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Gene expression profiles in granuloma tissue reveal novel diagnostic markers in sarcoidosis

George P. Christophi [a,*,1], Tiffany Caza [b,1], Christopher Curtiss [b,1], Divya Gumber [d], Paul T. Massa [c], Steve K. Landas [b]

[a] Department of Gastroenterology, Washington University School of Medicine, St. Louis, MO, United States
[b] Department of Pathology, SUNY Upstate Medical University, Syracuse, NY, United States
[c] Department of Microbiology & Immunology, SUNY Upstate Medical University, Syracuse, NY, United States
[d] Department of Internal Medicine, Cleveland Clinic, Cleveland, OH, United States

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ABSTRACT

Sarcoidosis is an immune-mediated multisystem disease characterized by the formation of non-caseating granulomas. The pathogenesis of sarcoidosis is unclear, with proposed infectious or environmental antigens triggering an aberrant immune response in susceptible hosts. Multiple pro-inflammatory signaling pathways have been implicated in mediating macrophage activation and granuloma formation in sarcoidosis, including IFN-γ/STAT-1, IL-6/STAT-3, and NF-κB. It is difficult to distinguish sarcoidosis from other granulomatous diseases or assess disease severity and treatment response with histopathology alone. Therefore, development of improved diagnostic tools is imperative. Herein, we describe an efficient and reliable technique to classify granulomatous disease through selected gene expression and identify novel genes and cytokine pathways contributing to the pathogenesis of sarcoidosis. We quantified the expression of twenty selected mRNAs extracted from formalin-fixed paraffin embedded (FFPE) tissue ($n = 38$) of normal lung, suture granulomas, sarcoid granulomas, and fungal granulomas. Utilizing quantitative real-time RT-PCR we analyzed the expression of several genes, including IL-6, COX-2, MCP-1, IFN-γ, T-bet, IRF-1, Nox2, IL-33, and eotaxin-1 and revealed differential regulation between suture, sarcoidosis, and fungal granulomas. This is the first study demonstrating that quantification of target gene expression in FFPE tissue biopsies is a potentially effective diagnostic and research tool in sarcoidosis.

Introduction

Sarcoidosis is a multisystemic immune-mediated granulomatous disease of uncertain etiology with varying relationships to environmental exposures, demographic factors, and genetic predisposition (Iannuzzi et al., 2007). Sarcoidosis predominately involves the lungs and the lymphatic system is diagnosed by a combination of clinical, radiologic, and pathologic findings. Sarcoidosis remains a diagnosis of exclusion, with a delay in diagnosis due to non-specific disease criteria and need for invasive tissue biopsy. The disease is typically diagnosed in the third or fourth decade of life, and incidence of sarcoidosis is three times higher in blacks than whites (36 compared to 11 patients per 100,000) (Rybicki et al., 1997). Two-thirds of sarcoid patients go into remission before three years of disease involvement, while the remaining one-third develops chronic disease. Mortality in patients with sarcoidosis is higher than that of the general population, mainly due to pulmonary fibrosis. Treatment of sarcoidosis is limited, with corticosteroids being the mainstay of management (Iannuzzi and Fontana, 2011).

Sarcoidosis has a genetic predisposition evident by familial clustering and higher concordance among monozygotic compared to diazygotic twins (Grunewald, 2008; Rybicki et al., 1997, 2001). Genome-wide associations studies (GWAS) have identified multiple genes involved in sarcoidosis including the butyrophillin-like 2 (*BTNL2*) gene, a member of the B7 costimulatory receptor family (Valentonyte et al., 2005) and annexin A11, a calcium-dependent phospholipid-binding protein involved in cell division and vesicle trafficking (Hofmann et al., 2008). Importantly, mutations within NOD2, which induces constitutive activation of the transcription factor NF-kB, were identified in early-onset sarcoidosis (Kanazawa et al., 2005). Other candidate genes predicting susceptibility to sarcoidosis include genes involved in antigen processing and presentation by macrophages, factors affecting macrophage activation, T cell activation, and in injury repair (Kriegova et al., 2011). Candidate

* Corresponding author at: Washington University School of Medicine, Department of Gastroenterology, 660 S. Euclid Ave., Box 8121, St. Louis, MO 63110, United States.
E-mail address: gchristo@dom.wustl.edu (G.P. Christophi).
[1] Authors contributed equally to manuscript preparation.

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antigens that may trigger granulomatous inflammation in sarcoidosis include bacterial proteins from *Mycobacterium tuberculosis* and propionibacteria and β-glucan, a cell wall constituent of fungi (Ishige et al., 1999; Song et al., 2005; Terčelj et al., 2014).

The pathogenic hallmark of sarcoidosis is non-caseating granulomas that accumulate in multiple affected organs and are pathogenic through mass effect or tissue destruction. Sarcoid granulomas contain central macrophages that aggregate and differentiate into epitheliod histiocytes that fuse to form multinucleated giant cells (Saidha et al., 2012). Oligoclonal $\alpha\beta^+$ $CD4^+$ lymphocytes have been predominately located in the center and $CD8^+$ T cells at the periphery of granulomas (Saidha et al., 2012). Granuloma formation in sarcoidosis is postulated to be a complex process involving initially antigen processing and presentation with activation of $CD4^+$ T cells by macrophages followed by additional macrophage accumulation and continuous production of inflammatory mediators (Iannuzzi et al., 2007).

Sarcoidosis is a systemic disease evident by the upregulation of inflammatory molecules in serum that are secreted from peripheral leukocytes (Zhou et al., 2012). Microarray analysis from peripheral leukocytes of sarcoidosis patients revealed a unique gene expression signature involving genes implicated in T cell differentiation and activation and cytokine signaling (Zhou et al., 2012). In addition, inflammatory mediators have been elevated in the bronchoalveolar lavage fluid (BALF) of sarcoid patients including the cytokine IFN-γ and the chemokine IP-10 (Miotto et al., 2001; Terčelj et al., 2014).

