Development of a Sarcoidosis Murine Lung Granuloma Model Using *Mycobacterium* Superoxide Dismutase A Peptide

Carmen M. Swaisgood^{1,2}, Kyra Oswald-Richter⁴, Stephen D. Moeller¹, Jennifer M. Klemenc², Lisa M. Ruple¹, Carol F. Farver³, John M. Drake⁵, Daniel A. Culver^{1,2}, and Wonder P. Drake^{4,6}

¹Department of Pulmonary, Allergy, and Critical Care Medicine, Respiratory Institute, ²Department of Pathobiology, Lerner Research Institute, and

³Department of Anatomic Pathology, Cleveland Clinic, Cleveland, Ohio; and ⁴Department of Medicine, School of Medicine, ⁵School of Law, and

⁶Department of Microbiology and Immunology, School of Medicine, Vanderbilt University, Nashville, Tennessee

Sarcoidosis is characterized by noncaseating granulomas containing CD4⁺ T cells with a Th1 immunophenotype. Although the causative antigens remain unknown, independent studies noted molecular and immunologic evidence of mycobacterial virulence factors in sarcoidosis specimens. A major limiting factor in discovering new insights into the pathogenesis of sarcoidosis is the lack of an animal model. Using a distinct superoxide dismutase A peptide (sodA) associated with sarcoidosis granulomas, we developed a pulmonary model of sarcoidosis granulomatous inflammation. Mice were sensitized by a subcutaneous injection of sodA, incorporated in incomplete Freund's adjuvant (IFA). Control subjects consisted of mice with no sensitization (ConNS), sensitized with IFA only (ConIFA), or with *Schistosoma mansoni* eggs. Fourteen days later, sensitized mice were challenged by tail-vein injection of naked beads, covalently coupled to sodA peptides or to schistosome egg antigens (SEA). Histologic analysis revealed hilar lymphadenopathy and noncaseating granulomas in the lungs of sodA-treated or SEA-treated mice. Flow cytometry of bronchoalveolar lavage (BAL) demonstrated CD4⁺ T-cell responses against sodA peptide in the sodA-sensitized mice only. Cytometric bead analysis revealed significant differences in IL-2 and IFN- γ secretion in the BAL fluid of sodA-treated mice, compared with mice that received SEA or naked beads ($P = 0.008$, Wilcoxon rank sum test). ConNS and ConIFA mice demonstrated no significant formation of granuloma, and no Th1 immunophenotype. The use of microbial peptides distinct for sarcoidosis reveals a histologic and immunologic profile in the murine model that correlates well with those profiles noted in human sarcoidosis, providing the framework to investigate the molecular basis for the progression or resolution of sarcoidosis.

Keywords: lung; sarcoidosis; granuloma; *Mycobacterium* soda; mouse

Although sarcoidosis granulomatous inflammation can involve any organ, pulmonary involvement is evident in more than 90% of patients with sarcoidosis. Non-necrotizing granulomas comprise the histologic feature of the disease, with variable degrees of Th1-lymphocytic inflammation or fibrosis. The granuloma is compact and organized. It consists of a core of epithelioid and multinucleated giant cells, derived from the fusion of monocytes and macrophages. The macrophages are infiltrated and surrounded predominantly by T-helper (CD4⁺) cells, with some

CLINICAL RELEVANCE

The use of a microbial peptide, sodA, distinct for sarcoidosis revealed a histologic and immunologic profile in a murine model that correlated well with those profiles in human sarcoidosis. This murine model of sarcoidosis provides a framework for investigating the molecular basis of sarcoidosis progression or resolution.

aggregates of T-suppressor (CD8⁺) cells, and a collagenous band with interspersed fibroblasts. The diagnosis is usually based on the presence of noncaseating granulomas, with a consistent clinical presentation and negative studies for active infection or other causes of granuloma formation.

Recent studies from independent laboratories revived the association of sarcoidosis with infectious antigens. In Japanese patients with sarcoidosis, quantitative differences in *Propionibacterium acnes* DNA among sarcoidosis and control subjects were detected (1). Among American patients with sarcoidosis, the presence of genetically distinct mycobacterial nucleic acids in sarcoidosis granulomas was reported (2). Song and colleagues (3) used matrix-assisted laser desorption/ionization time of flight mass spectrometry to demonstrate the presence of mycobacterial catalase-peroxidase (katG) proteins in sarcoidosis granulomas. Mycobacterial heat-shock proteins were also evident in Polish sarcoidosis granulomas (4). Major histocompatibility complex (MHC) Class II alleles are associated with protection or susceptibility in patients with sarcoidosis (5, 6). Th1 cellular immune responses to the mycobacterial virulence factors ESAT-6 and katG were reported in sarcoidosis peripheral blood mononuclear cells and bronchoalveolar lavage (7, 8). Mycobacterial antigens that are recognized in the context of MHC Class II human leukocyte antigen (HLA)-DR were reported (9). Most recently, antigen-specific immune responses to mycobacterial virulence factors were reported to be present in sarcoidosis bronchoalveolar lavage (BAL) at the time of diagnosis, as mediated via Toll-like receptor 2 signaling (10). The molecular analysis of sarcoidosis granulomas for microbial virulence factors led to the identification of genetically distinct sequences of mycobacterial superoxide dismutase A (sodA; GenBank accession number DQ768096) (11). Unlike other proteins reported in sarcoidosis granulomas, these sequences had not been previously associated with other mycobacterial infections such as tuberculosis. Taken together, these studies indicate a key role of mycobacterial proteins in the pathogenesis of sarcoidosis in American patients.

Progress toward the development of an animal model has been hindered by a lack of antigens that induce the sarcoidosis Th1 immunophenotype. Using the distinct sodA peptides isolated from patients with sarcoidosis only (GenBank accession number

(Received in original form September 23, 2009 and in final form February 18, 2010)

This work was supported by National Institute of Health grants HL080953 (C.M.S.), HL069765 (K.O.-R.), HL081538 (D.A.C.), HL083839, and AI065744, and Vanderbilt University grant RR-00095 (W.P.D.).

