Pulmonary Immune Responses to *Propionibacterium acnes* in C57BL/6 and BALB/c Mice

Joshua G. McCaskill, Kelly D. Chason, Xiaoyang Hua, Isabel P. Neuringer, Andrew J. Ghio, William K. Funkhouser, and Stephen L. Tilley

Department of Medicine, Division of Pulmonary Diseases and Critical Care Medicine, and Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill; and National Health and Environmental Effects Research Laboratory, Environmental Protection Agency, Research Triangle Park, North Carolina

Propionibacterium acnes (PA) is a gram-positive anaerobic bacterium implicated as a putative etiologic agent of sarcoidosis. To characterize the pulmonary immune response to PA, C57BL/6 and BALB/c mice were intraperitoneally sensitized and intratracheally challenged with heat-killed bacteria. C57BL/6 mice challenged with PA developed a cellular immune response characterized by elevations in Th1 cytokines/chemokines, increased numbers of lymphocytes and macrophages in lung lavage fluid, and peribronchovascular granulomatous inflammation composed of T- and B-lymphocytes and epithelioid histiocytes. T-lymphocytes in the lung lavage fluid showed a marked CD4⁺ cell predominance. In contrast, C57BL/6 mice challenged with *Staphylococcus epidermidis* (SE), another gram-positive commensal of human skin, and BALB/c mice challenged with PA, showed only a modest induction of Th1 cytokines, less pulmonary inflammation, and no granulomatous changes in the lung. Enhancement of Toll-like receptor expression was seen in PA-exposed C57BL/6 mice within 24 h after exposure, suggesting that induction of innate immunity by PA contributes to the robust, polarized Th1 immune response elicited by this bacterium. These findings suggest that PA-induced pulmonary inflammation may be a useful model for testing the contributions of both bacterial and host factors in the development, maintenance, and resolution of granulomatous inflammation in the lung.

Keywords: inflammation; granuloma; lung; mouse; *Propionibacterium acnes*

Sarcoidosis is a systemic disease of unknown cause characterized by noncaseating granulomata in organs and tissues. The disease most frequently involves the lung and thoracic lymph nodes, but other organs, including the liver, spleen, skin, and eyes, are commonly involved. Pathologically, the granulomas of sarcoidosis are composed of a central core of macrophages and dendritic cells. Both surrounding and admixed with these antigen-presenting cells (APCs) are T- and to a lesser extent B-lymphocytes (1). Ultrastructural examination of the sarcoid granuloma has shown an intimate association and interdigitation of the cell walls of lymphocytes and APCs, suggesting cross talk between these cells (2). Oligoclonal expansion of distinct T-cell subsets has been reported in patients with sarcoidosis (3, 4). Based on these observations, the pathogenesis of sarcoidosis is believed to be antigen-driven. Despite the collective data suggesting an antigen-induced

immune response, the definitive antigen or antigens of sarcoidosis have remained elusive. Many studies have suggested putative infectious agents, but the data from these investigations have been insufficiently robust to unequivocally confirm that a particular agent is causative (5).

In Japan, a series of microbiological and molecular investigations have suggested that *Propionibacteria* spp. may be etiologically linked to sarcoidosis (6–10). *Propionibacteria* are gram-positive anaerobic bacteria that are well recognized as part of the normal microbiota of the human skin (11). Recently, *propionibacteria* have been cultured from peripheral lung tissue and mediastinal lymph nodes from patients without sarcoidosis, suggesting that they may be commensal in the lung as well (12). In human skin, an immune response is not generally mounted against resident *Propionibacteria* spp. or other flora such as *Staphylococcus epidermidis* (SE). However, in acne, the most common disease affecting the skin, an inflammatory response to *Propionibacterium acnes* (PA) is believed to contribute to disease pathogenesis (13). Thus, an exaggerated immune response against a bacterium present in organs frequently affected by sarcoidosis, such as the lung and skin, is a plausible theory of pathogenesis.

In the 1970s *Corynebacterium parvum*, genetically identical to and now known as PA, was tested in mice as an immunostimulant adjuvant therapy for cancer. In addition to anti-tumor effects, mice inoculated intravenously with heat-killed PA developed hepatosplenomegaly with granulomatous inflammation in the livers, spleens, and lymph nodes, establishing the capacity of this organism to induce granulomatous inflammation. Peripheral lymphopenia, increased antibody response, and increased resistance to infection were additional features observed after intravenous challenge with these anaerobic coryneform bacteria (14). Interestingly, all of these features have been reported in humans with sarcoidosis (15).

More recently PA has been used as a priming agent for LPS-induced acute liver injury. In this model, PA priming before LPS challenge profoundly enhances the degree of hepatic injury after LPS exposure (16). This liver injury model has been extensively characterized, including the priming phase by intravenous PA, which results in granuloma development in the murine liver (17–19). In contrast, little is known about the pulmonary immune response to PA. Given the increasing interest in this organism as a putative etiologic agent of sarcoidosis, we have investigated the pulmonary immune response to PA in C57BL/6 and BALB/c mice.

