Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a heterogeneous, prevalent, chronic autoimmune disease characterized by painful swollen joints and significant disabilities. Symptomatic relief can be achieved in up to 50% of patients using biological agents that inhibit tumor necrosis factor (TNF) or other mechanisms of action, but there are no universally effective therapies. Recent advances in basic and preclinical science reveal that reflex neural circuits inhibit the production of cytokines and inflammation in animal models. One well-characterized cytokine-inhibiting mechanism, termed the "inflammatory reflex," is dependent upon vagus nerve signals that inhibit cytokine production and attenuate experimental arthritis severity in mice and rats. It previously was unknown whether directly stimulating the inflammatory reflex in humans inhibits TNF production. Here we show that an implantable vagus nerve-stimulating device in epilepsy patients inhibits peripheral blood production of TNF, IL-1β, and IL-6. Vagus nerve stimulation (up to four times daily) in RA patients significantly inhibited TNF production for up to 84 d. Moreover, RA disease severity, as measured by standardized clinical composite scores, improved significantly. Together, these results establish that vagus nerve stimulation targeting the inflammatory reflex modulates TNF production and reduces inflammation in humans. These findings suggest that it is possible to use mechanism-based neuromodulating devices in the experimental therapy of RA and possibly other autoimmune and autoinflammatory diseases.

vagus nerve | rheumatoid arthritis | inflammatory reflex | tumor necrosis factor | cytokines

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation in the musculoskeletal joints resulting in cartilage degradation and bone destruction with consequent disability (1). The prevalence exceeds 1.3 million adult cases in the United States, with attributable medical costs estimated between $19–39 billion (2, 3). Standard therapies include glucocorticoids, methotrexate, monoclonal antibodies, and other pharmacological agents targeting inflammatory mechanisms (4). Despite these treatment options, many RA patients fail to respond, instead persisting with poor health, shortened life span, and significant impairments in quality of life affecting work, leisure, and social functions (5, 6). Thus, there remains a significant need for alternative therapeutic approaches.

Recent advances at the intersection of immunology and neuroscience reveal reflex neural circuit mechanisms regulating innate and adaptive immunity (7, 8). One well-characterized reflex circuit, termed the "inflammatory reflex," is defined by signals that travel in the vagus nerve to inhibit monocyte and macrophage production of tumor necrosis factor (TNF) and other cytokines (7). Electrical stimulation of the vagus nerve in animals (e.g., mouse, rat, and dog) stimulates choline acetyltransferase-positive T cells to secrete acetylcholine in spleen and other tissues (9). Acetylcholine is the cognate ligand for α-7 nicotinic acetylcholine receptors (α7nAChR) expressed on cytokine-producing monocytes, macrophages, and stromal cells (7, 10, 11). Ligand binding inhibits the nuclear translocation of NF-κB and inhibits inflammasome activation in macrophages activated by exposure to lipopolysaccharide (LPS), other Toll-like receptor (TLR) ligands, and other proinflammatory stimulating factors (12, 13).

Inflammatory reflex signaling, which is enhanced by electrically stimulating the vagus nerve, significantly reduces cytokine production and attenuates disease severity in experimental models of endotoxemia, sepsis, colitis, and other preclinical animal models of inflammatory syndromes (7, 8, 14–16). In experimental collagen-induced arthritis, vagotomy or selective disruption of α7nAChR worsened disease severity, and administration of nicotine or other selective α7nAChR agonists, ameliorated disease severity (17, 18). Vagus nerve stimulation delivered once daily for 60 s with an

Significance

Rheumatoid arthritis (RA) is a chronic, prevalent, and disabling autoimmune disease that occurs when inflammation damages joints. Recent advances in neuroscience and immunology have mapped neural circuits that regulate the onset and resolution of inflammation. In one circuit, termed "the inflammatory reflex," action potentials transmitted in the vagus nerve inhibit the production of tumor necrosis factor (TNF), an inflammatory molecule that is a major therapeutic target in RA. Although studied in animal models of arthritis and other inflammatory diseases, whether electrical stimulation of the vagus nerve can inhibit TNF production in humans has remained unknown. The positive mechanistic results reported here extend the preclinical data to the clinic and reveal that vagus nerve stimulation inhibits TNF and attenuates disease severity in RA patients.

