HHS Public Access

Author manuscript

Brain Stimul. Author manuscript; available in PMC 2023 July 10.

Published in final edited form as:

Brain Stimul. 2023 ; 16(3): 703–711. doi:10.1016/j.brs.2023.04.003.

Focused ultrasound neuromodulation of the spleen activates an anti-inflammatory response in humans

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Abstract

Focused ultrasound stimulation (FUS) activates mechanosensitive ion channels and is emerging as a method of noninvasive neuromodulation. In preclinical studies, FUS of the spleen (sFUS) activates an anti-inflammatory neural pathway which suppresses acute and chronic inflammation. However, the relevance of sFUS for regulating inflammatory responses in humans is unknown. Here, we used a modified diagnostic ultrasound imaging system to target the spleen of healthy human subjects with 3 min of continuously swept or stationary focused pulsed ultrasound, delivered at three different energy levels within allowable safety exposure limits. Potential anti-inflammatory effects of sFUS were assessed by measuring sFUS-elicited changes in endotoxin-induced tumor necrosis factor (TNF) production in whole blood samples from insonified subjects. We found that stimulation with either continuously swept or focused pulsed ultrasound has an

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2023.04.003.

anti-inflammatory effect: sFUS lowers TNF production for >2 h, with TNF returning to baseline by 24 h following sFUS. This response is independent of anatomical target (i.e., spleen hilum or parenchyma) or ultrasound energy level. No clinical, biochemical, or hematological parameters are adversely impacted. This is the first demonstration that sFUS suppresses the normal inflammatory response in humans, with potential implications for noninvasive bioelectronic therapy of inflammatory disorders.

Keywords

Inflammation; Cytokines; Ultrasound; Spleen; Cholinergic anti-inflammatory pathway; Bioelectronic medicine

1. Introduction

Diagnostic ultrasound is a safe, noninvasive technology widely used for visualizing deep anatomical structures. The physical basis of ultrasound technology relies on the production of mechanical pressure waves within tissues. Recent studies have shown that focusing ultrasound energy within the mandated safety exposure limits produces mechanical forces sufficient to activate mechanosensitive ion channels, e.g., TRPA1, TRP4, MEC-4, Piezo1 and two-pore-domain K+ [1–9]. As these channels are expressed in a cell- and tissue-specific manner, and ultrasound energy can be focused on defined targets, precise anatomical locales can be selectively stimulated in a noninvasive manner. For example, the activation of mechanosensitive channels in cell membranes depolarizes targeted individual neurons, leading to increased neuronal activity [10] and specific behavioral responses, including activation of eye [11] or limb movements [12], production of visual and somatosensory sensations [13,14], or relief from pain [15,16]. As abdominal organs receive extensive autonomic innervation, specific nerves controlling organ function can be precisely activated using focused ultrasound (FUS) at either the organ or sub-organ level [1,17–21]. For example, we recently showed in animal models of diabetes mellitus that FUS targeting sensory nerve fibers expressing mechanosensitive TRPA1 receptors within the porta hepatis of the liver modulates the transmission of glucose-related signals to the brain, thereby improving glucose homeostasis [1].

Additional evidence indicates that focused ultrasound can powerfully reduce inflammation. We and others have shown that FUS targeting the spleen (sFUS) activates the cholinergic anti-inflammatory pathway, a neuro-immune mechanism involved in the regulation of inflammatory processes [17,19,20]. In this pathway, neurons from abdominal celiac ganglia project axons to the spleen through the splenic nerve and terminate in the white pulp around splenic T cells. Upon signaling, these nerve fibers release norepinephrine (NE) which subsequently triggers the release of acetylcholine (ACh) from specialized splenic T-cells. Acetylcholine, in turn, inhibits the production of pro-inflammatory cytokines from splenic macrophages in an α7 nicotinic acetylcholine receptor (α7 nAChR)-dependent manner [22]. Mechanistically, targeting the spleen using FUS elicits splenic NE and ACh release, which inhibits interleukin 1β (IL-1β) and TNF production from splenic macrophages and suppresses inflammation in animal models of acute [18,20] and chronic inflammation [17].

Deficiency of acetylcholine producing T cells or of α7 nAChR in genetically engineered mice or pharmacologic ablation of the splenic neural network impairs modulation of TNF by sFUS [19], consistent with sFUS-induced suppression of inflammation through activation of the cholinergic anti-inflammatory pathway. Noninvasive sFUS suppresses cytokine responses to endotoxin to the same degree as invasive implant-based cervical vagus nerve stimulation (VNS), and at the same time lacks the cardiovascular and metabolic off-target effects of VNS [19].

Despite the abundant preclinical data, it is currently unknown whether sFUS in humans can activate the anti-inflammatory pathway and inhibit TNF production by monocyte. For example, the size of the human spleen and its distance from skin are significantly different than those in rodents, and both factors could limit sFUS efficacy. Here, we describe the first in-human modulation of the inflammatory response using non-invasive sFUS. In a preregistered study, we delivered sham or ultrasound stimulation to the spleen of healthy subjects, in either a continuously swept or focused mode, at a single or three different sub-organ locations, and at three power levels. To assess for potential anti-inflammatory effects, we measured TNF production by lipopolysaccharide-stimulated immune cells in whole blood samples obtained from the insonified subjects. We found that both continuously swept and focused ultrasound energy decreases TNF production compared to sham insonification, independently of sub-organ target location and ultrasound power. These findings demonstrate the ultrasound energy can be precisely targeted to the spleen to activate anti-inflammatory effects in humans.

2. Methods

2.1. Subjects

The study enrolled physically active, non-pregnant, healthy individuals between 18 and 45 years of age with a body mass index score of less than 29 and without a significant past medical history. The subjects were not on any medications and did not have any medical conditions that would render them at a greater risk of harm, interfere with the study intervention, or study results. Individuals were required to abstain from alcohol consumption starting 4 days prior to the ultrasound delivery and lasting through the follow-up visit; abstain from recreational drugs, tobacco, and nicotine product usage at least 1 month prior to the screening visit and throughout study participation; and refrain from food and drink (besides water) for at least 8 h prior to the baseline visit and follow-up visit and lasting until completion of each day's study procedures. The study was performed in accordance with the Declaration of Helsinki, the International Conference on Harmonization Good Clinical Practice Guidelines, and the applicable United States Code of Federal Regulations. The study was approved by the Northwell Health Institutional Review Board (FWA: 00002505). Written informed consent was obtained from all study participants prior to implementation of study procedures. The study was preregistered in http://www.clinicaltrials.gov: NCT03548116.

Study timeline.—Between April 2018 and January 2019, individuals who met initial eligibility criteria for enrollment based on the screening visit results (including medical

history, physical and neurological assessments such as demographics and vital signs, hematology and clinical chemistry blood results, and urine pregnancy test results for women of childbearing potential), were randomized into one of several groups. The study was carried out on two consecutive days (day 0 and day 1) (Fig. 1a). On day 0, a physical and neurological assessment was performed, and blood was collected for hematology, chemistry, catecholamines, and tumor necrosis factor (TNF) assessment ex vivo. The subjects then underwent the ultrasound procedure as per the group to which they were randomly assigned. Blood was collected at the 1 h and 2 h time points post-ultrasound intervention for ex vivo TNF assessment. Levels of catecholamines, hematology and chemistry were monitored to assess the safety of the procedure. On day 2, 24 h post ultrasound intervention, blood was collected for ex vivo TNF assessment and catecholamine measurement. Safety of the procedure was assessed by physical and neurological assessments such as vital signs and blood collection for hematology and chemistry.

