Indigenous Pulmonary *Propionibacterium acnes* Primes the Host in the Development of Sarcoid-Like Pulmonary Granulomatosis in Mice

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Although many cases of sarcoidosis are self-limiting with spontaneous remission, uncontrolled pulmonary granulomatosis with fibrosis produces intolerable long-term respiratory symptoms in a minority of patients. Individuals with chronic pulmonary sarcoidosis require an alternative therapy to corticosteroidal treatment because of its insufficient effectiveness. Although many researchers have considered infection as the triggering factor for this disease, the mechanisms by which the candidate causative organisms induce this disorder remain unclear. We report here that extrapulmonary sensitization to *Propionibacterium acnes*, which is one of the candidates to date, induced pulmonary Th-1 granulomas mainly in the subpleural and peribronchovascular regions often observed in sarcoidosis. These granulomas appear to be caused by indigenous *P. acnes* pre-existing in the lower respiratory tract of the normal lung, which is believed to be germ-free, and by an influx of *P. acnes*-sensitized CD4⁺ T cells from the circulation. Importantly, the eradication of indigenous *P. acnes* with antibiotics alleviated the granulomatous lung disease. This is the first report to present clear evidence of the contribution of an indigenous pulmonary bacterium to the formation of granulomatous lesions in the lung. We propose that treatment targeting indigenous *P. acnes* in the lung may be a possible remedy for pulmonary sarcoidosis. (Am J Pathol 2004; 165:631–639)

Sarcoidosis is one of the best-known systemic granulomatous diseases; however, despite a number of intensive investigations, its etiology has remained unresolved for more than 100 years.¹ The lung is the organ that is most

commonly affected by this disorder. Although uncontrolled pulmonary granulomatosis only occurs in a minority of patients, these individuals are at risk of irreversible lung fibrosis and have a poor prognosis owing to interference with gaseous exchange. As the lung is constantly confronted with airborne substances, including pathogens, many researchers have considered infection as the trigger, and have tried to identify possible causative transmissible agents and their contribution to the mechanism of formation of pulmonary sarcoid granuloma.^{2,3}

Because of their clinical and immunopathological similarities, it has been suggested that the most common mycobacterial infection, tuberculosis, might be related to sarcoidosis. However, despite the use of bacterial culture systems, and histochemical and polymerase chain reaction (PCR)-based methods, an association between *Mycobacterium tuberculosis* and sarcoidosis remains controversial.^{4–6} *Propionibacterium acnes*, which is an anaerobic nonspore-forming gram-positive rod bacterium that exists indigenously on the skin or mucosal surfaces,⁷ has been reported as one of the suggested causative antigens of sarcoidosis.⁸ Some studies using quantitative PCR have revealed markedly higher levels of *P. acnes* genomes in the mediastinal or superficial lymph nodes (LNs) of sarcoid patients than in those of controls, suggesting that there is an intrinsic infection because of *P. acnes* in patients with sarcoidosis.^{9–11}

The triggering process in pulmonary granuloma formation is thought to consist of airborne or blood-borne antigens anchoring in the lung, and antigen-presenting cells (APCs), such as macrophages or dendritic cells,¹² accumulating and surrounding them for phagocytosis and subsequent antigen presentation.¹³ Based on this view, a number of animal models of pulmonary granuloma have been proposed, notably murine schistosomiasis, with antigen embolization to hold antigens in the lung.^{14,15} However, long-term antigen deposition on the pulmonary interstitium is not suitable for clinical studies, and it is unlikely that disseminated blood-borne antigens are responsible for all cases of pulmonary granuloma.

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We observed that an immune response against indigenous *P. acnes* already exists in the normal lung, which is believed to be germ-free. We showed that expansion of the numbers of recirculating *P. acnes*-primed cells, produced by extrapulmonary sensitization, can specifically cause granulomatous changes of the lung sharing several similarities with pulmonary sarcoidosis, even in the absence of antigen anchoring. Furthermore, antibiotic treatment to eliminate pre-existing *P. acnes* substantially reduced pulmonary dysfunction. Based on these observations, we propose a novel view of the pathogenesis of unresolved pulmonary granulomatosis such as sarcoidosis, as well as a potentially effective treatment for this condition by means of common antibiotics.