Correspondence and requests for reprints should be addressed to Carmen M. Swaisgood, Ph.D., Department of Pulmonary, Allergy, and Critical Care Medicine, Respiratory Institute, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195. E-mail: swaisgc@ccf.org

Am J Respir Cell Mol Biol Vol 44, pp 166–174, 2011

Originally Published in Press as DOI: 10.1165/rncmb.2009-0350OC on March 26, 2010

Internet address: www.atsjournals.org

ABG68026), we developed a pulmonary model of sarcoidosis granulomatous inflammation. At present, no clear understanding exists of the factors that influence the natural history of sarcoidosis or govern its relative degrees of inflammation, granuloma formation, or fibrosis. The development of an animal model of sarcoidosis immunopathogenesis, using distinct microbial peptides associated with sarcoidosis granulomas, will enable us to identify molecular events that contribute to clinical outcomes in sarcoidosis. Some of the results of these studies were previously reported in the form of an abstract (12).

MATERIALS AND METHODS

Animals

Six-week-old female C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions, and all *in vivo* manipulations were performed under protocols approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Synthesis of *Mycobacterium* Peptides

The sodA peptide, AAAIAGAFGSFDKFR, was synthesized as described previously (11). Each peptide was synthesized by solid-phase F-moc chemistry (Genemed Synthesis, San Diego, CA) to a purity of greater than 70%. Identity was confirmed by mass spectroscopy, and purity was assessed according to high-performance liquid chromatography.

Sensitization and Induction of Granuloma

We used the timeline of the murine lung granuloma model described by Chensue and colleagues (13), with some modifications. Mice were sensitized by a subcutaneous injection of 20 µg of *Mycobacterium* sodA peptide, incorporated into 0.25 ml of incomplete Freund's adjuvant (IFA; Sigma, St. Louis, MO) on Day 1. Control groups included untreated mice (baseline), mice with no sensitization (ConNS), mice sensitized with IFA only (ConIFA), or mice injected intraperitoneally with 3,000 *Schistosoma mansoni* eggs suspended in 0.5 ml of PBS. Fourteen days later, ConNS and ConIFA mice were challenged by tail-vein injection with 6,000 Sepharose 4B naked beads (in 0.5 ml of PBS; Sigma). The sodA-sensitized and *Schistosoma* egg-sensitized mice were challenged in the same manner with beads covalently coupled to sodA or soluble *Schistosoma* egg antigens (SEAs; Schistosome Biological Supply Center, Theodor Bilharz Research Institute, Giza, Egypt), respectively.

Histologic and Morphometric Analysis of Mouse Lung Granulomas

After BAL, PBS-perfused lungs were excised, excluding the trachea and major bronchi. The left lung was fixed in Histochoice (Amresco, Solon, OH), processed, and paraffin-embedded. Paraffin sections (5-µm thickness) were stained with hematoxylin and eosin (H&E) for histologic examination and determination of granuloma size. Granulomas were measured blindly, using computerized morphometry with an Olympus BX41 microscope (Olympus America, Inc., Center Valley, PA). A minimum of 10 granulomas per lung, sectioned through the central bead nidus, was used in measurements.

Immunohistochemistry of Mouse Lung Granulomas

Immunohistochemistry of sodA-elicited lung granulomas was performed for the presence of T cells, macrophages, and B cells. Paraffin sections of left lungs of mice were deparaffinized, rehydrated, and permeabilized with Trilogy (Cell Marque Corporation, Rocklin, CA) for 30 minutes in a steamer, rinsed in fresh hot Trilogy for 30 minutes, and cooled for 5 minutes. *In situ* peroxidase activity was quenched with a methanol/30% H₂O₂ solution for 20 minutes. After a brief rinse in water, nonspecific binding was prevented by incubating tissue sections in serum-free, ready-to-use Protein Block (DAKO, Carpinteria, CA) for 5–10 minutes at room temperature. Purified rat anti-mouse Mac-2 monoclonal antibody (1:500, clone M3/38 by Cedarlane; Accurate Chemical and Scientific Corp., Westbury, NY), polyclonal rabbit anti-mouse CD-3 (1:1,000, ab5690; Abcam, Inc., Cambridge, MA),

and polyclonal rat anti-mouse CD45R (also B220; 1:50, clone RA3-6B2; B.D. Biosciences, San Jose, CA) were used for the identification of macrophages, general T cells, and general B cells, respectively. Sections were incubated in primary antibody diluted in AB Diluent (DAKO) for 60 minutes at room temperature and overnight at 4°C, and then rinsed twice in PBS for 5 minutes. Sections were incubated in Biotin-SP-conjugated AffiniPure Mouse Anti-Rat IgG or Goat Anti-Rabbit IgG (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies diluted in AB Diluent for 60 minutes at room temperature, and rinsed as already described. Sections were incubated in AB Complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, and rinsed as already described. Peroxidase activity was developed by incubation in DAB (Vector) according to the manufacturer's instructions. Sections were counterstained in hematoxylin, dehydrated, and mounted in Richard-Allan Mounting Medium (Fisher Scientific, Houston, TX).

For the identification of CD4⁺ T cells, 5-µm sections of frozen tissues embedded in optimal cutting temperature (OCT; Tissue-Tek, Sakura Japan) were placed on charged slides, fixed for 5 minutes in acetone, rinsed in distilled water, and placed in PBS. Endogenous peroxidase was quenched with 0.03% hydrogen peroxide, and samples were treated with a casein block before addition of the primary antibody. Tissues were incubated with biotinylated rat anti-CD4 (catalogue number 553649; B.D. Pharmingen, San Jose, CA), diluted 1:50, and incubated overnight at 4°C. The Vectastain ABC Elite System (Vector Laboratories, Inc.) and DAB⁺ (DAKO) were used to produce localized, visible staining. Slides were lightly counterstained with Mayer's hematoxylin, dehydrated, and coverslipped.