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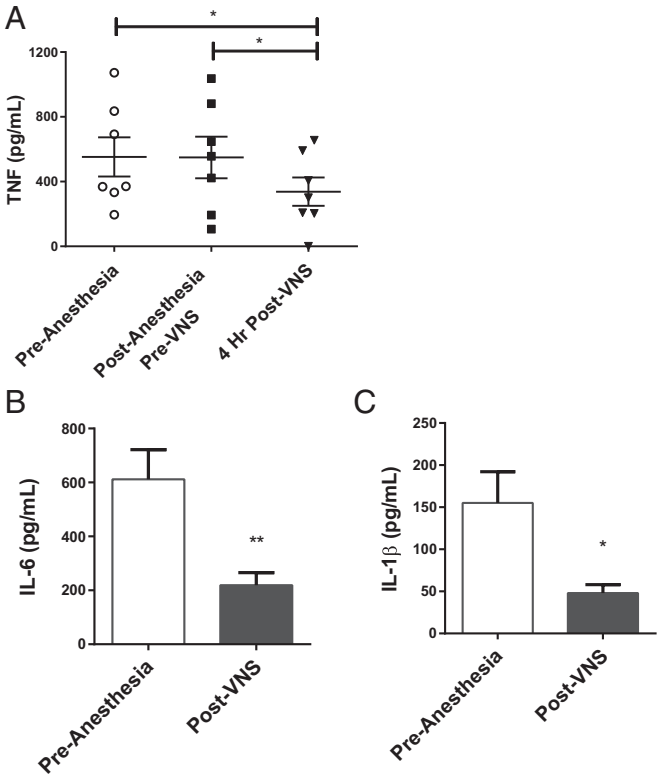

Fig. 1. Inflammatory reflex activation reduces whole-blood LPS-induced TNF production in epilepsy patients. Electrical stimulation of the vagus nerve in humans inhibits whole-blood LPS-induced TNF release. Blood was obtained from epilepsy patients ($n = 7$) undergoing implantation of a vagus nerve-stimulation device at different time points: before anesthesia induction and before vagus nerve stimulation; after anesthesia induction and before vagus nerve stimulation (pre-VNS); and 4 h after vagus nerve stimulation (post-VNS). Whole blood was incubated with LPS and TNF (*A*), IL-6 (*B*), and IL-1β (*C*) levels in plasma were determined after 4 h in culture. The significance of the differences between mean values at each time point was tested by unpaired ANOVA (*$P < 0.05$, **$P < 0.01$). Data are shown as mean ± SEM.

implanted device attenuated joint swelling, inhibited cytokine production, and conferred significant protection against synovitis and periarticular bone erosions (19, 20). Accordingly, we reasoned that it might be possible to modulate cytokine levels and inflammation using an active implantable medical device in humans (20).

Vagus nerve-stimulating devices have been used for decades in patients with refractory epilepsy and have been used more recently in patients with depression. These devices have been implanted in more than 100,000 patients, are relatively well tolerated, and have not been associated with immunosuppression or long-term complications (21, 22). We implanted a cohort of epilepsy patients with a vagus nerve-stimulating device and observed that transient delivery of electrical current during general anesthesia significantly inhibited TNF production in peripheral blood monocytes. A subsequent study of 17 RA patients in an 84-d open-label trial also revealed significantly decreased TNF production and significantly improved clinical signs and symptoms of disease.

Results

To determine whether vagus nerve stimulation inhibits TNF production in humans, we studied seven epilepsy patients [five male, two female; mean age 35 y (range 25–43 y)] who were implanted with a vagus nerve-stimulating device using a coiled cuff electrode (Cyberonics) on the left cervical vagus nerve. These patients had no history of inflammatory or autoimmune disorders. Peripheral blood was collected before, during, and after vagus nerve stimulator implantation surgery. Endotoxin was added to the whole blood to stimulate the production of TNF by monocytes for 4 h (13, 23). The application of current-controlled electrical pulses (single 30-s stimulation at 1.0-mA output current, 20-Hz pulse frequency, 500-μs pulse duration) significantly inhibited whole-blood TNF production compared with baseline levels before electrical stimulation (Fig. 1*A*). The inhibition of TNF release following vagus nerve stimulation during general anesthesia cannot be attributed to a placebo effect, because the subjects were unconscious and were not aware of the nerve stimulation. Whole-blood production of interleukin (IL)-6 and IL-1β was also inhibited significantly by vagus nerve stimulation (Fig. 1 *B* and *C*). To our knowledge, this is the first report that the delivery of electric current applied directly on the cervical vagus nerve to stimulate the inflammatory reflex inhibits the endotoxin-induced release of TNF, IL-1β, and IL-6 in humans.

