

# Rapid Communication

## Peroxisome Proliferator–Activated Receptor $\gamma$ Activity Is Deficient in Alveolar Macrophages in Pulmonary Sarcoidosis

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The ligand-activated transcription factor, peroxisome proliferator–activated receptor  $\gamma$  (PPAR $\gamma$ ), has pleiotropic effects on lipid and glucose metabolism as well as modulating immune activity. In Th1-predominant models of inflammatory bowel disease and arthritis, PPAR $\gamma$  ligands can ameliorate clinical disease severity, partly by downregulating a range of inflammatory cytokines. However, PPAR $\gamma$  has not been evaluated in chronic sarcoidosis, a disease characterized by persistent activation of Th1 immune responses in alveolar macrophages. We hypothesized that a deficiency of PPAR $\gamma$  activity contributes to ongoing inflammation in pulmonary sarcoidosis via failure to repress proinflammatory transcription factors. To address this, we studied eight patients with active sarcoidosis and nine healthy control subjects by bronchoscopy. Bronchoalveolar lavage specimens from patients revealed a striking reduction of PPAR $\gamma$  activity by electrophoretic mobility shift assay in alveolar macrophages compared with healthy control subjects, with a concomitant upregulation of nuclear factor (NF)- $\kappa$ B activity. Immunostaining and real-time polymerase chain reaction demonstrated reductions of PPAR $\gamma$  nuclear protein and gene expression. The data show for the first time that alveolar macrophages from patients with active sarcoidosis exhibit activation of NF- $\kappa$ B and deficiency of PPAR $\gamma$ . Although these results do not demonstrate a direct causal effect, they are consistent with the hypothesis that insufficient PPAR $\gamma$  activity contributes to ongoing dysregulated inflammation in pulmonary sarcoidosis by failing to suppress NF- $\kappa$ B.

Sarcoidosis is a multisystem granulomatous inflammatory disease of unclear etiology. The lungs, lymphoreticular system, skin, and eyes are most commonly affected (1). Sarcoid inflammation is mediated by complex interrelationships among macrophages, T-lymphocytes, and a host of cytokines, leading to formation of granulomas and variable degrees of fibrosis. A Th1 immune phenotype, with exuberant production of interleukins 2, 12, and 18 and interferon  $\gamma$

(IFN- $\gamma$ ), is characteristic (2, 3). Immune dysregulation is thought to account for disease persistence in chronic sarcoidosis. The specific factors that govern resolution versus persistence of sarcoidosis are poorly understood.

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated nuclear hormone receptors that have pleiotropic immune modulating effects. Ligand-activated PPAR $\gamma$  forms a heterodimer with the retinoid X receptor and can regulate gene transcription by binding to specific DNA response elements. A major mechanism of PPAR $\gamma$ 's anti-inflammatory activity is likely due to sequestration or inactivation of the coactivators for key proinflammatory transcription factors, including nuclear factor (NF)- $\kappa$ B, activator protein (AP)-1, STAT-1, and Ets, leading to transrepression of inflammatory genes (4–6). Although not evaluated in lung disease, ligands of PPAR $\gamma$  may have salutary effects on several chronic inflammatory diseases, including inflammatory bowel disease, atherosclerosis, and experimental autoimmune encephalomyelitis (7–9).

In healthy lungs, PPAR $\gamma$  is constitutively expressed in epithelial cells, smooth muscle cells, and alveolar macrophages (10–12). Abnormally low PPAR $\gamma$  expression has been demonstrated in pulmonary alveolar proteinosis and pulmonary hypertension (11, 13). Because sarcoidosis is characterized by ongoing inflammation, we hypothesized that PPAR $\gamma$  expression may be deficient in active pulmonary sarcoidosis, a disease characterized by a Th-1 pattern of immune activation. We also hypothesized that an important mediator of inflammation, NF- $\kappa$ B, would be activated in sarcoid alveolar macrophages.