MATERIALS AND METHODS

Mice

Pathogen-free C57BL/6 and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME), bred in our animal facility, and used between 3 and 6 mo of age. Exposed and control mice were housed in filter-top cages and kept in a negative-pressure, ventilated cubicle in the Keck Animal Models Facility at the University of North Carolina, Chapel Hill. All animal experiments complied with the Institutional

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Correspondence and requests for reprints should be addressed to Stephen Tilley, Pulmonary Immunobiology Lab, 8033 Burnett-Womack Bldg. CB# 7219, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7219. E-mail: stephen_tilley@med.unc.edu

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Animal Care and Use Committee guidelines of University of North Carolina as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Bacteria

PA cultures (ATCC #6919) were grown on sheep blood agar in an anaerobic chamber (COY Laboratory Products, Inc., Grass Lake, MI) at 37°C in the microbiology laboratory at UNC Hospitals. A control gram-positive bacterium, SE (ATCC #12228), was also grown on sheep blood agar at 37°C. After 48 h, the cultures were examined for growth and used for experiments when colonies became confluent. The identity of the PA was verified by colony morphology, indole positivity, and Gram staining.

Inoculation of Mice with Bacteria

PA colonies were isolated from blood agar plates and suspended in PBS. The bacteria were heat-killed by autoclaving at 121°C and then intraperitoneally injected (0.5 mg; 0.25 ml of the 2 mg/ml suspension) into mice. Two weeks after intraperitoneal injection, PA-sensitized mice were challenged with heat-killed PA (0.5 mg; 0.05 ml of 10 mg/ml suspension) intratracheally. C57BL/6 and BALB/c mice sensitized and challenged with PBS (PBS/PBS) were used as controls. To determine the specific impact of sensitization alone as well as challenge alone, some C57BL/6 mice were either sensitized to PA but not challenged (intraperitoneal PA/intratracheal PBS), or nonsensitized but challenged (intraperitoneal PBS/intratracheal PA). As an additional control to determine whether the observed immune response was specific to PA or common to gram-positive bacteria, some mice were intraperitoneally sensitized with heat-killed SE and subsequently challenged with heat-killed SE using a dosing scheme identical to that described above for PA. The total numbers of animals examined in each of these groups are detailed in Tables E1 and E2 in the online supplement. Mice were killed on Days 1, 3, 5, 7, 14, 21, 28, 35, 42, and 54 after challenge by an overdose of pentobarbital sodium (150 mg/kg), and organs and cells were harvested for histologic, cytokine, and RNA analysis.

Whole Lung Lavage

The trachea was exposed and cannulated with a 19-gauge blunt-tipped cannula and the lungs were lavaged with 1 ml of sterile Hank's Balanced Saline Solution (HBSS). After removal the lavage fluid was immediately placed on ice and centrifuged at 1,500 rpm for 5 min at 4°C. After centrifugation, the supernatant was removed and stored at -80°C until further use for analysis of cytokines. The cell pellet was resuspended in 0.5 ml of cold HBSS for determination of total and differential cell counts. Total cells were counted with a hemocytometer. Cells were cytopun onto glass slides using a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) at 1,000 rpm for 6 min and stained with a Hema 3 stain (Fisher Scientific, Hampton, NH) for determination of cellular differentials.

Flow Cytometry

Lung lavage cells from mice sensitized and intratracheally challenged with PA and SE were examined 7 and 21 d after challenge. Lavage fluid from three mice in each group were pooled, centrifuged, and resuspended in FACs Wash (HBSS, 2% FBS; Invitrogen, Grand Island, NY) containing FC Block (FcγIII/II) (0.25 μl/ml) at a concentration of 1×10^5 cells/100 μl. After incubation for 15 min, cells were aliquoted into 96-well plates at a concentration of 100,000 cells/well. Five microliters of diluted FITC-CD4 rat anti-mouse L3T4 clone (RM4-5) and PerCP-CD8a rat anti-mouse Ly-2 clone (55-6.7) (BD Biosciences, San Diego, CA) were added to each well. After 5 min incubation, cells were washed three times with FACs Wash, resuspended in FACs Fix (HBSS, 10% neutral buffered formalin [NBF]), and stored at 4°C until FACs analysis.

Lung Histology

After whole lung lavage, the lungs were inflated with 10% NBF at 20 cm H₂O. Lungs and heart were removed en bloc and immersed in 10% NBF for 24 h before paraffin embedding. All lobes of the lung were dehydrated, paraffin-embedded, and one 5-μm section was cut across each lobe. Sections were stained with hematoxylin and eosin (H&E) for histologic evaluation.

Immunohistochemistry

Tissue sections were cut, floated on a protein-free water bath, mounted on saline-treated slides (Fisher, Raleigh, NC), and air-dried overnight. The slides were heat-fixed at 60°C in a slide dryer (Shandon Lipshaw, Pittsburgh, PA) for 10 min and cooled to room temperature. Sections were then deparaffinized and hydrated to 95% alcohol (xylene for 10 min, absolute alcohol for 5 min, and 95% alcohol for 5 min). Endogenous peroxidase activity was blocked with 0.6% H₂O₂ in absolute methanol for 8 min. Slides were rinsed in 95% alcohol for 2 min, placed in deionized H₂O, and washed in PBS. After treatment with Cyto Q Background Buster (Innovex Biosciences, Richmond, CA) for 10 min, slides were incubated with the primary antibodies against CD68 (Dako, Carpinteria, CA), CD4 (Santa Cruz Biotechnology, Santa Cruz, CA), and CD45R/B220 (BD Pharmingen, San Diego, CA) diluted in 1% BSA for 45 min at 37°C in PBS at a dilution of 1:25. Slides were incubated with biotinylated linking antibody from Stat-Q Staining System (Innovex Biosciences) for 10 min at room temperature, washed with PBS, and peroxidase enzyme label from Stat-Q Staining System (Innovex Biosciences) applied. After incubation for 10 min at room temperature and washes with PBS, tissue sections were developed with 3,3'-diaminobenzidine-tetrahydrochloride for 3 min at room temperature. Sections were counterstained with hematoxylin or methylene green, dehydrated through alcohols, cleared in xylene, and coverslipped using a permanent mounting media.