We next studied the effects of vagus nerve stimulation in patients with RA. At enrollment the 18 study patients had active disease, with at least four tender and four swollen joints (of a 28-joint count), despite methotrexate therapy for at least 3 mo on a stable dose. One patient from cohort I, who fulfilled the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification for RA, was later diagnosed with Whipple disease and was excluded from the efficacy analysis. This patient is included in the baseline patient characteristics (Table 1) and adverse-event data (Table S1). The RA patients with active disease were studied in two cohorts. Cohort I ($n = 7$) included patients with active disease despite therapy with methotrexate. They had never received a biological TNF antagonist or had previously failed treatment with TNF antagonists because of drug toxicity. Cohort II ($n = 10$) included patients who had failed conventional therapy with methotrexate and also had failed treatment with at least two biological agents differing in mechanisms of action (e.g., anti-TNF, anti–IL-6 receptor, anti-CD20 antibodies, and/or T-cell costimulation inhibitor). There were no deaths, serious adverse events, withdrawals from the study because of adverse events, or infections in either cohort. In agreement with known risks of the procedure, nine patients experienced mild or moderate adverse events associated with implanting the vagus nerve stimulator on the left cervical vagus nerve (Table S1).

The study design schematic is shown in Fig. 2*A*. The vagus nerve was stimulated during surgery (day −14) to measure electrode impedance and to verify device function. During the 14-d postoperative recovery period (day −14 to day 0), the device was turned off, and no current was delivered to the vagus nerve. On the first treatment day (study day 0), patients received a single 60-s stimulation with electric current pulses of 250-μs duration at 10 Hz and an output current between 0.25–2.0 mA, as tolerated. No further stimulation was delivered for 7 d. On study day 7, the output current was adjusted to the highest amperage tolerated, up to 2.0 mA; this level of current was subsequently delivered once daily for 60 s in 250-μs pulse widths at 10 Hz. Current escalation up to the highest tolerated amperage (up to 2.0 mA) was repeated weekly until day 28. At that visit the frequency of daily stimulation events was increased to four times daily in patients who had not achieved a moderate or good clinical response according EULAR criteria (24). On day 28, the output current delivered was comparable in both cohorts: cohort I output current was 1.29 ± 0.37 mA (mean ± SD); cohort II output current was 1.60 ± 0.36 mA. In cohort I, two of seven patients received electric current pulses four times daily. In cohort II, 6/10 patients received electric current pulses four times daily.

We observed that TNF production in cultured peripheral blood obtained from the combined RA study cohort on day 42 was significantly reduced from baseline day −21 (TNF = 2,900 ± 566 pg/mL on day −21 vs. 1,776 ± 342 pg/mL on day 42, $P < 0.05$) (Fig. 2*B*). On day 42 the vagus nerve stimulator was turned off. After a 14-d hiatus, it was restarted on day 56, and patients were followed through day 84. After the vagus nerve stimulator was turned off, TNF production

Table 1. RA patient baseline demographics, medication history, and disease severity

Demographics	Cohort I	Cohort II	Combined
Total, *n*	8	10	18
Enrollment by country			
Bosnia	3	0	3
Croatia	2	0	2
The Netherlands	3	10	13
Mean age in years (range)	55 (36–69)	48 (36–56)	51 (36–69)
Sex, % female	50	100	78
Ethnicity, % Caucasian	88	100	94
Mean no. of years since RA diagnosis (SD)	9.9 (5.7)	11.8 (6.3)	11.0 (5.9)
No. rheumatoid factor-positive patients (%)	7 (88)	5 (50)	12 (67)
No. anti-citrullinated peptide Ab^+ patients (%)	6 (75)	6 (60)	12 (67)
No. patients receiving prior nonbiologic disease-modifying antirheumatic drugs (%)			
0 drugs	1 (13)	1 (10)	2 (11)
1 drug	2 (25)	3 (30)	5 (28)
2 drugs	2 (25)	2 (20)	4 (22)
3 or more drugs	3 (37)	4 (40)	7 (39)
No. patients receiving prior biologic disease-modifying antirheumatic drugs (%)			
0 drugs	3 (38)	0	3 (17)
1 drug	4 (50)	0	4 (22)
2 drugs	1 (12)	0	1 (6)
3 drugs	0	3 (30)	3 (17)
4 drugs	0	4 (40)	4 (22)
5 drugs	0	2 (20)	2 (11)
6 drugs	0	1 (10)	1 (6)
DAS28-CRP (SD)	6.05 (0.87)	5.94 (0.72)	5.99 (0.77)
High-sensitivity CRP, mg/L (SD)	17.5 (10.0)	17.5 (18.5)	17.5 (14.9)

increased significantly by day 56; when the stimulator was turned on again, TNF production again decreased significantly by day 84 (1,776 ± 342 pg/mL on day 42 vs. 2,617 ± 342 pg/mL on day 56 and 1,975 ± 407 pg/mL on day 84, $P < 0.01$ for both). This finding indicates that active electrical stimulation of the vagus nerve inhibits TNF production in patients with RA.

