Disclosure of potential conflict of interest: H. Movassagh has a patent pending through the University of Manitoba. J. F. McConville has provided expert testimony on behalf of the US government (Domino vs. United States, 15 C 5579); has received grants from the US Department of Health and Human Services, Health Resources and Services Administration (grant no. 1 T0BHP285660100); and has received payment for lectures from the American College of Chest Physicians. A. S. Gounni has received a grant from the Canadian Institutes of Health Research and has a patent pending through the University of Manitoba. The rest of the authors declare that they no relevant conflicts of interest.

REFERENCES

1. Hekking PP, Bel EH. Developing and emerging clinical asthma phenotypes. J Allergy Clin Immunol Pract 2014;2:671-80; quiz 81.
2. Kumanogoh A, Kikutani H. Immunological functions of the neuropilins and plexins as receptors for semaphorins. Nat Rev Immunol 2013;13:802-14.
3. Shim EJ, Chun E, Kang HR, Cho SH, Min KU, Park HW. Expression of semaphorin 3A and neuropilin 1 in asthma. J Korean Med Sci 2013;28:1435-42.
4. Nkyimbeng-Takwi EH, Shanks K, Smith E, Iyer A, Lipsky MM, Detolla LJ, et al. Neuroimmune semaphorin 4A downregulates the severity of allergic response. Mucosal Immunol 2012;5:409-19.
5. Mizutani N, Nabe T, Yoshino S. Semaphorin 7A plays a critical role in IgE-mediated airway inflammation in mice. Eur J Pharmacol 2015;764:149-56.
6. Movassagh H, Shan L, Halayko AJ, Roth M, Tamm M, Chakir J, et al. Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration. J Allergy Clin Immunol 2014;133:560-7.
7. Bartemes KR, Kita H. Dynamic role of epithelium-derived cytokines in asthma. Clin Immunol 2012;143:222-35.
8. Movassagh H, Saati A, Nandagopal S, Mohammed A, Tatari N, Shan L, et al. Chemorepellent semaphorin 3E negatively regulates neutrophil migration in vitro and in vivo. J Immunol 2017;198:1023-33.
9. Movassagh H, Shan L, Mohammed A, Halayko AJ, Gounni AS. Semaphorin 3E deficiency exacerbates airway inflammation, hyperresponsiveness, and remodeling in a mouse model of allergic asthma. J Immunol 2017;198:1805-14.
10. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000;162:2341-51.
11. Chakir J, Dube J, Laviolette M, Goulet F, Germain L, Auger F, et al. Isolation and characterization of human airway fibroblasts in culture. Methods Mol Med 2000;44:53-65.

Available online May 12, 2017.
http://dx.doi.org/10.1016/j.jaci.2017.04.031

A short course of gamma-tocopherol mitigates LPS-induced inflammatory responses in humans *ex vivo*

To the Editor:

Currently, there is an unmet need for nonsteroidal treatments for chronic inflammatory diseases such as asthma, and vitamin E has been proposed as one such treatment to combat asthma-related airway inflammation. Although alpha-tocopherol (αT), the vitamin E isoform found in most supplements, decreased pulmonary T_H2 cytokines and improved airway responsiveness to methacholine in patients with asthma following allergen challenge, no change was seen in allergen-induced bronchoconstriction or airway cellularity.[1] In addition, vitamin E supplements may increase bleeding risk.[2] γ-Tocopherol (γT), the predominant isoform in the US diet, and its metabolite 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) possess unique properties that αT does not, including inhibition of cyclooxygenase-2 (COX-2) and lipoxygenase activity,[3] trapping of reactive nitrogen species,[4] and inhibition of nuclear factor kappa B and inflammatory cytokines,[5] making it an attractive candidate for combating asthma-associated inflammation.