Lung Homogenates

Lungs were snap-frozen in liquid nitrogen and stored at -80°C until homogenization. Lungs were homogenized in lysis buffer (300 mM NaCl, 15 mM Tris, 4 mM MgCl₂, 0.1% Triton-X 100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, pH 7.4 and filter sterilized) and centrifuged at $1,500 \times g$ for 15 min at 4°C. Supernatant was removed, mixed, aliquoted, and stored at -80°C until use.

ELISA

The concentrations of TNF-α, IFN-γ, IL-4, IL-12 p40, IL-12p70, IL-13, and monocyte chemoattractant protein-1 (MCP-1) were measured by ELISA, according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN).

RNA Analysis by Quantitative Real-Time PCR

Lungs and livers were removed and snap-frozen in liquid nitrogen, then stored at -80°C. Total RNA was extracted using RNA-bee (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RNA was then treated with RQ1 DNase (Promega, Madison, WI) and reverse-transcribed with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Expression levels TNF-α, IFN-γ, IL-12p35, MCP-1, angiotensin-converting enzyme (ACE), Toll-like receptor (TLR) 1, TLR2, TLR4, TLR5, and TLR9 were determined by Quantitative Real-Time PCR on the ABI 7900HT Real-Time PCR System (Applied Biosystems). Taqman Universal PCR Master Mix and the following primers and probes for PCR were purchased from Applied Biosystems: Mouse β-actin Endogenous Control (4352341E), Taqman Gene Expression Assays for TNF-α (Mm00443258_m1), IFN-γ (Mm0080178_m1), IL-12p35 (Mm00434165_m1), MCP-1 (Mm00441242_m1), ACE (Mm00802048_m1), TLR1 (Mm00446095_m1), TLR2 (Mm00442346_m1), TLR4 (Mm00445274_m1), TLR5 (Mm00546288_s1), and TLR9 (Mm00446193_m1). Relative mRNA expression, normalized to the mouse β-actin gene, was calculated by the comparative Ct method, and compared to RNA expression in the lungs of control mice (PBS/PBS) not exposed to bacteria.

Statistical Analysis

All data are presented as means ± SE. Statistical significance was assessed by the two-tailed, unpaired Student's *t* test.

RESULTS

Lung Histopathology after Sensitization and Challenge with PA

Lung histology was evaluated in sensitized C57BL/6 mice 1, 3, 5, 7, 14, 21, 28, 35, 42, and 54 d after intratracheal challenge

Figure 1. Lung histopathology 7 d after intratracheal challenge with PA and SE. C57BL/6 were intraperitoneally sensitized and challenged 2 wk later with heat-killed PA (A, B, C). Lung histopathology was compared with C57BL/6 mice sensitized/challenged with SE (D, E, F) and BALB/c mice sensitized/challenged with PA (G, H, I). Representative H&E-stained sections from each group are shown at magnifications of $\times 2$ (A, D, G), $\times 10$ (B, E, H), and $\times 60$ (C, F, I).

with heat-killed PA. Twenty-four hours after intratracheal challenge, a peribronchovascular (PBV) neutrophilic infiltrate and patchy acute pneumonia was observed (Figure E1 in the online supplement). On Day 3 a mixed neutrophilic and lymphocytic PBV infiltrate was seen, with areas of epithelioid histiocytes and loose granulomas (Figure E2). By Day 5 a lymphocyte-predominant PBV infiltrate with admixed histiocytes was present along with rare loose granulomata (Figure E3). Peak inflammation was observed on Day 7 after intratracheal challenge with PA, composed of lymphohistiocytic PBV infiltrates around most airways and vessels (Figures 1A and E4). Loose granulomata were present in the PBV stroma and lung parenchyma (Figures 1B, 1C, and E4). By Day 14, PA-exposed mice showed variably dense lymphohistiocytic PBV infiltrates, which were less prominent than those seen 7 d after exposure. Granulomata were observed in some sections but were infrequent (Figure E5). On Day 21, PA-exposed mice showed focal lymphoplasmacytic infiltrates without granulomata (Figure E6). Subsequent time points showed mild PBV lymphocytic infiltrates consistent with bronchus-associated lymphoid tissue (BALT) aggregates and only rare PBV granulomata (Figure E7).

