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Interferon-gamma depresses human intestinal smooth muscle cell contractility: Relevance to inflammatory gut motility disturbances

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ABSTRACT

Aim/background: In addition to absorptive disturbances, inflammatory bowel diseases (IBD) perturb normal contractility of intestinal smooth muscle. Such motility disturbances may reflect both nervous alterations and increased abundance of cytokines (*e.g.* IL-1β, TNF-α, and IFN-γ), which impair normal intestinal smooth muscle structure and function. In a previous study, we reported that IL-1β decreased mesenteric muscular contractility, consistent with cytokine-mediated changes in contraction present in IBD. Here, we considered the impact of pro-inflammatory cytokines on human intestinal smooth muscle cell (HISMC) contractility *in vitro*, which might provide a method for evaluating treatments for IBD.

Materials and methods: We used an *in vitro* tonic contraction assay to study how HISMC contractility was affected by cell density, serum, and cytokines (IL-1β, TNF-α, and IFN-γ). MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) and wound healing analyses were also used to measure cell proliferation and migration in HISMC in response to IFN-γ.

Key findings: We found that IFN-γ (but not IL-1β or TNF-α) significantly depressed HISMC tonic contractility over six days. IFN-γ also decreased HISMC proliferation, migration, and smooth muscle F-actin expression in a dose-dependent manner (studied at 4 days).

Significance: Our studies indicate that IFN-γ dose and time-dependently reduces normal HISMC contractility, motility and proliferation which may contribute to dysmotility observed in GI inflammatory disorders and that IFN-γ therapeutics might restore normal HISMC contractility impaired in IBD.

1. Introduction

Gastrointestinal (GI) motility disorders produce clinical symptoms including diarrhea, constipation, abdominal pain, and nausea. GI motility disorders can both reduce or increase intestinal motility [1] and small intestine dysmotility can delay bolus transit, cause bacterial overgrowth and constipation leading to destructive changes in the microbiome and nutrient deficiencies [2]. Intestinal dysmotility in IBD may be caused by nervous and hormonal dysregulation as well as by an imbalance of cellular mediators (*e.g.* cytokines) present, recruited or induced in the gut during infection or inflammation. Common GI motility disorders also include chronic idiopathic intestinal pseudo-obstruction, irritable bowel syndrome (IBS), and Scleroderma [3]. Inflammation derived from inflammatory bowel diseases (IBD), and postoperative ileus also cause GI dysmotility [4,5].

Inflammation itself can alter the contractile capacity of human intestinal smooth muscle cells (HISMC) triggering both hypo- and hyper-contractility. Reduced contractility of smooth muscle has been reported in IBD patients with either ulcerative colitis (UC) or Crohn's disease (CD) [6,7]. Smooth muscle hypo-contractility is found in animal models of gut inflammation induced by postoperative ileus, in response to dextran sulfate sodium (DSS) or trinitrobenzene sulphonic acid (TNBS) induced models of colitis, following intestinal manipulation and mesenteric ischemia-reperfusion injury [8–10]. In rats, postoperative ileus decreases jejunal and colonic circular smooth contractions which are associated with decreased GI transit linked to increased tumor necrosis

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factor-α (TNF-α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [11,12]. TNBS-induced gut inflammatory responses are often mediated by Th1 cytokines including interleukin-1β (IL-1β), TNF-α, and IL-12 [13–16].

Conversely, nematode-induced inflammation is associated with Th2 cytokine bias and often produces a hypercontractile state in gut smooth muscle which supports worm expulsion. These effects are mediated by IL-4 and IL-13 and STAT-6 activation that drives PGE_2 formation [17–19]. These differing responses on intestinal motility dysfunction reflect the region involved, the specific inflammatory stimulus, the stage of inflammation [20], smooth muscle hypertrophy or hyperplasia [21] and the Th1/2 balance [22].

Because links between IBD and GI motility are still unclear but could be important to improved outcomes, the aims of our study were 1) to develop an *in vitro* model of GI motility using HISMC, 2) to study the influence of Th1 pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) on HISMC tonic contractility and 3) to characterize the effects of cytokines on HISMC proliferation, migration, and α -smooth muscle actin expression. IBD (*i.e.* CD and UC) are chronic diseases affecting 1.6 million people in the United States. According to the Crohn's and Colitis Foundation, the annual burden for IBD patients in the United States exceeds 2.2 billion dollars. The current study is aimed at understanding mechanisms underlying IBD induced GI dysmotility, which may lead to the development of new therapeutic targets.

2. Methods

HISMC were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in smooth muscle cell medium (SMCM, ScienCell) supplemented with 5% FBS (Gemini Bio-Products, West Sacramento, CA) smooth muscle cell growth supplement (ScienCell), and 1% penicillin/streptomycin (ScienCell). These HISMC are primary cells retrieved from the small intestinal wall (duodenum, jejunum, and ileum). HISMC were cultured in the carry flasks that were previously coated with poly-L-lysine (0.015 M, Sigma Aldrich, and St. Louis, MO). Other materials include: Recombinant human IL-1β, IFN-γ, and TNF-α; which were all purchased from Thermo/Fisher (Waltham, MA, USA). These reagents were added to supplemented smooth muscle cell medium (SMCM) during the polymerization period of the HISMC/collagen gels. A Nikon D90 digital camera was used to capture images of the HISMC/collagen gels for at least 6 days. MTT was purchased from Sigma-Aldrich. Dexamethasone and Nω-Nitro-L-Arginine-Methyl Ester (L-NAME) was purchased from Sigma Aldrich. NS-398 (COX-2 inhibitor), AG490 (JAK2/JAK3 inhibitor) and Ruxolitinib (JAK1/JAK2 inhibitor) were purchased Cayman Chemical.