Despite these properties, some have proposed a proinflammatory role for γT primarily on the basis of work with animal models, some of which demonstrated increased airway inflammation, and observational studies in humans, suggesting a negative correlation between plasma γT levels and lung function.[6,7] In contrast, human intervention trials conducted at our center have shown that a 1-week course of a mixed tocopherol preparation of γT inhibited LPS-induced cytokine production by PBMCs *ex vivo*[5] and reduced *in vivo* neutrophil-predominant airway inflammation in response to inhaled endotoxin (LPS) challenge, a potent innate immune stimulus that also promotes T_H1 responses.[8,9]

In this proof-of-concept study, we examined a γT-enriched supplement to determine whether treatment over 24 hours results in significant anti-inflammatory activity that could potentially counter the effects of an innate immune stimulus (LPS). An effective, rapid-onset dosing regimen could then be explored *in vivo* as "rescue" therapy using models of acute neutrophilic airway inflammation.

We conducted an open-label pilot study in 10 healthy volunteers aged 18 to 45 years with no more than mild asthma. Participants consumed 2 γT-enriched geltabs (~1200 mg of γT) every 12 hours for 3 doses. Blood samples were collected at baseline, before dose 3 (24 hours), and 6 hours after dose 3 (30 hours) to measure plasma tocopherols and blood coagulation parameters. PBMCs were isolated from blood collected at baseline and 30 hours for *ex vivo* LPS stimulation and mRNA extraction (see the Methods section in this article's Online Repository at www.jacionline.org). The University of North Carolina Institutional Review Board approved the study.

All 10 subjects completed the study. Half of the subjects reported a history of mild asthma, and 80% were atopic (demographic characteristics summarized in Table E1 in this article's Online Repository at www.jacionline.org). By 30 hours, plasma γT and γ-CEHC concentrations increased significantly from baseline ($P = .002$ for both) (Fig 1, *A*). Serum αT concentrations decreased from baseline by 30 hours ($P < .0001$), while its primary metabolite, α-CEHC, increased significantly ($P = .002$) (see Table E2 in this article's Online Repository at www.jacionline.org).

PBMCs were stimulated with 0 and 0.1 ng/mL of LPS for 24 hours, and supernatants were analyzed as planned *a priori* for production of T_H1 cytokines: IL-1β, IL-6, IL-8, and TNF-α. After γT supplementation, we found a significant decrease in LPS-induced production of IL-1β and IL-6 compared with PBMCs collected before supplementation ($P = .002$ and .04, respectively) (Fig 1, *B*). Although no change was seen in LPS-induced IL-8 production, we found a negative correlation between change in plasma γ-CEHC concentration and change in LPS-induced IL-8 production ($r = -0.75$; $P = .01$) (Fig 2).

As an exploratory measure, we used Nanostring technology to measure differential expression of a panel of 594 genes related to immune responses in isolated PBMCs. Of the genes evaluated, *IL6*, *IL1β*, and *PTGS2* (encoding COX-2) were identified as genes most impacted by γT supplementation. Analyses demonstrated a reduction in LPS-induced expression of *IL6* ($P = .05$) as well as a modest reduction in *IL1β* and *PTGS2* expression ($P = .1$ and .06, respectively) (see Fig E1 in this article's Online Repository at www.jacionline.org). γT was well tolerated with no alteration in international normalized ratio, prothrombin time, or activated partial thromboplastin time.