2.2. Study design

Eligible individuals were initially randomized into 6 groups of 10 subjects each, for a total of 60 subjects. These groups were selected to evaluate the power and targeting dependency of sFUS. Groups 1–3 received sFUS at a single site, the hilum of the spleen (see Fig. 1B, Supplementary Fig. 1a), at the following energies (see Supplementary Table 1): continuously swept stimulation at 11.7 mW/cm^2/MI of 1.2 (group 1), focused pulsed stimulation at 225.4 mW/cm^2/MI of 0.7 (group 2, target depths <3.5 cm) or 174.8 mW/cm^2/MI of 0.846 (group 2, target depths >3.5 cm), and focused pulsed stimulation at 223.9 mW/cm^2/MI of 1.4 (group 3, target depths <3.5 cm) or 119.9 mW/cm^2/MI of 1.4 (group 3, target depths >3.5 cm). Groups 4–6 received sFUS at three sites, upper, middle, and lower third of the spleen (see Supplementary Fig. 1a), at the same corresponding energy levels to Groups 1–3 (see Supplementary Table 1). Based on the results from these six groups, an additional group of ten subjects (group 0) was added between November 2019 and March 2020 to evaluate sham ultrasound exposure. Subjects in this group had the same ultrasound probe placement and movements over the splenic location but did not receive any applied ultrasound energy (i.e., the ultrasound power to probe was turned off; Fig. 1b). All subjects (Groups 0–6) were blinded to the type of intervention received.

2.3. Imaging accuracy assessment

Ultrasound images were acquired and saved for every imaging frame and every push-pulse delivered during the complete dosing sessions. Offline analysis was performed to determine the accuracy of targeting of the delivered dose to the intended organ location. Targeting accuracy was affected by manual selection and placement of the region-of-interest (ROI) by the sonographer, variability of the subject's anatomy, and technical limitations of the modified software on the imaging console for controlling the depth and angle of the delivered push-pulses. (see Supplementary Figs. 1a, b, c).

2.4. Probe and region of interest (ROI) placement

Each subject was positioned in the right decubitus position with their left arm extended over their head. The ultrasound probe was placed intercostal between the lower ribs slightly posterior to the midaxillary line to locate the spleen within the displayed B-mode image.

The sonographers then adjusted the angle and depth of a ROI box on the displayed image to target the portion of the spleen intended to receive the stimulation dose. In group 0, subjects received similar imaging ultrasound movements with a disconnected probe (i.e., no ultrasound energy was transmitted from the probe). This was followed by simulated non-imaging ultrasound of the single splenic target with the disconnected probe. The connection point to the probe was hidden so that the subject was not aware of the probe status. In this group, the simulated ultrasound probe movements (with no delivered ultrasound energy) were made over the hilum for 3 repetitions of four 15-s-long intervals, with 15-s breaks between intervals and 2-min breaks between repetitions. Groups 1–3 subjects received imaging ultrasound to locate the single splenic target as shown in Fig. 1b. This was followed by continuously swept or focused pulsed ultrasound stimuli at the group-specific power level. Groups 4–6 subjects received imaging ultrasound to locate the three splenic targets as shown in Fig. 1b. This was followed by continuously swept or focused pulsed ultrasound stimuli at the group specific power level.

2.5. Subject variability

The subjects varied in spleen size, shape, and depth from the body surface. Based on the shape and size of the individual's spleen, the sonographers determined the best placement of the probe and ROI for the particular dose to be delivered. The distribution of the depth of the hilum target ranged from 4 to 8 cm in subjects from groups 1–3, (Supplementary Fig. 1b.i). For groups 4–6, the range of the lower, middle, and upper depths varied from approximately 2 cm–9 cm (Supplementary Figure 1b, ii, iii and iv). Inter-subject variability of probe placement and ROI selection was quantified and reported in Supplementary Table 2.

2.6. Software configuration

The software configuration on the LOGIQ E9 with the C1–6-XDclear curvilinear array probe (GE HealthCare, Milwaukee WI) was modified to deliver the push-pulses for research study purposes with the Elastography Mode of the commercially available system (see Supplementary Table 1). Within this software setting, the sonographer positioned a single ROI box on the screen using the track ball interface, the ultrasound system delivered the push pulses to either 2 or 3 simultaneous focal spots depending on the depth of the center of the ROI placement, based on the available Elastography Mode software settings. If the depth of the center of the ROI was less than 3.5 cm, the ultrasound system produced 3 simultaneous focal spots at a fixed depth of 3 cm with one focal spot aligned to the angle of the ROI and two other focal spots, one on the left of the ROI box and one on the right (Supplementary Fig. 1c.i.). If the depth of the center of the ROI was greater than 3.5 cm, the system produced 2 simultaneous focal spots at a fixed depth of 6 cm with one focal spot on the left of the ROI box and one on the right (Supplementary Fig. 1c.ii.).

The location of each focal spot delivered was determined in an offline imaging accuracy assessment of each saved image frame (corresponding to each push-pulse delivered). The number of focal spots delivered to the splenic tissue or falling outside of the boundary of the splenic tissue were qualitatively scored. A single imaging accuracy score was determined as the percent of ultrasound pulses delivered to the splenic tissue for each subject (see

Supplementary Table 2). The accuracy of the focal spot placement was scored in a blinded manner by two observers. For example, the focal spots intended to be delivered to the middle of the spleen as shown in Supplementary Fig. 1c.iii would be scored at 100% with all of the focal spots falling into the spleen tissue and the focal spots intended to be delivered to the hilum as shown in Supplementary Fig. 1c.iv would be scored at 50% with one of the focal spots falling into the spleen tissue and the other falling to the lower right region outside the spleen.

2.7. Whole blood assay

Two different methods were utilized for whole blood assay. In the first method ("Custom method", Fig. 1c), venous blood was drawn into sodium heparin tubes, immediately aliquoted (hold time did not exceed 1 h) and stimulated with endotoxin. Endotoxin [lipopolysaccharide (LPS), *Escherichia coli* 0111:B4, Sigma Aldrich, USA) was re-suspended to 5 mg/ml, sonicated for 30 min, vortexed, and diluted with phosphate buffered saline (PBS) to generate a working 1 mg/ml stock. This stock was serially diluted with 1x PBS to final concentrations of 0 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml in 500 μL blood aliquots. Microfuge tubes aliquoted with blood and endotoxin were incubated on a rocking platform at 37 °C with 5% CO_2 for 4 h. In a second method (Fig. 1d), a separate aliquot of blood was assessed using the TruCulture® system (Myriad RBM, USA). Venous blood was drawn intoTruCulture tubes directly containing endotoxin at 0 ng/ml (NULL tube) or 100 ng/ml and incubated at 37 °C for 4 h. After a 4 h incubation in both methods, plasma was collected by centrifugation [5 min, 2000 g (5000 rpm in Microfuge 5415C; Brinkmann, Westbury, NY)] and frozen at −20 °C for future analysis. All samples were performed in duplicate. TNF levels in the plasma were quantitated using commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

2.8. Laboratory assessments

Laboratory assessments were performed on specimens collected at different time points as indicated. Serum and plasma were aliquoted and stored at −80 °C until analysis. Commercial laboratory assessments including blood chemistry, cortisol, hemoglobin, and catecholamines were conducted at the Northwell Core Laboratories, Manhasset, NY. Heart rate, blood pressure, and blood oxygen (O_2) were monitored at each visit.

