CHEST

Original Research

DIFFUSE LUNG DISEASE

Nicotine Treatment Improves Toll-Like Receptor 2 and Toll-Like Receptor 9 Responsiveness in Active Pulmonary Sarcoidosis

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Background: **New evidence links nicotine to the regulation of T cell-mediated inflammation via α7 nicotinic cholinergic receptor activation, and chronic nicotine exposure (smoking) reduces the incidence of granulomatous diseases. We sought to determine whether nicotine treatment was well tolerated while effectively normalizing immune responses in patients with active pulmonary sarcoidosis.**

Methods: **Consenting adults with symptomatic sarcoidosis (n = 13) were randomly assigned to receive 12 weeks of nicotine treatment plus conventional therapy or conventional therapy alone. Obtained blood cells were evaluated for their responsiveness to selected Toll-like receptor (TLR) and nucleotide oligomerization domain-like receptor ligands and T cell surface marker expression before and after nicotine treatment. Asymptomatic patients (n = 6) and disease-free subjects (n = 6) served as comparative control subjects. Adverse events were monitored for the duration of the study.**

Results: **Compared with the asymptomatic group, symptomatic patients had impaired peripheral responses to TLR2, TLR4, and TLR9 ligands (anergy) and reduced peripheral populations of $CD4^+FoxP3^+$ regulatory T cells (Tregs). Nicotine treatment was associated with restoration of TLR2 and TLR9 responsiveness, and expansion of Tregs, including the $CD4^+CD25^-FoxP3^+$ phenotype. There were no serious adverse events or signs of nicotine dependency.**

Conclusions: **Nicotine treatment in active pulmonary sarcoidosis was well tolerated and restored peripheral immune responsiveness to TLR2 and TLR9 agonists and expansion of $FoxP3^+$ Tregs, including a specific "preactivated" ($CD25^-$) phenotype. The immune phenotype of patients with symptomatic sarcoidosis treated with nicotine closely resembled that of asymptomatic patients, supporting the notion that nicotine treatment may be beneficial in this patient population.**

Trial registry: **ClinicalTrials.gov; No.: NCT00701207; URL: www.clinicaltrials.gov**

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Abbreviations: α7 nAChR = nicotinic cholinergic receptor α7 subunit; IFN-γ = interferon-γ; NLR = nucleotide oligomerization domain-like receptor; PBMC = peripheral blood mononuclear cell; PPD = purified protein derivative; qRT-PCR = quantitative real-time polymerase chain reaction; Th = T helper cell type 1; TLR = Toll-like receptor; TNF-α = tumor necrosis factor-α; Treg = regulatory T cell

Sarcoidosis is a common interstitial lung disease of unknown cause for which highly effective, inexpensive, and well-tolerated treatments are lacking. For instance, corticosteroids, the mainstay of sarcoidosis treatment, are of unclear long-term clinical benefit,[1] and their use is independently associated with a reduction in patient-perceived quality of life.[2] Thus, there is a need for novel disease-modifying treatments that are better tolerated.

The standard approach to treating symptomatic pulmonary sarcoidosis aims to suppress T cell-mediated immune responses and is based on the premise that the disease is a manifestation of a hyperactive T helper cell type 1 (Th1)-type immune response to environmental

antigens, as demonstrated in the lungs of patients with active pulmonary sarcoidosis.[3,4] Paradoxically, these patients often exhibit peripheral anergy, which is believed to relate to impaired regulatory T cell (Treg) activity.[5] Indeed, Treg dysfunction has been incriminated as a potential cause of chronically active pulmonary sarcoidosis.[6,7]

A growing body of evidence indicates that nicotine has potent immunomodulatory actions, including suppression of Th1-type immune responses, through its interaction with the nicotinic cholinergic receptor α7 subunit (α7 nAChR).[8,9] Simulation of α7 nAChR is shown to increase the suppressive actions of Tregs[10] and attenuates Th1-type immune responses.[8,9,11] In this regard, tobacco smoke, the primary source of nicotine consumed by humans, inhibits T cell-mediated immune responses[12] and reduces bronchoalveolar fluid T-cell CD4:CD8 ratios in patients with pulmonary sarcoidosis.[13] Recognizing that cigarette smoke contains many potentially immune-modulating molecules other than nicotine,[14] it is noted that chronic nicotine exposure is sufficient to induce T-cell anergy.[11] The immunomodulatory actions of nicotine are linked to altered sensing of antigens by Toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs),[15,16] receptors incriminated in the pathogenesis of idiopathic granulomatous diseases, such as sarcoidosis and Crohn's disease.[17,18] Suppression of Th1-type immune responses by nicotine may explain the observed twofold increased incidence of active TB in Taiwanese smokers[19] and the protective effects of smoking with respect to developing noninfectious granulomatous diseases such as sarcoidosis[13,20] and hypersensitivity pneumonitis.[21,22]

The immunomodulatory effects of nicotine are under investigation for the treatment of various inflammatory diseases, including Crohn's disease,[23] ulcerative colitis,[24] and rheumatoid arthritis.[25,26] Preliminary studies indicate that nicotine suppresses tissue inflammation and reduces disease-specific symptoms in patients with Crohn's disease,[23] a disorder of the intestines that is histopathologically indistinguishable from sarcoidosis, and inhibits the development of hypersensitivity pneumonitis in experimental models.[27] By suppressing granuloma formation in response to environmental antigens (eg, TLR and NLR ligands), we reason that nicotine may be beneficial for the treatment of sarcoidosis. In advance of a definitive clinical trial, we sought to determine whether nicotine treatment was well tolerated while normalizing immune cell antigen responses and immune cell populations in patients with active pulmonary sarcoidosis.

