured in a subset of animals 12 hours after surgery, is shown in Figure 1F. The geometric center was significantly decreased in the GM group compared with the Sham group (5.57 ± 0.57 vs 3.24 ± 0.68, $P = .039$).

3.2 | Changes in MLC phosphorylation and edema

MLC phosphorylation, the rate-limiting step for smooth muscle contraction, was measured in intestinal smooth muscle. Representative blots and the ratio of MLC phosphorylation are shown in Figure 2A,B. Phosphorylated serine 19 was significantly decreased in the GM group 24 hours after surgery compared with the Sham group (0.95 ± 0.08 vs 0.52 ± 0.07, Sham vs GM, $P = .0006$).

Wet to dry weight ratios, indicating the development of interstitial intestinal wall edema, increased significantly from 4 to 24 hours after surgery in the GM group compared with the Sham group (Figure 2C). In addition, wet to dry weight ratios increased significantly from 4 to 24 hours after surgery in the GM group compared with the 0 time point.

3.3 | Effects of conditioned media from macrophages on contractile activity

We determined the effects of mechanical stimulation of macrophages on contractile activity and cytokine production. We collected "conditioned media" from macrophages subjected to CCS or ECS, mimicking intestinal wall mechanical stimuli under normal or edematous conditions, respectively. As shown in Figure 3A, conditioned media had no significant effects on contractile activity of intestinal sections collected from naïve rats. In contrast, conditioned media collected from macrophages subjected to ECS, but not CCS, inhibited the intestinal contractile activity in Sham animals (subjected to laparotomy only) (Figure 3B). The effects of conditioned media collected from macrophages subjected to CCS showed no differences compared with media alone. Intestinal contractile activity was still inhibited by ECS conditioned media, after oxyhemoglobin treatment to oxidize nitric oxide, suggesting that nitric oxide was not causing the inhibition of contractile activity (Figure 3B).

