Research paper

Therapeutic ultrasound attenuates DSS-induced colitis through the cholinergic anti-inflammatory pathway

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1. Introduction

Ulcerative Colitis (UC) is an inflammatory bowel disease (IBD) that affects the colon and rectum, characterized by a disease course that includes diarrhea, weight loss, fatigue, anaemia and blood in the stools. There is no specific cause for UC; however, genetic, environmental and microbiome factors altogether are known to unbalance the immune system, eventually leading to IBD [1–3]. IBD incidence has been increasing rapidly over the last few decades in newly industrialized countries, whereas the highest prevalence is reported in Europe and North America [4]. To date, there is no cure for UC, a disease that results in poor quality of life, increased risk of colorectal cancer, and morbidity/mortality associated with colectomy for possible symptomatic relief [5,6]. Despite recent advances and the development of biological therapies, a major fraction of patients does not respond to treatment. There is currently no drug available to provide sustained remission of IBD [7,8]. Therefore, a possible non-pharmacological approach to mitigate UC would be vagal nerve stimulation (VNS), which leads to activation of the cholinergic anti-inflammatory pathway (CAIP) and an anti-TNFα response [9]. It has been previously reported that UC patients may present with vagal nerve (VN) dysfunction, regardless of disease activity and previous colectomy history [10], making VNS a possible adjunct or alternative to pharmacological or biological therapeutic approaches.

Activation of CAIP through VNS acts on the spleen (SN) that causes the secretion of

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norepinephrine (NE). When released, NE binds to α7 nicotinic acetylcholine receptor, dextran sulphate sodium, vagus nerve, splenic nerve, mucosal immunity, ulcerative colitis, inflammatory bowel disease, acetylcholine, norepinephrine, macrophages.

Added value of this study

Our finding that therapeutic ultrasound attenuates Dextran Sulphate Sodium (DSS)-induced colitis through activation of the cholinergic anti-inflammatory pathway adds a valuable treatment option for Inflammatory Bowel Disease (IBD) patients. As a non-invasive and easily accessible technique, therapeutic ultrasound was shown here to change the immunological profile of a murine IBD model, leading to clinical and pathological changes that attenuated DSS-induced acute colitis.

Implications of all the available evidence

Including all evidence available, the future of IBD treatment may include valuable novel options. The TUS treatment here used could be easily translated to the clinic, considering it has been used for decades. The development of a multiple transducer system that could be worn by patients over their abdominal area could significantly improve their quality of life. As a non-invasive low intensity ultrasound treatment, for the first time is reported here that TUS becomes an alternative treatment for IBD patients.
movement would interfere with the treatment. Considering that a 5 cm² transducer was used on the mouse, the area treated was the entire right side between the axilla and the hip. TUS treatment is not focused; therefore, the actual volume of tissue treated or how each organ was affected is undetermined. Calibration of the ultrasound transducer was performed by measuring the effective transducer output utilizing a needle-type hydrophone (HNA series; Onda, Sunnyvale, CA) in degassed water at RT (Supplementary Fig. 1). Mice receiving DSS alone were anesthetized on days 4–10. Mice received 2% DSS in drinking water from days 0 to 7.

2.4. Clinical activity

Mice (n = 10–15/group) were daily evaluated for clinical symptoms, as previously described [30,31]. Briefly, animals were clinically evaluated for weight loss, stool consistency and blood in the stool, where which parameter varied from a score of 0–4, totaling a Disease Activity Index (DAI) of 12 when presenting severe colitis. The specific criteria for DAI are presented in Table 1. Upon euthanasia, colons were collected, cleaned with PBS (Phosphate Buffered Saline) 1×, weighted and measured before processed for histological and proteomic analysis. Spleens, mesenteric lymph nodes (MLN) and colons for further analysis.

2.5. Proteomics

Colonic samples (n = 5–6/group per time point) were collected from C57BL/6 female mice receiving 2% DSS or 2% DSS + TUS. Collection was performed at days 0, 3, 5, 7, 9, 11 and 14. After PBS 1× cleaning, samples were snap frozen and processed for further proteomic analysis. Briefly, frozen colonic samples were homogenized in cell lysis buffer (1 mM EDTA, 150 mM NaCl, 0.05% Tween-20 and 20 mM Tris-HCl in ultrapure water) containing Pierce Protease Inhibitor Tablets (Thermo Scientific, Waltham, MA) and 1.0 mm Zirconium Beads. Homogenates were centrifuged at 14,000 rpm at 4 °C for 20 min and the supernatant was collected. The process was repeated two times and aliquots were stored at −80 °C. Bicinchoninic acid assay (BCA – Thermo Scientific, Waltham, MA) was used for protein quantification and samples were further diluted to 1 mg/mL of total protein. MILLIPLEX Map Mouse Cytokine/Chemokine Panel (EMD Millipore, Billerica, MA) was used for proteomic analysis of colonic homogenates according to manufacturer specifications in a Bio-Plex 200 (Bio-Rad Laboratories, Hercules, CA). The same control samples (day 0) were used for multiplex ELISA experiments in the 2% DSS and 2% DSS + TUS exposed mice. Further analysis of colonic TGFβ (Thermo Scientific, Waltham, MA) and HSP70 (R&D Systems, Minneapolis, MN) were performed using ELISA Streptavidin-HRP assay.