2.9. LPS-induced acute inflammation model in rats

To determine appropriate experimental conditions and assay parameters before subjecting human subjects to the experimental protocol, experiments were performed using adult male Sprague–Dawley rats 8–12 weeks old (250–300 g; Charles River Laboratories). These were housed at 25 °C on a 12-h light/dark cycle and acclimatized for 1 week, with handling, before experiments were conducted to minimize potential confounding measures due to stress response. Water and regular rodent chow were available ad libitum. Experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of GE Global Research. LPS (from *Escherichia coli*, 0111: B4; Sigma–Aldrich) was administered to animals (10 mg/kg), which corresponds to an approximate LD_{75} dose, via intraperitoneal (IP) injection. Spleen and blood samples were harvested 90 min after LPS administration. Spleen was homogenized in PBS containing phosphatase (0.2

mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 mM benzamidine, 1-mM sodium orthovanadate, and 2 μM cantharidin) and protease (1 μL per 20 mg of tissue as per Roche Diagnostics) inhibitors. A targeted final concentration of 0.2 g tissue per ml PBS solution was applied in all samples. Blood samples were stored with the anticoagulant disodium (ethylenedinitrilotetraacetic acid; EDTA) to prevent coagulation of samples. Samples were then stored at −80 °C until analysis. Samples were analyzed by ELISA for changes in TNF (Abcam). Ultrasound stimulation in the rodent endotoxemia model was performed as previously described.[29]

2.10. Whole blood assay in rats

Naïve Sprague Dawley rats were anesthetized with 2% sodium isoflurane and subjected to ultrasound stimulation as previously described[29], except stimulation was performed on LPS naïve animals. In addition, instead of using the previously reported pre-clinical single element ultrasound transducer for stimulation[29], the LOGIQ E9 with the C1–6-XDclear curvilinear array probe was utilized by placing a gel ultrasound standoff between the animal and the probe. The thickness of the stand-off was sized such that the 6.5 cm focal depth aligned with the depth of the spleen (which could be visualized during image-based alignment of the probe). Peripheral blood was collected in sodium heparin tubes before and after intervention, in both shamand sFUS-exposed animals. LPS (*Escherichia coli* 0111:B4) was suspended to 5 mg/ml, sonicated for 30 min, vortexed, and diluted with PBS to generate a working 1 mg/ml stock. Working stock was used to generate serial dilution samples with 1x PBS to final concentrations of 0 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml in 500 μL blood aliquots. Microfuge tubes aliquoted with blood and endotoxin were incubated on a rocking platform at 37 °C with 5% CO_2. All samples were performed in duplicate. TNF levels in plasma were quantitated using commercial ELISA (R&D Systems).

2.11. Statistical analysis

All statistical analyses were conducted by the R software (version 3.6). The Mixed ANOVA analysis performed on the primary outcome and safety biomarker measures was conducted using the rstatix R package. The Wilcoxon rank-sum test, a non-parametric statistical hypothesis test, was used to compare any two related sample distributions unless otherwise stated.

3. Results

3.1. Splenic focused ultrasound suppresses TNF production in response to in vivo and ex vivo LPS challenge in rats

We have previously shown, in anesthetized rats, that 3 min of continuous splenic focused ultrasound (sFUS) suppresses LPS-stimulated pro-inflammatory cytokine production.[17] However, to maintain targeting of the spleen in awake humans, sFUS has to be administered over relatively short periods delivered during breath holds. To determine whether a total 3 min of several shorter stimulating periods are sufficient to activate an anti-inflammatory response, we performed preclinical studies in rats.

After intraperitoneal administration of LPS (10 mg/kg; LD75 dose), animals were subjected to total of 3 min of sFUS applied in several 15- or 30 s-long periods, interspersed with 30 s-long periods with the ultrasound turned off. Whole blood and spleen samples were collected 90 min after sFUS, at which timepoint peak TNF levels are expected following LPS challenge[17]. We observed that sFUS, delivered in either 15- or 30-s long periods, significantly suppresses splenic (Supplementary Fig. 2a) and blood (Supplementary Fig. 2b) TNF levels, compared to shamtreated animals. Therefore, the anti-inflammatory effect of intermittent sFUS delivered over short periods is comparable to that of the continuous 3 min treatment previously reported[17].

Next, we used the custom ex vivo whole blood assay to evaluate the anti-inflammatory effects of sFUS administered to healthy animals not challenged with LPS in vivo. Animals were subjected to either sham ultrasound or intermittent sFUS delivered over 3 min at an estimated ~375 mW/cm^2. Peripheral blood was collected and incubated with increasing concentrations of LPS to stimulate the production of TNF by monocytes for 4 h (as shown previously[11] and used herein in the clinical study). sFUS significantly attenuates whole-blood TNF production in the ex vivo cytokine assay compared to the sham stimulated group (Supplementary Fig. 2c).

3.2. Enrolment of subjects in human study

To determine whether ultrasound energy (either continuously swept or pulsed FUS) targeting the spleen affects TNF production in humans, seventy healthy subjects were recruited between April 2018 and March 2020. Baseline characteristics of the study population are shown in Table 1. The average age was 29.6 years with 36 (51%) males and 34 (49%) females. Subjects were randomized to one of 7 groups (Suppl. Table 1). Subjects assigned to groups 1–3 received imaging ultrasound to locate the single splenic target, the hilum of the spleen (Suppl. Figure 1a). This was followed by continuously swept (group 1) or focused pulsed ultrasound stimuli at the group-specific power level (group 2: low mechanical index (MI), group 3: high MI), all applied to the hilum of the spleen (Fig. 1b). Subjects assigned to groups 4–6 received imaging ultrasound to locate the 3 splenic targets (Suppl. Figure 1a). This was followed by continuously swept (group 4) or focused pulsed ultrasound stimuli at the group specific power level (group 5: low MI, group 6: high MI) (Fig. 1b). Subjects assigned to group 0 were subjected to a sham procedure with the machine turned off. Subjects of Group 0 were enrolled en bloc, in a non-randomized manner, as a true sham group, after subjects in groups 1–6 had completed the study and analysis showed an effect on TNF production in all insonified subjects. Based on the analysis of the imaging data, it was confirmed that each subject indeed received the assigned intervention based on their assigned group. All enrolled subjects completed the study. During analysis of the primary endpoint measures, nine subjects were excluded (Supplementary Table 3). The sixty-one included subjects had a mean (± SD) age of 30.2 ± 6 years, mean body mass index of 23.8 ± 2.4 kg/m^2, and mean physical activity of 6.8 ± 4.3 h (Table 1).

3.3. Splenic focused ultrasound suppresses TNF production in whole blood samples in humans

Changes in inflammatory response were evaluated using 2 ex vivo whole blood assays.[11] First, venous blood was collected immediately before (baseline) and at 3 time points after intervention (1-h, 2-h and 24-h post-ultrasound stimulation). Then, samples were "challenged" in vitro with LPS using either a custom assay (Fig. 1c), at 5 different concentrations of LPS (from 0 to 100 ng/ml), or a commercially available assay, i.e., TruCulture®, with either 0 or 100 ng/ml LPS concentration (Fig. 1d).