Lung Immunohistochemistry of Human Granulomas

Immunohistochemistry of a sarcoidosis granuloma containing the unique sodA sequence was performed for the presence of CD3⁺ cells, CD4⁺ cells, CD8⁺ cells, macrophages, and B cells. The CD4⁺ histologic stain worked suboptimally on the tissue specimen and the control, and was not included in the analysis. For CD3, CD8, CD68, and CD20 staining, 5-µm sections were placed on charged slides and rehydrated. Sections were rehydrated and placed in heated Target Retrieval Solution (DAKO). Endogenous peroxidase was neutralized with 0.03% hydrogen peroxide, followed by a casein-based protein block (DAKO) to minimize nonspecific staining. Sections were incubated with CD3 (sc-1127; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted 1:250 for 60 minutes. The Vectastain Elite ABC Kit and DAB were used to produce localized, visible staining. When staining for CD8⁺ T cells, sections were incubated with CD8 (sc-7970; Santa Cruz Biotechnology, Inc.), diluted 1:300 for 60 minutes. When staining for CD68⁺ cells, sections were incubated with CD68 (M 0814; DAKO), diluted 1:300 for 60 minutes. For the staining for CD20⁺ cells, sections were incubated with CD20 (ab9475; Abcam, Inc.), diluted 1:35 for 60 minutes. When staining for CD8, CD68, and CD20, the Envision+ HRP/DAB System (DAKO) was used to produce localized, visible staining. All slides were lightly counterstained with Mayer's hematoxylin, dehydrated, and coverslipped.

Leukocyte Recruitment to the Airways

BAL was performed as described previously (14), by infusing 1 ml of PBS through a tracheal cannula. The BAL was centrifuged (700 × g, 5 min, 4°C), and the fluid phase was aliquoted and frozen at –70°C for cytokine measurements. After the hypotonic lysis of red blood cells in the pellet, BAL cells were resuspended in 1 ml of PBS and counted using a hemocytometer. Differentials were performed in BAL cells by the cytocentrifugation of 100 µl containing 10³ cells/ml onto glass slides, using Wright stain (EM Science, Gibbstown, NJ), and counting by light microscopy. The percentage of different leukocyte populations was determined in duplicate by counting 200 cells in fields selected randomly under a low-power magnification (×2) at which leukocytes cannot be distinguished, and the total number of each leukocyte population was calculated according to the volume of the BAL.

Flow Cytometric Analysis of BAL Cells

BAL was performed as described above. After centrifugation, BAL cells were counted and analyzed by flow cytometry. Intracellular

staining was performed to identify IFN- γ -secreting T cells in response to microbial stimulation. For the intracellular cytokine assay, BAL was pooled from 3–6 mice, and $0.5\text{--}1 \times 10^5$ BAL cells were incubated in RPMI-1640-supplemented medium with or without sodA (20 $\mu\text{g}/\text{ml}$). Staphylococcal enterotoxin B (SEB, 10 $\mu\text{g}/\text{ml}$; Sigma) stimulation served as a positive control at 37°C under 5% CO_2 for 60 minutes before the addition of B.D. GolgiStop (B.D. Biosciences). To demonstrate that the immune response was MHC class II-specific, BAL cells from sodA-treated mice were first incubated at room temperature for 30 minutes with a monoclonal anti-MHC II antibody (1 $\mu\text{g}/\text{ml}$ of blocking antibody, clone m5/114.15.2; eBioscience, San Diego, CA) or the corresponding isotype control (rat IgG2b, κ) before antigen stimulation. After 6 hours of incubation at 37°C under 5% CO_2 , cells were washed and stained with the surface antibodies anti-CD3 and anti-CD4 (B.D. Biosciences) at 4°C for 30 minutes. After washing, fixation, and permeabilization, using a Fix and Perm Kit according to the manufacturer's instructions (B.D. Biosciences), anti-IFN- γ (B.D. Biosciences) was added at 4°C for 45 minutes. Cells were washed and analyzed via flow cytometry. IFN- γ frequencies were defined as the percentage of stimulated $\text{CD}3^+\text{CD}4^+$ T cells minus their unstimulated background frequency. The medium used in all experiments was RPMI-1640 (Cellgro, Mediatech, Inc., Manassas, VA) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA), penicillin (50 U/ml; Cellgro), streptomycin (50 mg/ml; Cellgro), sodium pyruvate (1 mM; Cellgro), and glutamine (2 mM; Cellgro).

Cytokine Analysis in BAL Fluid

To measure Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-5) cytokine levels in BAL, fluid was collected from 5–6 mice from each group: baseline, SEA-treated, or sodA-treated mice. Cytokine levels were measured using a cytometric bead array (CBA; B.D. Biosciences) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses of differences between groups were performed using a Kruskal-Wallis test or ANOVA as appropriate, followed by Dunnett's multiple comparison test (JMP Statistical Discovery Software; SAS, Cary, NC). Statistical analyses of distinctions in cytokine expression between baseline, SEA-treated, and sodA-treated mice were performed using the Wilcoxon rank sum test at an α -level of 0.05.