Materials and Methods

Mice

Female 5- to 7-week-old C57BL/6J mice were obtained from CLEA Japan (Shizuoka, Japan) or Japan SLC (Tokyo, Japan), and kept under specific pathogen-free conditions in the animal facility of the Department of Molecular Preventive Medicine, University of Tokyo School of Medicine, Tokyo, Japan. All animal experiments complied with the guidelines of the University of Tokyo.

Immunostaining

The following anti-mouse monoclonal antibodies were used: CD4 (clone; RM4-5), biotinylated interferon (IFN)- γ (XM1.2), and biotinylated interleukin-4 (BVD6-24G2), all from BD PharMingen (San Diego, CA); biotinylated F4/80 (Cl:A3-1) and CD11c (N418), both from Serotec (Oxford, UK); DEC-205 (NLDC-145; BMA Biomedicals, Augst, Switzerland); and mouse monoclonal antibody to *P. acnes* recognizing lipoteichoic acid of the plasmalemma.¹⁶ The secondary antibodies were as follows: an alkaline phosphatase-labeled anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA) or hamster (Cedarlane, Ontario, Canada) IgG (IgG), or avidin (Nichirei, Tokyo, Japan), and a horseradish peroxidase-labeled anti-rat (BioSource, Camarillo, CA) or mouse (DAKO, Carpinteria, CA) Ig.

Single and double immunostaining were performed by the indirect immunalkaline phosphatase and immunoperoxidase methods.¹⁷ For double-immunofluorescence staining, acetone-fixed 6- μ m fresh-frozen tissue sections were incubated with anti-CD4 and then with Alexa Fluor 488 anti-rat Ig (Molecular Probes, Eugene, OR). They were next incubated with biotinylated IFN- γ or biotinylated interleukin-4 followed by Alexa-594-conjugated avidin (Molecular Probes), and were observed using fluorescence microscopy.¹⁸

Reverse Transcriptase (RT)-PCR

Samples of 1 μ g of total RNA were isolated from the lungs and secondary LN specimens of the lung, liver, skin, and pancreas of specific pathogen-free mice using Trizol (In-

vitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. The RNA samples were then reverse-transcribed into complementary DNA (cDNA)^{16,19} and amplified. PCR products of the 16s ribosomal RNA of *P. acnes* were electrophoresed on 2.5% agarose gels. Bands visualized by ethidium bromide staining were of the expected size for each mRNA product. The oligonucleotide primers for *P. acnes*, *M. tuberculosis*, and *Propionibacterium granulosum* were designed as described previously:¹⁰ *P. acnes*, forward 5'-GCGTGAGTGACGGTAATGGGTA-3' and reverse 5'-TTCCGACGCGATCAACCA-3'; *M. tuberculosis*, forward 5'-TCCTATGACAATGCACTAGCCG-3' and reverse 5'-GCCAACTCGACATCCTCGAT-3'; and *P. granulosum*, forward 5'-ACATGGATCCGGGAGCTTC-3' and reverse 5'-ACCCAACATCTCACGACACG-3'. Contamination by *P. acnes* during the course of the experiment was checked with buffer controls (data not shown). The primers for GAPDH as an internal control were described previously.¹⁹ The PCR conditions were denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 90 seconds, with a final step of 72°C for 10 minutes.

Antigen-Specific Proliferation Assay

In vitro cell-proliferation assays were performed as described previously.²⁰ Briefly, peribronchial, inguinal, hepatic, and pancreatic LN cells (10^5 cells/190 μ l/well) from normal mice were stimulated with antigens (*P. acnes* and OVA; 10 μ g/10 μ l of culture medium) at 37°C for 72 hours. After incubation, cell numbers were measured using the Premix WST-1 cell-proliferation assay system (Takara Bio, Shiga, Japan), according to the manufacturer's instructions.