RA signs and symptoms are measured using a standard disease activity composite score [the 28-joint C-reactive protein (CRP)-based disease activity score, DAS28-CRP] derived from counting swollen joints and tender joints, a patient-defined visual analog score of disease activity, and serum CRP levels (25). We observed that DAS28-CRP values at day 42 were significantly improved (i.e., lower) from baseline day −21 in the combined cohorts (DAS28-CRP = 6.05 ± 0.18 on day −21 vs. 4.16 ± 0.39 on day 42, $P < 0.001$), when the device was delivering current (Fig. 2*C*). Within days after receiving electrical stimulation of the vagus nerve, the DAS28-CRP improved significantly in some patients (Fig. S1). When the device was turned off (at day 42), the DAS28-CRP increased significantly within 14 d (4.16 ± 0.39 on day 42 vs. 4.96 ± 0.31 on day 56, $P = 0.001$). Restarting the device (day 56) significantly reduced the DAS28-CRP (Fig. 2*C*). Linear regression analysis comparing the mean change in the DAS28-CRP and the percentage change in TNF release from baseline day −21 to day 42 revealed a highly significant correlation ($r = 0.384$, $P < 0.0001$) (Fig. 2*D*). The temporal pattern of TNF production in the combined cohort correlated with the DAS28-CRP (Fig. 2*E*).

We assessed the fraction of patients who improved from baseline to achieve ACR 20%, 50%, and 70% clinical responses and also the number of patients who improved from baseline sufficiently to meet the definition for EULAR response and remission. The ACR response is defined as the percentage improvement in disease activity between two time points (ACR20 is ≥20%, ACR 50 is ≥50%, and ACR70 is ≥70% improvement). The EULAR response depends on the change in the DAS28-CRP and the absolute level achieved after treatment (24). As shown in Fig. S2, the ACR and EULAR response criteria were fulfilled in a large subset of patients in both cohorts. At the primary endpoint (day 42) the percentages of patients fulfilling the ACR response criteria for 20%, 50%, and 70% improvement were 71.4%, 57.1%, and 28.6%, respectively, for cohort I and were 70.0%, 30.0%, and 0.0%, respectively, for cohort II. The percentages of patients achieving DAS28 remission (DAS28-CRP <2.6) on day 42 in cohorts I and II were 28.6% and 0.0%, respectively. Improvement was observed in all constituent components of the composite end points (tender joint count, swollen joint count, patient's assessment of pain, patient's global assessment, physician's global assessment, and CRP) (Table S2). Together, these data indicate that vagus nerve stimulation inhibits TNF and significantly attenuates RA disease severity.

We measured a panel of serum cytokines to assess further the mechanisms of this experimental therapeutic intervention. Most, including serum TNF, IL-10, IL-12p70, IL-13, IL-1α, IL-1β, IL-2, IL-4, IL-5, and TNF-β, were below 1 pg/mL (unreliable limits of detection). Serum IL-6 levels in subjects who improved by EULAR criteria were significantly decreased compared with subjects who failed to improve: IL-6 levels were 15.4 ± 2.4 pg/mL in nonresponders ($n = 5$) vs. 5.0 ± 1.4 pg/mL in responders ($n = 12$) ($P = 0.001$) (Fig. 3*A*). Decreased IL-6 levels in the patients who responded to therapy correlated with improvement in disease severity between day −21 and day 42 ($r = 0.707$, $P = 0.002$) (Fig. 3*B*). The IL-6 responses are specific, because IL-8 and IL-17 levels did not change significantly [IL-8: 25.6 ± 9.1 pg/mL in nonresponders ($n = 5$) vs. 13.7 ± 1.7 pg/mL in responders ($n = 12$), $P = 0.29$ (Fig. 3*C*); IL-17: 2.8 ± 1.1 pg/mL in nonresponders ($n = 5$) vs. 1.8 ± 0.2 pg/mL in responders ($n = 12$), $P = 0.18$ (Fig. 3*E*)] and did not correlate to clinical response (Fig. 3 *D* and *F*).