RESULTS

Mycobacterium sodA Elicits Lung Granuloma Formation in Mice

To determine if *Mycobacterium* sodA peptides isolated from human sarcoid granulomas could elicit granuloma formation in mouse lungs, mice were sensitized with an emulsion of IFA and sodA on Day 1 and challenged on Day 14 with sodA-coupled beads, which emulsified to the lungs (Figure 1). Lungs and BAL fluid were collected 4 days after the challenge, on Day 18 (Figure 1). A gross examination of the euthanized mice detected the presence of extensive hilar lymphadenopathy and parenchymal infiltrates. No gross abnormalities of the heart, liver, or spleen were evident, and no beads or granulomas were found in these tissues. Histologic analyses of H&E-stained lung sections showed extensive granuloma formation throughout the lungs of sodA-treated mice (Figure 2A). Most granulomas were concentric to the bead (Figure 2A, *inset*), and localized to the broncho-arterial bundle. The presence of hilar lymphadenopathy and noncaseating granulomas consisting of multinucleate giant cells and lymphocytic infiltrate correlates well with observations in human sarcoid lung histology (Figure 2B). Multinucleated giant cells and lymphocytic infiltration were present in the granulomas of sodA-treated mice (Figures 2A, *inset*, and 2C). Consistent with previous reports, SEA produced large granulomas with extensive lymphocytic infiltrate (13), as well as extensive extracellular matrix deposition and fibrosis (Figure 2D). In contrast to both

sodA-treated and SEA-treated mice, untreated mice had no granulomas. No sensitization (ConNS; Figure 2E) or sensitization with IFA alone (ConIFA; Figure 2F), followed by challenge with naked beads, led to the formation of significantly smaller foreign-body granulomas consisting of a few cell layers (Figure 2E, *inset*). An examination of the size of granuloma formation between ConNS, ConIFA, sodA-treated, and SEA-treated mice revealed significant differences between treatments. The median size of sodA-elicited granulomas was $32,045 \mu\text{m}^2$ (25th and 75th percentiles, $23,061 \mu\text{m}^2$ and $39,634 \mu\text{m}^2$, respectively; Figure 3), which was larger than the size elicited in ConNS mice (median, $11,247 \mu\text{m}^2$; 25th and 75th percentiles, $6,425 \mu\text{m}^2$ and $17,510 \mu\text{m}^2$, respectively; $P < 0.001$, according to Kruskal-Wallis test; Figure 3) and ConIFA mice (median, $15,199 \mu\text{m}^2$; 25th and 75th percentiles, $12,414 \mu\text{m}^2$ and $21,714 \mu\text{m}^2$, respectively; $P < 0.001$, according to Kruskal-Wallis test; Figure 3). In contrast, SEA-elicited granulomas (median area, $61,215 \mu\text{m}^2$; 25th and 75th percentiles, $42,423 \mu\text{m}^2$ and $95,263 \mu\text{m}^2$, respectively) were approximately twofold larger than sodA-elicited granulomas ($P < 0.001$ according to Kruskal-Wallis test; Figure 3). Dunn's multiple comparison test revealed no differences between the lung granuloma sizes of ConNS and ConIFA mice. However, significant differences were found between sodA-elicited and SEA-elicited granulomas versus those in ConNS mice ($P < 0.001$ for both) and ConIFA mice ($P < 0.01$ and $P < 0.001$, respectively).

Cellular Profile of Murine Pulmonary Granuloma Initiated by sodA

The cellular profile of sarcoidosis granulomas is well-characterized. To compare the cellular profile of sodA-elicited mouse lung granulomas with those of patients with sarcoidosis, we assessed for the presence of macrophages, T cells, and B cells. Analogous to what occurs in human sarcoidosis granulomas containing the unique sodA peptide, murine macrophages were interspersed throughout the granuloma (Figures 4A and 4B). However, in the murine model, they were most abundant in the innermost layers, closest to the beads (Figure 4A). In addition, general staining for T cells showed them to be interspersed in the middle layers of both human and mouse granulomas, as expected (Figures 4C and 4D). Specific staining for $\text{CD}4^+$ T cells showed them to be abundant in the middle layers of mouse granulomas (Figure 4C, *inset*). Although B cells were present in both murine and human sarcoidosis granulomas, they did not comprise a significant proportion of the lymphocytic population (Figures 4E and 4F, respectively). Control, serially cut sections, treated with secondary antibody only, showed no staining.

Figure 1. Timeline of murine model of lung sarcoidosis. C57Bl/6 mice were not sensitized (ConNS) or were sensitized subcutaneously on Day 1 with an emulsion of either incomplete Freund's adjuvant (IFA) + superoxide dismutase A (sodA) peptide, IFA + saline (ConIFA), or intraperitoneally with 3,000 *Schistosoma mansoni* eggs. Sensitized mice were challenged 14 days later via tail-vein injection of 6,000 Sepharose beads, covalently bonded to sodA or schistosome egg antigens (SEA), or by injection of naked beads (ConNS and ConIFA). Four days after challenge, BAL and lungs were collected and analyzed.

Figure 2. Lung granuloma formation in mice 4 days after challenge. Lungs were paraffin-embedded, cut, and stained with hematoxylin and eosin. (A) Lung of a mouse sensitized and challenged with sodA ($n = 16$). *Inset*: Higher magnification of a granuloma in A reveals a multinucleated giant cell (arrow). (B) Lung granulomas of patient with sarcoidosis. *Inset*: High magnification of a lung granuloma from another patient with sarcoidosis. (C) Multinucleated giant cell (arrow) in mouse lung granuloma induced by sodA. (D) Lung granuloma from mice sensitized with *Schistosoma mansoni* eggs and challenged with SEA-coated beads ($n = 4$). (E) Lung from a nonsensitized (ConNS) mouse challenged with naked beads ($n = 20$). *Inset*: Higher magnification of granuloma in E. (F) Lung from a mouse sensitized with IFA only (ConIFA) and challenged with naked beads ($n = 9$). *b*, beads. Scale bars = 50 μm in A, C, D, E, and F; and 100 μm in B, *inset* in B, and *inset* in E.