Adoptive Transfer of *P. acnes*-Primed Helper T Cells

P. acnes-sensitized CD4⁺ T cells were obtained from the inguinal LN of the mice immunized three times. Immunization was performed by subcutaneous injection of 400 μ g of heat-killed *P. acnes* (ATCC 11828; American Type Culture Collection, Manassas, VA) with complete Freund's adjuvant (CFA) (Difco, Detroit, MI) or CFA alone into the footpad at 2-week intervals. CD4⁺ cells were isolated using the MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The purity of the CD4⁺ cell populations was 94% or more, as confirmed by immunofluorescence flow cytometry. Isolated CD4⁺ cells [2×10^6 cells/200 μ l of phosphate-buffered saline (PBS)] were injected into the tail vein of normal mice, and 2 weeks later the lungs were examined histologically.

Induction of Chronic Pulmonary Granulomatosis

Lung granulomatosis was induced by multiple immunizations, which were performed by subcutaneous injection

of 400 μg of heat-killed *P. acnes* with CFA into the footpad at 2-week intervals.

Flow-Cytometric Analysis of Cells

Bronchoalveolar lavage (BAL) cells were collected by five injections of 0.8 ml of sterile PBS containing 2% fetal calf serum (Sigma, St. Louis, MO) and 2 mmol/L ethylenediaminetetraacetic acid. The total number of BAL cells was counted with a hemocytometer. BAL cells were analyzed using an EPICS Elite instrument (Beckman Coulter, Miami, FL) and preincubated with rat anti-mouse CD16/CD32 (clone 2.4G2) monoclonal antibody to block FcR-mediated binding, followed by incubation with fluorescein isothiocyanate-conjugated anti-CD4 (H129.19) and phycoerythrin-conjugated anti-CD8 α (53-6.7) monoclonal antibodies (both from BD PharMingen) for 25 minutes at 4°C.

Serological Analysis

Serum calcium levels were determined using a Fuji DRI-CHEM 5500V (Fuji Medical System, Tokyo, Japan), and angiotensin-converting enzyme activities were measured by angiotensin-converting enzyme color (Fuji REBIO, Tokyo, Japan) according to the manufacturer's instructions.

Antigen-Preloading Experiment

Doses of 10 μg , 1 μg , and 0.1 μg of *P. acnes* and PBS were intratracheally administered to mice 1 week before the first immunization of the lung granulomatosis model; 1 μg of *P. acnes* contained 2.5×10^5 organisms. In the control experiment, *Lactobacillus gasseri* (ATCC 33323) was used as the antigen in place of *P. acnes*.

Antibiotic Treatment

Minocycline hydrochloride (MINO) (Wyeth Lederle, Tokyo, Japan), clindamycin (CLDM) (Pharmacia, Tokyo, Japan), and gentamicin sulfate (GM) (Schering-Plough, Osaka, Japan) were used. Briefly, 133 μg of MINO, 1.6 mg of CLDM, and 53 μg of GM were intratracheally administered on day 1. Thereafter, the same dose of each antibiotic was injected intraperitoneally every day for 1 week before immunization, followed by intraperitoneal injections three times per week. Twenty μg of prednisolone sodium succinate (Shionogi, Osaka, Japan) was intraperitoneally injected three times per week as a positive control. Short-term treatment with MINO and CLDM were started at the day of the third immunization. Throughout the experiment, the mice were given water containing each antibiotic at the indicated dose.

Delayed-Type Hypersensitivity Reaction Test

Mice were immunized with 50 μg of *M. tuberculosis* (Difco) included in CFA via their footpads. Two weeks after immunization, 10 μg of purified protein derivative

from *M. tuberculosis* (BCG, Tokyo, Japan) was injected into the ear of each mouse, along with injections of PBS into the opposite ear. Measurements of ear swelling were performed 48 hours after the purified protein derivative challenge.