Whole blood challenge with LPS results in a concentration-dependent increase in TNF levels, measured using the custom method, in all groups and at all time points (Supplementary Fig. 3; examples of individual subject data are shown in Fig. 2a and b). The inflection point of the dose-response curve is at LPS concentration of 1 ng/ml (Supplementary Fig. 3), which was used in subsequent analyses. To quantify the effect of sFUS on TNF levels in an individual subject, fold change in TNF levels at a given time point was calculated by subtracting the logarithm to base 2 (log2) TNF values (in response to 1 ng/ml LPS) at different time-points from baseline log2 TNF levels (Fig. 1e; Fig. 2c and d).

The effect of sFUS on TNF levels assessed using the custom whole blood assay in different treatment groups is presented in Fig. 2e–g. We observed no significant change in TNF production in the sham-stimulated group (Group 0) at any time point compared to baseline (Fig. 2a and c; Fig. 2e–g). At 1- and 2-h time points, TNF is significantly suppressed in groups 1–3 (i.e., groups in which sFUS was delivered for 3 min at a single site of splenic hilum), with a trend for suppression in groups 4–6 (i.e., groups in which sFUS was delivered for 3 min sequentially at three different locations within the spleen) (Fig. 2e and f, Table 2). In all ultrasound-stimulated groups, TNF production returns to baseline levels by the 24-h time point (Fig. 2g). In two-way mixed ANOVA, a significant and independent effect of treatment group on TNF level is observed in samples exposed to 1 ng/ml LPS, but not in samples exposed to other LPS concentrations, as part of the custom assay (Table 2). A significant and independent effect of time point after sFUS is observed in samples exposed to any LPS concentration (except 0 ng/ml) (Table 2). These findings indicate that 3 min-long ultrasound using either continuous (Group 1) or pulsed sFUS targeted at the spleen at a single site (Groups 2, 3) inhibits LPS-induced TNF production in healthy subjects.

Next, we carried out post-hoc, pairwise comparisons of TNF levels measured with the custom assay. Compared to the sham-stimulated group (Group 0), a significant suppression of TNF is observed in groups in which stimulation occurred at a single site (Groups 1–3), and a trend for suppression in groups in which the stimulus was spread across 3 splenic locations (Groups 5–6). In pairwise comparisons between single ultrasound-stimulated groups (Groups 1–6), no significant differences in TNF levels are observed at any time point after the intervention (Supplementary Fig. 4). In addition, no difference in TNF levels at the time of maximal response (2-h time point) is observed in pairwise comparisons between all subjects that received continuously swept (pooled groups 1 and 4), targeted pulsed low-power ultrasound (pooled groups 2 and 5) and targeted pulsed high-power ultrasound (pooled groups 3 and 6) (Supplementary Fig. 12). These findings indicate that the effect of

ultrasound on TNF is independent of the type (i.e., continuously swept vs. pulsed targeted) or power level of insonification (i.e., low vs. high mechanical index).

Having found no significant effects between the different ultrasound stimulation groups, we decided to pool all the stimulated groups (Groups 1–6) together and compare the effect of ultrasound stimulation vs. sham stimulation (Group 0). Comparable TNF levels are observed between sham stimulated group and the pooled ultrasound group at baseline (Fig. 3). In contrast, sFUS results in significant reduction in TNF levels, compared to sham, at 1-h and 2-h post-stimulation time points; TNF levels in the sFUS and sham groups are no different at 24-h post-stimulation (Fig. 3). A time-dependent decrease in TNF from baseline is observed at 1-h and 2-h post-stimulation in the sFUS group and TNF levels return to baseline levels at 24-h post-ultrasound. In contrast, TNF in the sham group does not change with time (Fig. 3). Notably, differences in TNF levels are significant only at the responsive point of the LPS dose-response curve (i.e., at 1 ng/ml LPS concentration) (Supplementary Fig. 3): a significant difference in TNF levels between sham and sFUS groups is observed at an LPS concentration of 1 ng/ml in the custom assay, but not at lower (0.1 ng/ml) or higher LPS concentrations (10, 100 ng/ml) at any of the time points analyzed (Supplementary Fig. 5).

In addition, we evaluated the utility of a commercially available whole blood assay, the TruCulture® method, to assess the effects of splenic sFUS on TNF levels. TruCulture tubes are pre-loaded with cell culture media and the inflammatory stimulant (LPS). Peripheral blood was loaded directly into two sets of TruCulture tubes containing either no endotoxin (Null tube) or 100 ng/ml endotoxin (LPS tube) and tubes incubated at 37 °C for 4 h; TNF was measured using ELISA, similarly to the custom assay. At baseline, comparable TNF levels are observed between sham-stimulated group and the (pooled) ultrasound-stimulated group (Supplementary Fig. 6). In contrast to the custom whole blood assay, the TruCulture method detects no significant difference in TNF levels after ultrasound stimulation (Supplementary Fig. 6). A two-way mixed ANOVA analysis (Table 2) showed that only time represents a significant independent variable ($p = 2 \times 10^{-4}$) with no significant effect of treatment group ($p > 0.05$). This is in agreement with the lack of significant differences between groups in the custom assay at the comparable LPS concentration (100 ng/ml) (Table 2).

3.4. Splenic focused ultrasound does not produce meaningful changes in clinical chemistry or hematology tests

To assess the safety profile of sFUS, we measured several clinical and laboratory parameters at baseline and at different time points post-stimulation, in all treatment groups (Table 3). None of the subjects reported perception of the sFUS stimuli. Most of the analyzed parameters, including heart rate, electrolytes, and hematological and biochemical markers are not affected by either the type of insonification (i.e., group 1–6) or the application of ultrasound energy or the post-stimulation time point (Table 3). Conversely, statistically significant changes are observed in some of the parameters at different time points (Table 3), including hemoglobin ($p = 4.1 \times 10^{-4}$) and aspartate aminotransferase ($p = 0.02$) (Supplementary Figs. 7 and 8). Blood glucose ($p = 4.9 \times 10^{-5}$), carbon dioxide ($p = 0.012$) and cortisol ($p = 5.4 \times 10^{-13}$) levels were significantly reduced at 1-h and 2-h

post-stimulation time points relative to baseline (Supplementary Figs. 9, 10, and 11). Most of these variables have prominent diurnal variations (nadir at midnight), e.g., hemoglobin, carbon dioxide, cortisol and glucose, so these changes were deemed not clinically significant by the clinical team, as average values remained within normal clinical limits. Furthermore, no adverse events were reported during the study.

4. Discussion

To our knowledge, this is the first-in-human study to assess the capability of non-invasive ultrasound stimulation of the spleen (sFUS) to suppress pro-inflammatory cytokine production. We show that targeting the spleen using either continuously swept or pulsed focused ultrasound stimuli significantly reduces endotoxin-stimulated TNF production in whole blood samples obtained from insonified subjects. Furthermore, this effect is independent of ultrasound intensity when targeting a single splenic site, i.e., the hilum, for 3 min (Supplementary Table 1). The anti-inflammatory effect was found to be less pronounced when the stimulus was spread across 3 different splenic locations, delivered for 1 min at each site. Importantly, this first clinical feasibility test of the anti-inflammatory effects of sFUS appears to be safe, as no adverse effects on several clinical, biochemical and hematological parameters (Table 3) were observed in any intervention group.