BAL Cell Profile in sodA-Treated Mice

BAL was performed to identify and quantify the different cell types present. No significant difference was evident between the

Figure 3. Morphometric analysis of lung granulomas elicited by different treatments. Granuloma area was determined for ConNS and ConIFA mice challenged with naked beads, sodA-sensitized and challenged mice, and SEA-sensitized and challenged mice, as described in MATERIALS AND METHODS. Median and 25th and 75th percentile bars are shown; values were derived from analyses of 3–4 mice per treatment, totaling 24–125 granulomas. Statistical differences were determined using the Kruskal-Wallis test followed by Dunnnett's multiple comparisons test. * $P < 0.001$. † $P = \text{NS}$. § $P < 0.001$.

total numbers of leukocytes in the BAL of ConNS, ConIFA, or sodA-treated mice (Table 1). To determine if sodA affected a specific leukocyte population, differentials were performed on BAL cells. Although the number of macrophages and neutrophils was similar among all groups studied, sodA caused a significant increase in lymphocytes compared with the control groups ($P < 0.025$; Table 1). The ratio of CD4:CD8 T cells in sodA-treated BAL cells was determined by FACS analysis to be 2.3:1. Eosinophils did not constitute a significant cell type in any of the treatments used.

Specific CD4⁺ T-Cell Responses in BAL of sodA-Treated Mice Exhibit a Th1 Immunophenotype

A hallmark of sarcoidosis immunology is a Th1 immunophenotype. To determine if the murine model was similar immunologically to human sarcoidosis, we assessed for the immune recognition of sodA by T cells from the BAL of untreated,

TABLE 1. LEUKOCYTE RECRUITMENT TO THE AIRWAYS IN CONTROL AND SUPEROXIDE DISMUTASE A-TREATED GROUPS

	Total	Macrophages	PMNs	Lymphs	Eos
conNS ($n = 8$)	132 $\pm$ 13.5	85.4 $\pm$ 17.6	22 $\pm$ 8.4	9.1 $\pm$ 1.8	1 $\pm$ 0.7
conIFA ($n = 3$)	138.3 $\pm$ 26.2	113.7 $\pm$ 23.1	19.2 $\pm$ 4.5	5.4 $\pm$ 1.3	0.2 $\pm$ 0.2
sodA ($n = 6$)	138.3 $\pm$ 12.3	98.8 $\pm$ 11.1	21.4 $\pm$ 3.5	18.2 $\pm$ 3.7*	0

Definitions of abbreviations: conIFA, mice sensitized with incomplete Freund's adjuvant only; conNS, mice with no sensitization; sodA, mice sensitized with superoxide dismutase A; PMNs, polymorphonuclear leukocytes (neutrophils); Lymphs, lymphocytes; Eos, eosinophils.

Data are expressed as mean $\pm$ SEM.

* $P < 0.025$ versus conNS and conIFA, according to ANOVA.

Figure 4. Immunohistochemical analyses of leukocytes present in lung granulomas of sodA-treated mice (A, C, and E) and human patients with sarcoidosis (B, D, and F). (A and B) Macrophages. (C and D) CD3⁺ T cells. *Inset in C:* CD4⁺ T cells. (E and F) B cells. Scale bars = 25 μ m in A, C, *inset in C*, and E, and 50 μ m in B, D, and F.

Figure 5. BAL fluid from sodA-treated mice demonstrates sodA-specific CD4⁺ Th1 immune response. Intracellular cytokine staining for IFN- γ was performed on BAL cells from baseline, SEA-treated, and sodA-treated mice, after stimulation with sodA peptide or staphylococcal enterotoxin B (SEB). Dot plots represent percentage of cells expressing IFN- γ within CD3⁺CD4⁺ gated subsets. These data are representative of three sets of mice ($n = 9$ per set).

SEA-treated, and sodA-treated murine populations. BAL cells stimulated by staphylococcal enterotoxin B (SEB) served as the positive control, and BAL cells alone served as the negative control (Figure 5). Appropriate responses occurred in all three murine populations to SEB, and none to a lack of peptide. BAL cells from sodA mice displayed a twofold $CD3^+CD4^+$ IFN- γ response to sodA, compared with no response in baseline or SEA-treated mice (Figure 5). Strong $CD3^+CD4^-$ T-cell responses were also evident, suggesting that the animal model demonstrates $CD8^+$ T-cell responses to mycobacterial antigens that were also in samples of human sarcoidosis (8, 10). Cytokine analyses of the BAL fluid demonstrated statistically significant differences in the expression of Th1 and Th2 cytokines between untreated, SEA-treated, and sodA-treated mice. The BAL fluid from sodA-treated mice contained significantly higher concentrations of IL-2 compared with baseline mice and SEA-treated mice ($P = 0.008$ and $P = 0.02$, respectively; Wilcoxon rank sum test) (Figure 6A). IFN- γ concentrations were also significantly different compared with baseline and SEA control mice (both $P = 0.008$, Wilcoxon rank sum test) (Figure 6B). No significant differences in IFN- γ concentrations were evident between untreated and SEA-treated murine populations. No significant expression of Th2 cytokines occurred among untreated and sodA-treated murine populations. However, a significant expression of both IL-4 and IL-5 cytokine responses was evident in SEA-treated mice ($P = 0.008$ for IL-4 and IL-5, Wilcoxon rank sum test) (Figures 6C and 6D).

Antigen-Specific Recognition in sodA-Treated Murine BAL Cells is MHC Class II-Dependent

Previous reports noted an association between MHC Class II alleles and susceptibility to sarcoidosis. According to a recent report, the mycobacterial antigens presented by these MHC Class II alleles are recognized by sarcoidosis $CD4^+$ T cells (15). Although BAL cells from ConNS or ConIFA mice did not elicit a response against sodA peptide, BAL cells from sodA-treated mice showed $CD4^+$ IFN- γ responses that were 5.3 and

6.5 fold higher, respectively, than those of BAL cells from these mice (Figure 7). To assess whether the antigen-specific recognition exhibited by $CD4^+$ T cells from sodA-treated mice was secondary to sodA antigens presented in the context of MHC Class II alleles, BAL cells were incubated with α -MHC II or isotype antibody for 30 minutes before antigen stimulation. The blocking of MHC II abolished the immune recognition of sodA by $CD4^+$ T cells, whereas the isotype antibody did not (Figure 7).