Next, we investigated whether this inflammatory response in the lung was unique to PA or whether it was a general response to gram-positive bacteria. We chose to test SE, another commensal gram-positive organism of human skin. Mice were intraperitoneally sensitized with SE and intratracheally challenged 2 wk later with the same organism. Lung histology was evaluated at the same time points as the PA-sensitized and -challenged mice described above. Twenty-four hours after intratracheal inoculation with SE, PBV acute inflammation and patchy acute pneumo-

nia was observed and was similar in magnitude to PA-exposed mice (Figure E1). A mixed neutrophilic and lymphocytic PBV inflammation was seen 3 d after exposure (Figure E2). These infiltrates included rare eosinophils without histiocytes or granulomata. On Day 5, a lymphocyte-predominant PBV infiltrate was present with some admixed histiocytes. Infiltrates were less dense than those observed in PA-exposed mice (Figure E3). Seven days after exposure a lymphocytic PBV infiltrate was seen. Admixed histiocytes were rare in SE-exposed mice (Figures 1D–1F), but frequent in the PA-exposed animals (Figures 1A–1C). On Day 14, SE-exposed mice showed only focal scant PBV lymphocytic infiltrates with rare histiocytes, and no granuloma formation (Figure E5). On Day 21 SE-exposed mice showed no significant inflammation, with rare single plasma cells noted in the PBV stroma (Figure E6). Subsequent time points showed mild PBV lymphocytic infiltrates consistent with BALT aggregates, as was observed in PA-exposed mice. Granulomas were not observed in SE-treated animals. Overall, PA-exposed mice showed a denser PBV infiltrate than SE mice at each time point, and a higher incidence of granulomata. The inflammation in both PA and SE mice peaked in intensity at Day 7, and decayed back to normal by Day 21 in SE mice and by Day 42 in PA mice.

Next, we evaluated lung morphology in BALB/c mice sensitized and intratracheal challenged with PA to determine whether background strain was important to lung phenotype. Similar to C57BL/6 mice challenged with PA, a PBV and alveolar neutrophilic infiltrate was observed 1 day post intratracheal challenge. By Day 3, PBV mixed acute and chronic inflammation was observed. On Days 5 and 7 after PA challenge, only PBV chronic

inflammation was observed. In contrast to PA-challenged C57BL/6 mice, no significant histiocytic component was noted in these infiltrates, and no granuloma formation was observed (Figures 1G–1I). These histopathologic findings suggest that PA possesses unique qualities that render the organism capable of eliciting granulomatous inflammation in the murine lung, and that the capacity to mount a granulomatous response to PA is also dependent upon the genetic characteristics of the mouse.

Inflammatory Cells in Lung Lavage Fluid

Next, we examined the inflammatory cell composition of the lung lavage fluid at each time-point evaluated histologically. Figure 2A shows total cells in the lung lavage fluid at each time point after intratracheal challenge with PA or SE. An increase in total cells was observed in response to both PA and SE in C57BL/6 mice, 1 and 3 d after exposure. Total cells remained elevated in the PA group through Day 21. In contrast, cell counts in SE-exposed mice returned to normal by Day 5. Both organisms produced an acute rise in neutrophils that persisted through Day 7 in the PA group and through Day 5 in the SE group; however, neutrophil numbers were significantly greater in PA-exposed mice on Days 5 and 7 (Figure 2B). A marked lymphocytosis developed in PA-exposed mice by Day 5 and persisted through Day 21 (Figure 2C). A trend toward increased numbers of lymphocytes was still observed up to 28 d after challenge ($2.6 \pm 1 \times 10^4$ versus $1.85 \pm 1.1 \times 10^4$ cells/ml, $P = 0.09$). Only a modest increase in lymphocytes was observed in the SE group. The number of macrophages was also increased in the lung lavage fluid of PA-challenged mice on Days 5, 7, and 14 after intratracheal challenge. No such increase in macrophage number was seen in any of the SE-challenged mice.

BALB/c mice were examined through 14 d after intratracheal challenge with PA. While total cells increased in the lavage fluid of BALB/c mice, numbers were significantly lower than those in similarly treated C57BL/6 animals (Figure 2A). The inflammatory response in BALB/c mice was characterized by an early rise and rapid decline in neutrophils, followed by an increase in lymphocytes (Figures 2B–2D). However, the magnitude and duration of neutrophilia and lymphocytosis was significantly lower than that observed in C57BL/6 mice exposed to PA.

Next we were interested in determining whether PA could elicit a similar immune response in the lungs of C57BL/6 mice without prior sensitization. Total cells and differentials were examined 7 and 14 days after intratracheal exposure to PA or PBS, in groups with and without sensitization. As shown in Figure 3A, a significant increase in total cells at Day 7 was only observed in mice sensitized and challenged intratracheally with PA, and was due to increased numbers of lymphocytes, macrophages, and neutrophils. A modest but statistically significant increase in lymphocytes was seen on Day 7 in PA-challenged mice that were not sensitized ($2.1 \pm 0.25 \times 10^4$ versus $0.68 \pm 0.14 \times 10^4$ cells/ml, $P = 0.02$); however, this response was significantly lower than that seen in PA-exposed mice receiving sensitization ($25.4 \pm 7.2 \times 10^4$ versus $2.1 \pm 0.25 \times 10^4$ cells/ml, $P = 0.02$). By Day 14, neutrophilia had resolved but macrophages and lymphocytes remained significantly elevated in mice sensitized and intratracheally challenged with PA, but not in nonsensitized mice (Figure 3B). These results show that sensitization significantly enhances the recruitment of leukocytes to the lung after PA challenge.

Characterization of the Lymphohistiocytic Inflammation in PA-Exposed Mice

Robust increases in lung lavage fluid lymphocytes and a corresponding aggregation of epithelioid histiocytes and lymphocytes

Figure 2. Time course and characterization of leukocytes in the lung lavage fluid after sensitization and challenge with PA. PA sensitized/challenged C57BL/6 mice (solid bars) are compared with SE sensitized/challenged C57BL/6 mice (shaded bars) and PA sensitized/challenged BALB/c mice (open bars). Mice treated with intraperitoneal PBS and intratracheal PBS were used as controls (striped bars). (A) Total cells. (B) Neutrophils. (C) Lymphocytes. (D) Macrophages. Data are representative of three independent experiments and are expressed as mean cell number $\pm$ SEM. * $P < 0.05$ versus control mice of the same genetic background, + $P < 0.05$ versus PA-exposed C57BL/6 mice ($n = 3$ –6 mice per group).