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Materials and Methods

Overview of the Study Design

Please refer to e-Appendix 1 for additional methodologic details. Adult patients (≥ 18 years old) were enrolled in the study after first obtaining written informed consent in compliance with The Ohio State University Biomedical Sciences Institutional Review Board (#2008H0006). In addition, the use of nicotine as an investigational new drug for sarcoidosis was approved by the US Food and Drug Administration. The diagnosis of sarcoidosis was based on established criteria, including biopsy confirmation and exclusion of other potential causes.[28] In this regard, all subjects were purified protein derivative (PPD) skin test negative and had no evidence of infectious organisms by tissue acid-fast bacillus and Grocott methenamine silver staining. The study design was unblinded, randomized, and controlled, with specifically identified patient inclusion and exclusion criteria. All patients were diagnosed as having sarcoidosis; however, the nicotine treatment study was restricted to those patients with symptomatic (active) granulomatous lung disease (radiographic stage II or III), including chronic cough and/or dyspnea,[29] of at least 6 months' duration following diagnosis. Patients with any of the following characteristics: active smokers, those with previous splenectomy, prisoners, and those who required high-dose immunosuppression (ie, ≥ 0.2 mg/kg/d prednisone [or equivalent] or > 15 mg/wk methotrexate or required second-line cytolytic agents [eg, cyclophosphamide, azathioprine] or anti-tumor necrosis factor [TNF] treatments [eg, thalidomide, anti-TNF antibodies, and so forth]) to control disease activity were excluded. The study further excluded patients at high risk of complications relating to the use of nicotine, including patients with known intolerance of nicotine or those with active cardiac or CNS disease who were at higher

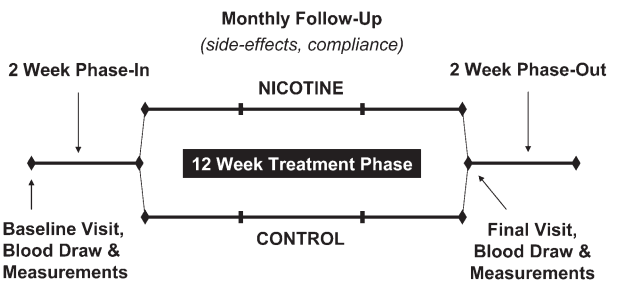

Figure 1. Summary schematic of the patient nicotine treatment protocol.

Table 1—*Human TLR and NLR Ligands Used in the Whole Blood Assay Designed to Test Peripheral Immune Cell Responsiveness in Patients With Pulmonary Sarcoidosis*

Target TLR/NLR Receptor	Ligand Name	Ligand Description	Ligand Dose
TLR2	PGN-K12	Peptidoglycan from *Escherichia coli K12*	5 μg/mL
	PI-LAM	*Mycobacterium tuberculosis* antigen	2 μg/mL
	RvLM	*M tuberculosis* antigen	2 μg/mL
TLR3	Poly I:C	Polyinosinic-polycytidylic acid, synthetic analog of dsRNA	1 μg/mL
TLR4	UltraPure LPS	UltraPure lipopolysaccharide from *Escherichia coli* 0111:B4 strain	100 ng/mL
TLR7	Imiquimod	Synthetic antiviral imidazoquinoline amine analog	5 μg/mL
TLR8	ssRNA40/ LyoVec	Single-stranded RNA GU-rich oligonucleotide complexed with the cationic lipid, LyoVec	1 μg/mL
TLR9	ODN2336 CpGA	Type A CpG oligonucleotide	1.5 μM
NOD1 (NLRC1)	Tri-DAP	Peptidoglycan-like L-Ala-γ-D-Glu mDAP	2 μg/mL
NOD2 (NLRC2)	MDP	Muramyl dipeptide	1 μg/mL

NLR = nucleotide oligomerization domain-like receptor; TLR = Toll-like receptor.

risk of cardiac arrhythmias or seizures, respectively. Finally, patients with extensive pulmonary fibrosis (based on lung biopsy or high-resolution CT scan) or those who were unable to provide informed consent were also excluded. Patients were then assigned to symptomatic (n = 13) and asymptomatic (n = 6) groups based on their reported symptoms at the time of enrollment. Although diagnosed with radiographic evidence of biopsy-proven pulmonary sarcoidosis, asymptomatic patients were symptom-free, were not receiving any form of sarcoidosis-directed therapy, and, thus, did not receive nicotine but served as a reference cohort according to the study design. Symptomatic patients who met exclusion criteria demonstrated signs of disease progression (eg, altered lung function [pulmonary function tests]) with developed symptoms of cough and dyspnea in accordance with study inclusion criteria and remained so despite receipt of conventional first-line therapies. In addition, these are patients who would be considered candidates for escalation of corticosteroids and/or steroid-sparing agents (eg, methotrexate)[30] but elected instead to participate in the current study.