2.6. Flow cytometry

Spleens (n = 6/group) were collected at days 0 and 14 from C57BL/6 female mice receiving 2% DSS or 2% DSS + TUS. Tissue dissociation was performed in Ammonium-Chloride-Potassium (ACK) lysing buffer (Lonza, Walkersville, MD) using two frosted glass slides for cell isolation. Cells were washed in PBS 1× (1,500 rpm, 4 °C, 10 min), filtered through a 70 μm filter and washed one more time. Samples were fixed in 10% NBF (neutral buffered formalin) for 35 min at 4 °C, washed in PBS 1× and stored at 4 °C until further analysis. Cells were incubated with specific antibodies for 35 min in Stain Buffer (BD Pharmigen, San Jose, CA) on ice and protected from light, following manufacturer instructions. Flow cytometry was performed using a V-bottom 96-well plate in Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA) and analysed through Flowjo (Ashland, OR) software. Immune cell population of the spleen was characterized using the following antibodies: FITC CD3 (Rat, 0.5 mg/mL, BD Pharmigen, cat. 555274), APC CD4 (Rat, 0.2 mg/mL, BD Pharmigen, cat. 553053), PE CD8α (Rat, 0.2 mg/mL, BD Pharmigen, cat. 553033), PE CD25 (Rat, 0.2 mg/mL, BD Pharmigen, cat. 553866), FITC F4/80 (Rat, 0.5 mg/mL, eBioscience, cat. 11–4801-82) and Alexa Fluor 488 B220 (Rat, 0.5 mg/mL, Biolegend, cat. 103225). F4/80− and CD3− CD4+ CD25− spleen populations were enriched prior to flow cytometry analysis through Magnetic Cell Separation MicroBeads (MACS - Miltenyi Biotec, Bergisch Gladbach, Germany).

Table 1

| Score | Weight loss | Stool consistency | Bleeding |
|-------|-------------|--------------------|---------|
| 0     | None        | Normal stool       | No bleeding |
| 1     | 1–5%        | Slightly loose stool | Few blood-tinged stools |
| 2     | 5–10%       | Loose stools       | Slight bleeding |
| 3     | 10–15%      | Watery stool       | Gross bleeding |
| 4     | >15%        | Severe diarrhoea   | Blood filling the whole colon |

Fig. 1. Schematics of the methods section. Mice were shaved over the abdomen and a water-based ultrasound gel was applied. The 5 cm² transducer was placed on top of the gel and held motionless by a clamp holder while the animal was under isoflurane anaesthesia. The treatment lasted for 7 min at 1 MHz (2 W/cm², 10% duty cycle, ~250 kPa) and was repeated once a day from days 4 to 10. Mice received 2% DSS in drinking water from days 0 to 7.
following manufacturer instructions. Each sample was analysed for 10,000 events and results are shown as mean ± SD percentage of the total number of cells. Isotypes were also analysed, and flow cytometry gating is represented in Supplementary Fig. 2.

2.7. Histological and immunohistochemistry evaluation

Colons were collected on days 0, 7 and 14 from C57BL/6 female mice receiving 2% DSS or 2% DSS + TUS (n = 5/group per time point), and at day 14 for C57BL/6 splenectomised or α7nAChR KO female mice receiving either 2% DSS or 2% DSS + TUS (n = 10/group). Freshly collected samples were washed with PBS 1×, longitudinally cut, positioned as a Swiss Roll in 10% NBF and incubated at RT (room temperature) for 24 h. Afterwards, all tissue samples were kept in PBS 1× until embedded in paraffin. Colons were sectioned at 3 μm, deparaffinized and stained with H&E (Guills II haematoxylin and Eosin-Y) for histological grading. Samples were analysed by two independent reviewers blinded to the different cohorts, where the whole area of the colon was scored and one section per animal was processed. The analysis was performed as previously described [32], including grade of inflammation (0–3), extent within the intestine layers (0–3), regeneration (0–4), crypt damage (0–4) and percentage of involvement (0–4). Detailed scores are shown in Table 2. Images were acquired with a 20× air objective from Leica Aperio ScanScope CS (NA = 0.75, Leica Microsystems, Buffalo Grove, IL) and using Aperio ImageScope software.