Figure 3. Effect of sensitization on leukocyte recruitment to the lung of C57BL/6 mice. Seven (A) and 14 (B) d after intratracheal challenge, mice sensitized and challenged with PA (black bars) were compared with mice intratracheally challenged but not sensitized (dark gray bars), mice sensitized but not challenged (light gray bars), and control mice that were neither sensitized nor challenged with PA (open bars). Data represent mean cell number recovered from each animal, $\pm$ SEM. * $P < 0.05$ versus intraperitoneal PBS/intratracheal PBS control mice, + $P < 0.05$ versus intraperitoneal PBS/intratracheal PA control mice ($n = 3-4$ mice per group).

into loose granulomata in C57BL/6 mice sensitized and intratracheally challenged with PA suggested that PA-induced lung inflammation should be further investigated to determine its full potential as an animal model for sarcoidosis. To further characterize the cell populations infiltrating the lung after PA exposure, we performed immunohistochemistry on lung slices 7 d after exposure. As shown in Figure 4, a mixed population of CD68+ (tissue macrophages and dendritic cells), CD4+ T lymphocytes, and CD45/B220+ (B cells) are present in the peribronchovascular infiltrates. Serially cut control sections treated with the secondary antibody only showed no staining. To further characterize the T cell population, flow cytometry was performed on lung lavage fluid lymphocytes. As described above, more lymphocytes were recovered from the lungs of PA-challenged than from SE-challenged animals as determined by cellular differentials of cytopun lavage fluid. Forward and side scatter properties by flow cytometry confirmed this visual observation, showing 20% and 29% of the total lavage fluid cells in the lymphocyte gate in PA-exposed mice on Days 7 and 21, respectively. In contrast, only 11% (Day 7) and 4% (Day 21) of total lavage fluid cells in the SE-exposed group were lymphocytes. The majority of the T-lymphocytes from PA-exposed mice expressed CD4 rather than CD8, with a CD4/CD8 ratio of 2:1 on Day 7 and >10:1 on Day 21 (Figure E8). These results show that PA results in both B and T cell recruitment to the murine lung, and that the vast majority of T cells are of the CD4+ T-helper subtype.

Proinflammatory Cytokines and Chemokines in PA-Exposed Mice

TNF- α , IFN- γ , and IL-12 are three cytokines believed to be critical for the development of granulomatous inflammation in

Figure 4. Cell types present in peribronchovascular infiltrates of PA-exposed mice. Immunohistochemistry was performed on sections from formalin-fixed, paraffin-embedded lung from mice 7 d after intratracheal challenge with PA using antibodies against CD68 (A), CD4 (B), and CD45/B220 (C). Biotinylated secondary antibody was used with Streptavidin-HRP/DAB detection system with hematoxylin (A, B) and methylene green (C) counterstains.

sarcoidosis. After intratracheal inoculation with PA, we found increases in mRNA expression of TNF- α , IFN- γ , and IL-12p35, the rate-limiting subunit of the IL-12p70 complex (Figure E9). To determine the functional significance of these changes in gene

expression at the protein level, these cytokines were measured in the lung lavage fluid and whole lung homogenates from PA- and SE-exposed C57BL/6 mice as well as in PA-exposed BALB/c mice by ELISA. Twenty-four hours after intratracheal challenge, TNF- α levels increased in lung lavage fluid from all mice and was greater in SE-challenged animals ($2,565 \pm 209$ versus $1,421 \pm 263$ pg/ml, $P = 0.01$; Figure 5A). However, on Days 3, 5, and 7 after challenge, TNF- α levels were significantly higher in the PA-challenged C57BL/6 group relative to the SE group (272 ± 91 versus 14 ± 14 pg/ml, $P = 0.04$; 128 ± 18 versus 0.8 ± 0.4 pg/ml, $P = 0.0008$; 131 ± 32 versus 0.8 ± 0.7 pg/ml, $P = 0.01$). Similar trends in TNF- α levels were seen in lung homogenates (Figure 5B). BALB/c mice showed a more modest increase in TNF- α in response to PA (Figure 5A,B). IFN- γ levels rose markedly in lung lavage fluid of C57BL/6 mice 24 h after PA challenge (759 ± 177 pg/ml), and remained very high through Day 7 (Figure

5C). Similar changes were observed in lung homogenates (Figure 5D). In contrast, levels of IFN- γ rose modestly 1 d after SE challenge before returning to baseline levels on Day 3. Only a modest induction of IFN- γ was also observed in BALB/c mice. IL-12p40, a subunit of both the IL-12 p35p40 complex as well as the IL-23 p19p40 heterodimer, was also found to be markedly higher in PA- versus SE-exposed mice 3–7 d after intratracheal challenge (Figures 5E and 5F). Despite its lability, the heterodimeric 70-kD protein was also detected and significantly higher in PA-treated animals (Figures 5G and 5H).

In contrast to the observed marked elevations of Th1 cytokines, levels of IL-4 and IL-13 did not increase in lungs of C57BL/6 mice after challenge with PA (Figures 6A and 6B). IL-13 levels were very low in the lungs of PA-exposed BALB/c mice as well (Figure 6B); however, they were ~ 2 -fold higher than IL-13 levels in C57BL/6 mice.