The effect of sFUS on TNF production in humans is consistent with that in animals [17,18,20,23], and can be explained by activation of a neuroimmune pathway at the spleen termed the cholinergic anti-inflammatory pathway. Several previous studies have targeted the neuroimmune pathway at the spleen using sFUS (Supplementary Table 4). Previously, the Okusa group demonstrated that activation of that pathway using continuously swept ultrasound stimuli, i.e., using a standard ultrasound imaging protocol, prevented renal ischemia reperfusion injury in a mouse model of acute kidney injury [24,25]. Additionally, the Lim group recently reported that sFUS reduces disease severity in a mouse model of inflammatory arthritis [17]. Our group has followed a different stimulation technique, in which image-based targeting is used to localize pulsed ultrasound stimuli to sub-organ splenic locations [19]. In that animal study, splenic ultrasound was found to have a virtually equivalent, dose-dependent effect on TNF suppression in acute endotoxemia as electrical vagus nerve stimulation [19]. Splenic ultrasound induced a dose-dependent increase in splenic norepinephrine and acetylcholine levels, whereas catecholamine depletion or blocking of α7nACh receptor, both essential signaling steps in the cholinergic anti-inflammatory pathway, prevented cytokine suppression by ultrasound [19].

Consistent with preclinical data, the results of the current study show that sFUS in humans is capable of inhibiting the TNF response over a range of ultrasound energy intensities (i.e., 11.7–225.4 mW/cm^2). These ultrasound parameters are available within imaging (continuously swept) or elastography (focused pulsed stimuli) settings on the LOGIQ E9 with the C1–6-XDclear curvilinear array probe, and within the FDA limits of exposure (i.e., <720 mW/cm2). Although TNF tends to be suppressed in all insonified groups (1–6), the effect was statistically significant only in the 3 groups in which the 3-min stimulus was applied to a single location (i.e., the splenic hilum). This result has precedence in previous preclinical studies; experiments in which ultrasound energy was swept over the

entire spleen have required longer stimulation times to elicit activation of the cholinergic anti-inflammatory pathway [17,24, 25], while studies in which ultrasound was targeted to a single location within the spleen required as little as 1 min of stimulation [1,18,19,23]. These results suggest that a dose-response study (i.e., on volume of tissue stimulated versus stimulus duration) with more subjects is warranted.

Although the mechanism of ultrasound neuromodulation of peripheral nerves is still under investigation, previous studies have established differential excitatory and inhibitory effects of ultrasound stimulation depending on the intensity and duration of insonification, both in vivo and ex vivo [26,27]. Recently, we demonstrated dependence of ultrasound-induced neuromodulation on activation of TRPA1 channels, a type of mechano-sensitive ion channel, in animals, and in neuron cultures [1]. The Shapiro group has shown that direct excitation of cultured neurons by ultrasound pulses is dependent on calcium-selective mechanosensitive ion channels [2], while the Chalasani group showed the ability to confer ultrasound sensitivity to mammalian cells by expressing the human homolog of TRPA1 [28]. However, in addition to neurons, multiple other cell types express TRPA1 ion channels, including T cells and macrophages, which are involved in regulation of cytokine production and immune function [29]. For example, activation of TRPA1 in $CD4^+$ T cells is known to improve experimental colitis [30]. As these cells are components of the cholinergic anti-inflammatory pathway, it is possible that ultrasound may also directly modulate these cells to activate anti-inflammatory effects. Further studies will be needed to evaluate this possibility.

This preregistered, randomized, controlled study was designed to examine whether spleen ultrasound has an anti-inflammatory effect, by lowering the LPS-induced TNF response in whole blood, and whether the method of delivery (continuously swept or focused pulse, at high and low mechanical index) and the insonified sub-organ location (hilum or multiple sites of the spleen parenchyma) impact the TNF-suppressing effect. One limitation of the study is that the sham group, in which no ultrasound energy was delivered to the spleen, was added to the study en bloc (in a non-randomized manner), months after data collection from the first 6 groups was completed and analysis of the results showed that a TNF-lowering effect was present in all insonified groups. The level of baseline TNF (before intervention) in the sham group was no different than that in the insonified groups (Fig. 3), indicating that the different time at which TNF measurements occurred in the sham group did not impact the results. The clinical staff that interacted with patients in the sham group was the same as with the insonified groups, which minimizes variability introduced by differences in the clinician-patient interaction. As expected, sham insonification had no effect on TNF (Fig. 3).

Another limitation of the study is that TNF was the only cytokine on which the effect of sFUS was assessed. For example, several pro-inflammatory cytokines are suppressed by activation of the neuro-immune pathway at the spleen, including IL-1beta, IL-6 and IL-8 [31]. In this study, we focused on a single cytokine outcome, LPS-induced production of TNF, for several reasons. First, TNF is an essential, and the best characterized, pro-inflammatory cytokine released by immune cells in the spleen in response to an acute inflammatory stimulus, including LPS [32]. In human immune phenotyping studies, TNF in response to LPS was among the cytokines with the largest fold-change and the smallest spread in values among individuals [33]. Second, the time course of TNF production in

response to LPS is well-characterized in healthy individuals [34]. That time course was used during the design of our study to specify the time points at which samples would be collected. In contrast, the time course of other inflammatory cytokines is more extended and variable, and would not be sampled appropriately by the time points chosen in this study [35]. Third, several preclinical studies have established that the mechanism of action of anti-inflammatory neuromodulation therapies that target the neuroimmune pathway at the spleen, including vagus nerve stimulation and spleen focused ultrasound (sFUS), includes suppression of the release of TNF from myeloid cells in the spleen [18,22]. In our own preclinical studies in which sFUS was administered around an inflammatory stimulus, we have observed robust suppression of TNF, whereas suppression of other cytokines was less robust ([19,20], and Supplementary Fig. 2 of this study). Future studies, possibly with larger sample sizes due to the required statistical corrections for multiple comparisons, will be needed to establish the effects of sFUS neuromodulation on different pro-inflammatory and anti-inflammatory cytokines released from white blood cells.

Notably, we found that the TNF-suppressive effect of sFUS depends on the sensitivity of the ex vivo assay used to trigger the release of TNF in whole blood samples. When different LPS concentrations were tested, only 1 ng/ml LPS concentration is found to be the inflection point of the TNF response, while lower and higher LPS concentrations fall within the saturation range of the dose-response curve (Supplementary Fig. 3) and therefore may not allow the detection of the effect of sFUS on TNF production (Table 2). Similarly, the TruCulture assay, which uses a pre-determined LPS dose of 100 ng/ml, does not detect the effect of sFUS on TNF production, even though it reports an effect of time point of measurement, in both ultrasound- and sham-stimulated subjects (Table 2, and Supplementary Fig. 6). It is important to appreciate that the incubation time of the assay is a critical parameter which was not extensively explored in our study. In its current form, the TruCulture assay has been developed to determine maximum (total) cytokine secretion for a given stimulus, hence the long (24–48 h) recommended incubation time. In this study, we were interested in LPS signaling of a sub-maximum secretion rate which is likely more sensitive to neuromodulatory influences. This was achieved by a short incubation time after a moderate LPS signal. The TruCulture system could potentially assess ultrasound-mediated effects using very short incubation times, but this approach would likely have increased potential variability due to procedure-related slight differences in very short incubation times. The fact that the effect of sFUS on TNF production was detected in only one concentration of the LPS stimulus of the custom assay, but not in other LPS concentrations or in the TruCulture assay, is a major limitation of our study. Despite that limitation, the prospective, randomized, controlled and double-blinded study design, and the 4- to 5-fold suppression of TNF in the insonified groups compared to sham, creates confidence that the effect of sFUS is robust and, potentially, clinically significant.