Statistical Analysis

Differences were evaluated using two-factor factorial analysis of variance and Fisher's protected least significant difference. *P* values <0.05 were considered to be statistically significant.

Results

Presence of *P. acnes* in the Alveolar Space of the Healthy Murine Lung

If there is a pre-existing immune response to *P. acnes*, it should be possible to detect this bacterium in the healthy lung. We therefore performed an immunohistochemical test for *P. acnes* on fresh frozen lung sections from C57BL/6 mice. We observed positive staining in groups of two to five round granules. All of these had been phagocytosed by lung cells, most of which lay adjacent to the alveolar space (Figure 1, A and B). Double immunostaining revealed that the *P. acnes*-positive cells expressed F4/80 (Figure 1C), which is a known macrophage marker, but not the dendritic cell markers CD11c (Figure 1D) and DEC205 (Figure 1E).^{21,22} In addition, RT-PCR analyses of normal lungs with removal of the trachea and the main-stem bronchus, showed a graded distribution of *P. acnes* genomes (Figure 1F). By contrast, the genomes of *M. tuberculosis* and *P. granulosum*, which are mucosal commensal bacteria similar to *P. acnes*, were not detected in the normal lungs (Figure 1G).

Steady-State *P. acnes*-Specific Immune Response of Regional LN Lymphocytes

Peripheral APCs transport antigens to draining LNs for presentation,^{22,23} even in the steady state. Our results in Figure 1 suggest that *P. acnes* exists indigenously on the normal alveolar surfaces as well as the skin and mucosal surfaces of the oral cavity and intestine.⁷ To test for a *P. acnes*-specific immune response in normal pulmonary LNs, we first established the presence of *P. acnes*-genomes in normal pulmonary LNs as well as other LNs by RT-PCR (Figure 2A). This result indicated the steady-state transport of indigenous *P. acnes* from the periphery to the regional LNs of the lung. Therefore, we subsequently tested whether a specific immune response to *P. acnes* had been established in these LNs. As expected, we found that normal peribronchial LN lymphocytes proliferated specifically in response to *P. acnes*, as did cells in the inguinal, hepatic, and pancreatic LNs (Figure 2B).

Figure 1. Presence of *P. acnes* in the healthy murine lung. **A** and **B**: Immunostaining of *P. acnes* (brown) in the alveolar spaces of the normal murine lung. **B** is a higher magnification view of *P. acnes*-bearing cells. **C–E**: Double staining of *P. acnes* (brown) and F4/80 (**C**), CD11c (**D**), and DEC205 (**E**) (both blue). Only F4/80-expressing cells phagocytosed *P. acnes*. **F** and **G**: Detection of the 16S rRNA of *P. acnes* in the lower respiratory lung of naïve mice. We used total RNA extracted from live *P. acnes* as a positive control. Normal peripheral blood mononuclear cells (PBMs) were used to exclude technical contamination. **G** shows the absence of *M. tuberculosis* and *P. granulosa* in the normal specific pathogen-free lungs. Data shown are from one representative of more than three independent experiments. $n = 5$. Mice are numbered 1 to 5. Scale bars: 5 μm (**A**); 20 μm (**B**, **C**, **E**).

Pulmonary and Hepatic Granulomas Are Induced by Adoptive Transfer of *P. acnes*-Primed T Cells in Naïve Mice

Interactions between antigen-bearing APC and Th cells are essential in the development of granuloma, which is enhanced by an influx of circulating antigen-primed T cells.^{13,24} We next determined whether the introduction of circulating *P. acnes*-primed T cells resulted in granuloma formation in the normal lung. We obtained *P. acnes*-sensitized CD4⁺ T cells from the draining LN of a footpad that had been repeatedly immunized with *P. acnes* using CFA, and injected them into the tail veins of normal mice. Two weeks after the transfer of 2×10^6 T cells, we observed granulomatous changes, in the form of aggregations of epithelioid and mononuclear cells, in the lung and the liver (Figure 3A), whereas the adoptive transfer of CFA-primed T cells produced no such effects (Figure 3B). Furthermore, the mice treated with *P. acnes*-primed T cells showed increased numbers of total cells and CD4⁺ T cell counts of both BALs and peribronchial LNs compared with the control group, which supported the histological findings (Figure 3, C and D).