Figure 5. Th1 cytokines in lung lavage and lung homogenates after sensitization and challenge with PA. TNF- α (A, B), IFN- γ (C, D), IL-12p40 (E, F), IL-12p70 (G, H) were measured by ELISA in the supernatant obtained from a 1-ml whole lung lavage (A, C, E, G) and in 5-ml lung homogenate protein extracts (B, D, F, H) from PA-sensitized/challenged C57BL/6 (solid bars), SE-sensitized/challenged C57BL/6 (shaded bars), and PA-sensitized/challenged BALB/c mice (open bars). Data represent mean cytokine level $\pm$ SEM. * $P < 0.05$ versus respective controls, + $P < 0.05$ versus C57BL/6 PA-exposed mice ($n = 3$ –6 mice per group).

Figure 6. Th2 cytokines in the lung after sensitization and challenge with PA. Cytokine levels were measured in lung homogenate protein extracts by ELISA on the indicated days after challenge. IL-4 (A) and IL-13 (B) levels in C57BL/6 mice sensitized/challenged with PA (solid bars), C57BL/6 mice sensitized/challenged with SE (shaded bars), and IL-13 levels (B) in BALB/c mice sensitized/challenged with PA (open bars). Data represent mean cytokine level $\pm$ SEM. * $P < 0.05$ versus respective controls, + $P < 0.05$ versus C57BL/6 PA-exposed mice ($n = 3$ –6 mice per group).

MCP-1 is a C-C chemokine responsible for attracting monocytes and macrophages to sites of inflammation. To evaluate the contribution of this chemokine to PA-induced pulmonary inflammation, we measured mRNA and protein levels of MCP-1 in the lungs of PA-treated mice. As shown in Figure 7A, significant up-regulation of mRNA expression was found during the first 7 d after challenge with PA. MCP-1 protein levels in lung correlated well with these observed changes in gene expression (Figures 7B and 7C). MCP-1 protein levels were also elevated in the lungs of SE-exposed C57BL/6 mice and PA-exposed BALB/c mice, but were significantly lower than PA-exposed C57BL/6 animals. Taken together, these data show that PA potently induces Th1 cytokines and chemokines necessary for granuloma development in C57BL/6 mice, and provides a mechanistic explanation for the differential pulmonary immune response to these two gram-positive organisms, as well as the differences observed in PA-induced pulmonary inflammation between C57BL/6 and BALB/c mice.

ACE Expression in the Lungs of Mice with PA-Induced Inflammation

In addition to its constitutive production by the vascular endothelium, ACE can be ectopically secreted by granulomas in sarcoidosis and other granulomatous diseases. Therefore we measured ACE mRNA expression in the lungs of PA-challenged mice. ACE expression was several fold higher than that of control lungs, 4.5-fold higher 21 d after challenge (Figure E9D).

PA-Induced Changes in TLR Expression

To further examine the mechanisms by which PA induces such a polarized and robust Th1 cytokine milieu and subsequent gran-

Figure 7. MCP-1 chemokine expression in the lung after sensitization and challenge with PA. MCP-1 mRNA expression (A), protein expression in lung homogenates (B), and protein levels in lung lavage fluid (C) in mice sensitized/challenged with PA (solid bars) and mice sensitized/challenged with SE (shaded bars). MCP-1 protein levels were also measured in BALB/c mice sensitized/challenged with PA (B, open bars). Data in A are expressed as fold-expression over controls, as determined by quantitative real-time PCR (comparative C_T method), whereas data in B and C are MCP-1 levels as measured by ELISA ($n = 3$ –6 mice per group; * $P < 0.05$ versus respective controls, + $P < 0.05$ versus C57BL/6 mice sensitized/challenged with PA).

ulomatous response, we measured changes in TLR expression in C57BL/6 mice sensitized/challenged with PA and SE. Twenty-four hours after intratracheal challenge with PA, TLR1, TLR2, TLR4, TLR5, and TLR9 expression was measured in the lungs, and compared with TLR expression in the lungs of control (PBS/PBS) and SE (intraperitoneal SE/intratracheal SE)-treated mice. TLR5, which is triggered by flagellin, was included as a negative control, as both PA and SE lack this structure. TLR5 mRNA expression was not up-regulated in PA-exposed or SE-exposed mice (data not shown). As shown in Figure 8, significant increases in TLR1 expression were observed in PA-exposed mice relative to controls and SE-exposed mice. A trend towards increased expression of TLR2 (~ 40 -fold change, $P = 0.06$) and TLR9

Figure 8. TLR mRNA expression in the lungs of C57BL/6 mice sensitized and challenged with PA. TLR1 (A), TLR2 (B), TLR4 (C), and TLR9 (D) expression was determined by quantitative real-time PCR (comparative C_T method). Data are expressed as fold-expression over controls exposed to PBS. * $P < 0.05$ versus respective controls, + $P < 0.05$ versus C57BL/6 mice sensitized/challenged with SE ($n = 3-4$ mice per group).

(~ 30 -fold increase, $P = 0.087$) was also observed. A significant decrease in TLR4 expression was found ($P = 0.0009$). In contrast, only TLR2 showed up-regulated expression in SE-exposed mice (Figure 8). Messenger RNA expression was also measured in livers 1 wk after sensitization, revealing a similar profile but more modest induction of TLR1, TLR2, and TLR9 in PA- but not SE-exposed animals (Figure E10). These data show dramatic differences in TLR expression in response to PA versus SE in the murine lung, and provide one potential mechanism by which PA, but not SE, generates a polarized Th1 cytokine storm.