DISCUSSION

Sarcoidosis involves the lung in more than 90% of patients. The disease is characterized by Th1 alveolitis, accompanied by a $CD4^+$ T-cell predominance, leading to the formation of granulomas. No animal model, to the best of our knowledge, uses peptides that are unique to sarcoidosis. The mounting evidence for the involvement of mycobacterial antigens in the pathogenesis of sarcoidosis, and the specific identification of mycobacterial virulence factors in the BAL and granulomas of patients with sarcoidosis, led us to develop a murine model of pulmonary sarcoidosis. The administration of antigen-coated beads to elicit a delayed-type hypersensitivity reaction in mice has been widely used to study the cellular and molecular mechanisms for the selective recruitment of leukocytes to the airways (13, 16–18). After the embolization (via tail-vein injection) of antigen-coated beads, mice presensitized with the same antigen develop many features similar to human granuloma formation. For example, *Mycobacterium bovis* purified protein derivative (PPD)-sensitized and challenged mice develop lung granulomas that are largest 4 days after challenge, and that resolve 8 days after challenge (13). Similar to the situation in human pulmonary granulomas, sodA induced the formation of large granulomas throughout murine lungs. The murine model of sarcoidosis possesses the strongest pathologic hallmarks of sarcoidosis: hilar lymphadenopathy, lymphocytic infiltrates, the formation of giant cells, and a Th1

Figure 6. Cytokines present in BAL fluid from baseline, sodA- and SEA-treated mice. BAL fluid was collected and pooled from mice, and analyzed for (A) IL-2, (B) IFN- γ , (C) IL-4, and (D) IL-5. Statistical significance was determined using the Wilcoxon rank sum test. * $P = 0.008$. † $P = NS$. § $P = 0.02$. These data are representative of three sets of mice ($n = 9$ per set).

Figure 7. The *sodA*-specific CD4⁺ Th1 immune response is major histocompatibility complex (MHC) Class II-restricted. Intracellular cytokine staining for IFN- γ was performed on BAL cells from control and *sodA*-challenged mice, after stimulation with *sodA* peptide or SEB. Dot plots represent percentage of cells expressing IFN- γ within CD3⁺CD4⁺ gated subsets. These data are representative of three sets of mice ($n = 9$ per set).

immunophenotype (Figures 2 and 6). This model demonstrates an additional strength, in that peptides isolated only from sarcoidosis granulomas are used to incite granulomatous inflammation. This unique *Mycobacterium* *sodA* peptide sequence demonstrates the specificity of this peptide for eliciting the histologic features observed in human sarcoidosis.

In contrast, no sensitization (ConNS) or sensitization with IFA alone (ConIFA), followed by challenge with naked beads, led to the formation of small, foreign-body granulomas. To contrast the granuloma formation elicited by *sodA* to that elicited by a sarcoidosis-unrelated antigen, we used the SEA model developed by Chensue and colleagues (13). As was the case in *sodA*-treated mice, SEA elicited granulomas throughout the lungs, with the formation of giant cells. In contrast to *sodA*-elicited granulomas, SEA granulomas were larger, they developed fibrosis, and they were associated with a Th2 immunophenotype (Figures 2 and 6) (13). Taken together, these

studies show that *sodA* elicits lung granulomas in mice, with the histologic features of human sarcoidosis.

Next, we determined the leukocyte components of *sodA* murine lung granulomas by immunohistochemistry, and compared them with those in human sarcoid pulmonary granulomas. Macrophages were the most abundant cell type in the granulomas of both *sodA*-treated mice and human sarcoidosis. Macrophages are known to be key players in sarcoidosis pathogenesis, because of their ability to present foreign antigens to innate and adaptive cellular components. Other cell types that have been the focus of intense investigation for their possible role in granuloma formation include T lymphocytes. We used CD3, a general marker for both CD4⁺ and CD8⁺ T cells. T cells were found in the middle layers of granulomas in both murine and human lungs. In addition, we localized abundant CD4⁺ T cells to the same region as the CD3⁺ cells in the *sodA*-elicited murine lung granulomas. A recent study of

sarcoidosis BAL reported on CD4⁺ and CD8⁺ T-cell responses to mycobacterial antigens. We found similar responses in the murine model (Figure 5). Although the formation of granulomas was evident in SEA-sensitized mice, T cells isolated from their BAL did not recognize sodA peptides (Figure 5). Finally, B cells were also found sporadically in human and murine sarcoid lung granulomas. These observations are consistent with those of Minami and colleagues (19), Nishiwaki and colleagues (20), and McCaskill and colleagues (21), who studied murine immune pulmonary responses to *Propionibacterium acnes*. They found peribronchovascular granulomatous inflammation, composed of T and B cells and histiocytes, in response to *P. acnes*. Although *P. acnes* was implicated as a putative agent in Japanese and European patients with sarcoidosis, to the best of our knowledge, the molecular and immunologic evidence of its involvement in American patients with sarcoidosis was not previously reported.

Increasing evidence suggests that the degree of inflammation and fibroblast action/proliferation may be dependent on a balance of Th1-like and Th2-like cytokines expressed during the evolution of sarcoidosis (22). A study by Moller and colleagues (23) of BAL cells and fluid from sarcoid patients showed a predominant Th1 cytokine response, with an elevated gene and protein expression of IFN- γ and IL-12, but not IL-4 or IL-10. Moller and colleagues (23) suggested that a chronic deregulation of IL-12 (which induces the production of IFN- γ , and directs T-cell expansion down the Th1 pathway) is the driving force of granuloma formation in sarcoidosis. The importance of these cytokines in the formation of granulomas was shown when IL-12 and IFN- γ knockout mice failed to develop granulomas after being challenged with granulomatous agents (24, 25). The lymphocytes from sodA-treated mice also demonstrated the Th1 immunophenotype seen in patients with sarcoidosis, compared with the Th2 granulomatous inflammation in SEA-treated mice (Figure 6). Other early mediators include TNF- α , IL-1 β , and IL-6, which both amplify and maintain the formation of granulomas (26). The flow cytometry of murine BAL fluid demonstrates that no immune responses to microbial antigens occur at baseline, with an appropriate positive response to the positive control, SEB. BAL CD4⁺ T cells demonstrate antigen-specific responses to sodA-sensitized mice. A Th1 immunophenotype was demonstrated by the secretion of IL-2 and IFN- γ . A lack of recognition of sodA was evident in SEA-sensitized, ConNS, and ConIFA mice, as expected (Figures 5 and 7).