Table 2
Histological scoring. Colon samples were evaluated for inflammation, extent, regeneration, crypt damage and percent of involvement. Maximum scoring possible is 56 for severe colitis [32].

| Feature graded | Grade | Description |
|----------------|-------|-------------|
| Inflammation   | 0     | None        |
|                | 1     | Slight      |
|                | 2     | Moderate    |
|                | 3     | Severe      |
| Extent         | 0     | None        |
|                | 1     | Mucosa      |
|                | 2     | Mucosa and submucosa |
|                | 3     | Transmural  |
| Regeneration   | 0     | Complete regeneration or normal tissue |
|                | 1     | Almost complete regeneration |
|                | 2     | Regeneration with crypt depletion |
|                | 3     | Surface epithelium not intact |
| Crypt damage   | 0     | None        |
|                | 1     | Basal 1/3 damaged |
|                | 2     | Basal 2/3 damaged |
|                | 3     | Only surface epithelium intact |
| Percent involvement | 1 | 1–25% |
|                  | 2 | 26–50% |
|                  | 3 | 51–75% |
|                  | 4 | 76–100% |

Fig. 2. Clinical symptoms of 2% DSS colitis mice under TUS treatment. Experimental colitis was induced by DSS for 7 days in drinking water and TUS treatment was administered from day 4 to 10 over the abdomen. TUS attenuated clinical symptoms from day 9 and forward when measuring the (A) disease activity index (DAI), including (B) amelioration of stool consistency, (C) weight loss and (D) blood in the stool at different time points. *p < .05 compared to 2% DSS + TUS. Two-way ANOVA followed by Sidak post-hoc test. N = 15/group.
Colons, spleens and MLNs (n = 3–4/group) collected on days 0 and 14 from all groups were evaluated through immunohistochemistry for immune cell population. Samples were cut at 3 μm of thickness using a Leica Manual Microtome, left on adhesive slides at RT overnight and baked for 1 h at 65 °C. Antigen retrieval was achieved after 40 min of incubation at 100 °C in antigen unmasking solution (citrate-based, pH = 6.0; Vector Laboratories, Burlingame, CA), followed by 15 min incubation with SuperBlock Blocking Buffer (Thermo Scientific, Waltham, MA) at RT. After blocking, samples were incubated overnight at 4 °C with the following primary antibodies: CD4 (Rabbit, 0.623 mg/mL, Abcam, cat. ab183685), CD8 (Rabbit, 1 mg/mL, Abcam, ab203035), CD25 (Goat, 0.2 mg/mL, Invitrogen, cat. PA5–46922), F4/80 (Rabbit, 0.23 mg/mL, Novus Biologicals, cat. NBP2–12506), B220 (Rat, 0.5 mg/mL, Invitrogen, cat. 14–0452-81), GFAP (Rabbit, 1 mg/mL, Abcam, cat. ab211271) and α7nAChR (Goat, 0.5 mg/mL, Abcam, cat. ab110851). Next, samples were incubated for 5 min at RT with Peroxidized 1 (BioCare Medical, Pacheco, CA) followed by a 30 min incubation with their respective secondary HRP (Horseradish Peroxidase) antibodies. When co-staining for α7nAChR+F4/80+ cells, the secondary antibodies used were chicken anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 594. Isotypes were also analysed. Images were acquired at 10× magnitude on a Leica Aperio ScanScope CS using a 10× air objective (NA = 0.75, Leica Microsystems, Buffalo Grove, IL) and Aperio ImageScope software. Photomicrographs were taken from the whole area of the colon, spleen or MLN (30–40 images/organ for each animal) and analysed with ImageJ software (NIH, Bethesda, MD). Results are presented as mean ± SD. Images were taken with a 10× objective.
Fig. 4. Proteomic colon changes in 2% DSS colitis mice under TUS treatment. Experimental colitis was induced by DSS for 7 days in drinking water and TUS treatment was administered from day 4 to 10 over the abdomen. The colons were collected at days 5, 7, 9, 11 and 14, homogenized and later analysed by multiplex ELISA assay. Results demonstrate downregulation of colonic IL-5, IL-17 and Eotaxin, at different time points during TUS treatment (days 5 to 9); and upregulation of colonic IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70) and G-CSF at different time points starting at day 5 with TUS treatment. *p < .05 compared to 2% DSS. *p < .05 compared to 2% DSS. Two-way ANOVA followed by Sidak post-hoc test. N = 5–6/group at each time point. Results are presented as mean ± SD. Heat maps of these results are presented in Supplementary Fig. 4.