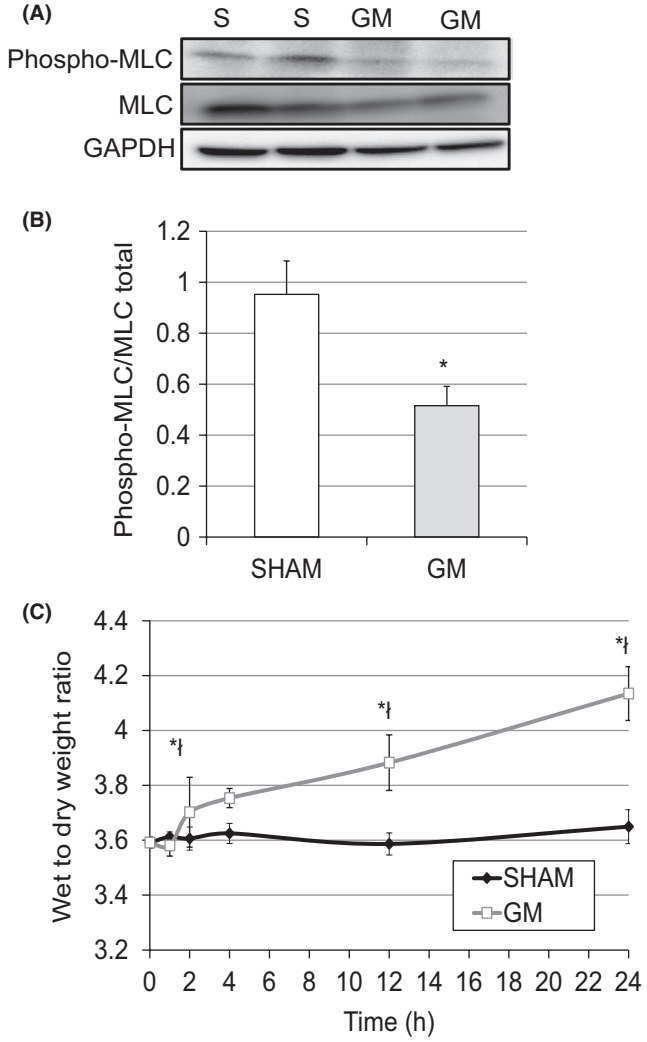

FIGURE 2 Myosin light chain (MLC) phosphorylation and the development of intestinal edema. A, A sample blot of phospho-MLC, MLC, and GAPDH in sham-operated animals (S) and after gut manipulation (GM) to induce ileus, measured in intestinal smooth muscle layers of the ileum 24 h after surgery. B, The ratio of phospho-MLC to MLC 24 h after sham operation (SHAM) or gut manipulation (GM) to induce ileus. (n = 11 per group; **, $P < .001$) C, Edema development, indicated by wet to dry weight ratios, was measured at 0, 1, 2, 4, 12, and 24 h after surgery to induce ileus (GM) or sham surgery (SHAM). (n = 31 for 0 h, 10 for 1 h groups, 10 for 2 h groups, 11 for 4 h groups, 6 for 12 h groups, and 25 and 16, respectively, for Sham and GM groups at 24 h; *, $P < .05$ vs sham at same time point; ł, $P < .0005$ vs 0 h time point)

Primary hISMC were treated with conditioned media from macrophages subjected to either CCS or ECS (Figure 3C). When hISMC were subjected to CCS, the conditioned media did not affect MLC phosphorylation. When primary hISMC were subjected to ECS, conditioned media from macrophages subjected to ECS significantly inhibited MLC phosphorylation compared with conditioned media from macrophages subjected to CCS.

Conditioned media from macrophages subjected to CCS or ECS were analyzed for cytokine content. In Figure 3D,3, cytokine levels in media are shown relative to quiescent cells. There are relatively small changes in the media of macrophages subjected to CCS. In contrast, several cytokines increase significantly in the media from macrophages subjected to ECS, including CXCL1, which was increased almost 3-fold.

3.4 | Cytokine production in vivo

To determine if in vivo changes in cytokines were similar to changes in macrophages subjected to mechanical stress in vitro, we collected intestinal tissue after gut manipulation or sham surgery and measured cytokine levels in the smooth muscle layers. Data are shown as a ratio of GM to SHAM. As shown in Figure 4A, CXCL1 was increased 8-fold within 1 hour after gut manipulation. The increased CXCL1 levels were sustained until at least 24 hours after gut manipulation. LIX (CXCL5) was also upregulated in smooth muscle tissue after gut manipulation in an early and sustained manner. At later time points, IL-1β and TNFα were upregulated at 4 and 12 hours, respectively.

The upregulation of CXCL1 was confirmed by ELISA. As shown in Figure 4B, CXCL1 was significantly increased within 1 hours after GM compared with the Sham group. We also determined circulating CXCL1 levels in severely injured trauma patients 48-72 hours after hospital admittance. Plasma CXCL1 levels were significantly upregulated in trauma patients who developed ileus compared to trauma patients who did not develop ileus ($P = .004$) (Figure 4C).

3.5 | Effects of CXCL1 on contractile activity

The effects of CXCL1 (200 ng/mL) on intestinal contractile activity were measured in an organ bath in animals subjected to sham surgery only (Figure 5A). CXCL1 induced a significant decrease in contraction amplitude and a significant increase in tone, compared with VEH treatment (0.1% bovine serum albumin in phosphate-buffered saline). Agonist-induced contractile activity was significantly decreased after treatment with CXCL1 (Figure 5B).

To further explore the effects of CXCL1 on contractile activity, we measured contractile activity after either sham surgery or gut manipulation in animals treated with a CXCR2 antagonist (SB265610, 3 mg/mL) or vehicle (3% DMSO). As shown in Figure 5C, treatment with the CXCR2 antagonist did not ameliorate the decreased contraction amplitude induced by gut manipulation. In contrast, inhibition of CXCR2 abrogated the decreased agonist-induced contractile activity induced by gut manipulation (Figure 5D).

4 | DISCUSSION

Our data suggest that gut manipulation-induced ileus is mediated, at least in part, by intestinal macrophage secretion of CXCL1. In support of these observations are the early and sustained increases in CXCL1 in intestinal smooth muscle, a loss of function experiment