2.1. Collagen gel contraction assays

Type 1 collagen matrices were prepared as previously described [23,24]. Briefly, tendons were manually removed from rat tails, rinsed in 100% isopropanol (Thermo/Fisher) and dissolved in sterile 4 mM acetic acid for 24 h at 4 °C with stirring. Collagen was then filtered through a 200 μm nylon filter (Corning Inc., Corning, NY, USA), aliquoted in 20 ml portions in 50 ml conical tube, snap-frozen and freeze-dried (Millrock Technology, Kingston, NY, USA) for later experimental use. Frozen aliquots not in use were stored at −20 °C. HISMC/collagen gels were prepared with modifications described before [23]. Prior to the day of the experiment, freeze-dried collagen was placed in cold 0.012 M hydrochloric acid (HCl) at 1.25 mg/ml final concentration and allowed to dissolve overnight at 4 °C with mild agitation. On the day of the experiment, 0.8 ml of cold 5× DMEM (containing 87.5 mM HEPES, pH 7.2) was added to 5 μl of 1 M NaOH and 3.2 ml of the dissolved collagen solution. Cultured HISMC were washed once with PBS-EDTA and then harvested with trypsin-EDTA (Sigma-Aldrich). The HISMC were then centrifuged at 1500 rpm for 5 min and suspended in SMCM (supplemented with 5% FBS, Pen/Strep, and SMCGS) or DMEM (supplemented with PSA and glutamine). HISMC were then counted, and in all experiments (except for the experiments with different cell density concentrations), 1.2×10^6 cells were added to 8 ml of SMCM to achieve 50,000 cells per gel. The final HISMC/collagen gel mixture (8 ml of cell suspension in 4 ml gel solution) was seeded into 0.5 ml aliquots into 24-well plates (Falcon) and incubated at 37 °C for 1 h to polymerize. HISMC were counted using a Coulter Z1 counter and suspended in SMCM medium cell densities up to 50,000 cells/gel. Gels were aliquoted in 0.5 ml aliquots in 24-well plates and polymerized at 37 °C for 1 h. After polymerizing, 1 ml of SMCM was added to each well achieving a final volume of 1.5 ml, and HISMC/collagen gels were detached from the wells using a sterile spatula. Gel contraction was monitored over 6 days. The gels were incubated at 37 °C and 7.5% CO_2 for 6 days. HISMC showed progressive gel contraction in all three dimensions. The area of each HISMC/collagen gel was measured over 6 days using images captured on a digital camera (Nikon D90, Tokyo, Japan) and analyzed using NIH ImageJ. Images were imported to ImageJ and the surface area of each gel was measured each day for the duration of the experiment. Surface areas of the gels of each day were then compared statistically.

2.2. Cytokine treatments

We studied effects of cytokines on HISMC contractility using cells mixed into collagen gels at 50,000 cells per gel. These gels were polymerized for 1 h at 37 °C and cytokines then added to SMCM as follows: IL-1β (20 ng/ml), TNF-α (20 ng/ml), IFN-γ (500 Units/ml), IL-1β (20 ng/ml) + TNF-α (20 ng/ml), IL-1β (20 ng/ml) + IFN-γ (500 Units/ml), TNF-α (20 ng/ml) + IFN-γ (500 Units/ml). After gels polymerized, 1 ml of cytokine treatments were added to the wells in each column for a final volume of 1.5 mls per well. Gels were then detached using a sterile pipet and photographed. In some experiments, 10^{-6} M Dexamethasone, 10^{-4} M Nω-Nitro-L-Arginine- Methyl Ester (Sigma), 10 μM of NS-398 (Sigma, COX-2 inhibitor), 50 μM AG490 (JAK2/JAK3 inhibitor, Sigma), and 10 μM Ruxolitinib (JAK1/JAK2 inhibitor, Sigma) were also used to attempt to reverse IFN-γ effects on HISMC contractility (see Table 1). (data not shown).

Table 1
Additional experiments conducted to restore HISMC contractility, and to further elucidate IFN-γ inhibition mechanism. Concentrations used were based on values near or at IC50 as previously reported by Al-Kofahi et al. [23] and Woolard et al. [54].

Additional reagents tested to restore IFN-γ induced inhibition of HISMC tonic contraction (data not shown)			
Reagents	Proposed mechanism of action	Experimental concentrations	Experimental effect
Dexamethasone	Anti-inflammatory	1 μM	No effect
NS-398	COX-2 inhibitor	10 μM	No effect
L-NAME	Nitric oxide inhibitor	100 μM	No effect
AG-490	JAK2/JAK3 inhibitor	50 μM	No effect
Ruxolitinib	JAK1/JAK2 inhibitor	10 μM	No effect

2.3. Gel contraction image analysis

Contraction of HISMC/collagen gels was determined as the fractional change in the gel surface area at each day compared to time = 0 gels. In most studies, day 4 was used for overall comparisons. Contractility was expressed as the fraction of gel area measured on day 0, normalized to internal controls [30]. All measurements were made using the NIH ImageJ analysis program [32]. At day 0, gel surface areas were equal to the well surface area, as contraction has not yet begun. Over 6 days, HISMC contraction reduces the respective gel area, which was found to show the greatest differences at day 4. On day 4, gel surface areas were measured, and area changes were determined as the gel area divided by the initial gel surface area on day 0. This value was subtracted from 1 to express it as a fractional change in area and then normalized to the contraction in control gels within each experiment, which was set as maximal (100%) contraction.

2.4. Cell proliferation assays

To study cell proliferation in response to cytokine treatments, HISMC were seeded in 12 well plates and incubated with supplemented SMCM in two different experiments at 20% confluency and allowed to attach to the wells overnight. The next day, the medium was replaced with 1 ml of medium containing no IFN-γ (control), 5 units/ml IFN-γ, 50 units/ml IFN-γ, 100 units/ml IFN-γ, 250 units/ml IFN-γ, 500 units/ml IFN-γ and 1000 units/ml IFN-γ. Cells treated for four days were used to run an MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. MTT is a yellow water-soluble solid that is reduced by live cells to a purple formazan product that is insoluble in aqueous solutions. On day 4, HISMC treated with IFN-γ in 12 well plates were washed with warm RPMI-1640 medium (Sigma R7509). 1 ml of an MTT solution (5 mg/ml in RPMI-1640) was added to each well in the 12 well plates and was allowed to incubate at 37 °C for 3 h. At the end of the incubation period, the MTT solution was removed. 1 ml of acidic isopropanol (0.04 M HCl in absolute isopropanol) was added to each well to solubilize the formazan product. After pipetting up and down each well, they were transferred to a 1.5 ml Eppendorf tube and centrifuged at 13, 000 rpm for 2 min. 200 μl of the supernatant was transferred in triplicate to a 96-well plate for each control and IFN-γ treatment. The absorbance of converted dye was measured at a wavelength of 570 nm with a background subtraction of 650 nm. Absorbance measured using the Bio-Rad Benchmark Plus spectrophotometer. Decreased absorbance correlates with the inhibition of growth of cells, and increased absorbance correlates with promoting the growth of the cells.