Fig. 5. Proteomic colon changes in 2% DSS colitis mice under TUS treatment. Experimental colitis was induced by DSS for 7 days in drinking water and TUS treatment was administered from day 4 to 10 over the abdomen. The colons were collected at days 5, 7, 9, 11 and 14, homogenized and later analysed by multiplex ELISA assay. Results demonstrate downregulation of colonic MCP-1, M-CSF, MIG, RANTES and TNFα at different time points during TUS treatment (days 5 to 9); and upregulation of colonic MIP-1α, MIP-1β and MIP-2 at different time points starting at day 5 with TUS treatment. In addition, results show reduction in colonic levels of TGFβ and HSP70 at days 7 and 14, respectively, under TUS treatment. *p < .05 compared to 2% DSS. *p < .05 compared to control. Two-way ANOVA followed by Sidak post-hoc test for multiplex ELISA and one-way ANOVA followed by Tukey post-hoc test for ELISA Streptavidin-HRP assay. N = 5–6/group at each time point. Results are presented as mean ± SD. Heat maps of these results are presented in Supplementary Fig. 3.
2.8. Statistical analysis

Software Prism 7 (Graph Pad Inc., La Jolla, CA) was used for all statistical analysis, which was performed through two-tailed Student’s t-test, two-way ANOVA followed by Sidak post-hoc test, or one-way ANOVA followed by Tukey post-hoc test. P < .05 was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM), unless otherwise indicated. The data was confirmed for normal distribution in Software Prism 7 (Graph Pad Inc., La Jolla, CA).

3. Results

3.1. Therapeutic ultrasound improves DSS colitis

Acute colitis was induced in C57BL/6 female mice by the addition of 2% DSS in drinking water for 7 days, while TUS was initiated to the abdomen on day 4 (when animals began presenting clinical symptoms) and was continued through day 10. Clinical scores were measured daily (Fig. 2) and demonstrated improvement of colitis severity from days 9 to 13 when TUS was applied (*p < .05 compared to 2% DSS). The greatest clinical improvement was observed in stool consistency from days 10 to 14, decreased weight loss at days 10 and 11, and diminished blood in the stools at day 5 (Fig. 2; *p < .05 compared to 2% DSS). Of note, blood in the stool was detected by day 4 in this IBD model, prior to the administration of TUS to the abdomen. TUS decreased the amount of

Fig. 6. Immune cell population changes in colon, spleen and MLN in 2% DSS colitis mice under TUS. (A–E) Colon IHC analysis revealed no differences amongst all groups regarding B220+ B cells, increased levels of CD4+ and CD8+ cells in comparison to controls, while CD8+ levels were decreased when comparing TUS treated animals to 2% DSS group and CD25+ T cells were increased in 2% DSS only group. (F–G) Spleen FACS analysis demonstrated no changes for CD4+ and CD25+ cells. Increase percentage was seen for CD8+ T cells and decrease in B220+ B cells when comparing 2% DSS to control. In addition, TUS treatment normalized CD8+ T cells and B220+ B cells when compared to 2% DSS. (K–O) MLN IHC analysis demonstrated no difference amongst all groups for CD8+, CD25+ and F4/80+ cells. TUS treatment increased CD4+ and B220+ levels compared to control. *p < .05 compared to control. #p < .05 compared to 2% DSS. One-way ANOVA followed by Tukey post-hoc test. N = 4/group for IHC analysis and N = 6/group for FACS analysis. Results are presented as mean ± SD.

Fig. 7. Clinical and histological analysis of splenectomised mice. There was no difference between the groups in (A) disease activity index, (B) stool consistency, (D) weight loss, (E) histological colonic damage, (F) colon length and (G) colon weight at day 14. TUS decreased the amount of (C) blood in the stools on days 6 and 7. H&E staining of the colons demonstrated destruction of the crypts, loss of the epithelial barrier, loss of goblet cells and high immune cell infiltration for both groups. *p < .05 compared to 2% DSS + TUS. Two-way ANOVA followed by Sidak post-hoc test for clinical analysis. Student’s t-test for histological scores and macroscopic measurements. N = 10/group. Results are presented as mean ± SD for histological scores. Images were taken with a 10× objective.
colon shortening, with no change in colon or spleen weights at day 14 (Fig. 3; p < .05 compared to 2% DSS). Histological scores revealed a decrease in colonic damage on day 14 with partial preservation of the epithelial barrier and goblet cells along with decreased destruction of the crypts and immune cell infiltration (Fig. 3; p < .05 compared to 2% DSS). Animals receiving TUS treatment over the abdominal area had a measured –2°C increase in their core temperature (Supplementary Fig. 2), assessed by rectal thermocouple (Omega Engineering Inc., Norwalk, CT). In preliminary experiments, lower power settings or shorter treatment courses for TUS did not modify colitis compared to mice receiving 2% DSS alone (data not shown).