Upon enrollment in the study, basic pulmonary function tests including a baseline FVC measurement were performed and blood samples were obtained from a peripheral vein. Symptomatic patients were then further randomized to receive 12 weeks of nicotine treatment by way of continuous-release transdermal patch in conjunction with their conventional therapy (n = 7) according to the basic protocol presented in Figure 1. The daily nicotine patch dose was incrementally increased from 7 mg to 14 mg to 21 mg at 1-week intervals as tolerated based on reported side effects. The patients were then maintained on the highest tolerated dose for the duration of the 12-week treatment period. Dyspnea and sarcoidosis-specific disease activity surveys (eg, St. George's Respiratory Questionnaire, the Sarcoidosis Health Questionnaire) were performed at baseline and repeated at monthly intervals for all patients. Follow-up peripheral blood samples and FVC measurements were obtained at the end of the 12-week treatment period, after which those receiving the nicotine patch underwent dose de-escalation in reverse of the escalation protocol. The patients were monitored for signs of nicotine dependency, as per the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* criteria, including inability to discontinue nicotine or signs of withdrawal.[31]

Whole Blood TLR/NLR Ligand-Immune Cell Response Assay

Ex vivo stimulation of fresh peripheral whole blood samples with specific TLR and NLR ligands (Table 1) for 24 h was performed using a previously established protocol[32] with slight modifications. Quantification of immune cell TNF-α and interferon-γ (IFN-γ) release at collected time points was determined by enzyme-linked immunoabsorbent assay according to the manufacturer's recommendations.

Lymphocyte Population Characterization by Flow Cytometry

Following RBC lysis, nucleated blood cells were isolated from fresh blood samples by centrifugation. They were examined for their T cell surface marker expression using flow cytometry, according to standard protocols.

Real-Time Polymerase Chain Reaction Determination of Peripheral Blood Mononuclear Cell Th1-Related Molecule Expression

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples by differential gradient centrifugation using standard methods. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to established techniques from isolated PBMC RNA. Patient sample results were compared with those obtained from disease-free control subjects (n = 6). Primers used to identify each Th1-related molecule transcript are detailed in e-Table 1.

Statistical Analyses

The data were derived from all patients in each group and expressed as mean ± SEM, and statistical significance was based on a value of $P \leq .05$. SigmaPlot 11.0 software (Systat Software

Table 2—*Patient Demographic Characteristics*

Groups	Age, y	Race[a]	Sex, Male (Female)	FVC, % Predicted
Control (n = 6)	48.8 ± 2.5	5/1/0	3 (3)	
Asymptomatic (n = 6)	48.0 ± 3.4	5/1/0	3 (3)	100.1 ± 4.6
Symptomatic (n = 13)	51.6 ± 2.6	9/4/0	4 (9)	87.3 ± 4.1
Nicotine-treated (n = 7)	53.0 ± 2.4	5/2/0	2 (5)	83.7 ± 6.9

[a]White/black/other.

Table 3—*Self-Reported Organ-Specific Symptoms Based on Survey Results From the Sarcoidosis Health Questionnaire*

Groups	Shortness of Breath	Cough	Skin Problems	Eye Problems	Joint Pain
Asymptomatic (n = 6)	5.5 ± 0.2	6.5 ± 0.2	5.8 ± 1.0	6.0 ± 0.6	3.5 ± 0.7
Symptomatic (n = 13)	2.3 ± 0.3[a]	3.0 ± 0.5[a]	5.5 ± 0.4	5.2 ± 0.4	3.5 ± 0.4
Nicotine-treated (n = 7)	2.4 ± 0.5[a]	3.0 ± 0.7[a]	5.1 ± 0.7	5.1 ± 0.6	3.3 ± 0.6

Each symptom was scored according to the following scale: 1 = severe; 7 = minimal.
[a]$P < .05$ compared with corresponding asymptomatic group symptom.

Inc) and SYSTAT 13.0 software (Systat Software Inc) were used to plot the data and carry out the statistical analyses, respectively.

Results

Patient Demographics and Clinical Findings in Patients With Symptomatic Pulmonary Sarcoidosis Treated With Nicotine

Patients with sarcoidosis and control subjects were relatively well matched in terms of age, race, and sex (Table 2). In terms of immunosuppressant treatment, symptomatic patients were receiving an average of 8 mg/d prednisone, and approximately 40% of these patients were also in receipt of 10 mg/wk of methotrexate on average. Additionally, one patient was being treated with hydroxychloroquine (200 mg/d). Overall, these patients were randomly distributed equally to receive or not receive nicotine. Although symptomatic patients had lower baseline FVC measurements compared with the asymptomatic group, they did not reach statistical significance. Symptomatic patients reported significantly greater cough and shortness-of-breath symptoms; however, there was no significant difference in perceived extrapulmonary disease manifestations (Table 3).

Patients were closely monitored for nicotine-related adverse events throughout the study period, particularly during the escalation and de-escalation phases of treatment. As expected, side effects were most common during the escalation phase and typically declined in intensity or completely resolved thereafter. Minor side effects occurring at a frequency exceeding 10% were headaches, abnormal dreams, agitation, insomnia, and local skin irritation. These symptoms are in keeping with previously documented side effects from transdermal nicotine delivery using the patch.[33] One patient was unable to complete the nicotine treatment protocol due to repeated local skin irritation. In summary, no serious adverse events were reported, and none of the patients demonstrated signs of dependency during the de-escalation phase.