We found that sFUS delivered at intensities at or below those used in abdominal imaging studies for just a few minutes causes a 4- to 5-fold reduction in TNF production from white blood cells. The effect size is likely to be clinically significant. For example, LPS-induced TNF production in whole blood samples from rheumatoid arthritis patients receiving electrical VNS stimulation was reduced by ~50% and was highly correlated with change in the disease activity score [36]. We also found that production of TNF returns to

pre-insonification baseline within less than 24 h, at least after a single session of sFUS. That suggests that, in practice, in chronic diseases, sFUS may need to be administered multiple times a week for a sustained anti-inflammatory effect. Future studies will determine whether further optimization of sFUS parameters, especially treatment frequency, can attain a larger and more long-lasting anti-inflammatory effect.

In summary, the results of the current study show that splenic ultrasound insonification can activate an anti-inflammatory response in healthy human subjects. The magnitude of this biological effect, measured by whole blood TNF production in response to ex vivo LPS, is similar to that previously reported by activation of the neuroimmune pathway at the spleen using invasive vagus nerve stimulation [36]. In addition, sFUS allows anatomically-specific activation of the same neuroimmune pathway without the off-target effects of VNS, thereby potentially providing a human-relevant method for precision autonomic neuromodulation [37]. Given the good safety profile and the noninvasive nature of spleen ultrasound stimulation, we suggest that sFUS should be further tested as an organ-targeted, non-invasive bioelectronic medicine alternative to implant-based VNS, and as potential therapy of chronic diseases in which inflammation is implicated, such as rheumatoid arthritis [36,38], irritable bowel disease [39], pulmonary hypertension [40] or to diagnose infectious conditions [20].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Conflict of interest declaration

JG, KW, VC, JA, and CP are employees of General Electric and declare that GE has filed US and international patent applications describing methods, devices and systems for precision organ-based ultrasound neuromodulation. SZ, JP, DN, RR, JM, MA, MB, KJT and SSC have received research funding from GE to investigate the effects of ultrasound on inflammatory responses in humans and in animal models. KJT holds patents broadly related to this work. He has assigned all rights to the Feinstein Institutes for Medical Research.

Abbreviations:

sFUS	splenic focused ultrasound stimulation
TNF	tumor necrosis factor
LPS	lipopolysaccharide

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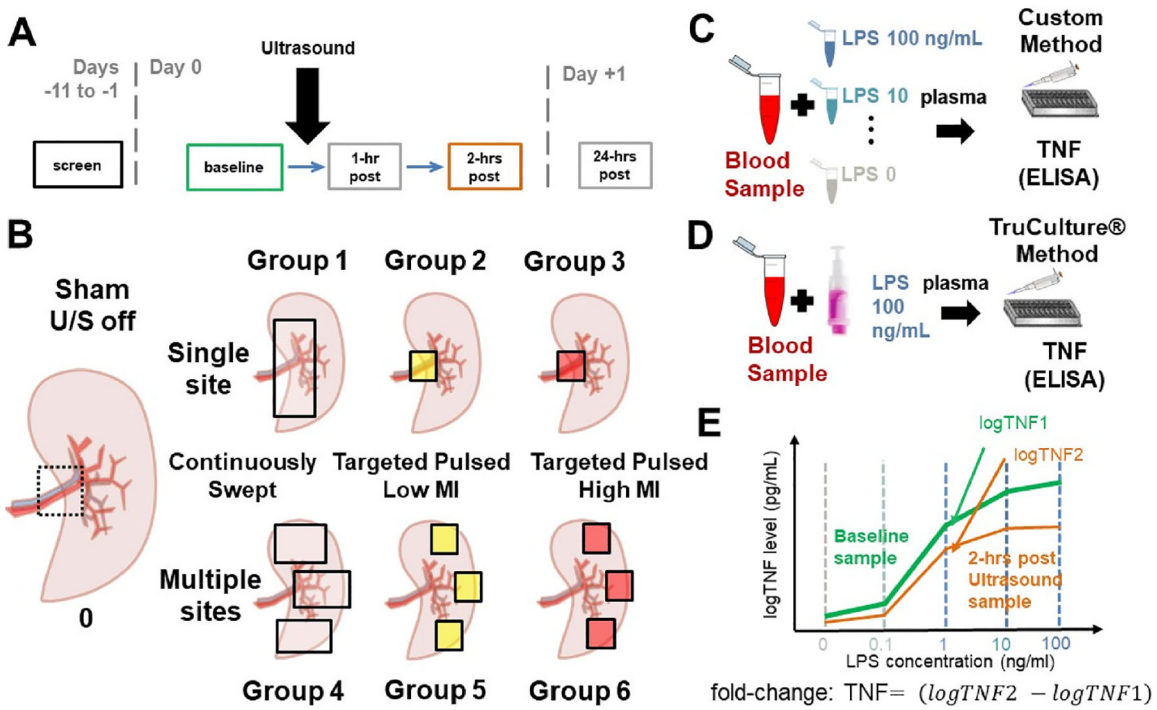

Fig. 1. Study methodology.

(**A**) Study design. Each subject was screened and randomly assigned to one of 6 groups, 1–11 days before the day of intervention; a seventh sham group was added at a later time. On the day of intervention (day 0), each subject interacted with investigators 4 times: at baseline, during which time a blood draw occurred and a physical exam (PE) was administered, at the time of intervention (i.e., ultrasound or sham), during which time the intervention corresponding to the assigned group was administered, and at 1- and 2-h post-intervention, during which time blood draws occurred. Approximately 24 h after the intervention, subjects were evaluated again, during which time a blood draw occurred and a PE was administered. (**B**) Intervention groups. Group 0: U/S machine was turned off for a sham intervention, with the probe placed on the abdomen over the spleen. Groups 1, 2, 3: spleen focused ultrasound (sFUS) was delivered at a single site, the hilum of the spleen, using continuously swept stimulation (group 1) or focused pulsed stimulation (group 2: low mechanical index (MI) pulses, group 3: high MI pulses) at the energy levels reported in Supplementary Table 1. Groups 4, 5, 6: sFUS was delivered sequentially at multiple sites, lower, middle and upper third of the spleen, with the same respective stimulation type and energy levels as groups 1, 2, and 3 (see Supplementary Table 1). (**C**) Whole blood assay: Custom method. A whole blood sample was collected and mixed with LPS solutions at different concentrations (0, 0.1, 1, 10 and 100 ng/ml). LPS induces release of TNF from macrophages in the blood sample. The amount of TNF released depends on the concentration of the LPS solution and on the activation status of macrophages at the time of blood collection, which is hypothesized to be affected by the sFUS intervention. TNF levels are measured in the plasma from each of the mixed blood-LPS samples using an ELISA method. (**D**) Whole blood assay: TruCulture method®. The blood is drawn directly into the TruCulture tubes and mixed with a fixed concentration of LPS solution (100 ng/ml).