### Materials and Methods

#### Study Population

Subjects with sarcoidosis ( $n = 8$ ) were recruited from patients undergoing routine clinical evaluation for initial diagnosis or confirmation of sarcoidosis. Patient characteristics are listed in Table 1. Patients with sarcoidosis were all characterized by active disease, as defined elsewhere (14). In brief, all patients were symptomatic, with either progressive deterioration of lung function, worsening infiltrates, or both. None had Löfgren's syndrome. The diagnosis was confirmed in all cases by histologic demonstration of non-necrotizing granulomas without evidence of infection. None had evidence for exposure to agents known to cause granulomatous lung disease. Bronchoalveolar lavage (BAL) cell populations were (mean  $\pm$  SD): macrophages 84%  $\pm$  13%, lymphocytes 11  $\pm$  8%, neutrophils 5  $\pm$  8%, and eosinophils 1  $\pm$  2%. One patient with

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Abbreviations: bronchoalveolar lavage, BAL; electrophoretic mobility shift assay, EMSA; interferon, IFN; nuclear factor- $\kappa$ B, NF- $\kappa$ B; peroxisome proliferator–activated receptor  $\gamma$ , PPAR $\gamma$ ; peroxisome proliferator response element, PPRE; whole cell extract, WCE.

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TABLE 1  
*Characteristics of patients with sarcoidosis*

Parameter	
Age, yr	42 ± 7
Sex	4 male/4 female
Race	4 white/4 black
Tobacco use	1 active, 3 former, 4 never
Duration, yr*	10 ± 9
Radiographic stage	
Stage I	2
Stage II/III	6
FVC, %pred	74 ± 20%
FEV <sub>1</sub> , %pred	63 ± 19%

Values are expressed as mean ± SD.

\* Radiologic or biopsy-confirmed disease duration.

sarcoidosis was taking prednisone 20 mg/d at the time of bronchoscopy; all others were free of corticosteroids or other immunosuppressives for > 2 mo (five patients had never received treatment). No patient was using inhaled corticosteroids, oral hypoglycemic agents, or nonsteroidal anti-inflammatory medications.

Healthy control individuals ( $n = 9$ ) were all nonsmokers with no history of lung disease and were not on medication at the time of the study. The mean BAL macrophage cell count for healthy control subjects was  $94 \pm 3\%$ . The protocol was approved by the Cleveland Clinic Foundation Institutional Review Board and written informed consent was obtained from all subjects. Not all experiments were performed on each sample due to variability in cell yield.

### Cell Collection and Culture

Whole BAL cell populations were used for this study. In brief, cells were collected by aspiration of warmed saline from segmental bronchi with a wedged bronchoscope (15). A modified Wright's stain was used on cytospin preparations to obtain differential cell counts. The mean viability of cells collected was greater than 95% as measured by trypan blue dye exclusion.

### Immunocytochemistry

PPAR $\gamma$  protein expression was evaluated in cytospin preparations from freshly-isolated alveolar macrophages from patients with sarcoidosis and healthy control subjects as previously described (11). The primary antibody was a rabbit polyclonal anti-PPAR $\gamma$  (1:1,000; Santa Cruz Corp, Santa Cruz, CA), and the secondary antibody an ALEXA-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Propidium iodide was used for nuclear localization and slides examined with confocal microscopy. Control experiments to ensure staining specificity were performed on all samples by omitting the primary antibody.

### Electrophoretic Mobility Shift Assay

Freshly isolated cells prepared from BAL fluid were centrifuged and resuspended in extraction buffer (20 mM Tris, pH 8.0; 150 mM NaCl; 1% Triton  $\times$  100) containing a protease inhibitor cocktail, and placed on ice for 20 min. The cell samples were then centrifuged at  $18,000 \times g$  for 20 min at 4°C to clear debris, and supernatants representing whole-cell extracts (WCEs) were col-

lected. Small-volume aliquots were stored at  $-80^{\circ}\text{C}$  until needed, to minimize damage from serial freeze-thaw cycles. The protein content of WCEs was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL).

For electrophoretic mobility shift assay, 10  $\mu\text{g}$  of the WCE were incubated in binding buffer (8 mM HEPES, pH 7.0, 10% glycerol, 20 mM KCl, 4 mM MgCl<sub>2</sub>, 1 mM sodium pyrophosphate) containing 1  $\mu\text{g}$  of poly(dI-dC) and 40,000-cpm oligonucleotide probe for 20 min at room temperature. The reaction mixture was then transferred to a 4% nondenaturing acrylamide gel. Competition experiments were done by incubating the extract with a 1000-fold molar excess of cold oligonucleotide. StormImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software were used for quantification of autoradiographs. Oligonucleotides representing the NF- $\kappa$ B and peroxisome proliferator response element (PPRE) binding sites were obtained from Invitrogen and Santa Cruz, respectively. Sequences were: 5'-AACTCCGGGAATTTCCCTGGCCC-3' for NF- $\kappa$ B, and 5'-CAAACTAGGTCAAAGGTCA-3' for PPRE. Antibody for PPAR $\gamma$  supershift was obtained from Santa Cruz.