3.2 Changes in colon proteomics by ultrasound treatment

Proteomic analysis comparing cytokines, chemokines and trophic factors (CCTF) of 2% DSS and 2% DSS + TUS groups was performed at different time points over 14 days. TUS decreased the levels of the following CCTF in the colon compared to 2% DSS alone: IL-5, IL-17, Eotaxin, monocyte chemoattractant protein 1 (MCP-1), macrophage-colony stimulating factor (M-CSF), monokine induced by gamma interferon (MIG), regulated on activation, normal T cell expressed and secreted (RANTES) and tumour necrosis factor alpha (TNFα) (Figs. 4 and 5; p < .05). TUS to the abdomen resulted in the abdominal area had a measured –2°C increase in their core temperature (Supplementary Fig. 2), assessed by rectal thermocouple (Omega Engineering Inc., Norwalk, CT). In preliminary experiments, lower power settings or shorter treatment courses for TUS did not modify colitis compared to mice receiving 2% DSS alone (data not shown).

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3.4 TUS treatment in splenectomised mice

Acute DSS colitis was induced in splenectomised C57BL/6 mice by adding 2% DSS in their drinking water for 7 days, and the animals were treated with TUS from days 4 to 10. There was no overall difference between 2% DSS and 2% DSS + TUS splenectomised groups when evaluating disease activity index, weight loss and stool consistency. However, TUS did diminish the presence of blood in the stools on days 6 and 7 compared to 2% DSS cohort (Fig. 7; p < .05). There was no difference observed between the groups when analysing colon length, colon weight and histological scores (Fig. 7). There were also no differences between the two groups in CD4⁺, CD8⁺, B220⁺ and F4/80⁺ cells in the colon. A percent increase in CD4⁺ cells across groups. However, CD8⁺ and B220⁺ cell levels were normalised at day 14 in 2% DSS + TUS group. The sonicated group also had increased levels of B220⁻ B cells compared to 2% DSS alone (Fig. 6; p < .05). MLN immunohistochemistry (IHC) analysis demonstrated no changes regarding CD8⁺, CD25⁺ and F4/80⁺ cells across the three groups. The percent increase in CD4⁺ and B220⁺ cells was detected in the 2% DSS + TUS group compared to controls (Fig. 6; p < .05). Representative IHC images are shown in Supplementary Figs. 5 and 6.

3.5 TUS treatment in α7nAChR KO mice

Acute DSS colitis was induced in WT C57BL/6 and α7nAChR KO mice, where one KO group received TUS treatment from days 4 to 10. There was worsening of disease activity index, weight loss, blood in the stools and stool consistency at different time points in the KO mice 2% DSS and 2% DSS + TUS groups when comparing to WT 2% DSS alone group (Fig. 9; p < .05). In addition, TUS treatment resulted in shorter colons at day 14 when compared to WT 2% DSS (Fig. 9; p < .05). No difference...
was seen amongst all groups for colon weight and histological scores, whereas spleen weight was increased in both KO groups compared to WT 2% DSS (Fig. 9; \( p < .05 \)). No difference was observed in CD4+, CD8+, CD25+, F4/80+ and B220+ cells in the colon (Fig. 10). Splenic levels of CD4+ were decreased in 2% DSS + TUS KO group (compared to 2% DSS KO; \( p < .05 \)). CD25+ T cells levels were not different from WT animals and were elevated in 2% DSS KO (compared to WT 2% DSS; \( p < .05 \)). CD8+ T cells were increased in both KO 2% DSS and KO 2% DSS + TUS groups. Decreased B220+ levels were seen in the KO 2% DSS group only (compared to WT 2% DSS; \( p < .05 \); Fig. 10). Representative IHC images are shown in Supplementary Figs. 9-11.

### 3.6. TUS activation of CAIP

To confirm the involvement of the cholinergic anti-inflammatory pathway, co-staining of histological sections for \( \alpha_7 \)nAChR and F4/80+ macrophages was performed in the spleen, MLN and colons for control, 2% DSS and 2% DSS + TUS animals (C57BL/6 WT). There was no difference amongst all groups regarding the spleen and MLN. However, TUS upregulated the levels of \( \alpha_7 \)nAChR+ F4/80+ cells in the colon at day 14 compared to control and 2% DSS exposed mice (Fig. 11; \( p < .05 \)). This difference was not observed in colons of splenectomised animals, whereas the levels of \( \alpha_7 \)nAChR+ F4/80+ cells in the MLN were increased under TUS treatment (Fig. 11; \( p < .05 \)). Moreover, to verify the possible activation of enteric glial cells, the levels of GFAP were measured in the colon of all groups, and no statistical difference was seen (Fig. 12).
4. Discussion