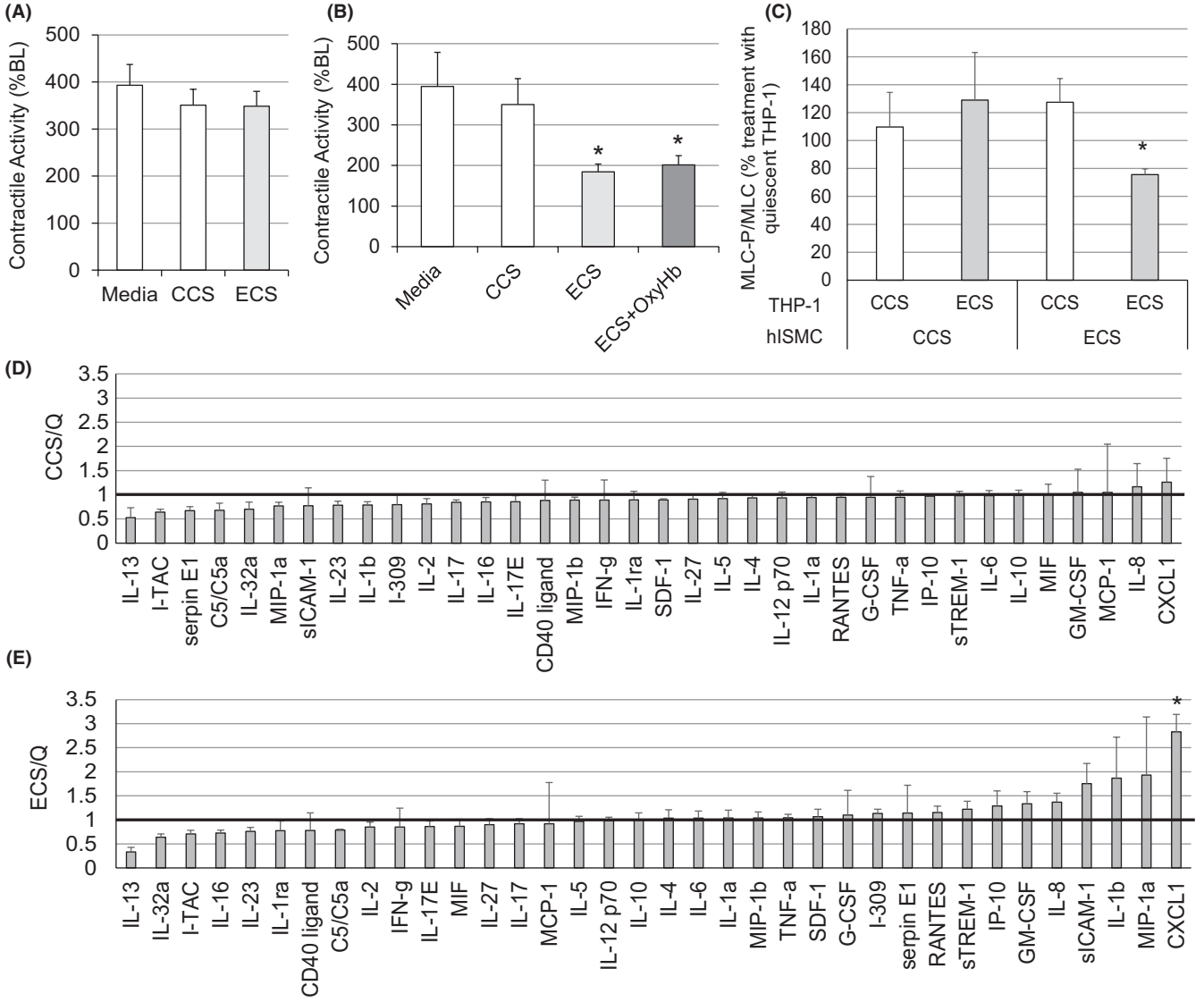

FIGURE 3 Effects of conditioned media from macrophages (activated THP-1 cells) on contractile activity and myosin light chain (MLC) phosphorylation. Intestinal sections were treated with "conditioned media" collected from macrophages subjected to control cyclical stretch (CCS) or edema cyclical stretch (ECS) conditions, mimicking intestinal wall mechanical stimuli under normal conditions or when the intestinal wall becomes edematous, respectively. A, Effects of conditioned media on contractile activity in intestinal sections collected from naïve (untreated, 0 h) animals. (n = 15-16 per group). B, Effects of conditioned media on contractile activity in intestinal sections collected 24 h after laparotomy only. In the ECS + Oxyhemoglobin group, the media was pretreated with oxyhemoglobin (30 μmol/L) for 5 min before use. (n = 12 per group for media, CCS, and ECS groups; n = 8 for the ECS + OxyHb group; *, $P < .05$). C, Effects of conditioned media on MLC phosphorylation in primary human intestinal smooth muscle cells. (n = 6 per group, *, $P < .05$) D-E, Cytokine profile in conditioned media after 4 h of CCS (D) or ECS (E). Data are shown as a ratio of stretched cells to control quiescent (Q) cells. The solid line indicated no change; below the line indicates decreases in response to the stretching protocol and above the line indicates increases. (n = 3 per group, *, $P < .05$ vs CCS) (Media = Media alone, CCS = media collected from THP-1 subjected to controlled cyclical stretch, ECS = media collected from THP-1 subjected to edema cyclical stretch program)

with CXCL1, and the attenuation of intestinal contractile dysfunction when CXCL effects were antagonized. Finally, trauma patients with ileus showed a similar increase in CXCL1 as our experimental model.