Figure 2. Immune response to *P. acnes* in normal peripheral LNs. **A**: Detection of the 16S rRNA of *P. acnes* in normal peripheral LNs. We used total RNA extracted from live *P. acnes* as a positive control. Data shown are from one representative of more than three independent experiments. **B**: Lymphocyte proliferation assay in response to *P. acnes* and control antigen. **White bar**, nonstimulated; **black bar**, *P. acnes*-stimulated; **striped bar**, OVA-stimulated. Data shown are from one representative of more than three independent experiments. $n = 7$. Data are means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$, versus both nonstimulated and OVA-stimulated groups.

Repeated *P. acnes*-Immunization Induces Pulmonary Granulomatosis

As a practical application of this transfer model, and to stimulate chronic pulmonary granulomatosis, we induced the continuous extrapulmonary expansion of *P. acnes*-primed T cells by repeated immunization via the footpad using CFA to expand the antigen-specific T cells efficiently. Distinct granulomas formed predominantly in the subpleural and peribronchovascular areas of the lung in mice that were treated in this way (Figure 4A). Immunohistochemical analyses showed that the typical granulomas consisted of central APCs, with CD4⁺ T cells on the periphery^{12,13} (Figure 4, B and C). Furthermore, these CD4⁺ cells expressed IFN- γ but not interleukin-4, indicating that the granulomas were of the Th1-type (Figure 4D).

We also estimated the degree of the granulomatous lesions by counting the BAL cells. The increase in the numbers of total cells and lymphocytes increased with the frequency of administration (Figure 5, A and B). These results were also consistent with the histological findings (data not shown), even though the proportion of CD4/8 cells was greatest in the group injected twice, the lungs of which showed signs of the beginning of granuloma formation (Figure 5B). In addition, these increases in the numbers of BAL cells were much higher than those observed in the group that received CFA alone (Figure 5C).

Because the results obtained in this experimental model matched the characteristics of patients with pul-

Figure 3. Transfer of *P. acnes*-primed T cells. **A** and **B**: H&E staining shows pulmonary (**left** and **center**) and hepatic (**right**) granulomas in mice injected with *P. acnes*-primed CD4⁺ T cells on day 14. **B** shows portions corresponding to **A** in the lung (**left**) and the liver (**right**) of mice injected with CD4⁺ T cells primed with CFA alone. **C** and **D**: BAL numbers and components in the recipient mice from **A** and **B**. The mice transferred with *P. acnes*-primed CD4⁺ T cells show increased numbers of total cells (**left**) and the CD4⁺ T cell (**right**) counts of BAL (**C**) and peribronchial LN (**D**) compared to those with only CFA-primed T cells. *n* = 3. Data are means ± SEM. TCC, total cell count. Scale bars, 100 μm.

monary sarcoidosis, we also investigated their serological similarity by evaluating serum calcium levels and angiotensin-converting enzyme activities, even though they are not specific for sarcoidosis but are indicators of granulomatous response.^{1,8,25} Serum calcium increased only in the group that was injected twice, whereas angiotensin-converting enzyme activity was higher in the group that received more than two immunizations (data not shown). Immunohistochemical analysis of the liver and spleen, both of which are frequently affected in sarcoidosis, revealed numerous granulomas in the liver (Figure 6A) and abnormal accumulations of CD4⁺ T cells in the red pulp of the spleen (Figure 6B) in the mice that were frequently immunized.