Discussion

To our knowledge, this study is the first to assess whether stimulating the inflammatory reflex by directly implanting an electronic device modulates TNF and other cytokines in humans. Historically the development of electrically active implantable medical devices has been primarily empiric, based upon observing effects of devices that deliver

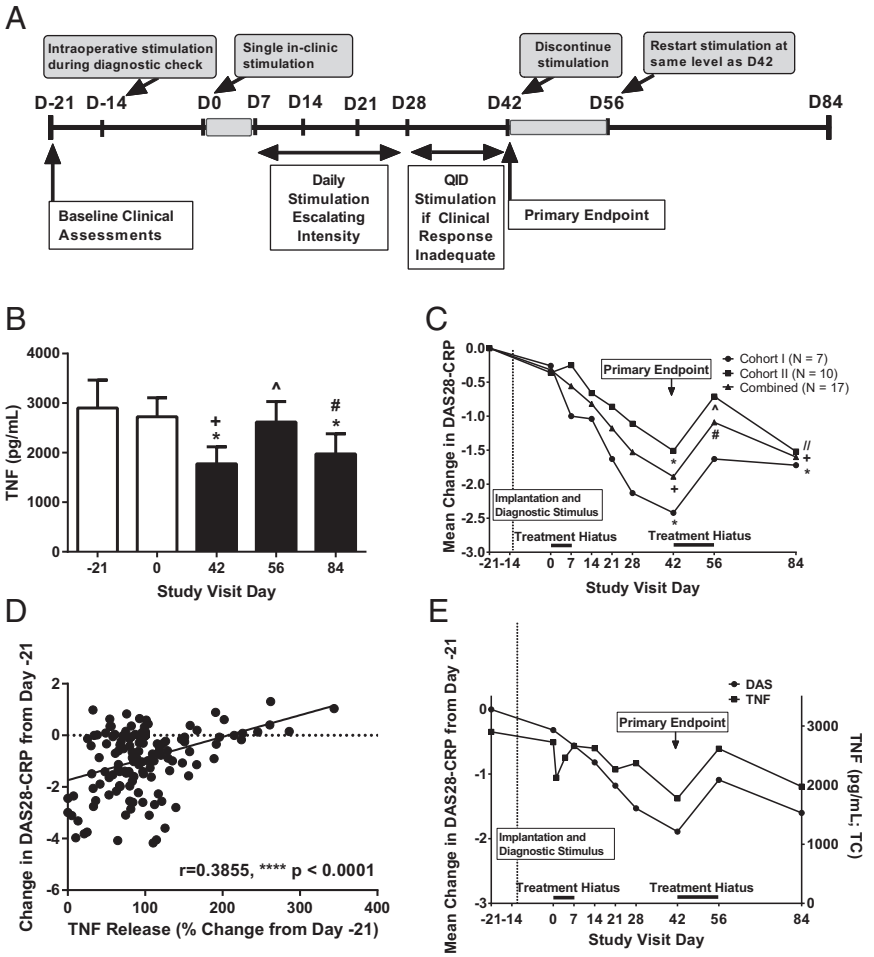

Fig. 2. The effects of inflammatory reflex activation on whole-blood LPS-induced TNF production and disease activity in RA patients. (*A*) Schematic of the RA study design. D −21 to D84 indicate study visit days. The stimulation schedule and timing of assessments are shown. (*B*) Mean LPS-induced TNF production in the combined RA cohort (n = 17) at study days −21, 0, 42, 56, and 84; visit means are designated by bars, and error bars indicate SEM. Differences in means were tested for significance by paired t test: $^{*}P < 0.05$ vs. d −21; $^{+}P < 0.01$ vs. d 0; $^{\wedge}P < 0.01$ vs. d 42; $^{\#}P < 0.01$ vs. d 56. (*C*) The mean change in DAS2 8-CRP from baseline by study visit day for cohort I (patients failing methotrexate treatment), cohort II (patients failing treatment by multiple biologic agents), and combined cohorts. The significance of the mean change by paired t test between visits is shown: $^{*}P < 0.05$ vs. d −21; $^{//}P < 0.01$ vs. d −21; $^{+}P < 0.001$ vs. d −21; $^{\#}P < 0.001$ vs. d 42; $^{\wedge}P < 0.05$ vs. d 42). (*D*) Linear regression analysis comparing the changes in the DAS28-CRP and the percent change in TNF release from study day −21 measured at each individual visit for each patient in the combined cohort. Changes in the DAS28-CRP and TNF release are significantly correlated by Pearson's test (r = 0.384, $P < 0.0001$). (*E*) Mean change in the DAS28-CRP and mean LPS-induced TNF release over time by study visit day. Changes in the DAS28-CRP and TNF release follow a similar temporal pattern in response to initial simulation, stimulation withdrawal, and stimulation reinitiation.