Specification of naïve $CD4^+$ T cells and macrophages to effector subset lineages is critical to skewing immune responses in response to antigenic triggers and involves specific activation of transcription factors. The immune response in sarcoidosis is primarily mediated by the type I inflammatory cytokine pathway, which is promoted by NF-kB and STAT1 dependent signaling (Iannuzzi and Fontana, 2011; Miotto et al., 2001). The transcription factor T-bet is elevated in sarcoidosis and promotes Th1 specification through IL-12 and IFNγ signaling (Kriegova et al., 2011). Importantly, IFNγ is elevated in sarcoidosis and mediates activation of signal transducer and activator of transcription-1 (STAT1) that is central in mounting a type I response by promoting expression of interferon-regulatory factor I (IRF-1) and downstream inflammatory mediators including the chemokine IP-10 and Nox2 (Miotto et al., 2001; Rosenbaum et al., 2009). Non-caseating epithelioid granulomas of sarcoidosis include Th1 $CD4^+$ lymphocytes that promote potent immune response via IL-2 and IFN-γ release and subsequent activation and differentiation of macrophages (Hunninghake and Crystal, 1981; Pinkston et al., 1983; Saltini et al., 1986).

The gold standard for diagnosing sarcoidosis is tissue biopsy and the presence of these non-caseating granulomas. However, non-caseating granulomas are non-specific and can be found in other pathologic conditions including infections such as tuberculosis and fungal infections or by environmental exposures (Iannuzzi and Fontana, 2011). The cellular morphology of granuloma from bacterial, fungal, or in sarcoidosis cannot be distinguished from one another by histology alone (Zaim et al., 1990). Interestingly, the biology of granuloma formation in sarcoidosis differs from reactions to foreign materials. Suture granulomas are formed by adsorption of plasma proteins to the suture material, local complement activation and cell apoptosis leading to macrophage recruitment, phagocytosis, and formation of multinucleated giant cells (Anderson et al., 2008).

This study aims to identify gene expression patterns that differentiate sarcoid granulomas from other causes of granulomatous inflammation. We postulated that the gene expression profile is unique between different granuloma types and wanted to perform the first study to examine the utility of gene profiling as a novel diagnostic and research tool. We quantified the expression of twenty selected mRNAs from formalin-fixed paraffin embedded (FFPE) tissue of normal lung, suture granulomas, sarcoid granulomas, and fungal granulomas. Utilizing quantitative real-time RT-PCR we analyzed the expression of several genes, including IL-6, COX-2, MCP-1, IFN-γ, T-bet, IRF-1, Nox2, IL-33, and eotaxin-1 and revealed differential regulation between suture, sarcoidosis, and fungal granulomas. This study introduces a novel potential diagnostic tool in sarcoidosis and furthers our current understanding of signaling pathways involved in granulomatous inflammation.

Materials & methods

Sample selection

The study was approved by the Upstate Medical University Institutional Review Board for the Protection of Human Subjects. Retrospectively reviewed archived FFPE biopsy and resection pathology specimens from the Department of Pathology at State University of New York Upstate Medical University diagnosed as sarcoid granulomas, infectious granulomas, suture granulomas, and normal (non-granulomatous) lung tissue were used for the study (Table 1). The following pathologic inclusion criteria were used to select samples for each diagnostic category: 1) normal samples (NS) included tissue with no evidence of necrotizing or non-necrotizing epithelioid granulomas, birefringent or foreign material, vasculitis, abundant background inflammation. Normal lung specimens were obtained from segments of normal lung that were procured from lung cancer resection specimens; 2) suture granuloma was composed of tissue with granulomatous inflammation surrounding suture material with no evidence of necrotizing granulomas, vasculitis, abundant background inflammation, nor demonstrable microorganisms on Ziehl–Neelsen (AFB) and Grocott's methenamine silver (GMS) stains; 3) sarcoid granulomas were composed of tissue with non-necrotizing

Table 1
Specimen characteristics. Specimens were divided into subgroups on the basis of the diagnosis, including normal lung tissue ($n = 12$), suture foreign body reactions ($n = 8$), sarcoid granulomas ($n = 13$), and infectious granulomas ($n = 5$). The age and gender are shown.

Category	Sample	Age	Gender	Tissue
Normal	1	78	F	Lung
	2	76	F	Lung
	3	64	F	Lung
	4	49	F	Lung
	5	70	M	Lung
	6	72	M	Lung
	7	60	M	Lung
	8	76	M	Lung
	9	70	F	Lung
	10	60	F	Lung
	11	69	M	Lung
	12	61	M	Lung
Suture	1	81	M	Peritoneum
	2	40	F	Thyroid
	3	16	M	Ureter
	4	59	M	Skin
	5	45	F	Soft tissue
	6	12	M	Soft tissue
	7	80	M	Skin
	8	62	F	Skin
Sarcoid	1	38	M	Lymph node
	2	74	M	Lymph node
	3	41	M	Lymph node
	4	70	F	Lymph node
	5	28	M	Lymph node
	6	46	M	Lymph node
	7	35	F	Liver
	8	46	F	Nasal sinus
	9	22	M	Skin
	10	44	F	Skin
	11	36	M	Lung
	12	48	F	Soft tissue
	13	58	F	Lymph node
Fungal	1	12	F	Lung
	2	51	F	Lung
	3	31	M	Liver
	4	48	M	Lung
	5	58	F	Lung