### RNA Expression

Total RNA was extracted from whole BAL cell populations with the RNeasy kit (Qiagen, Valencia, CA). RNA samples were stored at  $-80^{\circ}\text{C}$ . Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with the ABI Prism 7,000 Detection System (Applied Biosystems, Foster City, CA). Primers for PPAR $\gamma$  (#HS 00234592) and a housekeeping gene (GAPDH) (#4310884E) were obtained from ABI. All specimens were analyzed in duplicate and the amount of PPAR $\gamma$  mRNA present was normalized relative to the GAPDH expression in that sample. Relative quantification of mRNA was performed as previously described (16, 17). The data are expressed as a fold-change in mRNA expression relative to control values.

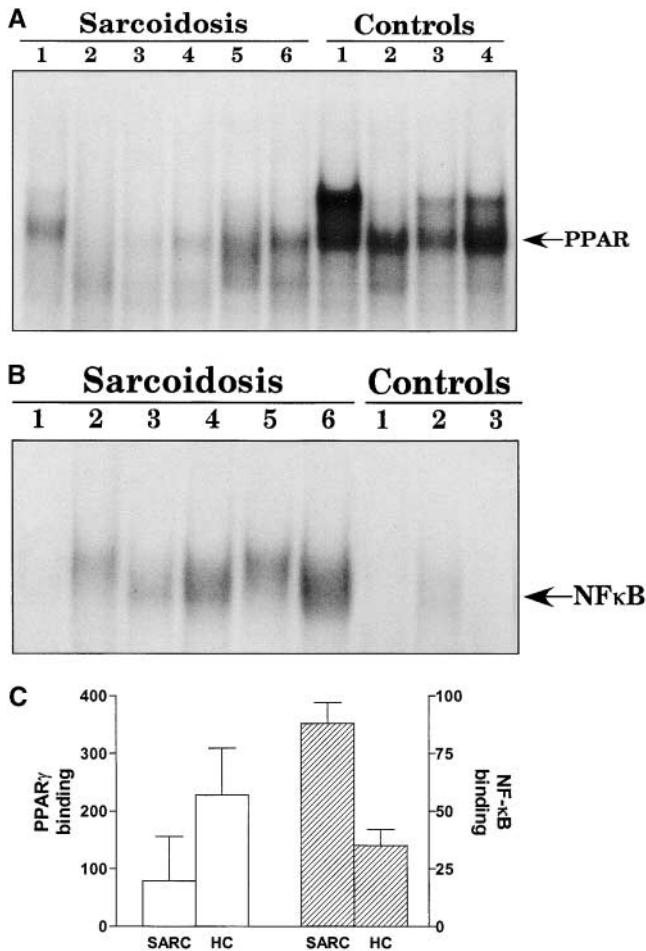
### Statistics

All statistics were calculated with Prism software (Graph Pad, San Diego, CA), with a prespecified significant  $P$  value of  $< 0.05$ . Comparisons between patients and healthy controls were analyzed by  $t$  test.

### Results

#### PPAR $\gamma$ Activation Is Reduced in Sarcoidosis

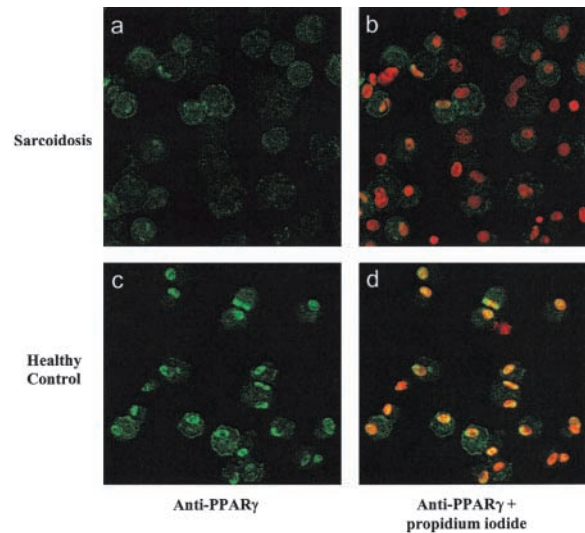
The functional status of PPAR $\gamma$  protein was assessed by electrophoretic mobility shift assay. Briefly, whole cell extracts from freshly isolated BAL cells of patients with sarcoidosis ( $n = 6$ ) and healthy control subjects ( $n = 4$ ) were incubated with oligonucleotides representing the PPAR $\gamma$  response element (PPRE). Figure 1A demonstrates decreased PPRE binding in the patients with sarcoidosis compared with control subjects. Control experiments with cold and mutant oligonucleotides confirmed binding specificity (data not shown). PPAR $\gamma$  specificity was confirmed by supershift with antibody to PPAR $\gamma$  (data not shown). Radioactivity quantification by phosphorimaging (calculated as arbitrary units) was less in sarcoidosis patients than in control subjects ( $79 \pm 17$  versus  $228 \pm 81$  U,  $P = 0.01$ ) (Figure 1C).