Although not considered a primary end point, there was no significant change in the reported sarcoidosis symptoms (eg, St. George's Respiratory Questionnaire, the Sarcoidosis Health Questionnaire) (Table 3) or the measured FVC (Table 2) following nicotine treatment compared with baseline.

Normalization of Peripheral T-Cell Population Subsets in Symptomatic Patients Treated With Nicotine

Whole blood samples obtained at baseline and after 12 weeks of nicotine treatment were processed to isolate nucleated blood cells and then analyzed using flow cytometry for their expression of cell surface markers specific to lymphocytes and various T-cell subsets. As expected based on previous studies,[34] lymphocytes represented a smaller fraction of the nucleated blood cell population in patients with symptomatic sarcoidosis compared with the asymptomatic group (Fig 2). The distribution of $CD4^+$ T cells was similar in all sarcoidosis groups (Fig 3A); however, certain regulatory T-cell subsets were altered in the symptomatic patients.

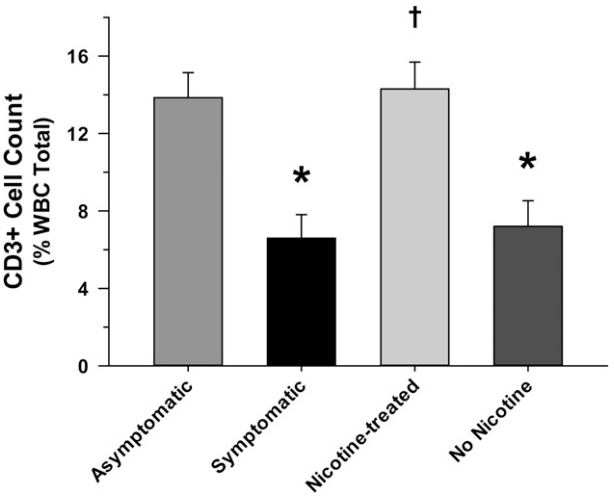

Figure 2. Lymphocyte differential of peripheral nucleated blood cells based on flow cytometry analysis. Patient nucleated blood cells isolated from peripheral whole blood samples were analyzed using flow cytometry to determine their lymphocyte distribution as indicated by $CD3^+$ cell surface expression (*$P < .05$, compared with the asymptomatic group; †$P < 0.05$, relative to the symptomatic and no nicotine groups, Wilcoxon rank-sum test). Asymptomatic and symptomatic group data represent baseline measurements, whereas the nicotine-treated and no nicotine group data denote measurements obtained posttreatment from those symptomatic patients randomized to receive or not receive nicotine, respectively.