Increased Numbers of Pre-Existing *P. acnes* Enhance Pulmonary Granuloma Formation

If the presence of indigenous *P. acnes* in the healthy lung leads to pulmonary granuloma, their numbers should influence the extent of the lesions. To test this hypothesis, we preloaded live *P. acnes* into healthy murine lungs before immunization. To exclude the possibility that the intratracheal injection alone induced granulomas, we as-

Figure 4. Pulmonary granuloma formation in mice that received repeated *P. acnes* immunization. **A**: H&E staining showed a large number of pulmonary granulomas, mainly in the peripheral (**left**) and peribronchovascular (**center**) areas of the lungs of mice immunized three times. **Right**: A higher magnification view of a typical granuloma. **B**, bronchus; **L**, lymphatics; **V**, pulmonary vessels. **B** and **C**: Cellular composition of the pulmonary granulomas. CD4⁺ T cells (brown) at the periphery of the granuloma, and F4/80⁺ (**B**) and CD11c⁺ (**C**) cells (both blue) in the center of the granuloma. **D**: Th1/2 cytokine expression in the pulmonary granuloma. Granuloma CD4⁺ T cells (green) expressed IFN-γ, but not interleukin-4 (both red). CD4⁺ IFN-γ⁺ cells (yellow) were peripheral to the layer of CD4⁺ T cells. Scale bars, 100 μm (**A**, **left** and **center**; **B**, **C**) and 50 μm (**A**, **right**).

certained that there were no granulomas in the control lungs from nonimmunized mice at either the initial stage or the end-point of the experiment (data not shown). Total counts of cells in the BAL, which were collected after three immunizations, depended on the dose of preloaded *P. acnes* (Figure 7A); the result of the histological examination of the lesions was consistent with this observation (Figure 7B). By contrast, there were no increases in the numbers of BAL cells in the control experiments using *L. gasseri* in place of *P. acnes* (Figure 7C).

Antibiotic Treatment Alleviates the Pulmonary Disorders Induced by Extrapulmonary *P. acnes* Immunization

To evaluate further the importance of pre-existing *P. acnes*, we reduced their numbers before immunization with the antimicrobial reagents MINO and CLDM, both of which are known to be effective drugs for acne vulgaris induced by *P. acnes*.^{26,27} GM, which is inhibitory to *M. tuberculosis* but not to *P. acnes*, was used as negative control.⁷ Two weeks after the third immunization, the

Figure 5. Cellular components of BAL in murine pulmonary granulomatosis. **A** and **B:** BAL numbers and components as a function of immunization frequency. Total BAL cells (**A**) and lymphocytes (**B**) increased with the frequency of immunization, whereas the CD4/8 ratio (**B**) was maximal in the group immunized twice. **C:** The numbers of total cells (**C, left**) and CD4⁺ T cell (**C, right**) counts of BAL in the mice that received two immunizations with or without *P. acnes*. *n* = 5. Data are means ± SEM (except in CD 4/8 ratio). Data shown are from one representative of more than three independent experiments.

MINO- and CLDM-treated mice, and even the short-term CLDM-treated mice, showed a marked reduction in total BAL cell counts (Figure 8A): the CD4⁺ BAL cell counts in these three groups were reduced by 53.5%, 42.1%, and 74.2%, respectively (Figure 8B). This effect was not observed in the mice treated with GM (Figure 8, A and B). Moreover, the doses of *P. acnes* genomes were altered in response to these antimicrobial effects (Figure 8C). Histological examinations also showed consistent improvement of granulomatous lesions in the mice treated with effective antibiotics (Figure 8E). Although immunomodulatory properties of antibiotics, especially MINO, have been described previously,²⁸ the tests for delayed-type hypersensitivity showed no nonspecific immune effects, at least in our experimental model using C57BL/6 mice (Figure 8D).