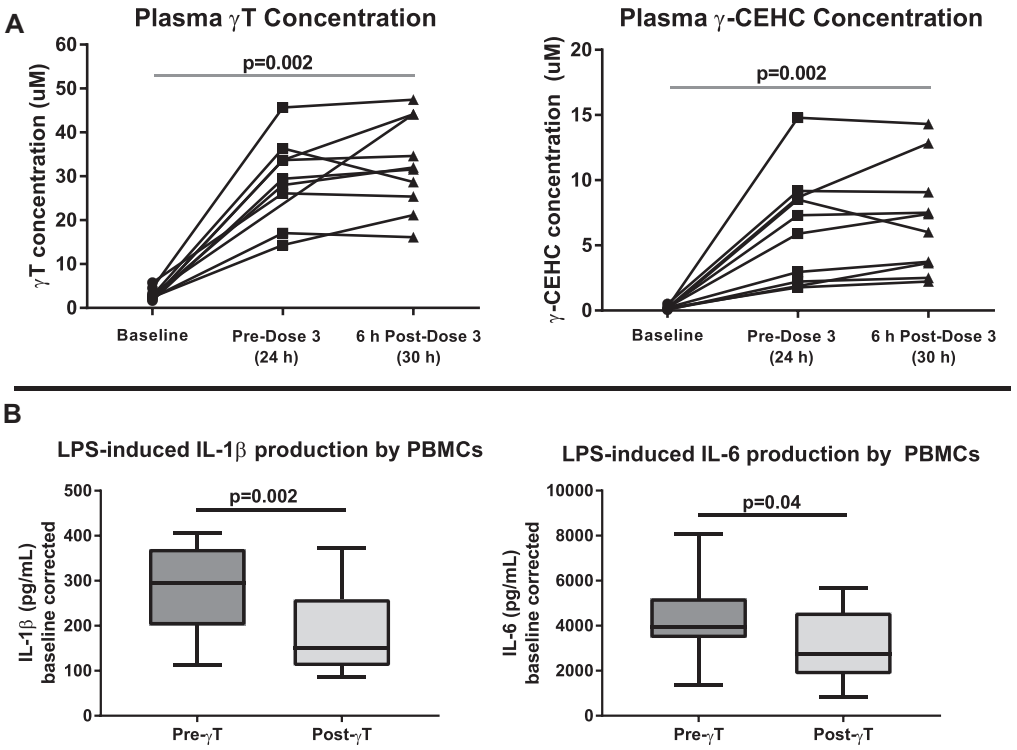

FIG 1. A, Plasma γT and γ-CEHC concentrations increased significantly from baseline at the 24- and 30-hour time points; analyses performed using Wilcoxon matched-pairs signed rank test (n = 10). **B,** PBMCs were isolated from blood samples at baseline and 30 hours and stimulated with LPS for 24 hours. LPS-induced IL-1β and IL-6 were significantly reduced after γT supplementation (data represented as medians and quartiles); analyses performed using paired *t* testing (n = 10).

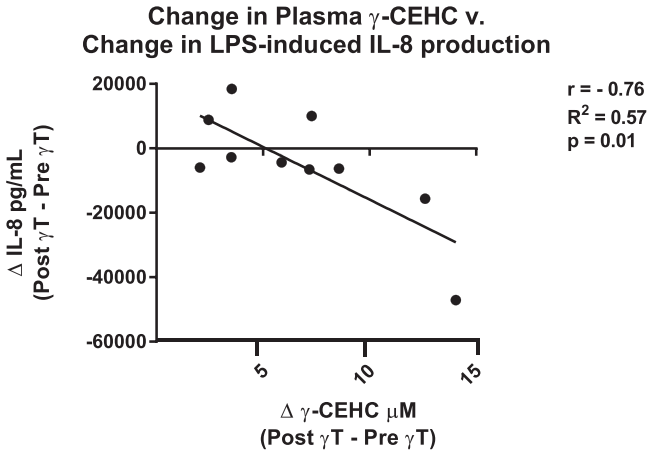

FIG 2. Change in plasma γ-CEHC concentration was significantly negatively correlated with change in LPS-induced IL-8 production by PBMCs ($r = -0.76$; $P = .01$), suggesting that a greater increase in γ-CEHC with γT supplementation results in reduced IL-8 production during LPS challenge.

Our results show that a short course of γT supplementation resulted in a significant increase in plasma γT and γ-CEHC concentrations and reduced LPS-induced IL-6 and IL-1β production by PBMCs. These findings are supported by reduced expression of *IL6* and, to a lesser extent, *IL1β*. We found a significant negative correlation between change in plasma γ-CEHC concentration and LPS-induced production of IL-8, a potent chemoattractant for neutrophils, which could account for the neutrophil-attenuating effects of γT on LPS-induced airway inflammation demonstrated in our previous studies.[8,9] γT has previously been shown to inhibit the COX-2 enzyme in the cellular environment.[3] Our results show that *PTGS2*, encoding COX-2, is among the genes most highly impacted by γT, which may regulate COX-2 at the transcriptional level as well.