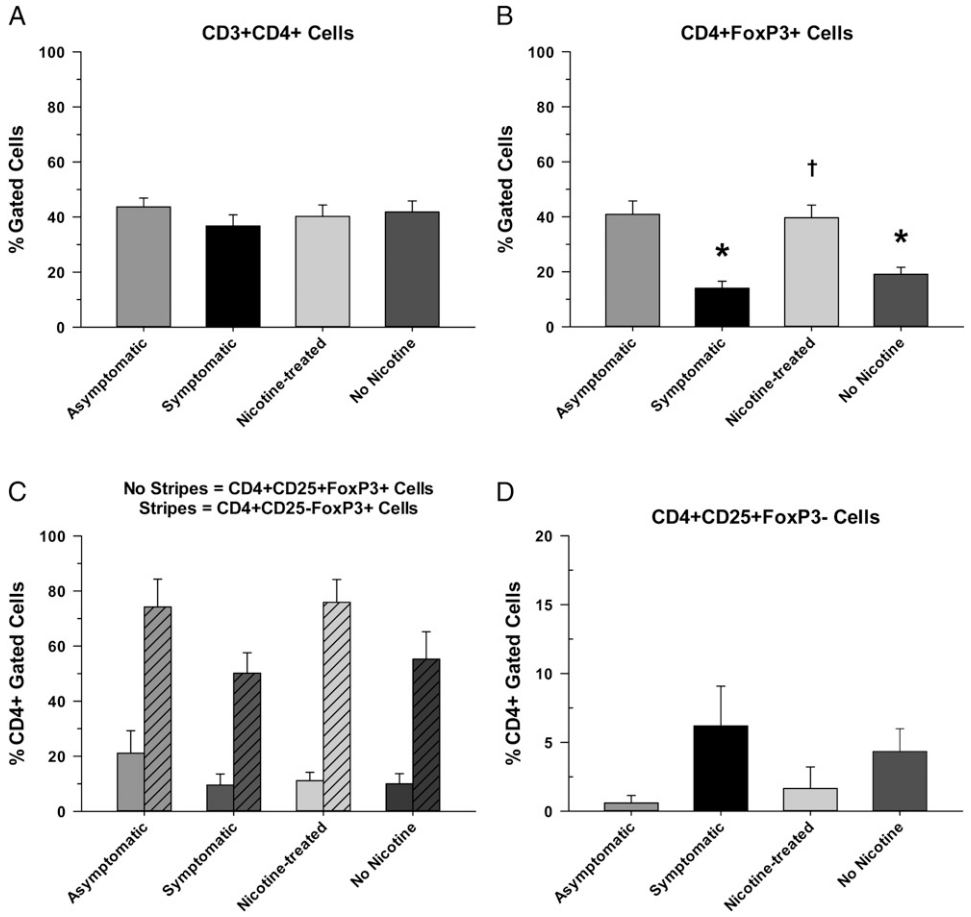

FIGURE 3. Peripheral nucleated blood cell T-cell population distribution in distinct sarcoidosis phenotypes and following nicotine treatment. Flow cytometry gated to focus on lymphocyte populations was used to analyze patient nucleated blood cells to determine T-cell subsets as indicated by their respective cell surface markers. Asymptomatic and symptomatic group data represent baseline measurements, whereas the nicotine-treated and no nicotine group data denote measurements obtained posttreatment from those symptomatic patients randomized to receive or not receive nicotine, respectively. A, The distribution of $CD4^+$ lymphocytes was similar for all groups. B, $CD4^+FoxP3^+$ regulatory T cells (Tregs) were significantly reduced in the symptomatic group and restored after nicotine treatment (*$P < .05$, compared with the asymptomatic group; †$P < .05$, relative to the symptomatic and no nicotine groups, Wilcoxon rank-sum test). C, Further analysis of the Treg populations shown in B demonstrated reductions in both $CD4^+CD25^+FoxP3^+$ (inducible) and $CD4^+CD25^-FoxP3^+$ (preactivated) Tregs in symptomatic patients compared with the asymptomatic group and restoration of these cell populations following nicotine treatment. D, Although not statistically significant, a marked trend toward increased "natural" $CD4^+CD25^+FoxP3^-$ Tregs was observed in symptomatic patients relative to the asymptomatic and nicotine treatment groups.

Most notably, symptomatic patients had a significantly lower distribution of $CD4^+FoxP3^+$ cells (Fig 3B), including $CD4^+CD25^+FoxP3^+$ (inducible regulatory) T cells and $CD4^+CD25^-FoxP3^+$ (preactivated regulatory) T cells (Fig 3C). There was a trend toward higher $CD4^+CD25^+FoxP3^-$ (natural regulatory) T cells in the symptomatic patients (Fig 3D). The effects of nicotine treatment on lymphocyte and T-cell subset populations could be summarized as a conversion to the asymptomatic phenotype, including increases in total lymphocyte and $FoxP3^+$ Treg populations. Those symptomatic patients randomized to not receive nicotine did not demonstrate any change from their baseline measurements (Fig 3).

The Effect of Nicotine Treatment on the Expression of Th1-Related Molecules

Based on previous studies indicating that Th1-related molecule expression is increased in patients with pulmonary sarcoidosis,[3,35] we sought to determine whether nicotine influenced Th1-related molecule expressions in unstimulated patient PBMCs. In general, PBMCs from patients with sarcoidosis expressed significantly higher levels of Th1-related molecule gene transcripts compared with disease-free control subjects. However, these Th1-related molecule expression levels did not differ significantly between the sarcoidosis groups or following nicotine treatment (Fig 4).

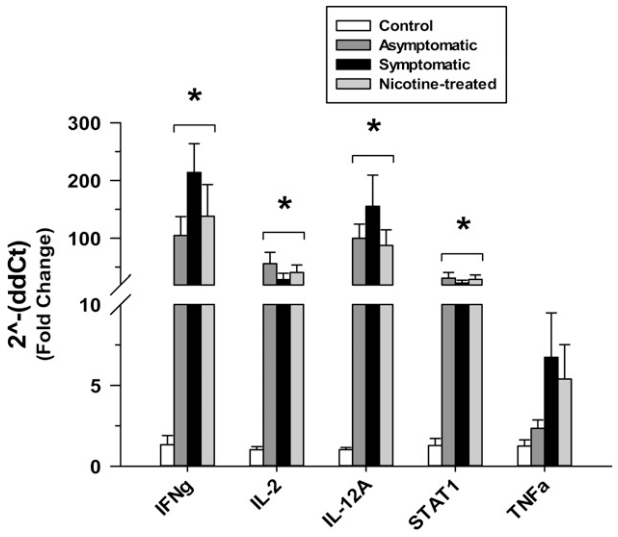

FIGURE 4. T helper cell type 1 (Th1)-related molecule gene expression as determined using quantitative real-time polymerase chain reaction (qRT-PCR) analysis of patient peripheral blood mononuclear cell (PBMC) RNA. Asymptomatic and symptomatic group data represent baseline measurements, whereas the nicotine-treated and no nicotine group data denote measurements obtained post-treatment from those symptomatic patients randomized to receive or not receive nicotine, respectively. qRT-PCR analysis of patient PBMC RNA samples demonstrated an increased expression of putative Th1 immune phenotype molecules in all sarcoidosis groups compared with disease-free control subjects (*$P < .05$, compared with control subjects, Wilcoxon rank-sum test). There was no statistical difference in the baseline (unstimulated) Th1-related molecule expression between the sarcoidosis groups or following nicotine treatment. IFN = interferon; TNF = tumor necrosis factor.