**Figure 1.** PPAR $\gamma$  activation is decreased and NF- $\kappa$ B activity is increased in active sarcoidosis. WCE were analyzed by electrophoretic mobility shift assay using radiolabeled oligonucleotides corresponding to the peroxisome proliferator response element for PPAR $\gamma$  (A) and NF- $\kappa$ B (B). Reaction specificity was confirmed by competition experiments with mutant and with excess unlabeled oligonucleotides (not shown). The same sarcoidosis patients were studied in both assays. C depicts the relative EMSA binding for PPAR $\gamma$  (clear bars, left axis) and NF- $\kappa$ B (striped bars, right axis) in arbitrary phosphorimaging units.

#### NF- $\kappa$ B Activity Is Elevated in Sarcoidosis

Because PPAR $\gamma$  is thought to antagonize inflammation in part by transrepression of proinflammatory transcription factors, we decided to assess whether NF- $\kappa$ B activation was upregulated in our patients. Figure 1B depicts an EMSA for the same six patients with sarcoidosis as in Figure 1A. NF- $\kappa$ B binding is upregulated in all patients, suggesting the presence of ongoing proinflammatory transcriptional activation. Phosphorimaging demonstrated a significant increase in binding for the sarcoidosis group compared with healthy controls ( $88 \pm 12$  versus  $35 \pm 7$  U,  $P = 0.02$ , Figure 1C). Interestingly, the patient with progressive disease despite corticosteroid therapy demonstrated reduced PPAR $\gamma$  and elevated NF- $\kappa$ B activity, similar to the untreated patients with active disease (Figures 1A and 1B, lane 5).



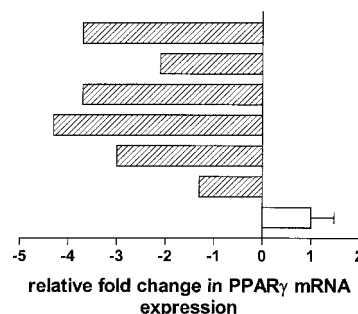
**Figure 2.** PPAR $\gamma$  protein is decreased in nuclei of alveolar macrophages. Alveolar macrophages from a patient with sarcoidosis (a and b) and healthy control subject (c and d) were stained for PPAR $\gamma$  protein expression. a and c depict PPAR $\gamma$  immunofluorescence (green); nuclear localization is confirmed in b and d, where images from propidium iodide-stained cells (red) are overlaid. Yellow staining in d represents nuclear PPAR $\gamma$  in healthy control subjects, which is absent in patients with sarcoidosis (b). Images were captured at  $\times 400$  magnification.

#### PPAR $\gamma$ Nuclear Protein Is Reduced in Sarcoid Alveolar Macrophages

The location of PPAR $\gamma$  protein was assessed by immunocytochemistry. Figure 2 depicts reduction of nuclear PPAR $\gamma$  staining in sarcoid alveolar macrophages (a and b) in contrast to a healthy control (c and d).

#### PPAR $\gamma$ Gene Expression Is Reduced in Sarcoid Alveolar Macrophages

To assess whether there was a concomitant reduction in gene expression, real-time RT-PCR was performed on BAL cells from six patients with active sarcoidosis. As demonstrated in Figure 3, PPAR $\gamma$  mRNA levels are reduced 2.8-fold compared with healthy control subjects ( $P < 0.03$ ).