The role of genetics in sarcoidosis pathogenesis demonstrates that MHC Class II alleles are associated with susceptibility to, or protection from, sarcoidosis (5, 27, 28). We were interested in the role of MHC Class II in the antigen presentation of sodA peptides in the murine model. A blockade of MHC Class II inhibited recognition of the sodA peptide, a result similar to what was described the recognition of mycobacterial antigens in patients with sarcoidosis (9). This particular feature of the model will allow us to identify the molecular basis for genetic associations with sarcoidosis outcomes.

Experimental models of granulomatous inflammation, characterized by either a Th1 or Th2 response (22), are also useful in delineating the mechanisms that maintain and resolve chronic granulomatous lung disease (29). For example, *in vivo* studies of the granuloma formation induced by *Mycobacterium* infection showed that IFN- γ and TNF- α are necessary for the progression of lesions (13). Moreover, McCaskill and colleagues (21) revealed a Th1 cytokine profile in the BAL fluid and lungs associated with *P. acnes*-induced granulomatous inflammation in murine lungs. In the present study, both IFN- γ and IL-2 were present in the BAL fluid of sodA-treated mice, consistent with the Th1 cytokine pattern observed in sarcoidosis BAL. On the

other hand, the formation of granulomas induced by *Schistosoma mansoni* was dependent on IL-4 (17, 30). In the SEA model, a shift in the cytokine profile occurred from Th1 to Th2 (i.e., IL-4, IL-5, and IL-10), which eventually resulted in fibrosis (13, 16). The activation of tissue remodeling, including the increased deposition and degradation of extracellular matrix, occurs simultaneously with the inflammatory response.

Thus, this murine model of sarcoidosis contains the gross, histologic, and immunologic features seen in active sarcoidosis. The work to date demonstrates that (1) mice can develop noncaseating granulomas from peptides unique to sarcoidosis; (2) the gross pathology and histology closely parallel those seen in human patients with sarcoidosis; (3) according to cell type and cytokine pattern, the immunology reflects observations in patients with sarcoidosis at presentation; and (4) MHC Class II alleles are also shown to be important in generating immune responses in this model. This model will serve as an important vehicle to facilitate the identification of mechanistic and immunologic contributors to the resolution of sarcoidosis or progression to fibrosis.

Author Disclosure: D.A.C. has received lecture fees from Takeda Corporation (less than \$5,000), industry-sponsored grants from the Takeda Corporation (\$10,001–\$50,000), Actelion (\$10,001–\$50,000), the Bard Corporation (\$50,001–\$100,000), Ortho-McNeil (\$50,001–\$100,000), and the Hollister Corporation (\$10,001–\$50,000). None of the other authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Nina Volokh, from the Department of Immunology at the Cleveland Clinic; Terri O'Brian, Matt Kohlmann, and Joseph A. Pangrace from the Center for Medical Arts and Photography at the Cleveland Clinic; and Melissa Downing from the Vanderbilt University Medical Center Immunohistochemistry Core Laboratory, for their technical assistance.

References

1. Ichikawa H, Kataoka M, Hiramatsu J, Ohmori M, Tanimoto Y, Kanehiro A, Nakata Y, Tanimoto M. Quantitative analysis of propionibacterial dna in bronchoalveolar lavage cells from patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2008;25:15–20.
2. Drake WP, Pei ZH, Pride DT, Collins RD, Cover TL, Blaser MJ. Molecular analysis of sarcoidosis tissues for *Mycobacterium* species DNA. *Emerg Infect Dis* 2002;8:1334–1341.
3. Song ZM, Marzilli L, Greenlee BM, Chen ES, Silver RE, Askin FB, Teirstein AS, Zhang Y, Cotter RJ, Moller DR. Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J Exp Med* 2005;201:755–767.
4. Dubaniewicz A, Dubaniewicz-Wybieralska M, Sternau A, Zwolska Z, Izycka-Swieszewska E, Augustynowicz-Kopec E, Skokowski J, Singh M, Zimnoch L. *Mycobacterium tuberculosis* complex and mycobacterial heat shock proteins in lymph node tissue from patients with pulmonary sarcoidosis. *J Clin Microbiol* 2006;44:3448–3451.
5. Rossman MD, Thompson B, Frederick M, Maliarik M, Iannuzzi MC, Rybicki BA, Pandey JP, Newman LS, Magira E, Beznik-Cizman B, *et al.* HLA-DRB1*1101: a significant risk factor for sarcoidosis in blacks and whites. *Am J Hum Genet* 2003;73:720–735.
6. Dubaniewicz A, Dubaniewicz A, Dubaniewicz A, Moszkowska G. DQA1*03011 allele: protective or an adverse effect on the development of sarcoidosis: preliminary study. *Respir Med* 2007;101:2213–2216.
7. Drake WP, Dhasan MS, Nadaf M, Shepherd BE, Vadivelu S, Hajizadeh R, Newman LS, Kalams SA. Cellular recognition of *Mycobacterium tuberculosis* ESAT-6 and katG peptides in systemic sarcoidosis. *Infect Immun* 2007;75:527–530.
8. Chen ES, Wahlstrom J, Song ZM, Willett MH, Wiken M, Yung RC, West EE, Mcdyer JF, Zhang Y, Eklund A, *et al.* T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J Immunol* 2008;181:8784–8796.
9. Hajizadeh R, Sato H, Carlisle J, Nadaf MT, Evans W, Shepherd BE, Miller RF, Kalams SA, Drake WP. *Mycobacterium tuberculosis* antigen 85A induces Th-1 immune responses in systemic sarcoidosis. *J Clin Immunol* 2007;27:445–454.
10. Oswald-Richter KA, Culver DA, Hawkins C, Hajizadeh R, Abraham S, Shepherd BE, Jenkins CA, Judson MA, Drake WP. Cellular responses to mycobacterial antigens are present in bronchoalveolar