Peripheral Anergy to Specific TLR Ligands in Symptomatic Patients Was Reversed by Nicotine Treatment

Whole blood samples obtained from patients in all sarcoidosis groups were analyzed for their response to specific TLR and NLR ligand treatments for 24 h. Symptomatic patients exhibited significant anergy to TLR2, TLR4, and TLR9 agonists, as indicated by significantly reduced levels of TNF-α and IFN-γ release (Figs 5A, 5B). Interestingly, nicotine treatment restored the responsiveness of the symptomatic group to TLR2 and TLR9 ligands in terms of both TNF-α and IFN-γ release such that their profile more closely resembled that of the asymptomatic group (Figs 5A, 5B). Presumably, nicotine-induced reversal of peripheral anergy reflected restoration of preactivated regulatory T cells (Fig 3C) in peripheral blood rather than direct suppression of Th1 cell activity (Fig 4). Those symptomatic patients randomized to not receive nicotine did not demonstrate any change from their baseline measurements (Figs 5A, 5B). The other ligands tested did not induce significantly different levels of TNF-α or IFN-γ release between the sarcoidosis groups or following nicotine treatment (Figs 5C, 5D).

Effects of Nicotine Treatment Upon the Expression of PBMC α7 nAChR

In general, patients with sarcoidosis exhibited significantly elevated PBMC α7 nAChR expression compared with disease-free control subjects (Fig 6). In addition, 12 weeks of sustained treatment with transdermal nicotine did not promote tachyphylaxis as α7 nAChR expression was not significantly reduced compared with the other sarcoidosis groups.

DISCUSSION

In addition to its well-established CNS dopaminergic actions,[36] research conducted over the past decade has demonstrated nicotine to be a potent modulator of inflammation, including Th1-type immune responses.[8,9,11] This may explain why chronic exposure to nicotine (ie, cigarette smoking) significantly reduces the chances of developing sarcoidosis[13,20] while increasing the risk for developing active TB infection.[19] When provided in a sustained-release form, nicotine levels are stable relative to levels in a typical cigarette smoker, thereby reducing its addictive properties.[37,38] Thus, we were not surprised that transdermal nicotine treatment of 12 weeks' duration in patients with symptomatic pulmonary sarcoidosis was well-tolerated with no reported major adverse events or evidence of addiction. Nicotine was shown to reverse anergy to specific TLR agonists (Figs 5A, 5B) while normalizing lymphopenia and a subpopulation of FoxP3$^+$ Tregs in peripheral blood, an immune profile closely resembling that of asymptomatic patients (Figs 2, 3). These findings suggest that nicotine could safely "reprogram" the immune status of patients with active pulmonary sarcoidosis toward that of patients with an inactive phenotype.

Studies indicate that nicotine may be an effective therapy for other chronic inflammatory diseases. Most notably, nicotine is shown to reduce tissue inflammation and disease-specific symptoms in patients with Crohn's disease,[23] an idiopathic, noninfectious granulomatous disorder of the intestines that is histopathologically similar to sarcoidosis.[39] Likewise, nicotine exposure suppresses granulomatous inflammation and delays the onset and severity of joint inflammation in experimental models of hypersensitivity pneumonitis[27] and rheumatoid arthritis,[25,26] respectively. The immunomodulatory actions of nicotine are related to stimulation of α7 nAChR and consequent activation of JAK-STAT signaling pathways.[40,41] Although nicotine treatment resulted in consistent trends toward reverting Th1-related molecule expression toward that of asymptomatic sarcoidosis, individual variation was quite large, and no statistical differences were observed. Moreover, long-term nicotine exposure did