Although αT supplementation is known to decrease plasma γT levels, to our knowledge, the converse has not been previously reported in humans. This finding could be the result of γT-driven increase in metabolism of αT, supported by the increase in plasma α-CEHC. Increased excretion of αT is another possible explanation, but this was not measured in our study. It is unknown whether plasma αT concentrations would decline further with continued γT treatment, whether tissue αT concentrations were affected, or what the consequences of this decline would be. Further study is required to determine the effects of longer courses of γT supplementation on αT levels.

Our study has several limitations including small sample size and lack of a control group. Anti-inflammatory effects measured were derived from *ex vivo* experiments with PBMCs, which may not reflect the behavior of airway lymphocytes and macrophages in patients with asthma. Further study is required to determine the efficacy of γT for airway inflammation in humans with asthma. Also, differential gene expression was measured following 24-hour LPS stimulation; the impact of γT on early response genes to LPS is unknown.

In conclusion, we have shown that a short course of γT supplementation significantly increased plasma γT and γ-CEHC concentrations and demonstrated anti-inflammatory effects. As a

next step, this regimen will be evaluated for its *in vivo* impact on models of neutrophilic airway inflammation in humans with asthma.

Allison J. Burbank, MD[a,b]*
Charity G. Duran, PhD[a]*
Martha Almond, RRT[a]
Heather Wells, BS[a]
Sha'Leema Jenkins, BA[a]
Qing Jiang, PhD[c]
Chao Yang, MPhil[c]
Ting Wang, MS[d]
Haibo Zhou, PhD[d]
Michelle L. Hernandez, MD[a,b]
David B. Peden, MD, MS[a,b]

From [a]the Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina, Chapel Hill, NC; [b]the Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, University of North Carolina, Chapel Hill, NC; [c]the Department of Nutrition Science, Purdue University, West Lafayette, Ind; and [d]the Department of Biostatistics, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC. E-mail: allison_burbank@med.unc.edu.

*Co-first authors.

This work was supported by grant numbers R01ES023349, P30ES010126, and US EPA CR 83578501. A.J.B. is supported by grant number 2T32GM086330. C.G.D. is supported by grant number 5T32ES007126-33. H.Z. and T.W. are supported by grant number R01ES021900. The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the US Environmental Protection Agency.

Disclosure of potential conflict of interest: C. G. Duran received Curriculum in Toxicology T32 Training Grant for this and other works and is employed by UNC Chapel Hill. M. Almond's institution received grant numbers R01ES023349, P30ES010126, and US EPA CR 83578501 for this work. Q. Jiang personally received a National Institutes of Health grant for this work and other works. M. L. Hernandez's institution received a grant from the National Institute of Environmental Health Sciences (NIEHS) for this work. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Hoskins A, Roberts JL III, Milne G, Choi L, Dworski R. Natural-source d-alpha-tocopheryl acetate inhibits oxidant stress and modulates atopic asthma in humans in vivo. Allergy 2012;67:676-82.
2. Miller ER III, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Ann Intern Med 2005;142:37-46.
3. Jiang Q, Ames BN. Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. FASEB J 2003;17:816-22.
4. Patel A, Liebner F, Netscher T, Mereiter K, Rosenau T. Vitamin E chemistry: nitration of non-alpha-tocopherols: products and mechanistic considerations. J Org Chem 2007;72:6504-12.
5. Wiser J, Alexis NE, Jiang Q, Wu W, Robinette C, Roubey R, et al. In vivo gamma-tocopherol supplementation decreases systemic oxidative stress and cytokine responses of human monocytes in normal and asthmatic subjects. Free Radic Biol Med 2008;45:40-9.
6. Berdnikovs S, Abdala-Valencia H, McCary C, Somand M, Cole R, Garcia A, et al. Isoforms of vitamin E have opposing immunoregulatory functions during inflammation by regulating leukocyte recruitment. J Immunol 2009;182:4395-405.
7. Marchese ME, Kumar R, Colangelo LA, Avila PC, Jacobs DR Jr, Gross M, et al. The vitamin E isoforms alpha-tocopherol and gamma-tocopherol have opposite associations with spirometric parameters: the CARDIA study. Respir Res 2014;15:31.
8. Wagner JG, Birmingham NP, Jackson-Humbles D, Jiang Q, Harkema JR, Peden DB. Supplementation with gamma-tocopherol attenuates endotoxin-induced airway neutrophil and mucous cell responses in rats. Free Radic Biol Med 2014;68:101-9.
9. Hernandez ML, Wagner JG, Kala A, Mills K, Wells HB, Alexis NE, et al. Vitamin E, gamma-tocopherol, reduces airway neutrophil recruitment after inhaled endotoxin challenge in rats and in healthy volunteers. Free Radic Biol Med 2013;60:56-62.