According to the Centers for Disease Control and Prevention, 2.8 million people were hospitalized for traumatic injuries in 2015.[22] Feeding intolerance affects more than 30% of moderate to severe trauma patients, and 25% of those patients are diagnosed with ileus.[3] Considering the large number of trauma patients reported by the CDC, feeding intolerance and ileus affect a very large number of people. The terms ileus and feeding intolerance are interrelated, with the main difference being the clinical diagnosis. In addition, ileus refers specifically to suppressed motility in the intestines, while feeding intolerance can be attributed to other

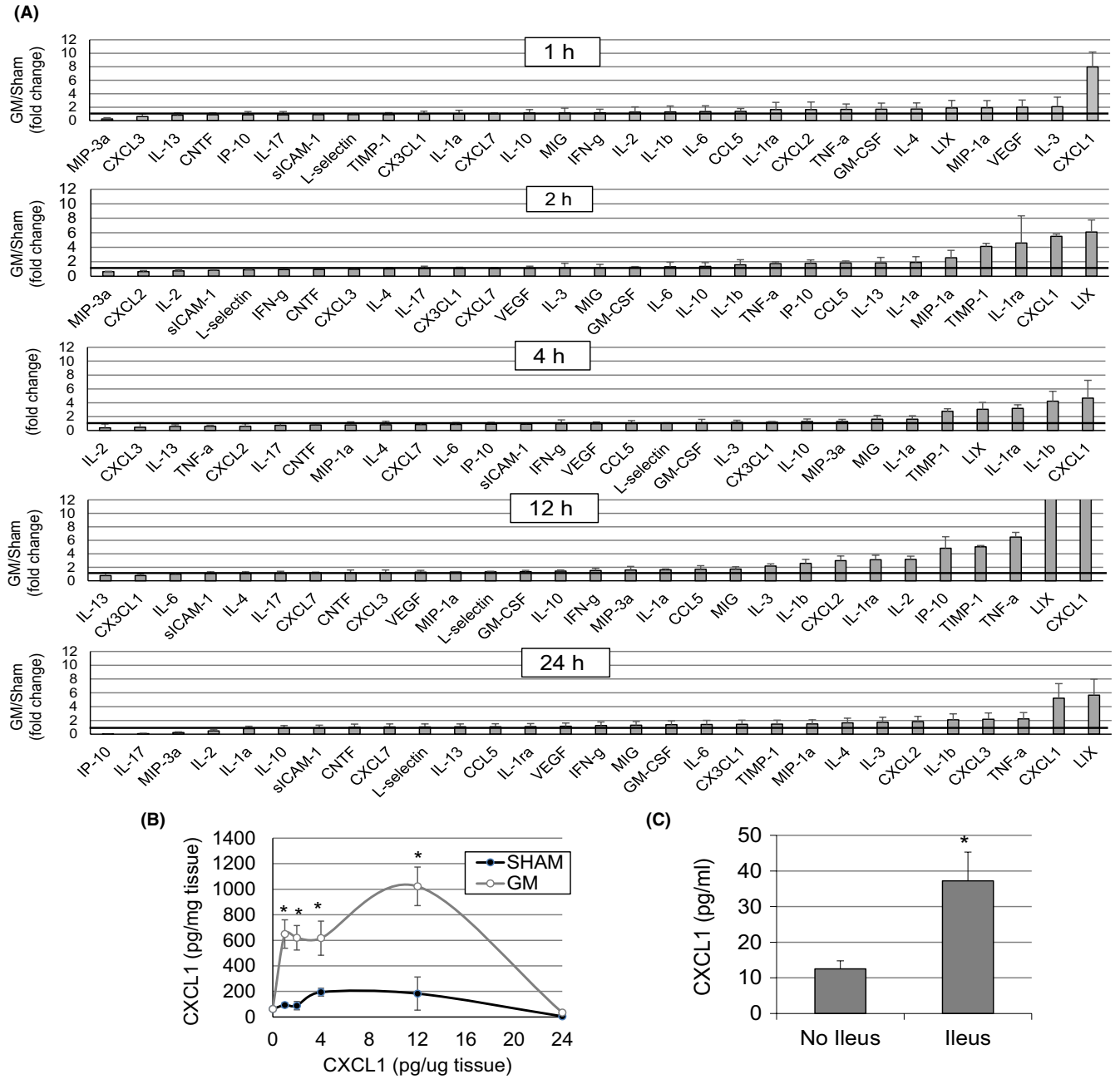

FIGURE 4 Cytokine profile in intestinal smooth muscle after induction of ileus and in human plasma after trauma. A, The cytokine profile is shown as a ratio of gut manipulation (GM) to sham surgery measured at the indicated time points. The solid line indicates no change; below the line indicates decreases in the GM group compared with the Sham group and above the line indicates increases. (n = 3 per group). B, CXCL-1 measured by ELISA in rat intestinal smooth muscle collected at 0, 1, 2, 4, 12, and 24 h after gut manipulation to induce ileus or sham surgery. (n = 3-4 per group; **, $P < .01$; *, $P < .05$). C, CXCL-1 levels in the plasma of human trauma patients measured between 48-72 h after hospital admittance. Trauma patients in the "no ileus" group had no symptoms of slowed gastrointestinal motility, whereas patients in the "ileus" group had documented ileus. (n = 15 and 13 for the no ileus and ileus groups, respectively; **, $P < .01$)

parts of the gastrointestinal tract. The salient point is that both ileus, and feeding intolerance are caused by depressed gastrointestinal motility. Unfortunately, there is a paucity of drugs to treat depressed gastrointestinal motility, and the available drugs have limited or no effectiveness in trauma patients.[23] Understanding the mechanism(s) by which gastrointestinal motility disorders develop in trauma patients is important in addressing the need for new and effective drugs to treat depressed gastrointestinal motility in trauma patients. Our results elucidate the mechanisms by which ileus develops in trauma patients.

Many of the drugs to improve postsurgical gastrointestinal motility target the central or ENS and some are principally designed to counter the effects of opioid-induced ileus. We show that both spontaneous contractile activity (contraction amplitude) and agonist-induced (carbachol-induced) contractile activity are decreased from 12-24 hours after surgery, even in the presence of tetrodotoxin