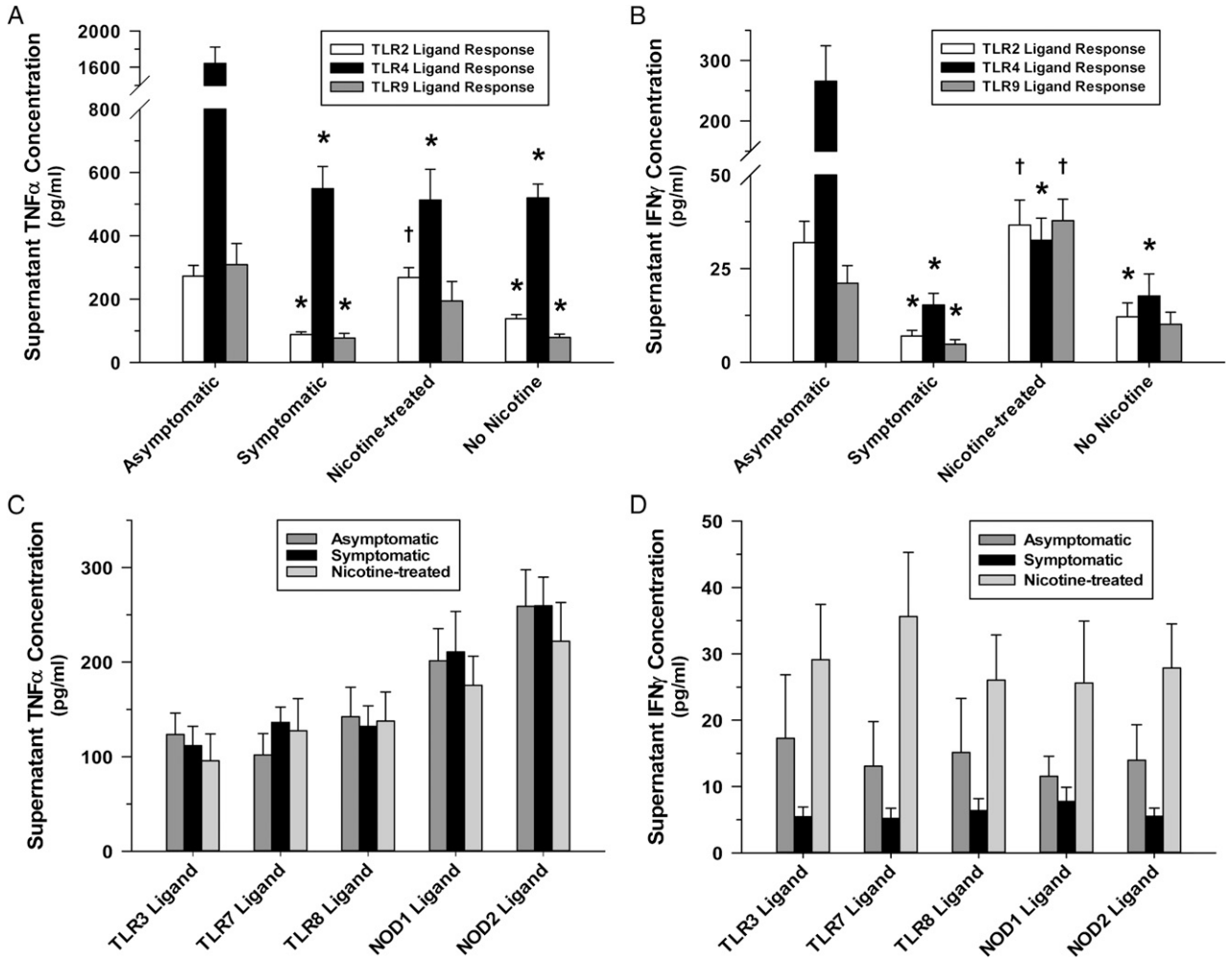

FIGURE 5. Ex vivo patient whole blood cell TNF-α and IFN-γ release in response to specific TLR and nucleotide oligomerization domain-like receptor (NLR) ligand treatment. Patient whole blood cell samples were incubated with various TLR and NLR ligands (Table 1) for 24 h, then supernatants were analyzed for TNF-α and IFN-γ release as determined by enzyme-linked immunoabsorbent assay. Asymptomatic and symptomatic group data represent baseline measurements, whereas the nicotine-treated and no nicotine group data denote measurements obtained posttreatment from those symptomatic patients randomized to receive or not receive nicotine, respectively. A, Compared with the asymptomatic group, TNF-α release in response to TLR2, TLR4, and TLR9 treatment was significantly diminished in the symptomatic patients. As reflected by TNF-α release, nicotine treatment significantly increased TLR2 responsiveness, and there was a strong trend toward restoration of the response to TLR9 treatment. However, nicotine appeared to have no effect on TLR4 responsiveness (*$P < .05$, relative to the corresponding asymptomatic group; †$P < .05$, compared with the matching symptomatic and no nicotine groups, Wilcoxon rank-sum test). B, Compared with the asymptomatic group, IFN-γ release in response to TLR2, TLR4, and TLR9 treatment was significantly reduced in the symptomatic patients. As reflected by IFN-γ release, nicotine treatment significantly increased TLR2 and TLR9 responsiveness but not the response to TLR4 treatment (*$P < .05$, relative to the corresponding asymptomatic group; †$P < .05$, compared with the matching symptomatic and no nicotine groups, Wilcoxon rank-sum test). C, TNF-α release in response to all the other TLR and NLR ligand treatments was not significantly different between the sarcoidosis groups. D, IFN-γ release in response to all the other TLR and NLR ligand treatments was not significantly different between the sarcoidosis groups. TLR = Toll-like receptor.

not alter the expression of α7 nAChR, suggesting that tachyphylaxis relating to changes in receptor density did not occur.

Despite the baseline increase in Th1-related molecule expression, peripheral anergy to specific environmental antigens was observed in patients with symptomatic sarcoidosis. Peripheral anergy is recognized to correlate well with signs of sarcoidosis disease activity, as reflected by more active pulmonary disease requiring escalation of immunosuppressants.[42,43] Indeed, peripheral anergy is typically confined to those with active sarcoidosis and is shown to be a sensitive and specific marker of disease acitivity.[43] In keeping with previous reports, patients with active pulmonary sarcoidosis exhibited peripheral anergy to mycobacterial (primarily TLR2) antigens[44,45] and TLR9 ligands[46] (Figs 5A, 5B). Mycobacterial antigens are of particular interest, as investigations have confirmed their presence in diseased sarcoidosis tissue,[47] presumably relating to impaired antigen clearance,[48] and it is