2.5. HISMC migration/wound healing assay

To study wound healing/migration in response to cytokine exposure, HISMC were plated on 96-well plates at 100% confluency. HISMC were allowed to attach to the plate overnight and the following day the middle of the surface was scratched displacing HISMC. The scratched surface area was the same in each well. Cytokine treatments were made in supplemented SMCM in the following concentrations: IFN-γ (500 units/ml and 5 units/ml) and control (supplemented SMCM). HISMC were treated for 4 days. On day 4, the medium was removed, and the cells were stained with 0.1% crystal violet in methanol for 15 min. Fresh methanol replaced the crystal violet and the absorbance of the cells in the wells was read at 620 nm using Bio-Rad Benchmark Plus spectrophotometer.

2.6. Western blotting for α-smooth muscle actin

HISMC were plated on 12-well plates at 100% confluency. HISMC were allowed to attach to the plate overnight. Cytokine treatments were made in supplemented SMCM in the following concentrations: IFN-γ (500 U/ml and 5 U/ml) and control (supplemented SMCM). The HISMC were treated for 4 days. On day 4, HISMC were lysed with 1 × Laemlli (Bio-Rad) with β-mercaptoethanol. 40 μl of cell lysate samples were loaded into each well and separated on 8% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (activated in methanol) using a transblot apparatus (Bio-Rad). After protein transfer, Ponceau S (Sigma-Aldrich) verified the transfer and photographed for normalization. PVDF membranes were blocked with 5% non-fat milk in TBST and were incubated with mouse anti- α-smooth muscle Actin (Sigma Aldrich, 1:500 in TBST) overnight at 4 °C. Anti-mouse antibody (Sigma-Aldrich, 1:1500) was added as the secondary antibody for 1 h in the cold room and 1 h at 25 °C. Blots were visualized using enhanced chemiluminescence with the Chemi-Doc MP Imaging System (Bio-Rad). Analysis of the resulting bands from the membranes and Ponceau S stain were performed using the NIH ImageJ analysis program.

2.7. Data analysis

Data are presented as mean ± SEM. When correlating two parameters (*i.e.* time and contractility) of experimental groups, repeated measures-two-way ANOVA with Bonferroni's testing was used. When comparing one parameter (*i.e.* contractility or absorbance) among three or more groups, repeated measures- one-way ANOVA with Bonferroni testing was used. The analysis was conducted in Prism 6 and Prism 7. Comparisons were considered significant at $P < 0.05$.

3. Results

3.1. HISMC cell density affects the rate and magnitude of gel contraction

Baseline values of the effect of cell density present within the gel on HISMC contractility are shown in Fig. 1A–E. The results were then normalized to control at Day 4 in Fig. 1F. Cell number concentration included: 0 cells (control), 1000 cells, 5000 cells, 10,000 cells, 25,000 cells, and 50,000 cells. These collagen gels with cells were stimulated with normal growth SMCM containing 5% FBS, 1% SMCGS, and 1% Pen/Strep. HISMC contractility increased as the cell number within the collagen gel increased in a time-dependent manner and at day 4 (normalized). 50,000 cells in the gel had the greatest amount of contraction. However, the contraction of 1, 000 cells at day 6, and the contraction 5000 cells at days 4, 5, and 6 were significant. Contraction of 10,000 cells was significant at days 2–6. Contraction of 25,000 cells and 50,000 cells were significant every day (1–6) of the experiment. For subsequent HISMC contraction assay experiments, collagen gels would contain 50,000 cells.

We selected day 4 as the normalization factor because previous research in our lab showed that tonic contractility was significant on day 4 [23]. 4 days of treatment was used for normalization and significance in subsequent experiments (proliferation, migration, and α-SMA expression).

3.2. HISMC cell density affects the rate and magnitude of gel contraction

FBS was added directly to the medium (DMEM with 1% glutamine and 1% Pen/Strep) surrounding the gels and not into the gels. Fig. 2A–E shows line graphs of the effects of different FBS concentrations on HISMC contractility over a 6-day time period. FBS concentrations included: 0 (control), 1%, 2.5%, 5%, 10%, and 20%. HISMC contractility increased as the percent of FBS concentration increased in a time-dependent manner and at day 4 (normalized) in Fig. 2F. 20% FBS caused the HISMC to contract the most. However, 2.5% and 5% FBS significantly increased HISMC contractility from day 2 through day 6. And 10% and 20% significantly increased HISMC contractility from day 1 through day 6. For subsequent HISMC contraction assay experiments, the medium mixed within and outside of the collagen contained 5% FBS along with 50,000 cells.