IBD are chronic gastrointestinal disorders that have been increasing rapidly in worldwide prevalence [4]. Despite recent advances, improvements in therapeutic options are needed since definitive remission is currently not achievable [7,8]. Previous studies have explored VNS using invasive approaches as a treatment for UC [19–22,23]. Our study explored the effects of TUS to the abdomen as a potential non-invasive treatment for DSS-induced acute colitis through activation of CAIP. We demonstrate that the application of TUS attenuated the severity of

Fig. 10. Colon, spleen and MLN immune cell changes in α7nAChR KO mice. There was no difference amongst all groups when analysing the colons for (A-E) CD4⁺, CD8⁺, CD25⁺, F4/80⁺ and B220⁺ cells. Splenic levels of (F) CD4⁺ were decreased (compared to 2% DSS) and (H) CD25⁺ T cells levels were not different from controls with TUS treatment. (G) CD8⁺ T cells were increased in both 2% DSS and 2% DSS + TUS KO groups. MLN levels of (M) CD25⁺ T cells were increased in both 2% DSS and 2% DSS + TUS KO groups, while decreased (O) B220⁺ levels were seen in the 2% DSS KO group only. There was no difference regarding splenic (I) F4/80⁺ and (J) B220⁺ cells, and no difference in MLN (K) CD4⁺, (L) CD8⁺ and (N) F4/80⁺ cells. *p < .05 compared to 2% DSS WT. #p < .05 compared to 2% DSS KO. One-way ANOVA followed by Tukey post-hoc test. N = 4/group for IHC analysis. Results are presented as mean ± SD.
colitis by improving clinical symptoms, colon shortening and histological damage that was dependent upon the response of the spleen and α7nAChR+ macrophages.

Evaluation of the proteomic changes induced by TUS in 2% DSS colitis mice revealed two different CCTF patterns when compared to 2% DSS mice: A) downregulation of colonic IL-5, IL-17, Eotaxin, MCP-1, LIF, M-CSF, MIG, RANTES and TNFα at different time points during TUS treatment (days 5 to 9); and B) upregulation of colonic IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70), G-CSF, LIX, MIP-1α, MIP-1β and MIP-2 at different time points starting at day 5 with TUS treatment. Decreased expression of IL-5 and Eotaxin may have contributed to decrease chemoattraction of eosinophils to the colon [33,34] during the injury phase, resulting in less tissue damage and mucosal healing [35]. Moreover, TNFα levels were decreased in TUS cohort, possibly due to activation of CAIP [15]. The decrease in TNFα could contribute to functional epithelial barrier and attenuation of clinical symptoms [36,37].

The decreased expression of IL-17, MCP-1, M-CSF and RANTES in the TUS treated cohort may also contribute to the attenuated pro-inflammatory response. These CCTF (i.e., IL17, RANTES, MIG) [38–40] may contribute to the reduced presence of CD8+ and CD25+ T cells in the colon; along with decreased M-CSF and MCP-1 in TUS-treated mice, they may have attenuated macrophage inflammatory functions [41,42] resulting in the reduction of tissue damage. We also detected a decrease in TGFβ in the TUS cohort treatment, which could have contributed to an immunosuppressive environment in the gut. TGFβ is known to induce the differentiation of Th17 cells [43,44] and to retain lymphocytes in the gut [44,45]. The decreased levels in our study could relate to reduced levels of IL-17 at day 9 and could have contributed to decreased levels of CD8+ and CD25+ T cells in the gut, translating in attenuation of the disease.

In comparison, the increase of IL-2, IL-4 and IL-9 with TUS treatment may have contributed to a more tolerogenic response to antigens in the gut and attenuated colitis. TUS increased expression of IL-7, G-CSF, MIP-1α, MIP-1β and MIP-2 that could modulate the influx of neutrophils and macrophages [43–48], resulting in improvement in colitis. G-CSF therapy has been shown to ameliorate DSS-induced colitis by increasing tropism of macrophages and stimulating a M2 macrophage phenotype [44]. Furthermore, increased levels of colonic IL-1β and IL-12 during the recovery phase could aid in the clearance of dead cells and debris [49,50], decreasing intestinal inflammation. Since the percentage of

Fig. 11. Colon, spleen and MLN analysis for F4/80+ α7nAChR+ cells. Photomicrographic images revealed (A) increased levels of F4/80+ α7nAChR+ cells in the colons of 2% DSS and 2% DSS + TUS, and even higher levels at the 2% DSS + TUS mice. No difference was seen across all groups in the (B) spleen and (C) MLN, in addition to the colons from (D) splenectomised animals. However, (E) TUS increased the levels of F4/80+ α7nAChR+ cells in the MLN of splenectomised animals. Images show staining for the nuclei (blue), F4/80 macrophages (green) and α7nAChR (red). Merged images demonstrate co-staining of F4/80+ α7nAChR+ macrophages in orange (insert). *p < .05 compared to control. #p < .05 compared to 2% DSS. One-way ANOVA followed by Tukey post-hoc test for WT C57BL/6 groups. Student’s test for splenectomised animals. N = 3/group. Results are presented as mean ± SD. Images were taken with a 20× objective, whereas inserts were taken with a 63× objective.
F4/80+ macrophages were not different when comparing 2% DSS and 2% DSS + TUS groups, the results suggest that the morphology and functionality of the macrophages were altered, but not necessarily their numbers. Further analysis is necessary to determine TUS induced polarization of M1 or M2 macrophages in DSS colitis. Overall, we observed an increase of pro-inflammatory cytokines/chemokines with TUS treatment towards the end of the disease course. Although counter-intuitive, our results suggest that TUS treatment induced a tolerogenic response in the gut during the injury phase, whilst promoting early resolution and recovery of DSS colitis.