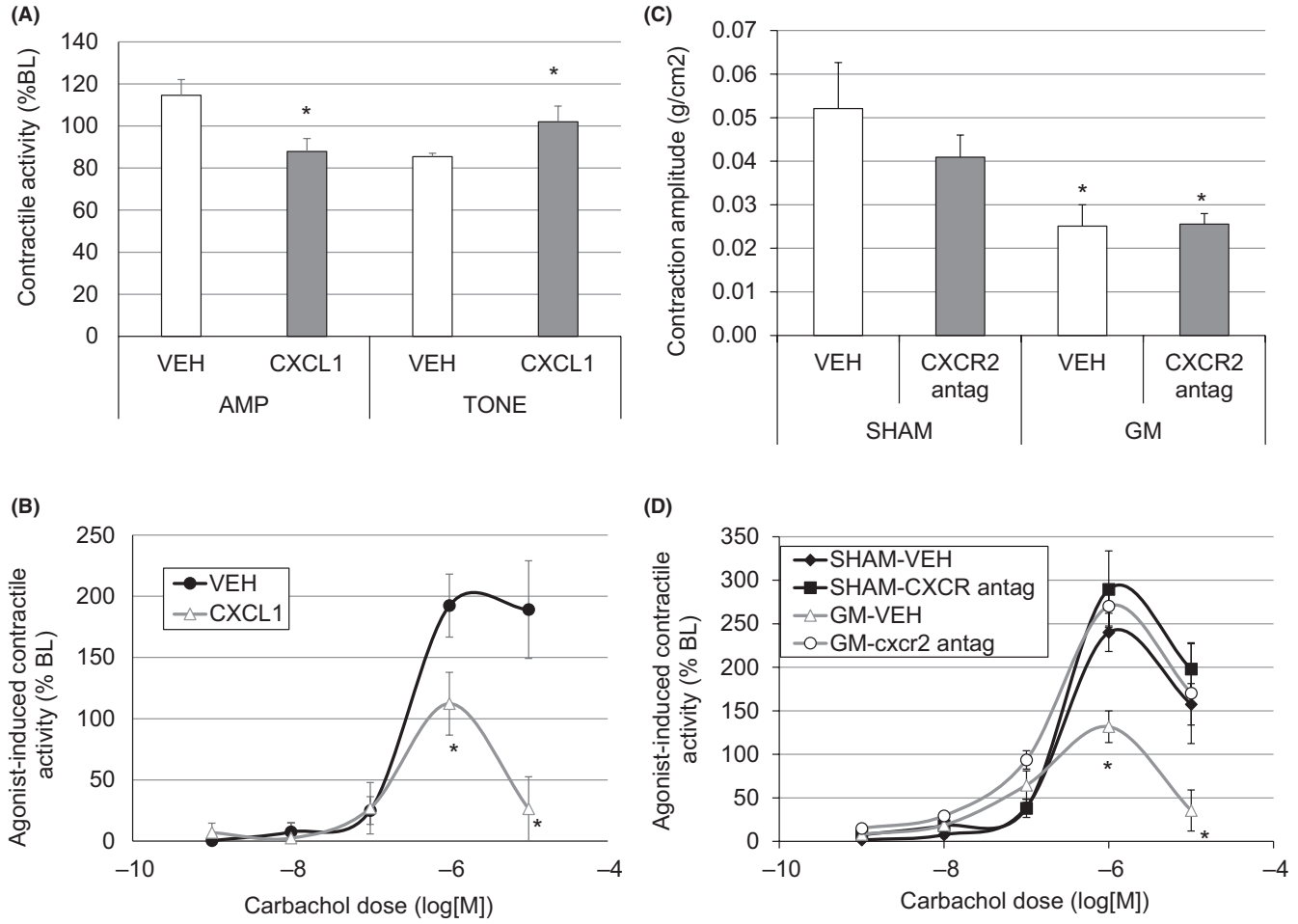

FIGURE 5 Intestinal contractile activity in response to CXCL1 and a CXCR2 antagonist. A, Changes in intestinal contractile activity, including both amplitude and tone, in response to CXCL1 treatment (200 ng/mL) or vehicle treatment (VEH, 0.1% BSA) of the intestinal segment (ex vivo). (n = 8 per group; *, $P < .05$ vs VEH treatment). B, Changes in agonist-induced intestinal contractile activity in response to CXCL1 treatment (200 ng/mL) or vehicle treatment (0.1% BSA) (n = 8 per group; *, $P < .05$ vs VEH treatment). C, Changes in intestinal contraction amplitude in response to CXCR2 antagonist treatment (SB265610, 3 mg/kg, IP) or vehicle (3% DMSO) immediately after sham surgery (SHAM) or induction of ileus by gut manipulation (GM). (n = 7 per group; *, $P < .05$ vs SHAM). D, Changes in intestinal agonist-induced contractile activity in response to CXCR2 antagonist treatment (SB265610, 3 mg/kg, IP) or vehicle (3% DMSO) immediately after sham surgery (SHAM) or induction of ileus by gut manipulation (GM). (n = 7 per group; *, $P < .05$ vs SHAM)

(Figure 1 A and D). Furthermore, MLC phosphorylation is decreased, as shown in Figure 2. These data support the hypothesis that smooth muscle dysfunction contributes to ileus, and, therefore, drugs targeting the ENS or CNS will be ineffective in improving gastrointestinal motility in trauma patients.

The smooth muscle dysfunction observed in our study agrees with Farro et al, in which impaired smooth muscle contractility after gut manipulation was also observed.[24] However, the time course of changes in smooth muscle function in our experiments was somewhat different from the Farro study. The suppressed smooth muscle dysfunction from 12-24 hours after gut manipulation in the Farro study was similar to our study. However, at earlier time points (1-4 hours) the differences in contraction amplitude measured in our study were tetrodotoxin sensitive, indicating that the differences in contractile activity were due to changes in the ENS; whereas, in the Farro study smooth muscle dysfunction was observed 1.5 hours after gut manipulation. In addition, we observed no significant changes in agonist-induced contractions until 12 hours after surgery, while Farro measured reduced agonist-induced contractions as early as 1.5 hours after surgery. The differences in the time course may reflect differences in the animal species. Our study was conducted in rats, whereas the Farro study was conducted in mice. Despite the differences, both studies are in agreement as to the smooth muscle dysfunction and the biphasic nature of the contractile dysfunction.