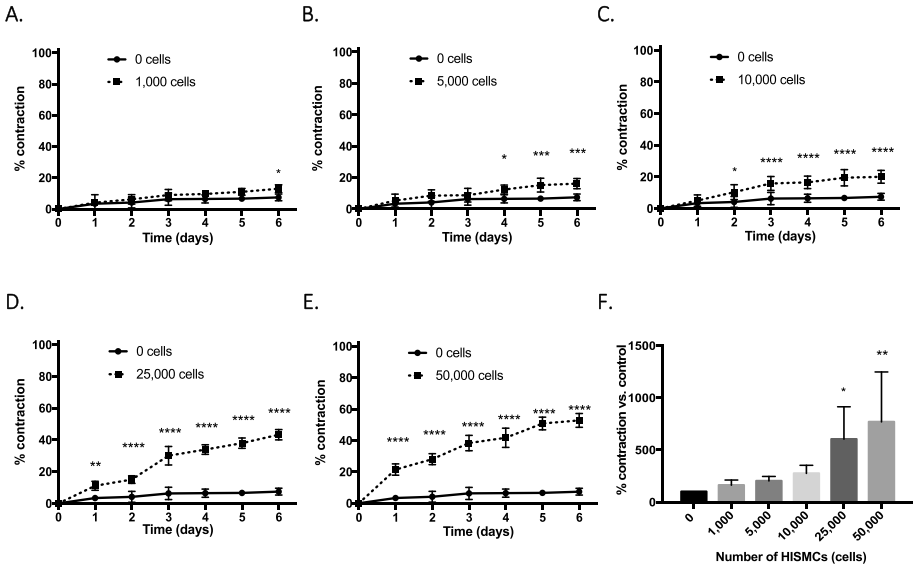

Fig. 1. The effects of cell density on HISMC contraction are shown over the course of six days using cell density of: A) 1000 cells, B) 5000 cells, C) 10,000 cells, D) 25,000 cells, E) 50,000 cells. Each cell number was compared the control (no cells). F) Effects of different cell densities on HISMC contractility, normalized to control (100% contractility) at Day 4. Statistical analysis was performed using two-way ANOVA for A–E and one-way ANOVA for F, n = 3. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

3.3. Cytokines affect the rate and magnitude of HISMC cell gel contraction

IL-1β (20 ng/ml), TNF-α (20 ng/ml), and IFN-γ (500 units/ml) were added to the collagen gels containing HISMC. These cytokines were also mixed with one another to form a combination treatment of two cytokines. Fig. 3A–F shows the time-dependent effects of the cytokines HISMC contractility. IFN-γ, IFN-γ + IL-1β, and IFN-γ + TNF-α significantly impaired HISMC contractility at days 1–6. These results were normalized at day 4 where IFN-γ, IFN-γ + IL-1β, and IFN-γ + TNF-α attenuated HISMC contractility (Fig. 3G). Since only IFN-γ had a significant effect on HISMC contractility, subsequent studies focused on it alone.

Since 500 units/ml of IFN-γ was sufficient to inhibit HISMC contractility, we determined whether lower concentrations also had an effect on HISMC contractility. Concentrations of IFN-γ used included: 62.5 U/ml, 125 U/ml, 250 U/ml, 500 U/ml, and 1000 U/ml. Contraction was measured over 6 days and shown in Fig. 4A–E. Each concentration was compared to the control. IFN-γ 62.5 U/ml (Days 1, 3–6), IFN-γ 125 U/ml (Days 1, 3–6), IFN-γ 250 U/ml (days 1–6), IFN-γ 500 U/ml (days 1–6) and IFN-γ 1000 U/ml (Days 1–6) significantly decreased HISMC contractility. These results were also normalized at day 4 and shown in Fig. 4F. For day 4, IFN-γ suppressed contraction at all concentrations.

3.4. Cytokines affect HISMC proliferation measured (MTT assay)

To further evaluate how IFN-γ might alter HISMC contractility we next studied its effects on HISMC proliferation using MTT proliferation

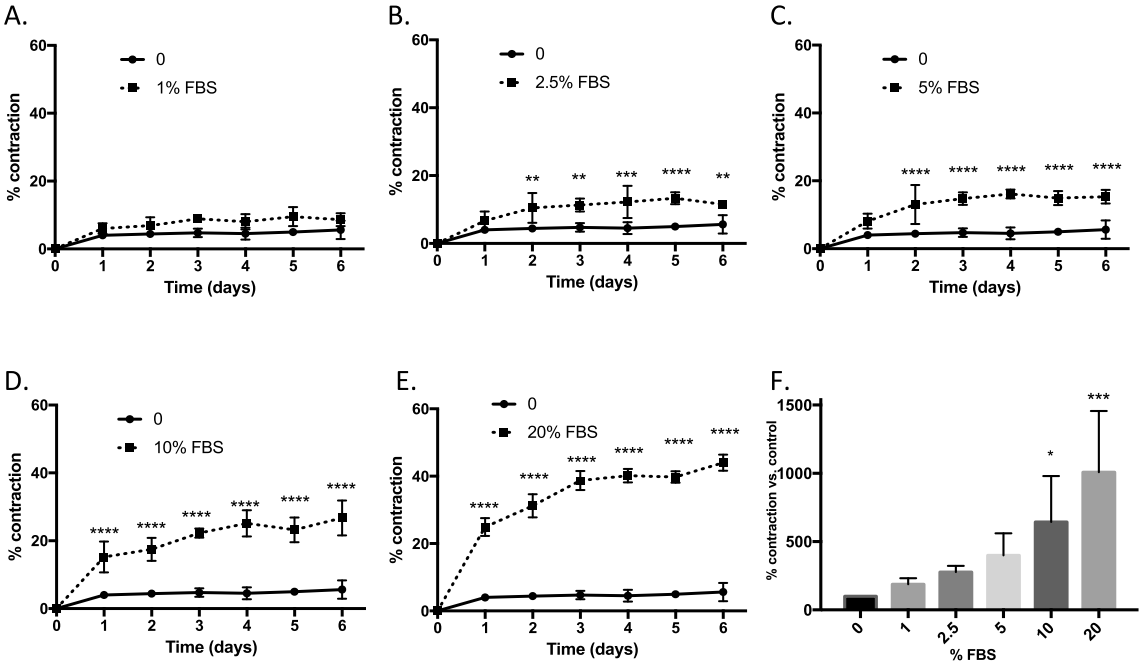

Fig. 2. Effects of serum concentrations on HISMC contraction shown over the course of six days using serum concentrations as follows: A) 1% FBS, B) 2.5% FBS, C) 5% FBS, D) 10% FBS, E) 20% FBS. Each concentration of FBS was compared to the control (no serum). F) Effects of different serum concentrations on HISMC contractility, normalized to control (100% contractility) at Day 4 Statistical was performed using two-way ANOVA, and one-way ANOVA for F, n = 3. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