DISCUSSION

PA has long been recognized as a potent immunostimulant capable of producing granulomatous inflammation in the reticuloendothelial organs of experimental animals after intravenous injection of heat-killed organisms. Although the mechanisms of hepatic granuloma formation in response to PA have been extensively investigated, very little has been published on the pulmonary immune response to this anaerobic bacterium. With increasing interest in PA as a putative etiologic agent of sarcoidosis, we have characterized the pulmonary immune response to this organism in mice. In this report we show that sensitization and challenge with PA can produce granulomatous inflammation in the lungs of C57BL/6 mice, in concert with an influx of CD4⁺ lymphocytes and elevations of Th1 cytokines, an immunologic profile similar to what has been reported in humans with sarcoidosis.

Acute lung inflammation after intratracheal challenge with PA has been described previously (20). Itakura and colleagues showed that nonsensitized C57BL/6 mice develop a neutrophilic alveolitis accompanied by a mild perivascular and peribronchial accumulation of lymphocytes, peaking 48 h after intratracheal inoculation. Granulomatous changes were not observed. Based on these observations, we reasoned that granulomas may take longer to form and may require sensitization before challenge. Indeed, by comparing leukocytes in the lavage fluid from sensitized and nonsensitized mice challenged intratracheally with PA, we found sensitization essential for the lymphocytosis and increase in macrophage numbers characteristic of animals that went on to develop pulmonary granulomas.

Pulmonary granulomatosis after intratracheal inoculation with PA has been described previously in rats. In this study, rats were sensitized intravenously with heat-killed PA and challenged 2 wk later intratracheally (21). Histopathology showed severe intra-alveolar and perivascular granulomatous inflammation, peaking 48–72 h after intratracheal challenge, with focal granulomas persisting for 7–14 d. Intravenous challenge with PA has also been reported to produce granulomatous inflammation in the lungs of sensitized rabbits and rats (21, 22).

Recently, Nishiwaki and colleagues have reported the development of pulmonary granulomas after multiple immunizations of PA into the footpad of mice (23). The authors propose that PA is indigenous to the lungs of mice, and that repeated immunization of the footpad stimulates an immune response to the same bacterium residing in the lung. While these results are intriguing, they may be confounded by the fact that complete Freund's adjuvant (CFA), known to contain cell-wall mycobacterial peptides, was required in conjunction with PA to elicit this response. In our studies, no other adjuvants were employed, ensuring that the pulmonary immune response elicited was entirely secondary to PA constituents.

To determine whether the robust pulmonary immune response was unique to PA or a general reaction to gram-positive organisms, we carried out parallel experiments with SE, another gram-positive commensal of human skin. We observed marked differences in the pulmonary immune response between these two bacterial strains. While the initial neutrophilic alveolitis was similar, the ensuing lymphocytic alveolitis was more severe and persistent in PA-challenged mice. Proinflammatory cytokines had largely returned to normal by 48 h in the SE group, but remained significantly elevated up to 1 wk in the PA-challenged mice. Granuloma formation was only observed in response to PA. Recently, the inflammatory response to these same two organisms has been compared in relation to their roles in the pathogenesis of acne (24). Cutaneous inflammation was evaluated *in vivo* by injecting both bacteria into the ears of CBA/J mice. Only PA was capable of eliciting inflammation in this model. Together, these findings suggest that PA possesses unique characteristics rendering them highly immunogenic.

Our data suggest that the mechanism for the differential effects of these two gram-positive bacteria in the lungs may be

the capacity of PA to potentially induce Th1 cytokines. Analysis of the genome sequence of PA has revealed several regions encoding major immune reactive proteins, many of which are orthologous to proteins found in *Mycobacterium tuberculosis* (25). However, the precise molecular mechanisms by which these “immune reactive” proteins of PA induce such a robust Th1 cytokine storm are poorly understood. It has become increasingly appreciated that innate immunity contributes substantially to the adaptive immune response. In this regard, APCs not only phagocytose bacteria for subsequent killing and presentation of peptide constituents of pathogens via MHCII to generate an adaptive immune response, but they also recognize a variety of molecular structures present in the invading pathogen. TLRs on the cell surface and within organelles of APCs discriminate these microorganism-associated molecular structures, and signal the cell to secrete Th1-inducing cytokines, enhance antigen presentation, and enhance co-stimulatory molecule expression (26). Thus, changes in TLR expression or signaling could influence the pulmonary immune response to PA. Consistent with this paradigm, we found striking differences in TLR expression in the lungs of PA- versus SE-exposed mice, which may explain, in part, the differential immune response elicited by these different gram-positive bacteria. While only TLR2 expression increased in the lungs of SE-exposed mice, several fold increases in expression of TLR1, TLR2, and TLR9 were observed in the lungs of all mice exposed to PA. TLR1 and 2 act in concert to recognize a variety of signals including peptidoglycan constituents of the cell wall of gram-positive bacteria, while TLR9 recognizes unmethylated CpG motifs of bacterial DNA. TLR9 has been localized to the endoplasmic reticulum of macrophages and dendritic cells, suggesting that this TLR may be activated by the intracellular persistence and slow degradation characteristic of PA (27). Since macrophage numbers in the lung lavage fluid of PA-exposed mice were 2-fold higher than SE-exposed animals, differences in TLR expression could be due, in part, to the number of cells present in the lungs at the time of analysis. However, nearly all PA-exposed mice showed more than 20-fold increases in mRNA expression of TLR2 and TLR9, suggesting that PA up-regulates the expression of these TLRs. Moreover, expression of some TLRs (TLR4) was significantly lower in the PA group, despite potentially greater numbers of macrophages in the lung. To our knowledge no other studies have examined PA-induced changes in TLR expression in the lung. Several studies have examined the role of TLRs in the enhancement of cytokine production after PA priming by intraperitoneal or intravenous injection, and suggested roles for TLR2, TLR4, and TLR9 (28–31). Other intracellular bacterial pathogens have also been shown to increase both TLR2 and TLR9 transcripts in infected organs (32). Taken together, these studies and ours suggest that both constituents of the bacterial cell wall (activating TLR1 and TLR2) and bacterial DNA (activating TLR9), coupled with the slow degradation of PA, may act in concert to produce the immune-enhancing effects of this bacterium.