Available online May 12, 2017.
http://dx.doi.org/10.1016/j.jaci.2017.04.030

Pulmonary inflammation in patients with chronic obstructive pulmonary disease with higher blood eosinophil counts

To the Editor:

Higher eosinophil counts in induced sputum and blood predict a greater clinical response to inhaled corticosteroids (ICS) in patients with chronic obstructive pulmonary disease (COPD).[1,2] This suggests a different profile of inflammation that is corticosteroid sensitive in patients with COPD with higher blood (or sputum) eosinophil counts. However, the nature of lung inflammation in patients with COPD with higher blood eosinophil counts is not well understood.

We have characterized pulmonary inflammation in patients with COPD with higher compared with lower blood eosinophil counts. We obtained induced sputum, bronchial mucosa, and bronchoalveolar lavage (BAL) samples to investigate whether patients with COPD with higher blood eosinophil counts have increased eosinophil numbers throughout the lungs. Furthermore, we investigated differences between groups in levels of inflammatory proteins and airway remodeling markers.

Patients with COPD older than 40 years with postbronchodilator FEV_1/forced vital capacity ratio of less than 0.7 and 10 or more pack-year smoking history were recruited. Skin prick testing to the common allergens house dust mite, cat hair, and grass pollen mix was performed; patients with a positive skin prick test result to any allergen were excluded. Patients with a previous diagnosis of childhood or adult asthma were excluded. All patients provided written informed consent using protocols approved by the Greater Manchester Ethics Committees (East, 05/Q1402/41 and South, 06/Q1403/156). Patients were classified according to blood eosinophil counts as eosinophil^{low} or eosinophil^{high} (<150 cells/μL or >250 cells/μL, respectively); patients with counts between 150 cells/μL and 250 cells/μL were excluded. Clinical assessments included symptom assessment, skin prick testing, exhaled nitric oxide, spirometry with reversibility, blood and sputum sampling, followed by bronchoscopy on a separate day. A total of 102 inflammatory proteins in the serum, sputum, and BAL were measured using a multiplex panel. IL-5, CCL11, and eosinophil-derived neurotoxin were measured in sputum and BAL supernatants. Endobronchial biopsy sections were stained with hematoxylin and eosin to quantify basement membrane thickness. Eosinophils were identified by Luna staining. Immunohistochemistry was performed with primary antibodies directed against neutrophils, macrophages, CD4 helper T cells, CD8 cytotoxic T cells, mast cells, basophils, and Tenascin (TNC). Reticular basement membrane (RBM) thickness and TNC staining thickness within the RBM were calculated. Differences between groups were analyzed using unpaired *t* tests, Mann-Whitney tests, and chi-square tests as appropriate. Significant differences in multiplex protein levels between groups were defined by median fold ratio (MR) of more than ±2 and *P* value of less than .05. Full details of the methods (including manufacturer details) and statistical analysis are provided in this article's Online Repository at www.jacionline.org.