While changes in contraction amplitude are likely to result in changes in intestinal transit, as demonstrated in Figure 1E, changes in tone are more difficult to interpret. Tone was calculated as the average minimum of the contraction cycle. All intestinal segments were adjusted to an average 0.5 g load when mounted in the organ bath chamber, allowed to warm for 10 minutes, then readjusted to the 0.5 g load. The intestinal segments were then allowed to relax with no more adjustments. In our experience, the tone and contraction amplitude change very little after the 30 minute equilibration period,

in the absence of any treatments. We observed a higher tone in the gut manipulation group compared with the sham group. This significantly altered tone was preserved after tetrodotoxin treatment, indicating that the changes in tone were myogenic, not neurogenic. In fact, at 24 hours after surgery, tone was significantly reduced in both the SHAM and GM groups in the presence of tetrodotoxin (Figure 1B), indicating that the ENS had a positive effect on tone, and the differences in tone due to myogenic effects were unmasked with TTX treatment. Farro et al demonstrate impaired relaxation of intestinal smooth muscle after induction of ileus.[24] The increased tone in our study may be due to impaired relaxation. Figure 1E shows that relaxation after agonist-induced contractions is indeed suppressed in the ileus group compared with the Sham group, supporting the idea that the ability of smooth muscle to both contract and relax is impaired.

Ileus in trauma patients or postoperative patients is thought to be induced, in part, by inflammatory injury to the smooth muscle layers. Kalff demonstrated that leukocytes infiltrate into the rat intestinal muscularis during the development of ileus.[12,18] Farro showed increased mRNA levels for a number of different inflammatory mediators in intestinal smooth muscle, including TNFα, Il1α, IL1β, IL6, and CXCL1.[24] We also observed increased inflammation in intestinal smooth muscle after induction of ileus. In addition to increased cytokine production, intestinal edema developed within 4 hours after surgery (Figure 2C). Edema development in the absence of vascular changes or hemodilution is indicative of inflammation.

We have shown previously that edema itself, in the absence of gut manipulation, can decrease intestinal contractile activity.[7,10] Cox et al have shown that edema also results in increased mechanical stress in the intestinal wall.[16] This increased stress decreases MLC phosphorylation in primary intestinal smooth muscle cells.[17] We speculated that increased mechanical stress may also affect the resident macrophages in the intestinal wall. Thus, we subjected activated macrophages to either control cyclical stretch (CCS), as the macrophages would experience under physiological conditions, or edema cyclical stretch (ECS), which intestinal macrophages would experience after edema development. We collected the "conditioned" media after stretching the macrophages. Interestingly, conditioned media from macrophages subjected to CCS or ECS did not affect intestinal contractile activity of intestinal strips collected from naïve animals. In contrast, conditioned media collected from macrophages subjected to ECS significantly decreased contractile activity of tissue sections after surgical stress (Figure 3). Pretreatment of the conditioned media with oxyhemoglobin had no effect; thus, the inhibitory effect of the conditioned media on contractile activity was not due to nitric oxide release from the macrophages. Furthermore, treatment of primary hISMC with conditioned media had similar results; conditioned media from macrophages subjected to ECS inhibited MLC phosphorylation in ISMC subjected to ECS, but not CCS. We conclude from these data, that when macrophages are subjected to mechanical stress, such as they would experience during intestinal edema development or gut manipulation, the macrophage secretome inhibits intestinal contractile activity. Furthermore, the macrophage secretome did not affect naive tissue, but only affected tissue already subjected to stress.

To identify the substance secreted from macrophages that inhibited intestinal contractile activity, we used a cytokine array to determine changes in a panel of cytokines in the conditioned media. In macrophages subjected to CCS, there were no significant changes in cytokines compared with quiescent cells. In media collected after macrophages were subjected to ECS, CXCL1 (GroA) was upregulated (Figure 3D,3). CXCL1 was also the predominant cytokine upregulated in vivo in intestinal smooth muscle after gut manipulation (Figure 4A). CXCL1 was upregulated within 1 hours after gut manipulation, and the upregulation was sustained for up to 24 hours (Figure 4F). Of note, CXCL1 was also upregulated in trauma patients who developed ileus versus trauma patients who did not develop ileus (Figure 4G). CXCL1 was upregulated very early after gut manipulation and was likely increased before leukocyte infiltration. Thus, CXCL1 was likely secreted by resident macrophages in the intestinal smooth muscle layers. The early release of CXCL1 is in agreement with the study by Farro, in which CXCL1 was significantly increased within the first 1.5 hours after gut manipulation.[24] However, unlike the Farro study, we did not see early increases in TNFα, IL-1β, or IL-1α. In our study, IL-1β increased 4 hours after gut manipulation and TNFα increased 12 hours after gut manipulation. These differences could have arisen for a number of different reasons. Firstly, Farro was measuring mRNA levels and we measure actual cytokine levels. Secondly, we measured the ratio of cytokines in GM to Sham. Thus, IL-1β and TNFα could have increased in both groups due to surgical stress. In the Farro manuscript, the cytokines are shown in the gut manipulation group only; the cytokine levels in the laparotomy only group are not shown.