While the pulmonary immune response after inoculation with PA revealed many similarities to the immunobiology of sarcoidosis, including (1) noncaseating granulomatous inflammation, (2) CD4+ lymphocytosis, and (3) a highly polarized Th1 cytokine profile, important differences in histopathology between PA-induced lung disease in mice and human sarcoidosis merit discussion. We observed peribronchovascular, and occasional parenchymal and subpleural granulomatous inflammation in the murine lung—a similar distribution seen in humans with sarcoidosis. However, the granulomatous inflammation showed significant morphologic differences. In sarcoidosis, well-circumscribed granulomata composed of a central core of epithelioid histiocytes, and multi-nucleated giant cells, are typically surrounded

by a rim of T-, and to a lesser extent, B-lymphocytes. In PA-induced granulomatous inflammation in the murine lung, looser collections of epithelioid histiocytes, T-lymphocytes, and B-lymphocytes was observed. There are several potential reasons for these histopathologic differences. First, it has not been unequivocally established that PA is etiologically linked to sarcoidosis. Thus, a triggering antigen other than PA may produce a different pathologic response. Second, if PA is a triggering antigen of sarcoidosis, mechanisms of sensitization are undoubtedly different than the single sensitization and single challenge with heat-killed bacteria performed in our studies. In addition, PA used in these studies may vary phenotypically from PA present in sarcoid granulomas. Finally, species differences between mice and humans may explain the pathologic characteristics of the inflammatory response. Indeed, histologic differences between the most commonly used murine models of asthma (ovalbumin) and pulmonary fibrosis (bleomycin) are well recognized. Nevertheless, these disease models have still contributed substantially to our understanding of the pathogenesis of these two pulmonary disorders.

In contrast to our findings in C57BL/6 mice, BALB/c mice sensitized and challenged with PA in an identical fashion failed to develop granulomata in the lung by 1 wk after exposure. Peribronchovascular chronic inflammation was observed in this strain, but no histiocytic component was noted in these infiltrates. A significantly less robust rise in granulomatogenic Th1 cytokines was observed, which may be responsible for the different pathologic findings. It is well recognized that Th2 cytokines dampen Th1 immune responses. Although IL-13 levels did not rise significantly after PA challenge in BALB/c mice, their increased levels relative to the C57BL/6 strain (~ 2-fold higher) could explain, in part, their attenuated Th1 cytokine response and granuloma formation. These strain differences with PA are similar to what has been reported in mice exposed to BCG. BCG-sensitized C57BL/6 mice produce 5- to 10-fold more IFN- γ and more TNF from spleen cells *in vitro* than BALB/c mice after challenge with BCG extract (33). Our findings in response to PA coupled with the well-recognized resistance of C57BL/6 and susceptibility of BALB/c mice to infection with *Mycobacteria* spp. is consistent with a model in which granuloma formation, independent of the stimulus, is significantly influenced by genetic background of the host (34). For example, MHC presentation of bacterial peptides may vary between strains, as C57BL/6 mice are of the H-2^b haplotype while BALB/c mice are H-2^d. Indeed, combined effects of both background (non-H-2) and H-2 genes have been shown to contribute to variations in the immune response to mycobacteria between these two inbred strains of mice (35). Should propionibacteria be further implicated in human sarcoidosis, then further investigation of inbred, recombinant inbred, and H-2 congenic strains of mice in this model could be useful for identifying host genes important to disease susceptibility, progression, and resolution.

Despite their tendency to incompletely recapitulate the precise histopathology of human diseases, murine models have significantly advanced our understanding of pathogenesis of many disorders, including those afflicting the lungs. The availability of numerous inbred strains of mice and the capacity to readily generate targeted mutations in the mouse genome has made murine models a powerful tool for dissecting pathophysiologic pathways, identifying genetic determinants influencing disease expression, and testing the potential of novel therapies. In this report we have shown that PA produces granulomatous inflammation in the murine lung, and that the immunologic profile produced by this exposure shares many features observed in humans with sarcoidosis. If data continue to implicate PA as a putative etiologic agent of this disease, mouse models of

PA-induced pulmonary inflammation may be useful for investigating the mechanisms by which this commensal bacterium may trigger both self-limited and chronic immune responses in the lung. Both bacterial and host factors could be tested in such a model, potentially identifying new paradigms for treating this enigmatic disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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