electrical current to depolarize neuronal or cardiac tissue. Absent appropriate biomarkers or mechanistic understanding, it has been difficult or impossible to develop or optimize the device parameters for current delivery, physiological effect in the targeted organ system, and clinical efficacy. Direct and accessible surrogate molecular markers of disease mechanism targeted by active implantable medical devices are uncommon. The discovery of the inflammatory reflex affords a unique opportunity for developing a neuromodulating device to regulate immune cell function by targeting a neural pathway that regulates cytokine production, a surrogate marker of molecular mechanism (26).

RA patients in cohort I are in early stages of disease not responding to therapy with methotrexate. These patients are frequently candidates for subsequent therapy with a biological agent that inhibits TNF. Cohort II patients are in later stages of disease, having failed multiple biological disease-modifying antirheumatic drugs. After electrical stimulation of the vagus nerve the DAS28-CRP improved significantly in both cohorts, and withdrawal of treatment significantly worsened the severity of disease. Reactivating the device on day 56 restored significant clinical improvement. The clinical responses were accompanied by significant reductions in TNF release during periods of disease remission and significant increases in TNF release during disease exacerbation. A large body of preclinical evidence has delineated the molecular and physiological mechanisms of the inflammatory reflex modulating TNF, IL-6, HMGB1, and other cytokines (7–9, 11–20). The molecular mechanisms of cytokine inhibition implicate acetylcholine derived from T_{ChAt} cells, the subset of choline acetyltransferase-positive T cells that we identified in the inflammatory reflex (9). In future clinical trials it should be interesting to study whether T_{ChAt} cells participate in mediating anti-inflammatory reflex mechanisms.

Vagus nerve stimulation has been used to treat medically refractory epilepsy in more than 100,000 patients, and it is generally well tolerated (21, 22). The adverse events reported here were mild to moderate in severity and were comparable in type and frequency to those seen in prior studies of vagus nerve stimulation therapy in epilepsy patients. These adverse events included transient hoarseness, postoperative hoarseness from neuropraxis, and transient intraoperative bradycardia during surgery. None of the patients developed infection. Larger clinical trials can be designed to