TUS reduced levels of CD8+ T cells, while the levels of CD25+ T cells were similar to control in the gut and spleen, contributing to decreased inflammation and epithelial damage. We have previously reported the increase of colonic CD8+ and CD25+ T cells in the DSS model of colitis related to disease worsening and increased histological damage [51], whereas both cell types were attenuated by TUS in the current study. We also observed an increase of CD4+ T cells and B220+ B cells in the MLN that may indicate a transition to a chronic state of the disease [52]. However, the upregulation of B220+ B cells in the spleen and MLN may contribute to gut homeostasis and attenuation of colitis while interacting with T regulatory cells [53]. Further studies are needed in a chronic relapsing DSS colitis model to determine if TUS treatment to the abdomen will result in decreased morbidity and durable positive clinical outcomes.

It has been previously shown that VNS ameliorated intestinal inflammation by activating CAIP directly in the gut, without involving the spleen or SN [54]. In the study of Matteoli et al. (2014), an invasive technique was used to directly electrical stimulate the right cervical vagus nerve in order to activate CAIP and decrease inflammation [54]. In comparison, the current study achieves similar downregulation of gut inflammation by applying TUS to the abdomen by activating the splenic nerve, splenocytes, vagal afferents, and/or vagal efferents resulting in elevation of the number of α7nAChR+F4/80+ in the colon. In addition, the current study demonstrated that there were no differences between the cohorts of splenectomised mice when comparing 2% DSS to 2% DSS + TUS cohorts. These observations would support the hypothesis that improvement of colitis requires the presence of the spleen and the splenic nerve, possibly involving activation of the VN. In our study, splenectomy by itself did worsen DSS-induced colitis (data not shown). This observation would suggest that CAIP is also a
normal response to DSS injury in the absence of TUS. If the ultrasound treatment to the abdomen did not involve activation of CAIP, amelioration of the disease would have occurred in the DSS colitis model in splenectomised mice. The VN stimulates the secretion of ACh by enteric neurons, which in turn reduces the production of pro-inflammatory factors by macrophages and induces a tolerogenic response in the gut [15]. However, VN innervation of the colon is limited [55]. In this study, TUS treatment in splenectomised mice resulted in a lack of significant clinical response, highlighting the importance of an intact spleen and SN. Reduced levels of MLN CD4+ and CD25+ T cells may indicate their migratory movement to the gut in response to colonic inflammation.

The interdependence of the spleen and VNS for activation of CAIP in the DSS colitis model has been previously reported [22]. VNS was ineffective in the treatment of intestinal inflammation in α7nAChR KO mice [54], or it promoted worsening of the disease when inducing DSS colitis [56]. VNS activates the SN through the celiac ganglion to release norepinephrine in the spleen. T cells respond by producing ACh that binds on splenic α7nAChR+ macrophages and inhibits the secretion of pro-inflammatory cytokines, specially TNFα [55,57,58]. The absence of α7nAChR abolished TUS therapeutic effects in DSS colitis, resulting in worsening of the clinical disease, where the immune cell profile reflected no differences in the colonic damage amongst all three groups. The decrease in body weight in α7nAChR KO mice was probably related to the further shortening of colon length detected in this group compared to 2% DSS KO. We were unable to point the exact cause of the colon damage; however, these results confirm the importance of α7nAChR in the context of TUS therapeutic effect. TUS treatment resulted in increases of colonic F4/80 α7nAChR+ cells compared to controls and 2% DSS WT, indicative of CAIP activation. The increased numbers of F4/80 α7nAChR+ cells in the colon might result from splenic egress, since we observed a trend towards reduced numbers of immune cells in the spleen overtime. In splenectomised DSS colitis mice, we observed an increase in F4/80 α7nAChR+ cells in the MLN but not in the colon. Since the VN also innervates the MLN [59], it is possible that the activation of CAIP occurred in the MLN, without altering the clinical and pathological disease outcomes. Our results in the 2% DSS + TUS group agree with the previous use of TUS, in which stimulation of the VN and the SN attenuated AKI through activation of CAIP, resulting in improvement of tissue morphology and function. No improvement in AKI was detected in splenectomised mice that were exposed to US to the abdomen, confirming the need for a spleen [27,29]. Lastly, VNS may also cause the activation of enteric glial cells (GFAP+ α7nAChR+) and support/preserve the epithelial barrier function [60–62]. We did observe a trend for increased GFAP+ cells in the colon with TUS treatment compared to 2% DSS WT animals.