Twenty-one eosinophil^{low} and 20 eosinophil^{high} patients were recruited with median blood eosinophil counts of 0.10×10^9/L and 0.41×10^9/L, respectively ($P < .001$; see Fig E1, *A* and *B*,

METHODS

Subjects

We recruited subjects who were aged 18 to 45 years and otherwise healthy without significant chronic medical conditions apart from mild asthma. Before study entry, subjects underwent a general health screen including a detailed medical history, physical examination, baseline laboratory evaluation, spirometry, allergy skin testing, and methacholine challenge to establish diagnosis of asthma. Subjects who were found to be pregnant, nursing an infant, regularly taking medications that modified immune/inflammatory responses (nonsteroidal anti-inflammatory drugs, antihistamines, anticoagulants, oral or inhaled corticosteroids, immune-suppressing medications), or had a history of abnormal blood cell counts or abnormal blood coagulation parameters were excluded. Subjects were asked to avoid taking any vitamin or herbal supplements during the study period except test agents administered for this study. This study was approved by the University of North Carolina Institutional Review Board and is listed on ClinicalTrials.gov, NCT02610829.

γT Supplement

Both active and placebo treatments were provided by Callion Pharma, LLC (Jonesborough, Tenn). The active treatment consisted of softgels containing 612 mg γT, 7 mg αT, 28 mg βT, and 8 mg δT. The placebo treatment consisted of 700 mg of safflower oil per softgel and contained 0.05 mg γT, 0.35 mg αT, 0.06 mg βT, and 0.01 mg δT. Both products were analyzed for microbial substances and heavy metal content. All analyses of product content were performed by independent laboratories (Medallion Labs, North Minneapolis, Minn; Microbac Laboratories, Louisville, Ky). A summary of analytic results was provided to the investigators by Callion Pharma, LLC.

Analysis of plasma tocopherols

Quantitation of tocopherols. γT, αT, and δT were measured by an HPLC assay with electrochemical detection. Briefly, tocopherols were extracted from plasma using a mixture of methanol/hexane (2:5, v/v). All extractions were carried out in the presence of 0.8 mM butylated hydroxytoluene. After centrifugation at 1000*g* for 10 minutes at 4°C, the top hexane layer was collected and evaporated under N_2, and the dried residue was redissolved in ethanol. Tocopherols were separated on a 150 × 4.6 mm, 5 μm Supelcosil LC-18-DB column, and eluted with 95:5 (v/v) methanol/water with final 25 mM of lithium acetate (pH 4.7) at a flow rate of 1.2 mL/min. Tocopherols were monitored by coulometric detection (Model Coulochem II, ESA Inc, Chelmsford, Mass) at 350 (upstream) and 450 mV (downstream electrode) using a Model 5011 analytic cell.

Quantitation of γ-CEHC and α-CEHC. γ-CEHC and α-CEHC were analyzed using LC-MS/MS. Plasma samples were extracted by a solvent mixture containing 6 volumes of working methanol (containing 0.2 mg/mL ascorbic acid) and 12 volumes of hexane via vigorous vortexing for 1 minute. After centrifugation at more than 12,000 rpm for 2 minutes, the methanol layer (90% to 95%) was transferred into a clean tube and the residual pellet was extracted 1 more time with 4 volumes of working methanol. After vortexing and centrifugation, the combined methanol layers were dried under nitrogen. During the extraction procedure, samples were protected from light. Before LC-MS/MS analysis, dried samples were reconstituted into methanol containing 0.2 mg/mL ascorbate and αT-5'-carboxychromanol (1 or 5 μM) was added as an internal standard. The LC-MS/MS analysis was performed with an Agilent 1200 LC system coupled to an Agilent 6460 QQQ mass spectrometer equipped with a jet stream electrospray ionization source (Santa Clara, Calif). The LC and electrospray ionization conditions are set as previously described. γ-CEHC was quantified using an external standard.

Ex vivo LPS challenge of PBMCs

LPS (*Escherichia coli* O111:B4; TCA-extracted, ≤1% protein content) was obtained from Sigma-Aldrich (St Louis, Mo). PBMCs were isolated from whole blood in BD Vacutainer CPT tubes as per the manufacturer's protocol. For each experimental condition, 300,000 cells were resuspended in RPMI with 10% FBS in a final volume of 500 μL in 5-mL polypropylene tubes. PBMCs were incubated for 24 hours at 37°C in media containing 0 or 0.1 ng/mL LPS. Each experimental condition was performed in triplicate. Cells were centrifuged, pooled, and resuspended in Ambion RNA Lysis buffer for RNA extraction per the manufacturer's protocol. Supernatants were reserved from centrifuged cells for cytokine analysis by ELISA (Mesoscale Discovery, Gaithersburg, Md).