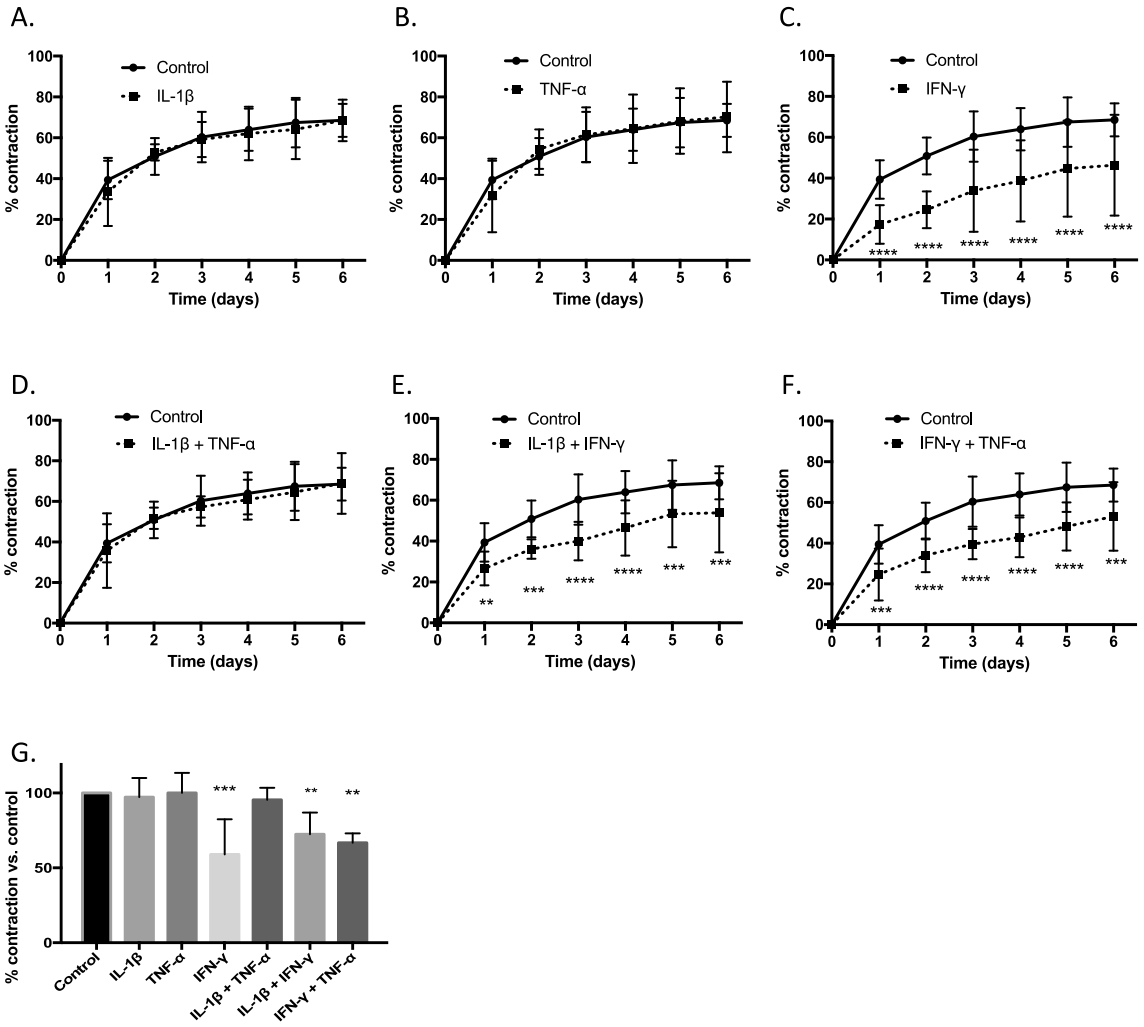

Fig. 3. Effects of pro-inflammatory cytokines on HISMC contraction are shown over the course of six days using cytokines or cytokine combinations of: A) IL-1β (20 ng/ml), B) TNF-α (20 ng/ml), C) IFN-γ (500 U/ml), D) IL-1β (20 ng/ml) + TNF-α (20 ng/ml), E) IL-1β (20 ng/ml) + IFN-γ (500 U/ml), and F) IFN-γ (500 U/ml) + TNF-α (20 ng/ml). Each cytokine treatment was compared to the control (no cytokines). G) Effects of different cytokines on HISMC contractility, normalized to control (100% contractility) at Day 4. Statistical analysis was performed using two-way ANOVA for A–F, and one-way ANOVA for G, n = 3. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

assays. Fig. 5A shows a concentration-dependent suppression of HISMC proliferation by IFN-γ (5 U/ml, 50 U/ml, 100 U/ml, 250 U/ml, 500 U/ml and 1000 U/ml) which show progressive reductions in MTT signal indicating that IFN-γ dose-dependently inhibits growth of HISMC *in vitro*.

3.5. Cytokines affect HISMC wound healing

Because we observed that IFN-γ decreased HISMC proliferation, we also evaluated its influence on HISMC migration/motility. HISMC were grown to confluency and cells in the center of each well were ablated and then stimulated with different concentration of IFN-γ (5 U/ml, 50 U/ml, 100 U/ml, 250 U/ml, 500 U/ml and 1000 U/ml). Again, we observed that IFN-γ suppressed HISMC migration in a concentration-dependent fashion (Fig. 5B).

3.6. Cytokines affect HISMC α- smooth muscle actin content

Because IFN-γ depressed HISMC tonic contractility, proliferation, and cell migration, we evaluated how it might affect α-SMA expression. α-SMA is a major structural protein that supports smooth muscle cell contraction but also proliferation, and migration. HISMC were stimulated with SMCM (control) medium, and medium with IFN-γ at concentrations of 5 U/ml and 500 U/ml for 4 days. Strikingly, we found that both concentrations of IFN-γ significantly decreased α-SMA expression in HISMC (Fig. 6).