Discussion

Although organisms are continuously exposed to foreign antigens, it has long been believed that the lower respiratory tract of the lung is an inviolable germ-free space, and that penetration of pathogens into the lung would cause pulmonary disorders. Based on this supposition, the animal models of pulmonary diseases have been based on the forced administration of antigens via the trachea, nasal cavity, or antigen-embolized pulmonary vessels.^{14,15,20,29} However, clinicians are often confronted with cryptogenic pulmonary disorders, primarily

Figure 6. Extrapulmonary lesions observed in murine pulmonary granulomatosis. **A** and **B:** A high frequency of immunization induced a large number of hepatic granulomas (**A**) and aberrant accumulation of CD4⁺ T cells (**arrows**) in the red pulp of the spleen (**B**). The specimens were obtained from mice immunized nine times. RP, red pulp; WP, white pulp. Scale bars, 100 μ m (**A**); 50 μ m (**B**).

interstitial lung disorders, such as pulmonary sarcoidosis, without any evident exposure to pathogens. We therefore speculated that there might be an indigenous organism in the healthy lung that can be pathogenic under certain conditions. Because of its distribution on the skin and mucosal surfaces of healthy individuals, and its remarkable influence on the pathogenesis of acne vulgaris²⁷ and on granuloma formation in experimental models,^{16,24} *P. acnes* seemed one of the candidate organisms. Indeed, previous reports have suggested the correlation between *P. acnes* and sarcoidosis.^{9–11}

In the present study, we identified *P. acnes* in normal murine alveolar cells by immunostaining (Figure 1, A and B). All of these *P. acnes* were taken up by lung cells, and the *P. acnes*-bearing cells expressed F4/80 rather than CD11c or DEC205 (Figure 1; C, D, and E), consistent with the known ability of macrophages to phagocytose and deliver antigens to dendritic cells in the lung.^{30,31} As far as the end of the airway, airborne organisms are impacted and eliminated by mechanical defenses including mucociliary clearance and coughing.³² Nevertheless, a small number of *P. acnes* might escape from this system and reside on the alveolar surface. The existence of *P. acnes*-bearing APCs in the healthy lung prompted us to examine whether there is an immune response against *P. acnes* in the pulmonary LNs of normal mice. Indeed, *P. acnes* genomes were detected in normal pulmonary LNs as well as the lungs (Figures 1F and 2A), and lymphocytes from LNs showed *P. acnes*-specific proliferation (Figure 2B), suggesting that these cells had already been exposed to *P. acnes* by lung-derived APCs and had established a memory response. Additionally, these re-

Figure 7. Effect of increasing *P. acnes*-colonization on granuloma formation. **A:** The total BAL cell counts in mice immunized three times. $n = 5$. Data are means $\pm$ SEM. Data shown are from one representative of more than three independent experiments. **B:** Histological findings by H&E staining. **C:** The total BAL cell counts in mice immunized three times with control bacteria, *L. gasseri*. $n = 5$. Data are means $\pm$ SEM. Scale bar, 100 μ m.

sults indicate that *P. acnes* were continuously transported to pulmonary regional LN in the steady state. Because of this constant delivery of antigens to the pulmonary LNs for a long period, the small number of indigenous *P. acnes* in the normal lung would be enough for a specific immune response, but not for the formation of the steady-state granuloma. Although mycobacterial, atypical mycobacterial, and other propionibacterial antigens were potential candidates as endogenous microorganisms triggering pulmonary granuloma formation, genomic analyses revealed an absence of these organisms in the lungs of specific pathogen-free C57BL/6 mice (Figure 1G and data not shown).

Because of this steady-state memory response against *P. acnes* in the lung, we subsequently hypothesized that an influx of *P. acnes*-sensitized T lymphocytes could cause lung inflammation even without artificial antigen-anchoring. The adoptive transfer of *P. acnes*-sensitized LN CD4⁺ T cells into naïve mice resulted in granulomatous changes in the lung (Figure 3A). This showed that extrapulmonary LN CD4⁺ T cells primed with *P. acnes* could interact with pulmonary resident cells via circulation and induce granuloma formation in the normal lung. We therefore hypothesized that a continuous supply of *P. acnes*-sensitized T cells should lead to chronic pulmonary granuloma formation, and consequently performed continuous remote sensitization of normal mice with *P.*