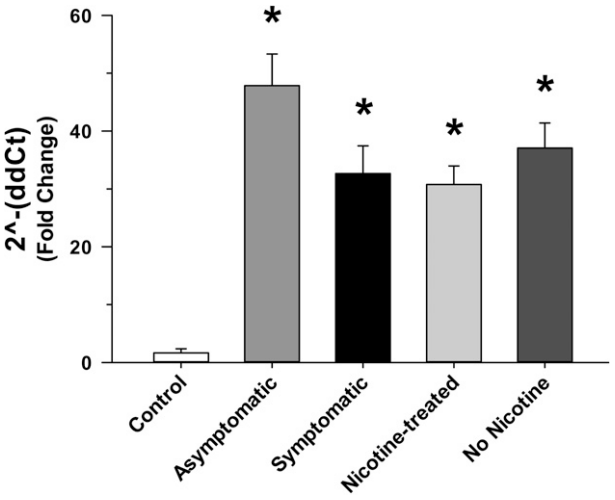

FIGURE 6. α7 nAChR expression as determined using qRT-PCR analysis of patient PBMC RNA. Asymptomatic and symptomatic group data represent baseline measurements, whereas the nicotine-treated and no nicotine group data denote measurements obtained posttreatment from those symptomatic patients randomized to receive or not receive nicotine, respectively. qRT-PCR analysis of patient PBMC RNA samples demonstrated an increased expression of α7 nAChR in all sarcoidosis groups compared with disease-free control subjects (*$P < 0.05$, compared with control subjects, Wilcoxon rank-sum test). However, there was no significant difference in expression between the sarcoidosis groups. α7 nAChR = nicotinic cholinergic receptor α7 subunit. See Figure 4 legend for expansion of other abbreviations.

further shown that a majority of patients with sarcoidosis are sensitized to these antigens.[47,49] The finding of impaired TLR2 and TLR9 responsiveness in the setting of active pulmonary sarcoidosis conforms to the notion that sustained granulomatous inflammation is a manifestation of altered immunity resulting in impaired antigen clearance.[48-50]

Tregs are considered to be major determinants of peripheral anergy in patients with active pulmonary sarcoidosis.[5] Our results support this premise and further indicate that nicotine treatment restores altered Treg populations in symptomatic patients toward those of asymptomatic patients. In addition to normalizing inducible $CD4^+CD25^+FoxP3^+$ Treg populations, nicotine treatment also promoted the expansion of $CD4^+CD25^-FoxP3^+$ (preactivated) Tregs (Fig 3C). This Treg subtype was characterized by Zelenay et al[51] as a "peripheral reservoir of differentiated Tregs that are recruited to the $CD25^+$ pool upon activation." Unlike active ($CD25^+$) Tregs, these $CD25^-$ Tregs lack suppressive function until they are IL-2 stimulated.[51] It is interesting to speculate that these cells would become activated at the site of inflammation (eg, the lungs) to suppress inflammation. In contrast, symptomatic patients appeared to have a trend toward higher expression of $CD4^+CD25^+$-$FoxP3^-$ (thymic) Tregs (Fig 3D), which are predominantly suppressive. We reason that the relative distribution of preactivated (lacking regulatory activity) and activated Tregs influences peripheral anergy to TLR ligands observed in patients with symptomatic sarcoidosis. Further investigations are required to elucidate the mechanisms through which nicotine restores Treg populations and whether Tregs are primarily responsible for selective peripheral anergy in patients with active pulmonary sarcoidosis.

There are limitations inherent to small clinical pilot studies, the primary consequence of which is to obscure potentially significant intergroup differences. As such, the observed statistical differences between asymptomatic and symptomatic as well as the demonstrated effects of nicotine are particularly noteworthy. Since concomitant immunosuppressant medications were not adjusted during the trial, the observed post-treatment changes of the immune profile in the setting of active pulmonary sarcoidosis are ascribed to nicotine. From a mechanistic standpoint, it is unclear whether peripheral blood immune cell responses are representative of immune cells participating more directly in granulomatous inflammation. To address these issues and to establish whether nicotine is an effective treatment of sarcoidosis, future studies should involve more patients and include an analysis of samples (eg, BAL) derived from diseased tissues. Finally, the use of PPD skin testing to screen for latent TB in patients with sarcoidosis was shown to be less sensitive than IFN-γ release assays.[52] Although the regional prevalence of TB is comparatively low (2.1/100,000 in Ohio compared with 3.6/100,000 in the United States from 2010 as per the Centers for Disease Control and Prevention,[53] it is possible that some of the patients who tested negative for PPD were previously exposed to TB, which could have influenced their TLR immune responses.

In conclusion, this study is the first to our knowledge to report that nicotine treatment is well tolerated and has potent immunoregulatory actions in patients with active pulmonary sarcoidosis. In particular, nicotine treatment was associated with reversal of peripheral anergy to TLR2 and TLR9 ligands and resulted in the suppression of Tregs, including a preactivated Treg subtype incriminated in the pathogenesis of autoimmune disease.[54] Following nicotine treatment, patients with active pulmonary sarcoidosis assumed the immune phenotype of asymptomatic patients. The implications of the nicotine-induced correction of peripheral anergy with respect to sarcoidosis-induced lung inflammation and related clinical end points remain to be established; however, these findings indicate that nicotine favors an immune phenotype corresponding to a benign disease phenotype.

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