4. Discussion

Tonic contractility mediates basal intestinal tone and maintains intestinal constriction once phasic contractions occur. Phasic contractions, which are directly involved in the propulsion of ingested material, are superimposed over tonic contractility [25]. Furthermore, tonic contraction is regulated similarly to phasic contraction by Ca^{2+} and actin filament controls. Therefore, loss of tonic contractility can diminish the speed and/or magnitude of phasic contractions. However, future studies will still be needed to evaluate this clinically. In this set of studies, we evaluated HISMC tonic contractility and its modulation by cell density, serum and during exposure to inflammatory cytokines as a function of time.

Our *in vitro* HISMC collagen gel assay showed that HISMC tonic contractility depended on cell density as well as serum (FBS)

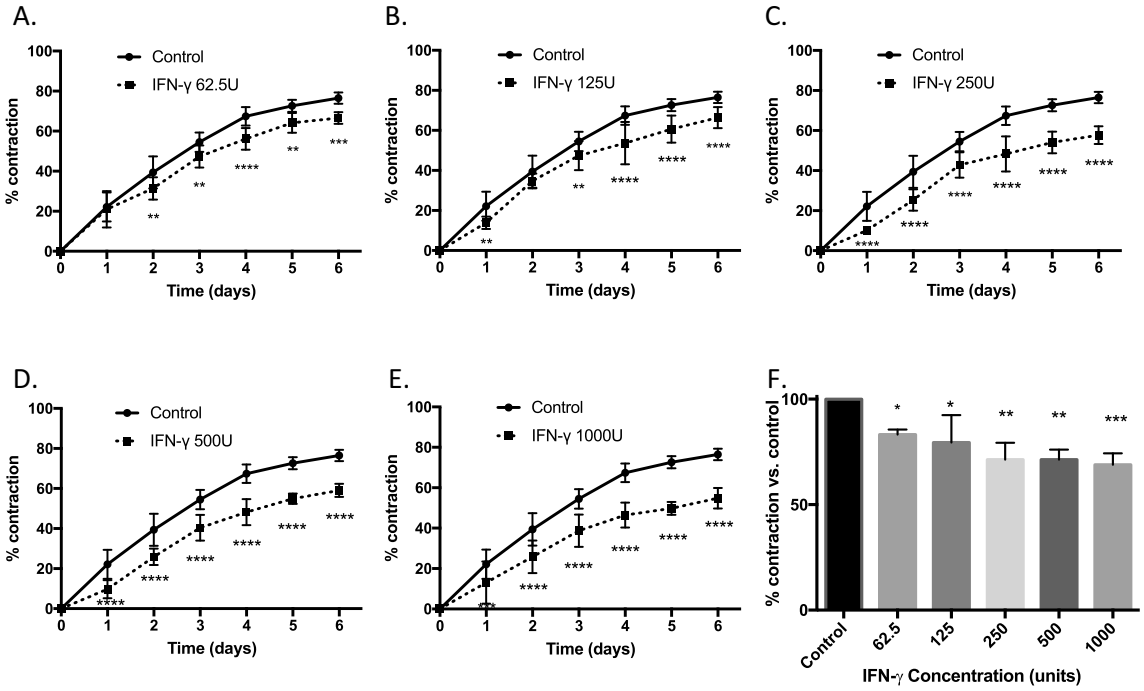

Fig. 4. Effects of IFN-γ on HISMC contraction are shown over the course of six days using different concentrations of IFN-γ: A) 62.5 U/ml, B) 125 U/ml, C) 250 U/ml, D) 500 U/ml, and E) 1000 U/ml. Each cytokine treatment was compared to the control (no cytokines). F) Effects of the different IFN-γ concentrations on HISMC contractility, normalized to control (100% contractility) at Day 4. Statistical analysis was performed using one-way ANOVA for A-E and two-way ANOVA for F, n = 3. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

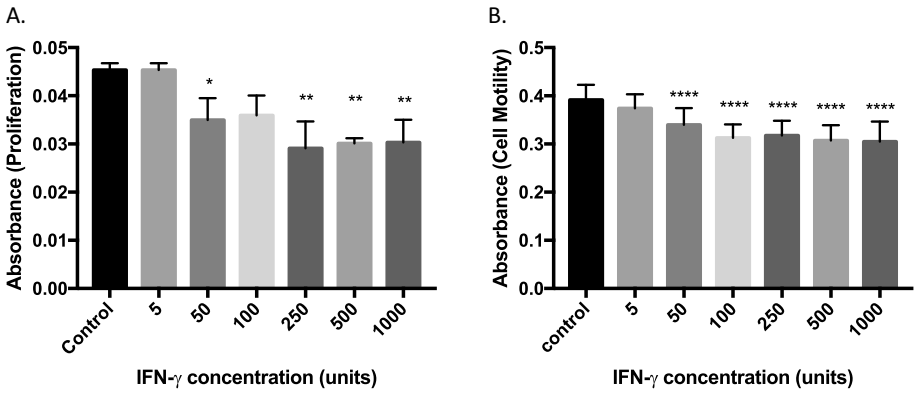

Fig. 5. A) Effects of the different IFN-γ concentrations on HISMC proliferation (MTT) after 4 days of treatment. B) Effects of the different IFN-γ concentrations on HISMC migration (crystal violet) after 4 days of treatment. Statistical analysis was performed using one-way ANOVA, n = 3. * = P ≤ 0.05, ** = P ≤ 0.01, **** = P ≤ 0.0001.