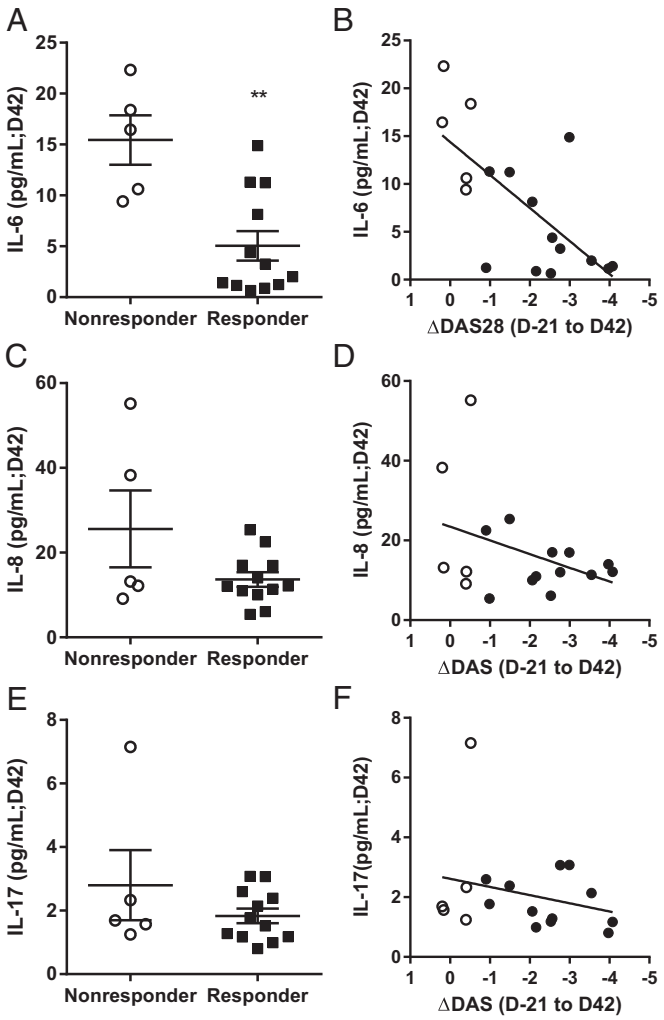

Fig. 3. Modulation of serum cytokines. Serum from each patient in the combined cohort was analyzed for multiple analytes at day 42. (*A*, *C*, and *E*) Individual patient values for EULAR nonresponders and responders are shown for IL-6 (*A*), IL-8 (*C*), and IL-17 (*E*) levels. The significance of differences between mean values at each time point was tested by unpaired *t* test (**$P < 0.01$). Horizontal bars indicate mean ± SEM. (*B*, *D*, and *F*) Linear regression analysis comparing analyte level at day 42 to the change in the DAS28-CRP from study day −21 to day 42. The change in the DAS28-CRP is significantly correlated to IL-6 release ($r = 0.707$, $P = 0.002$) (*B*) but not to IL-8 release ($r = 0.261$, $P = 0.31$) (*D*) or IL-17 release ($r = 0.384$, $P = 0.07$) (*F*).

determine the risk/benefit ratio for implantable electronic devices compared with the toxicity and side effects of pharmacological and targeted therapies for RA.

The electrical stimulation parameters used in this study were previously established to stimulate the inflammatory reflex in preclinical studies and differ significantly from the stimulation protocols used in epilepsy (19, 27). Here, electrical current (up to 2.0 mA) was delivered to the cervical vagus nerve for 60 s one to four times daily; the maximum time of electrical current flow for any patient in this study was 4 min daily. This stimulation protocol differs significantly from the protocols for treating epilepsy, in which current (up to 2.25 mA) is delivered at 60-s intervals, followed by an OFF interval of 5–180 min, repeated continuously. Thus, epilepsy patients may receive electrical current delivery for up to 240 min daily. Preclinical studies have established that stimulation of the inflammatory reflex for as little as 60 s confers significant inhibition of cytokine production for up to 24 h. The present study was not designed or powered to evaluate the relationship between specific electrical current dose–response and clinical outcomes or the longer-term durability of therapeutic benefit, and the effects of under- or overstimulation of the inflammatory reflex are also an important area for future study.

The primary objective of this study was to determine whether activating the inflammatory reflex with an implanted electronic device inhibits cytokine production in humans. It is reasonable to consider whether placebo mechanisms contribute to these findings, because some patients are aware when the device is delivering current. There are several arguments against a placebo effect explaining the observed inhibition of TNF and IL-6 and the significant clinical improvements. First, we observed that intraoperative vagus nerve stimulation significantly inhibited TNF release in epilepsy patients who were unconscious during the implantation. These patients could not be aware of the stimulation, indicating that the suppression of cytokine release immediately following vagus nerve stimulation cannot be attributed to a placebo effect. Second, we also observed that the suppression of TNF release during vagus nerve stimulation in RA patients occurred only when the device was functioning. It has been established previously that biomarkers are not modifiable by placebo effects in RA studies of this duration (28, 29). Third, we observed reduced TNF and IL-6 production and positive clinical responses in the subset of therapy-resistant patients who had failed both methotrexate therapy and treatment with multiple biologic agents with differing mechanisms of action. It has been established in prior studies that placebo response rates in drug-resistant cohorts are extremely low (ACR20 responses 5–11%). The findings here of significantly higher ACR20 responses (between 70% and 71.4%) argue strongly against a placebo effect being the mechanism. Fourth, a recent study reported clinical improvement using vagus nerve-stimulation therapy to treat another disease mediated by TNF, Crohn's disease (30). Although the investigators in that study did not measure the activity of the inflammatory reflex or cytokine production, they did examine endoscopic biopsies and observed that vagus nerve stimulation significantly inhibited inflammation in the colonic tissues, an objective histological tissue response that cannot be attributed to placebo effects. Finally, our recent prospective observational studies indicate that impaired constitutive vagus nerve activity precedes the development of clinically manifest RA (31). Therefore, when considered together with extensive preclinical data that identify molecular and neurophysiological mechanisms, the inhibition of TNF during electrical stimulation and the significant clinical responses shown give evidence that the clinical mechanism is mediated by the inflammatory reflex.