- lavage fluid used in the diagnosis of sarcoidosis. *Infect Immun* 2009;77:3740–3748.
11. Allen SS, Evans W, Carlisle J, Hajizadeh R, Nadaf M, Shepherd BE, Pride DT, Johnson JE, Drake WP. Superoxide dismutase A antigens derived from molecular analysis of sarcoidosis granulomas elicit systemic Th-1 immune responses. *Respir Res* 2008;9:36.
 12. Swaisgood CM, Richter K, Moeller S, Klemenc J, Drake WP. Development of a sarcoidosis murine model using mycobacterial antigens ESAT-6 and SodA. *Am J Respir Crit Care Med* 2009;179:A3996.
 13. Chensue SW, Warmington K, Ruth J, Lincoln P, Kuo MC, Kunkel SL. Cytokine responses during mycobacterial and schistosomal antigen-induced pulmonary granuloma-formation: production of Th1 and Th2 cytokines and relative contribution of tumor-necrosis-factor. *Am J Pathol* 1994;145:1105–1113.
 14. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA. The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. *Am J Pathol* 2000;157:177–187.
 15. Oswald-Richter K, Sato H, Hajizadeh R, Shepherd BE, Sette A, Sidney J, Newman LS, Drake WP. Mycobacterial ESAT-6 and katG are recognized by sarcoidosis CD4+ T cells when presented by the American sarcoidosis susceptibility allele, DRB1*1101. *J Clin Immunol* 2010;30:157–166.
 16. Chensue SW, Warmington KS, Ruth JH, Lincoln P, Kunkel SL. Cytokine function during mycobacterial and schistosomal antigen-induced pulmonary granuloma-formation: local and regional participation of IFN-gamma, IL-10, and TNF. *J Immunol* 1995;154:5969–5976.
 17. Chensue SW, Warmington K, Ruth JH, Lukacs N, Kunkel SL. Mycobacterial and schistosomal antigen-elicited granuloma formation in IFN-gamma and IL-4 knockout mice: analysis of local and regional cytokine and chemokine networks. *J Immunol* 1997;159:3565–3573.
 18. Chiu BC, Freeman CM, Stolberg VR, Komuniecki E, Lincoln PM, Kunkel SL, Chensue SW. Cytokine-chemokine networks in experimental mycobacterial and schistosomal pulmonary granuloma formation. *Am J Respir Cell Mol Biol* 2003;29:106–116.
 19. Minami J, Eishi Y, Ishige Y, Kobayashi I, Ishige I, Kobayashi D, Ando N, Uchida K, Ikeda S, Sorimachi N, et al. Pulmonary granulomas caused experimentally in mice by a recombinant trigger-factor protein of *Propionibacterium acnes*. *J Med Dent Sci* 2003;50:265–274.
 20. Nishiwaki T, Yoneyama H, Eishi Y, Matsuo N, Tatsumi K, Kimura H, Kuriyama T, Matsushima K. Indigenous pulmonary *Propionibacterium acnes* primes the host in the development of sarcoid-like pulmonary granulomatosis in mice. *Am J Pathol* 2004;165:631–639.
 21. McCaskill JG, Chason KD, Hua XY, Neuringer IP, Ghio AJ, Funkhouser WK, Tilley SL. Pulmonary immune responses to *Propionibacterium acnes* in C57BL/6 and BALB/c mice. *Am J Respir Cell Mol Biol* 2006;35:347–356.
 22. Kunkel SL, Lukacs NW, Strieter RM, Chensue SW. Th1 and Th2 responses regulate experimental lung granuloma development. *Sarcoidosis Vasc Diffuse Lung Dis* 1996;13:120–128.
 23. Moller DR, Forman JD, Liu MC, Noble PW, Greenlee BM, Vyas P, Holden DA, Forrester JM, Lazarus A, Wysocka M, et al. Enhanced expression of IL-12 associated with Th1 cytokine profiles in active pulmonary sarcoidosis. *J Immunol* 1996;156:4952–4960.
 24. Gudmundsson G, Hunninghake GW. Interferon-gamma is necessary for the expression of hypersensitivity pneumonitis. *J Clin Invest* 1997;99:2386–2390.
 25. Wang J, Wakeham J, Harkness R, Xing Z. Macrophages are a significant source of Type 1 cytokines during mycobacterial infection. *J Clin Invest* 1999;103:1023–1029.
 26. Kunkel SL, Chensue SW, Strieter RM, Lynch JP, Remick DG. Cellular and molecular aspects of granulomatous inflammation. *Am J Respir Cell Mol Biol* 1989;1:439–447.
 27. Grunewald J, Eklund A. Lofgren's syndrome human leukocyte antigen strongly influences the disease course. *Am J Respir Crit Care Med* 2009;179:307–312.
 28. Saltini C, Pallante M, Puxeddu E, Contini S, Voort CE, Drent M, Amicosante MM. M. avium binding to HLA-DR expressed alleles in silicon: a model of phenotypic susceptibility to sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2008;25:100–116.
 29. Kunkel SL, Lukacs NW, Strieter RM, Chensue SW. Animal models of granulomatous inflammation. *Semin Respir Infect* 1998;13:221–228.
 30. Chensue SW, Terebuh PD, Warmington KS, Hershey SD, Evanoff HL, Kunkel SL, Higashi GI. Role of IL-4 and IFN-gamma in *Schistosoma mansoni* egg-induced hypersensitivity granuloma-formation: orchestration, relative contribution, and relationship to macrophage function. *J Immunol* 1992;148:900–906.