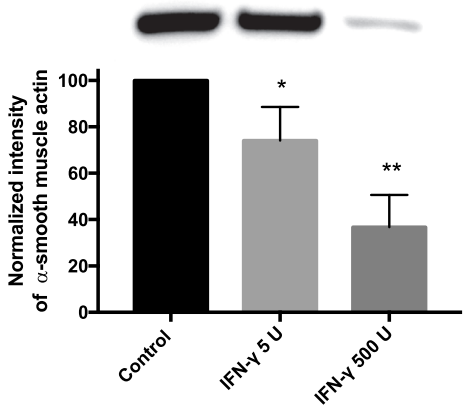

Fig. 6. α-SMA expression after 4 days of control and IFN-γ (5 U/ml and 500 U/ml) treatment. Statistical analysis was performed using one-way ANOVA (P ≤ 0.05), n = 3. Blots were normalized against Ponceau-S. * = P ≤ 0.05, ** = P ≤ 0.01.

concentrations. We showed that cell density and FBS concentration-dependently increased HISMC contraction overtime. FBS has been shown to dose-dependently increase muscle tone in canine airway smooth muscle cells [26]. Our model parameters, (similar to those employed by Al-Kofahi et al. [23]), evaluated HISMC contractility. We optimized cell density and FBS to 50,000 HISMC per well (24-well plate) and 5% FBS for subsequent measurements of HISMC tonic contraction.

We used lower concentrations of rat-tail collagen in gels, lower cell densities and FBS concentration compared to studies by Moore-Olufemi et al. [27]. Our model therefore limits the influence of serum on contraction, potentially allowing our cytokine treatments to exhibit more robust effects on contraction.

Many cytokines (IL-1 α/β, IL-6, IL-8, IL-12, IL-17, IL-18, TNF-α, and IFN-γ) are increased in IBD and in animal models of gut inflammation [28,29]. In this study of three major IBD-associated cytokines: IL-1β, TNF-α, and IFN-γ, only IFN-γ was seen to have a significant inhibitory effect on HISMC contractility. When IL-1β and TNF-α were applied individually, they had no effect on HISMC contractility (similar to

reports in isolated rat jejunal, ileal, and colonic smooth muscle cells [30]). IFN-γ, when combined with IL-1β or TNF-α did significantly decrease HISMC contractility, but this was not different from the response seen with IFN-γ alone.

IFN-γ has been shown to contribute to the pathogenesis of IBD. IFN-γ released from cultured intestinal lamina propria lymphocytes of CD patients was significantly more abundant than in lymphocytes from normal subjects, supporting a role for IFN-γ in CD [31]. Ito et al. [32] reported that IFN-γ was significantly upregulated (5 pg/100 mg of intestinal tissue) in the colons of DSS-induced colitis in wild-type mice (C57BL/6) who displayed weight loss, 60% mortality rate, bowel shortening and increased neutrophil abundance and serum haptoglobin levels. Importantly, they noted that IFN-γ −/− mice treated with DSS displayed no colitis and a 100% survival rate. IFN-γ also induces ICAM-1 expression in murine intestinal smooth muscle cells [33]. ICAM-1 expression is increased in tissue samples from subjects with CD and IFN-γ is thought to contribute to cytokine-induced suppression of human colonic intestinal smooth muscle contractility [34] suggesting potent links between IFN-γ and CD.

Hierholzer et al. [35] reported elevated iNOS production in a hemorrhagic shock mouse model. These effects, (along with increased ICAM-1 expression), reduced jejunal smooth muscle contractility and impaired gut motility. Selective inhibition of iNOS using l-N6-(1-iminoethyl) lysine was able to restore contractility and gut motility by reducing iNOS expression (and possibly normalizing ICAM). Although IFN-γ is a potent inducer of iNOS it was not clear to what extent IFN-γ participated in this effect [36]. Taken together, these observations along with our data suggest that IFN-γ plays a major role in the impaired contractility seen in IBD and GI motility disorders.

Based on these findings and reports from our lab [23], we tested several drugs for their ability to restore contractility diminished by IFN-γ including Dexamethasone, Ruxolitinib (a JAK1/JAK2 inhibitor), AG490 (a JAK2/JAK3 inhibitor), L-NAME (a pan-NOS inhibitor) and NS-398 (COX-2 inhibitor) [37–39] (see Table 1), however, none of these agents were able to restore HISMC gel contractility. IFN-γ might impair smooth muscle contractility through other pathways not tested here *e.g.* decreasing CPI-17, decreasing Ca^{2+} mobilization into the cell, or altering myosin ATP and myosin light chain activity.

Additionally, improved pharmacological agents that block IFN-γ more efficiently might reverse these effects. For example, Fontolizumab (HuZAF), a humanized anti-IFN-γ antibody that showed promise by increasing clinical response and remission rates of patients with active CD [40] might block these IFN-γ effects. We were unable to obtain this reagent and future studies may evaluate this approach. It has also been implicated that ustekinumab can reduce IFN-γ production by blocking IL-12 [41] which might also be useful in models where IFN-γ is being generated.

The inflammatory cytokines we tested on HISMC contractility have been measured in the serum of IBD patients. IFN-γ serum levels were measured as 0–6.87 pg/ml in UC serum, 6.24 pg/ml (1.06–13.6) in CD serum, and 0 pg/ml (0–1.67) in control patient serum [42]. In the same study, TNF-α serum levels were measured to be 7.59 pg/ml (5.34–10.16) in UC serum, 5.97 pg/ml (3.35–10.34) in CD serum, and 4.3 pg/ml (2.89–5.85) in control patient serum. These measurements were achieved using magnetic-bead-based immunoassay. Using ELISA, IL-1β was undetected in the plasma of UC and CD patients [43]. Therefore, in addition to IFN-γ we also tested TNF-α and IL-1b, however despite reports of these cytokines contributing to inflammation (particularly TNF-α [14,34]) we only observed alterations in contractility in response to IFN-γ. This was striking as we previously observed in mesenteric lymphatic smooth muscle that only IL-1β and not IFN-γ or TNF-α were able to suppress cell contractility in collagen gels. Therefore one possible finding of this study is that HISMC differ in their cytokine responses to IFN-g compared to other muscle cells tested [23].