This first-in-class study supports a conceptual framework for further studies of electronic medical devices in diseases currently treated with drugs, an approach termed "bioelectronic medicine" (32). Larger clinical trials in RA can be designed and powered to assess clinical efficacy, but our findings encourage pursuing this strategy in RA and in other cytokine-mediated autoimmune and auto-inflammatory disorders.

Materials and Methods

Study of Vagus Nerve Stimulation in Epilepsy Patients. The study of vagus nerve stimulation in epilepsy patients was performed at the Hofstra Northwell School of Medicine and was approved by the Clinical Research Center (CRC) and the Institutional Review Board. All patients provided informed consent before participation. The study population consisted of seven epilepsy patients being implanted with a Cyberonics Vagus Nerve Stimulation System (Cyberonics) according to the manufacturer's instructions as part of their standard care for the treatment of refractory epilepsy (Fig. S3). During the intraoperative diagnostic procedure, the pulse generator produces a 30-s stimulation at a 1.0-mA output current with pulse frequency of 20 Hz and pulse width of 500 μs. Blood samples were taken before anesthesia induction, after anesthesia induction but before intraoperative vagus nerve stimulation, and 4 h after intraoperative vagus nerve stimulation.

The LPS-induced cytokine release assay was performed as previously described (33). Cytokine levels were analyzed using the MSD multiplex cytokine assay (Meso Scale Discovery) per the manufacturer's instructions. TNF, IL-6, and IL-1β

release across time points was analyzed using the Prism analytical software package (GraphPad).

Study of Vagus Nerve Stimulation in RA Patients. The study of vagus nerve stimulation in RA patients was performed at one center in The Netherlands (the Academic Medical Center of the University of Amsterdam), at two centers in Bosnia and Herzegovina (the University Clinical Hospital in Mostar and Sarajevo University Clinical Center in Sarajevo), and at in one center in Croatia (Clinical Hospital Center Sestre Milosrdnice, Zagreb) and was approved by the respective national and institutional Ethics Committees. All patients provided informed consent before participation. The investigational study device was a Cyberonics Vagus Nerve Stimulation System, implanted as described above. The systems were treated as investigational study devices because of their off-label use in patients with RA. The study recruited two separate patient cohorts. Cohort I consisted of RA patients who had failed to respond to methotrexate and who were either TNF-antagonist naive or had previously failed treatment with a TNF antagonist because of safety reasons rather than lack of efficacy. Cohort II included patients who had not responded adequately to at least two biologic agents with at least two different mechanisms of action. Major inclusion and exclusion criteria are given in *SI Materials and Methods*. The use of prednisone at a stable daily dose of less than 10 mg and other nonbiological disease-modifying antirheumatic drugs at stable doses was allowed.

The design schematic of this single-arm study is shown in Fig. 2*A*. At the conclusion of the study, patients were offered the options of having the device surgically removed or left in place and inactivated or continuing treatment in a long-term extension study. All recruited subjects opted to continue in the extension study, which will be reported separately.

The primary study end point was mean change in the DAS28-CRP between visits on baseline day −21 and day 42 (25). Mean changes in the DAS28-CRP between day −21 and day 42 or day 84, and between day 42 and day 56 also were assessed for significance at $P < 0.05$ using a Student's paired t test in the SAS 9.2 statistical analysis package (SAS). Because this was an exploratory study, no formal statistical power calculations were performed, and no adjustments for multiple comparisons were made. Adverse events were collected from the day of implantation through the day 84 visit, coded using the Medical Dictionary for Regulatory Activities (MedDRA), and presented by MedDRA term as subject incidence rates.

Whole-Blood Cytokine Release Assay in the RA Study. The TruCulture system (Myriad RBM), an assay system suitable for use at clinical sites and scalable to larger studies, was used. Venous blood was drawn into tubes containing endotoxin at 100 ng/mL and was incubated at 37 °C for 24 h. Supernatant TNF was measured by ELISA (R&D Systems). Comparisons of changes in TNF release between baseline and subsequent visits [with three statistical outlier exclusions; robust regression and outlier removal (ROUT) method with the maximum false discovery rate at 1%] by paired t test and linear regression analysis of relationships between changes in the DAS28-CRP and TNF release were performed using the Prism analytical software package.

Serum Cytokines in the RA Study. Serum cytokine levels from day 42 were analyzed using the MSD multiplex cytokine assay as above. Analysis of IL-6, IL-8, and IL-17 release on day 42 and linear regression analysis of relationships (Pearson's test) between the change in the DAS28-CRP and serum cytokine release at day 42 were performed using the Prism analytical software package.

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