Nanostring analysis

RNA isolated from PBMCs was prepared at a concentration of 10 ng/mL in 10 μL for analysis with the Nanostring nCounter Immunology V2 gene expression panel. Raw data were normalized using internal positive control probes included with the assay to account for all platform-associated sources of variation. Samples were also normalized to housekeeping reference genes (ABCF1, EEF1G, POLR1B, POLR2A, PPIA, and TBP). The normalization factor for each sample was the overall average of the geometric means across all samples divided by the geometric mean of each sample to get a sample-specific normalization factor. Normalized values were then used for calculating fold changes in LPS-induced inflammatory gene expression with or without γT supplementation.

Statistical analysis

Comparisons between cytokine production with and without γT were conducted using paired *t* tests or Wilcoxon matched-pairs signed rank tests depending on whether the normality assumption was met. Pearson correlation coefficients were calculated to examine the linear relationship between change in cytokine production and change in plasma tocopherol concentrations. For analysis of gene expression changes, we used repeated-measures ANOVA and corrected for multiple comparisons by controlling for false-discovery rate. Criterion for significance was taken to be $P \leq .05$.

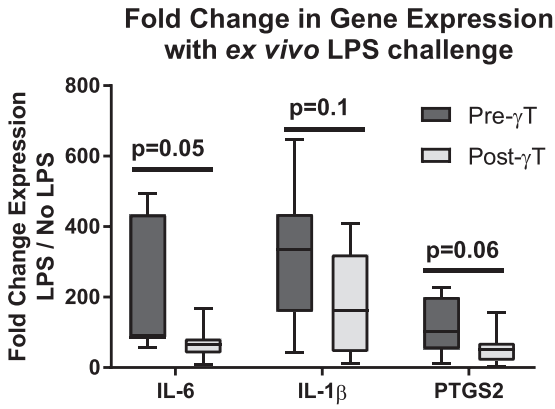

FIG E1. PBMCs demonstrated reduced gene expression of *IL6* following γT supplementation (n = 7). *IL1β* and *PTGS2* expressions were also modestly reduced (n = 7). Analyses were performed using repeated-measures ANOVA and corrected for multiple comparisons by controlling for false-discovery rate. *PTGS2,* Prostaglandin-endoperoxide synthase 2.

TABLE E1. Demographic characteristics of the 10 study volunteers

Characteristic	Value
Age (y), median (range)	34.5 (22-43)
Sex: female/male, n	3/7
Race, n	
White	8
Black	2
Ethnicity, n	
Hispanic	1
Non-Hispanic	9
Body mass index (kg/m^2), median (range)	24.25 (19.8-33.4)
Current asthma, n (%)	5 (50)
Atopic, n (%)	8 (80)

TABLE E2. γ-, α-, δ-tocopherols and α- and γ-CEHC concentrations in serum (represented as median [range]) of 10 volunteers at baseline and at 24 and 30 hours after the start of γT dosing

Concentration	Baseline	24 h	30 h
γ-tocopherol (μM)	2.44 (1.65-5.8)	29.43* (14.31-45.65)	31.77* (16.1-47.46)
α-tocopherol (μM)	21.42 (19.24-26.34)	15.15† (12.01-18.4)	14.05† (12.36-16.81)
δ-tocopherol (μM)	0.08 (0.03-0.51)	0.22 (0.07-0.58)	0.23 (0.09-0.54)
γ-CEHC (μM)	0.22 (0.06-0.51)	7.30* (1.78-14.8)	6.71* (2.22-14.3)
α-CEHC (μM)	0.008 (0.003-0.02)	0.029† (0.004-0.055)	0.022† (0.006-0.062)

*Significant change from baseline ($P < .05$) by Wilcoxon matched-pairs signed rank test.

†Significant change from baseline ($P < .05$) by paired t test.