We found that multiple concentrations of IFN-γ decreased HIMSC contractility. Our lowest concentration of IFN-γ was 67.5 U/ml (3.125 ng/ml) still higher than that seen in sera of IBD patients. However, *in vitro* human and animal studies often have used much higher cytokine concentrations to determine their influence on smooth muscle function. Human vascular smooth muscle cells (HVSMC) were treated with 100 ng/ml (2000 U/ml) of IFN-γ, which induced cell proliferation [44]. Human airway smooth muscle cells were treated with 100 ng/ml of IFN-γ alone or combined with 100 ng/ml TNF-α and/or 1 ng/ml IL-1β to induce COX-2 expression [45]. The IFN-γ concentrations (67.5–1000 U/ml) used in our experiments appear to be in the testable range for human cell responses. We reasoned that cytokine concentrations might be greater in the tissues during inflammation. Also, *in vitro* studies may require greater concentrations of cytokines seen in the serum levels of patients to elicit a response.

To determine the underlying causes of IFN-γ induced inhibition of HISMC contractility, we examined the effects of IFN-γ on HISMC proliferation and migration. We found that IFN-γ decreased HISMC proliferation and migration in a concentration-dependent manner. Hansson et al. [46] found a similar outcome of IFN-γ depressing rat arterial smooth muscle proliferation. It has also been reported that IFN-γ inhibits both TNF-α- and growth factor-induced HVSMC proliferation [47]. Recent reports have shown that IFN-γ stimulates HVSMC proliferation [44]. The opposing effects by IFN-γ on proliferation may be due to cell type or the route of administration of IFN-γ. In Hansson's [46] and Selzman's [47] studies, cells were cultured with IFN-γ; whereas, in Wang's [44] study, mice contained human coronary artery grafts in which they received an adenovirus with human IFN-γ. In the case of migration, IFN-γ has been shown to inhibit IL-4- and platelet-derived growth factor (PDGF)-induced human aortic smooth muscle cell migration [48]. PDGF has been implicated as a key regulator of the migration of HVSMCs [49]. To our knowledge, IFN-γ has not been implicated in inhibiting HISMC proliferation and migration, thus supporting the novelty of our findings.

Our study also determined that IFN-γ decreased α-smooth muscle actin expression and appears to decrease stress fibers in HISMC. We normalized our western blots for α-SMA performed to Ponceau S staining total protein of the membrane (since it has been determined as a better loading control than β-actin and GAPDH and since actin was the target protein of interest) [50,51]. Consistent with our findings, Hansson et al. [46] also found that IFN-γ decreased α-smooth muscle actin in rat vascular smooth muscle. IFN-γ also decreases α-SMA in human myofibroblasts, which have structural components similar to smooth muscle cells including α-SMA [52]. Interestingly, patients with deficient α-SMA develop functional intestinal obstructions as a result of normal intestinal motility [53].

Our data are consistent with IFN-γ (but perhaps not IL-1β and TNF-α) provoking a non-lethal de-differentiation in HISMC which changes these cells from a contractile to a non-contractile (and less proliferative and motile phenotype) which does not fully support intestinal contractility. Taken together, these functional changes and loss of the contractile apparatus components may be important potential causes underlying IFN-γ mediated disturbances of HISMC in IBD over and above those known to be produced by TNF-α and IL-1β (Fig. 7).

5. Conclusion

To our knowledge, this is the first study which assessed effects of cell density, serum, and cytokines on HISMC tonic contractility *in vitro*. This *in vitro* collagen gel assay provides a novel and useful model to study GI motility mechanisms. This model can also be used with cytokines, cell types and pharmacological agents. The main finding of our study is that only IFN-γ suppressed HISMC tonic contractility, proliferation, and migration by decreasing HISMC α-SMA content. Therefore IFN-γ released into the intestinal smooth environment may decrease GI motility, proliferation and smooth-muscle actin. Therapies targeting IFN-γ (Fontolizumab, Ustekinumab) have been used in CD clinical trials and may provide some benefit by maintaining GI motility

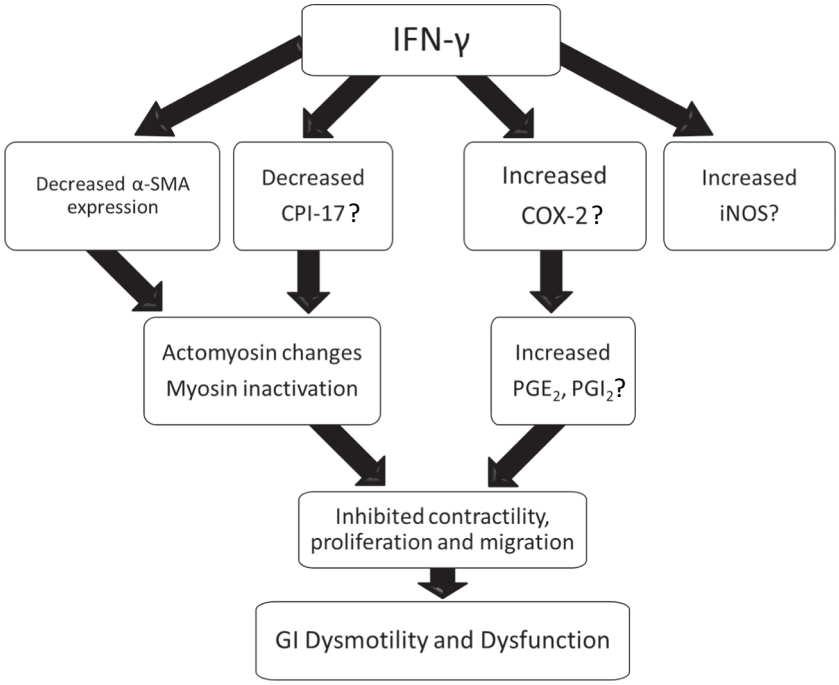

Fig. 7. Schematic of IFN-γ effects on HISMC leading to GI dysfunction.

depressed by IFN-γ. It seems likely that multiple biologic approaches that interfere with both IFN-γ and TNF-α could represent superior treatments for IBD dysmotility and other chronic inflammatory conditions.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Authors' contributions

CLF designed, interpreted, and executed the manuscript.

YW and JSA were involved project origin, planning, and development of manuscript.

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