In the current study, TUS exposure would result in a mechanical radiation force or peak rarefactive pressure of ~250 KPa, equal to a mechanical index (MI) of 0.25, below the food and drug administration (FDA) MI = 1.9 [63–65] for diagnostic US. In addition, TUS exposure to the abdomen resulted in ~2 °C rise in core body temperature (Supplementary Fig. 1). Hyperthermia has been previously reported to ameliorate intestinal inflammation when reaching core body temperatures of 42–43 °C maintained for 5 to 20 min, mainly by upregulating heat shock proteins (HSP) like HSP70 or HSP32 [66–68]. Although heat shock proteins have been shown to be protective in DSS colitis [69,70], our results demonstrated decreased levels of HSP70 by day 14 with TUS treatment. Interestingly, extracellular HSP70 has been considered as a pro-inflammatory factor since it stimulates the expression of pro-inflammatory genes in innate immune cells [71,72]. Therefore, the reduced levels seen in our study may contribute to the attenuation of clinical and pathological damage with TUS treatment. Further investigations are needed to determine how TUS directly causes CCTF changes, immune cell responses and stimulation of CAIP. Moreover, the complex response of immune cells and CCTF needs to be studied in KO mouse models and IBD patients, so we can better understand how to target these dynamic responses.

There were several limitations of this study that need to be addressed. This study was performed in female mice from one vendor and it is unclear if there was a gender bias associated with the response to TUS in the DSS colitis model. Male mice are more responsive to amelioration of DSS colitis with the use of α7 agonists, possibly due to hormonal influence [56]. Therefore, TUS effects in male mice require further investigation. It is also possible that the microbiome in the same strain of mice but obtained from different vendors, or the same vendor from a different location, may alter our observed results. Further studies would be needed to determine how TUS to the abdomen may alter the gut microbiome and how that could contribute to altering the DSS colitis model. We were also limited by small sample sizes for immunohistological analysis in the spleen and MLN, resulting in lack of differences between cohorts with and without TUS. In future experiments, we plan to use fluorescently marked splenic α7nAChR4/α80+ cells to track them during the 14 days of the disease. We would also stain the colon samples for eosinophils, which might be participating in TUS effects. We need to further investigate the beneficial effects of TUS as a function of decreased colonic epithelial barrier permeability, since the animals presented firmer stools along with decreased levels of TNFα. Reduced expression of adhesion
molecules could also contribute to decrease trafficking of immune cells and future studies would be needed confirm this hypothesis. In addition, proteomic analysis in the DSS colitis model of the colon for splenectomised and α7nACHR KO mice treated with TUS could also provide insight into the lack of a therapeutic response. Lastly, further experiments should be done to investigate the response of vagotomised mice to TUS, clarifying the specific involvement of the VN.

The results of this study demonstrate that TUS treatment decreased the severity of colitis most likely via stimulation of the splenic nerve, leading to CAIP activation (Fig. 13). We observed that the proteomic and immune cell profile in the gut was altered by TUS with decreased clinical symptoms and reduced histological damage during the recovery phase of DSS colitis. TUS exposures in α7nACHR KO or splenectomised mice receiving DSS confirmed that the activation of the CAIP and the spleen/splenic nerve were involved in TUS therapeutic effects. Since TUS is a non-invasive technique that has been used in the clinic for decades, it may be possible to use such an approach as an adjuvant in combination with current IBD treatments, aiming to improve clinical outcomes and reduce morbidity in IBD patients. In addition, our study is the first step to a possible future development of a multiple transducer-based system for low intensity ultrasound. The system could be potentially worn by IBD patients to stimulate changes in their immunological profile through sonication. Further investigation needs to be done to understand the involvement of the VN, the effects of TUS in chronic DSS colitis and in combination with pharmacological-biological IBD treatments.

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Declaration of competing interests

The authors have declared that no conflict of interest exists.

Authors' contributions

Frank JA, Paz AH and Nunes NS assisted in research design. Nunes NS carried out the research. Nunes NS and Frank JA analysed data and wrote manuscript. Paz AH and Visioli F assisted with histological grading. Chandran P, Sundby M and Gonçalves FC assisted with immunohistochemistry analysis. Chandran P assisted with flow cytometry analysis. Burks SR assisted with proteomic analysis. All authors reviewed and approved the manuscript.

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