**Figure 3.** PPAR $\gamma$  gene expression is decreased in sarcoidosis. Relative PPAR $\gamma$  mRNA expression was quantified by real-time RT-PCR by comparing the relative expression of PPAR $\gamma$  to a housekeeping gene, GAPDH. The data depict a significantly decreased PPAR $\gamma$  mRNA expression in sarcoidosis ( $n = 6$ ; striped bars) compared with healthy control subjects ( $n = 4$ ; open bars) ( $P < 0.03$ ). Bars denote SEM.

## Discussion

Our study provides evidence that: (i) PPAR $\gamma$  activity is decreased and NF- $\kappa$ B activity is increased in active pulmonary sarcoidosis; (ii) PPAR $\gamma$  gene expression and protein levels are decreased. The data are the first to show abnormal PPAR $\gamma$  and NF- $\kappa$ B activity in sarcoidosis alveolar macrophages. The increased NF- $\kappa$ B activity in the alveolar compartment is in accord with the prior description by Drent and coworkers of elevated NF- $\kappa$ B protein in sarcoidosis peripheral blood mononuclear cells (18). The overall pattern is consistent with the reported mutual antagonism between PPAR $\gamma$  and NF- $\kappa$ B (19). These findings have attractive theoretical implications in chronic sarcoidosis, a disease characterized by dysregulated activation of Th1 cytokines and pro-inflammatory transcription factors (18, 20). The persistence of low PPAR $\gamma$  activity in these patients despite ongoing inflammation and NF- $\kappa$ B activation suggests a defect in the ability of these patients to quench inflammation through PPAR $\gamma$  upregulation.

Several specific mediators of granulomatous inflammation in sarcoidosis are known to be influenced by PPAR $\gamma$  activity. For example, PPAR $\gamma$  activation *in vitro* inhibits the release of interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , interleukin-12p40, and IFN- $\gamma$  from LPS-activated macrophages and monocytes (5, 21–23). On the other hand, PPAR ligands upregulate GATA-3, a transcription factor that enhances countervailing Th2 responses (24). In the THP-1 human monocytic cell line, PPAR $\gamma$  ligands inhibit osteopontin and MCP-1 gene expression (20, 25). All of these molecules are thought to be important in the pathophysiology of sarcoidosis, and a deficiency of PPAR $\gamma$  activity could lead to their excessive production (20, 26–28).

Alveolar macrophages are a prominent source for production of inflammatory mediators in pulmonary sarcoidosis (29, 30). In the current investigation, we found deficiencies of PPAR $\gamma$  nuclear protein as well as activation in sarcoid alveolar macrophages. The mechanism for this deficiency may be at the transcriptional level, because the mRNA was also decreased in these patients. IFN- $\gamma$  could contribute to this effect, by downregulating PPAR $\gamma$  transcription via STAT-1 activation (31). Elevated IFN- $\gamma$  production is a well-described phenomenon in pulmonary sarcoidosis (20, 26). Additional mechanisms may also be operative, such as accelerated PPAR protein degradation, an effect that can also be mediated by IFN- $\gamma$  (32). Additional studies are planned to further investigate the mechanism(s) of decreased PPAR $\gamma$  activity in sarcoidosis.

Current understanding of PPAR $\gamma$ 's role in inflammation derives mainly from experimental models, usually conducted over short time-frames. The relative physiologic importance of various endogenous PPAR $\gamma$  ligands is unknown. The role of PPAR $\gamma$  deficiency in the pathophysiology of chronic inflammatory disease is unclear, given the short half-life of PPAR $\gamma$  (32). *In vitro* and *in vivo* experiments, however, have suggested that natural and synthetic PPAR $\gamma$  ligands can downregulate inflammation in colitis and experimental arthritis, diseases also characterized by chronic Th1 cytokine production (8, 24, 33). Although our data are correlative only, they support the hypothesis that

progressive sarcoidosis may be due in part to deficient PPAR $\gamma$  activity, facilitating an excessive Th1 response.

In summary, the results presented here suggest that PPAR $\gamma$  activity is deficient in the intra-alveolar compartment in active pulmonary sarcoidosis. Because of the diverse immunomodulatory effects of PPAR $\gamma$  activation, such a deficiency may have important implications for disease burden, pace, or resolution. Future investigations will aim to elucidate the mechanism of PPAR $\gamma$  dysfunction in active sarcoidosis.

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