According to Figure 4, CXCL1 was the first cytokine to increase after gut manipulation, and CXCL1 was, for the most part, the most increased cytokine. Thus, we investigated the effects of CXCL1 on intestinal contractile activity (Figure 5). When intestinal tissues from sham animals (laparotomy only) were treated with CXCL1, contraction amplitude decreased slightly but significantly and tone increased significantly compared with vehicle treatment (Figure 5A). CXCL1 substantially decreased agonist-induced contractile activity (Figure 5B). These effects of CXCL1 on intestinal contractile activity are similar to the effects of gut manipulation on intestinal contractile activity. If animals were treated with a CXCR2 antagonist immediately after gut manipulation, spontaneous contractile activity was unaffected (Figure 5C). In contrast, CXCR2 antagonism prevented decreases in agonist-induced contractile activity after gut manipulation (Figure 5D). One drawback of the study is that the CXCR2 antagonist has off target effects which may affect intestinal contractile activity.

CXCL1 is a member of the C-X-C chemokine family. CXCL1 is upregulated in many inflammatory processes, often in response to nuclear factor Kβ (NF-kβ) or CCAAT-enhancer-binding proteinsβ (C/EBPβ). The mechanism by which CXCL1 is increased in intestinal smooth muscle is unclear. However, CXCL1 was increased in the conditioned media of cyclically stretched macrophages

subjected to increased stretch (Figure 3E). These data suggest that CXCL1 is secreted by macrophages (or other cells types in the intestinal smooth muscle) in response to mechanical stimuli. We have shown previously that NF-kβ which can upregulate CXCL1, is increased in edematous intestinal smooth muscle (ie tissue that is experiencing increased mechanical stretch) resulting in decreased intestinal motility.[25,26] Thus, NF-kB may upregulate CXCL1 in response to increased stretch.

The mechanism by which CXCL1 can affect smooth muscle contractility is unclear. CXCL1 acts as a full agonist at the CXCR2 receptor. CXCR2 activation triggers a number of downstream pathways, including Akt signaling and PAK1 signaling.[27,28] We have shown previously that increased PAK1 activation can inhibit intestinal contractile activity via decreased MYPT1 phosphorylation.[17] Thus, CXCL1 may signal through PAK1 to decrease intestinal smooth muscle contractility. Interestingly, in vascular smooth muscle, Akt signaling was shown to increase smooth muscle tone in a manner that was uncoupled to MLC phosphorylation.[29,30] This may explain the disparate results concerning decreased contraction amplitude versus increased tone (Figure 1).

In summary, both spontaneous and agonist-induced intestinal contractile activities were decreased after gut manipulation. The decreased contractile activity resulted in decreased intestinal transit. Resident macrophages are a likely source of cytokine release early in the development of contractile dysfunction. Thus, we subjected macrophages to mechanical stress and showed that CXCL1 was released. CXCL1 also increased in intestinal smooth muscle after gut manipulation in a rodent model and in the circulation of trauma patients who develop ileus. CXCL1 decreased agonist-induced contractile activity; the suppression of agonist-induced contractile activity could be blocked with a CXCR2 antagonist. Taken together, these data suggest that CXCL1, released from macrophages during intestinal wall stress, can suppress intestinal contractile activity. CXCL1 is a potential target for treating decreased contractile activity in trauma and surgical patients.

CONFLICTS OF INTEREST

No competing interests declared.

AUTHOR CONTRIBUTIONS

KU, TD, CW, and CC contributed to study design and concept; TD, DB, AS, and KU contributed to acquisition of data; KU, TD, and DB contributed to analysis and interpretation of data; KU and TD contributed to drafting of the manuscript; KU, TD, CW, and CC contributed to critical revision of the manuscript for important intellectual content; KU contributed to statistical analysis; KU and CW Obtained funding.

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How to cite this article: Docsa T, Bhattarai D, Sipos A, Wade CE, Cox Jr CS, Uray K. CXCL1 is upregulated during the development of ileus resulting in decreased intestinal contractile activity. *Neurogastroenterol Motil*. 2020;32:e13757. https://doi.org/10.1111/nmo.13757