Figure 8. Reduction of pre-existing *P. acnes* with antibiotics. **A** and **B:** Total BAL cells (**A**) and CD4⁺ T cells (**B**) in mice immunized three times. "MINO short" and "CLDM short" refer to short-term treatment with these antibiotics soon after the third immunization. $n = 4$ to 6. Representative data shown are means $\pm$ SEM. Statistically significant reductions were observed in the numbers of BAL CD4⁺ T cells of MINO- and CLDM-treated group. **C:** Reduction of lung *P. acnes* genomes in the mice treated with susceptible antibiotics. Data shown are from one representative of more than three independent experiments. $n = 5$. **D:** Influence of the treatment with antibiotics on delayed-type hypersensitivity responses (**top**) and spleen indexes (**bottom**). The spleen index was calculated as $\sqrt{\text{spleen weight} \times 100/\text{whole body weight}}$. $n = 5$. Data are means $\pm$ SEM. Data shown are from one representative of more than three independent experiments. **E:** Histological findings by H&E staining. Scale bars, 100 μ m.

acnes. These mice exhibited distinct pulmonary granulomas, distributed in lymph-rich spaces³³ such as the subpleural, peribronchial, and perivascular areas (Figure 4A), and showed the typical cellular components of granuloma¹³ (Figure 4, B and C) and preferential Th1 cytokine expression (Figure 4D). These features are similar to those of pulmonary sarcoidosis.^{1,3,25} In addition, an elevated ratio of CD4 to CD8 BAL lymphocytes (Figure 5B) was observed in the group that were immunized twice, which was accompanied by increased serum calcium levels (data not shown). These observations are consistent with those of previous studies demonstrating an influence of activated macrophages on calcium metabolism³⁴ and a positive correlation between serum calcium levels and the BAL CD4/CD8 ratios in sarcoid patients.³⁵ Moreover, we found extrapulmonary lesions in the liver and spleen, both of which are frequently affected in sarcoidosis (Figure 6, A and B).^{1,8,25} Thus, this repeated *P. acnes*-immunization model, without any direct exposure of antigen to the lung, showed several similarities to the characteristics of sarcoid patients.

Because an influx of *P. acnes*-sensitized T cells into the lung can trigger granuloma formation (Figure 3A), we considered that interactions between indigenous *P. acnes*-loaded lung APCs and LN T cells would be essential for the development of granulomas. To confirm this, we examined whether changes in the number of pre-existing *P. acnes* cells in the lung had an effect on pulmonary granuloma formation. As expected, preloading of *P. acnes* exacerbated the pulmonary disorders (Figure 7, A and B), whereas reduction of the *P. acnes* population by antimicrobial treatment reduced the pulmonary lesions (Figure 8). These results suggest not only a pivotal role of normally localized *P. acnes* in the formation of pulmonary granuloma by extrapulmonary *P. acnes* sensitization, but also the potential clinical usefulness of antimicrobial eradication targeting lung-indigenous *P. acnes* for the treatment of pulmonary granulomatosis induced by similar pathogenesis.

The etiology of sarcoidosis remains to be resolved. Immunosuppressive, mainly corticosteroidal, therapy has been used for more than 50 years for this condition, but the long-term effects of steroidal treatment in chronic pulmonary sarcoidosis are still disputed,⁸ and the high relapse rate after treatment and the side effects of long-term use are often a clinical challenge.³⁶ However, the successful clinical report of cutaneous sarcoidosis treated with minocycline,³⁷ for example, has highlighted the usefulness of such an alternative therapy.

In this study, we have produced a novel murine pulmonary granuloma model with several features similar to those of pulmonary sarcoidosis. Sarcoidosis is a well-described clinical condition, and clearly the whole pathogenesis cannot be explained by this experimental model alone. However, if, as we have shown in mice, *P. acnes* also exists in the healthy human lung, people with a unique genetic background, as is reported in sarcoid patients,^{1,8,25} might readily form pulmonary lesions after excessive sensitization with *P. acnes* even at extrapulmonary sites, such as loci of acne vulgaris. We suggest that this new view of pulmonary sarcoidosis deserves further investigation and might provide the basis for novel therapeutic strategies.

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