TNF levels are measured in the plasma using the same ELISA method. (**E**) Method for calculating changes in TNF levels from baseline. TNF levels are measured at different LPS concentrations, at the baseline time point (green curve). TNF levels are again measured, at the same LPS concentrations, at the 2-hr post-intervention time point (orange curve). Change in logTNF (fold-change) was defined as the difference of logTNF (at 1 ng/ml LPS concentration), between the 2-hr post and baseline samples, normalized by logTNF at baseline.

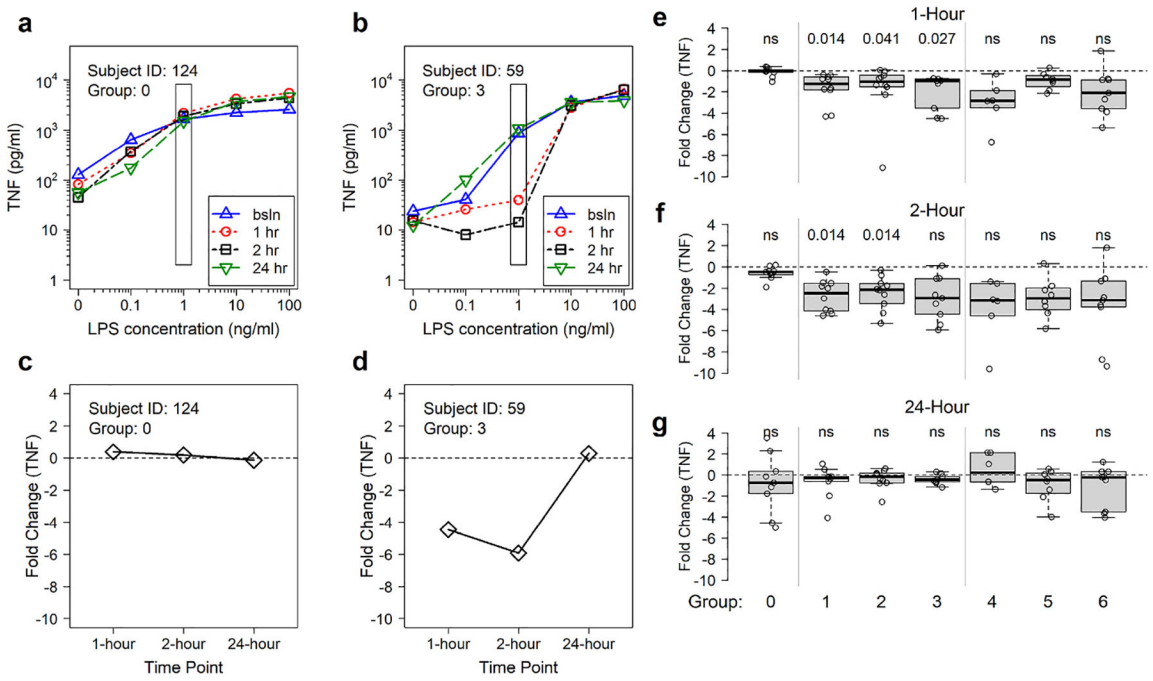

Fig. 2. TNF levels *in* different intervention groups, at different time points *after sFUS*.

(a–b) Examples of TNF levels in (a) a subject from group 0 (ID 124, sham intervention), and (b) another subject from group 3 (ID 59, 1 site, full power) measured at different LPS concentrations (0–100 ng/ml), in samples taken at different time points relative to intervention (at baseline (bsln) and 1, 2 and 24 h after intervention). LPS concentration of 1 ng/ml, denoted here with a box, is the inflection point of the LPS-TNF dose-response curve (see Suppl. Fig. 3) and was used in subsequent analyses. **(c–d)** Fold-change from baseline in log2 TNF level measured at 1 ng/ml LPS concentration at 1-, 2- and 24-h time points, in (c) subject ID 124 and (d) subject ID 59. **(e–g)** Overall fold-change of TNF level from baseline at (e) 1-h, (f) 2-h, and (g) 24-h after intervention, for all treatment groups. Each box and whisker plot represents the median as thick bold horizontal line, interquartile range as the box. The upper whisker is defined as the lesser of either maximum data value or the third quartile plus 1.5 times the interquartile range. The lower whisker is defined as the greater of either the minimum data value or the first quartile minus 1.5 times the interquartile range. The horizontal dashed line is at a zero-fold change (i.e., baseline value). Numbers above each box present p values from the Wilcoxon Sign test of the *f*old change in TNF between the timepoint and baseline. The p values have been corrected for multiple comparisons by the Bonferroni method. ns denotes a p value that is greater than 0.05. The number of subjects was 9, 10, 10, 9, 6, 8, and 9 for group*s* 0, 1, 2, 3, 4, 5, 6 respectively. Each group originally had *10* subjects, but a total of 9 subjects were excluded from the analysis of the primary endpoint measures (Supplementary Table 3).

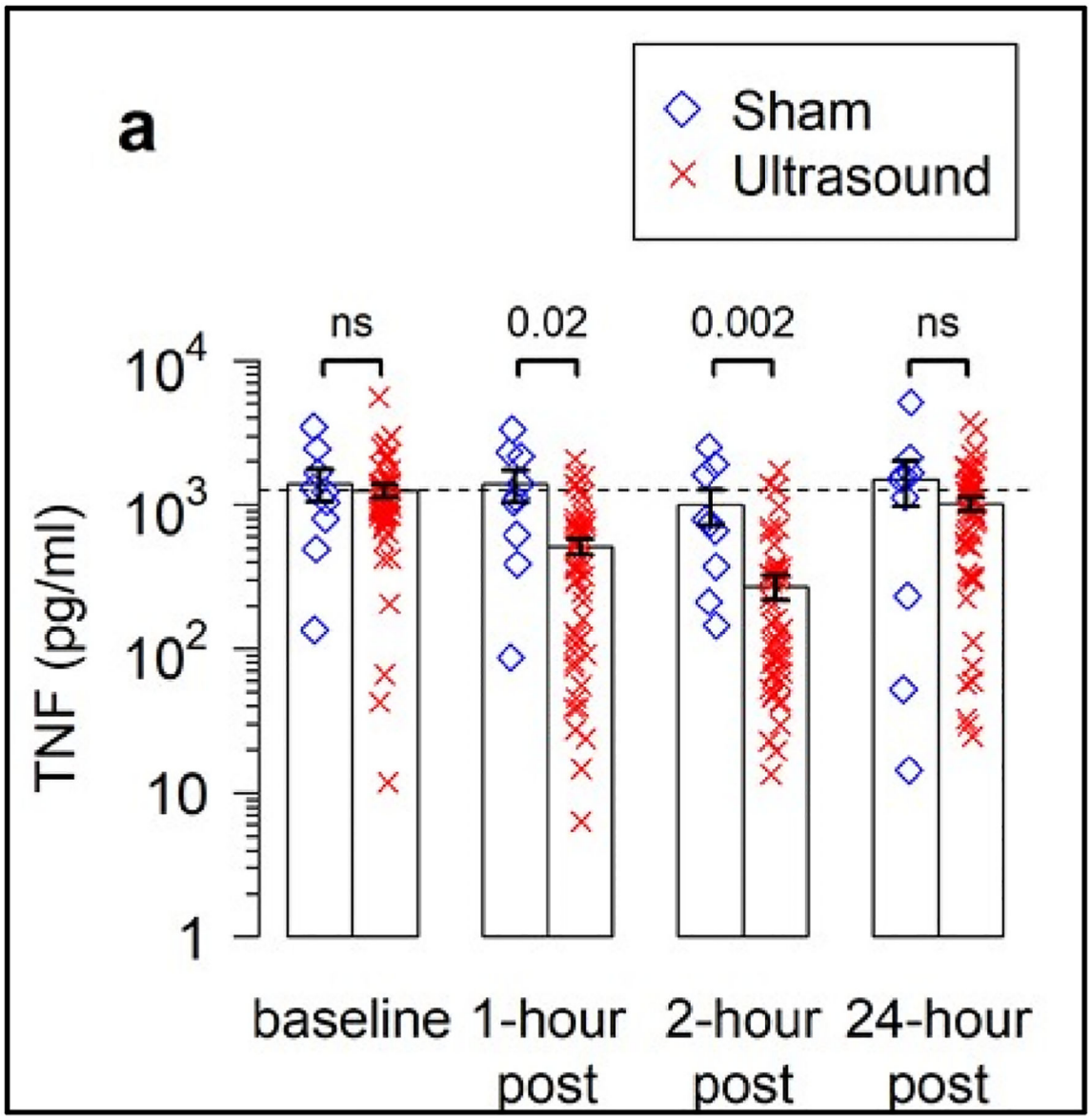

Fig. 3. TNF levels after sham vs. ultrasound stimulation of the spleen.

TNF levels from custom ex vivo 1 ng/mg LPS challenge for subjects in the sham group (N = 9) and in the stimulated group, i.e., the 6 ultrasound intervention groups combined (N = 52), at 4 time points (baseline, 1-h, 2-h, and 24-h post-intervention). Numbers above bars represent p values of comparing measures between the two groups at each time point (Wilcoxon signed rank test, corrected for multiple comparisons).

Table 1

Demographic characteristics of all initially enrolled subjects (n = 70) and after excluding 9 subjects from the primary TNF endpoint analysis (n = 61). Supplementary Table 3 lists the reasons for excluding nine subjects.

	Initially enrolled	After exclusions
Number of subjects	70	61
Age, years (mean ± SD)	29.6 ± 6.4	30.2 ± 6.3
Sex, N (%)		
Male	36 (51.4%)	29 (47.5%)
Female	34 (48.6%)	32 (52.5%)
Race, N (%)		
Asian	27 (38.6%)	21 (34.4%)
Black	4 (5.7%)	4 (6.6%)
Caucasian	26 (37.1%)	23 (37.7%)
Other	13 (18.6%)	13 (21.3%)
Body Mass Index, kg/m^2	23.7 ± 2.5	23.8 ± 2.4
Physical activity, hours/week	6.8 ± 4.5	6.8 ± 4.3

Table 2

Two-way mixed ANOVA analysis of primary endpoint measures.

Effect of time point and of intervention group on TNF levels. Results are presented for log2 TNF levels measured using the custom ex vivo LPS challenge assay, at each of 5 LPS concentrations (0, 0.1, 1, 10 and 100 ng/ml), for area under curve (AUC) of logTNF vs. LPS concentration, and for log2 TNF levels measured using the TruCulture assay (null and LPS tube). “Time point” variable includes 4 time points (baseline, 1-h, 2-h and 24-h time points). “Group” variable includes 7 groups (1 sham-stimulated group and 6 ultrasound-stimulated groups). Time points and groups are explained in Fig. 1A and B, respectively. “ns” denotes a p value greater than 0.05.

Dependent variable	**Independent variables**					
	Time point		**Group**		**Time point × Group**	
	F-stat.	**p value**	**F-stat.**	**p value**	**F-stat.**	**p value**
logTNF custom assay no LPS	0.37	ns	0.56	ns	1.35	ns
logTNF custom assay 0.1 ng/ml LPS	17.15	5.4E-07	0.54	ns	0.81	ns
logTNF custom assay 1.0 ng/ml LPS	40.14	9.0E-16	2.38	0.041	1.76	ns
logTNF custom assay 10.0 ng/ml LPS	10.59	3.5E-05	0.71	ns	1.32	ns
logTNF custom assay 100 ng/ml LPS	2.04	ns	0.42	ns	1.15	ns
logTNF custom assay AUC (Log TNF × LPS)	25.6	1.6E-11	0.62	ns	1.04	ns
LogTNF TruCulture assay null tube	1.69	ns	0.13	ns	0.77	ns
LogTNF TruCulture assay LPS tube	10.08	2.1E-04	1.77	ns	0.75	ns

Table 3
Two-way mixed ANOVA table of safety markers.

Effect of time point and treatment group on different clinical and laboratory parameters. p values are adjusted for multiple testing by the Bonferroni method; “ns” denotes a p value greater than 0.05. The superscript number next to each variable name denotes the number of repeated measures and time points: (#1) 3 measures (Screening, Baseline, 24-h follow-up); (#2) 2 measures (Screening, 24-h follow-up); (#3) 5 measures (Screening, Baseline, 1-h, 2-h and 24-h follow-up); (#4) 2 measures (Baseline, 24-h follow-up); (#5) 4 measures (Baseline, 1-h, 2-h and 24-h follow-up.).

Dependent variable	**Independent variables**					
	Time		**Group**		**Time × Group**	
	F stat.	**p value***	**F stat.**	**p value***	**F stat.**	**p value***
Heart rate[1]	1.11	ns	1.12	ns	0.48	ns
Systolic BP[1]	1.03	ns	1.74	ns	0.88	ns
Diastolic BP[1]	1.59	ns	1.21	ns	1.96	ns
WBC count[2]	1.45	ns	0.74	ns	2.79	ns
Hemoglobin[2]	22.88	4.1E-04	1.50	ns	1.08	ns
Blood glucose[3]	10.50	4.9E-05	1.55	ns	1.21	ns
Plasma creatinine[3]	1.35	ns	0.51	ns	1.34	ns
Sodium[3]	0.15	ns	2.91	ns	0.85	ns
Potassium[3]	0.57	ns	0.43	ns	1.13	ns
Chloride[3]	4.69	ns	0.80	ns	0.81	ns
Calcium[3]	0.85	ns	0.97	ns	0.68	ns
Carbon Dioxide[3]	6.05	0.012	0.98	ns	1.38	ns
Blood Urea Nitrogen[3]	5.96	ns	0.53	ns	0.90	ns
Alkaline phosphatase[3]	0.83	ns	0.93	ns	1.09	ns
Alanine transaminase[3]	1.53	ns	0.30	ns	1.69	ns
Aspartate aminotransferase[3]	6.29	0.02	0.64	ns	1.12	ns
Albumin[3]	1.04	ns	0.86	ns	0.74	ns
Prothrombin time[2]	0.13	ns	0.87	ns	0.21	ns
Creatine kinase[1]	1.17	ns	1.25	ns	0.26	ns
Lactate dehydrogenase[1]	2.34	ns	1.33	ns	2.15	ns
Amylase[4]	0.07	ns	1.01	ns	0.92	ns
Lipase[4]	0.22	ns	1.54	ns	0.78	ns
Cortisol[3]	26.70	5.4E-13	0.87	ns	0.45	ns
Epinephrine[5]	2.96	ns	0.13	ns	0.50	ns
Norepinephrine[5]	5.38	ns	1.77	ns	0.98	ns