Blockade of Th1 chemokine receptors ameliorates pulmonary granulomatosis in mice

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Running head: Targeted therapy of pulmonary granuloma

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Abstract

Sarcoidosis is a granulomatous disease of unknown cause. We identified immunological targets for the treatment of pulmonary granulomatosis using a murine model generated with Propionibacterium acnes (P. acnes).

Sensitization and challenge using heat-killed P. acnes and dendritic cells (DCs) were performed to produce pulmonary granulomatosis in C57BL/6 mice. Immunological analyses using the ELISA as well as a cDNA microarray were used to search for cytokines or chemokines associated with the formation of granuloma in the lungs.

Co-administration of P. acnes and DCs reproducibly induced the formation of pulmonary granulomas, which resembled sarcoid granulomas. The cDNA microarray assay demonstrated that the gene expression of CXCL9 and CXCL10, ligands for CXCR3, and of CCL4, a ligand for CCR5, was strongly up-regulated during the
granulomatosis. ELISA confirmed that levels of CXCL9 and CXCL10 as well as Th1 cytokines and chemokines including TNF-α and IFN-γ were elevated in BALF. The blockade of Th1 chemokine receptors using TAK-779, a dual blocker for CXCR3 and CCR5, demonstrated reduced numbers of CXCR3⁺CD4⁺ and CCR5⁺CD4⁺ T cells in BALF. Furthermore, the administration of TAK-779 ameliorated the granulomatosis.

The targeted inhibition of Th1 chemokines might be useful for inhibiting Th1-biased granulomatous diseases including sarcoidosis.

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Key words: pulmonary granulomatosis, Propionibacterium acnes, Th1 chemokines, CXCR3, CCR5
Text

Introduction

Sarcoidosis is a systemic granulomatous disease of unknown cause that mainly affects the lungs and lymphatic system.[1-4] Although spontaneous remission occurs in nearly two-thirds of cases, chronic and progressive courses are observed in 10 to 30% of patients.[1-4] Corticosteroids are used for the treatment of sarcoidosis, but their effects are controversial, particularly in cases complicated with lung diseases.[1-4] In addition, long-term therapy with corticosteroids can result in serious adverse events.[4] Therefore, a new approach to the treatment of sarcoidosis associated with intensive organ involvement is needed.

The sarcoid granuloma is characterized by noncaseating epitheloid cells with the dominant accumulation of CD4+ T cells and macrophages.[1-4] CD4+ T lymphocytes are divided into two subgroups, T helper (Th) 1 and Th2 cells, on the basis of cytokine production.[5] Based on the Th1/Th2 paradigm, sarcoidosis is
considered a typical Th1-dominant disease, since T lymphocytes in bronchoalveolar lavage fluid (BALF) and lymph nodes from patients with sarcoidosis predominantly produce interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α or β.[6-8] We and others also reported that levels of Th1 chemokines including CXCL9/ interferon γ (Mig), CXCL10/ Interferon-inducible protein-10 (IP-10) and CXCL11/ interferon-inducible T-cell α-chemoattractant (I-TAC) were elevated in BALF of patients with sarcoidosis.[9-13] However, it is still not clear whether these Th1-related molecules would be useful as targets in the treatment of pulmonary granulomatosis. The blockade of these molecules would likely benefit patients since in clinical studies with anti-TNF-α antibodies (infliximab and adalimumab), favorable results were obtained in some cases.[14,15]

To identify the novel molecular targets for the treatment of pulmonary granulomatosis, we here performed a global analysis of the mRNA expression of cytokines and chemokines
using a cDNA microarray in a model of pulmonary granulomatosis generated by the systemic administration of Propionibacterium acnes (P. acnes) and dendritic cells in mice, because P. acnes have been reported to be involved in the etiology of sarcoidosis.[16-18] We found that the Th1 chemokines CXCL9, CXCL10 and CCL4 had the highest levels among soluble factors tested during the course of pulmonary granulomatosis. Interestingly, the administration of TAK-779, an antagonist of CXCR3 and CCR5, significantly inhibited the formation of pulmonary granulomatosis by reducing the number of CXCR3⁺CD4⁺ T cells as well as CCR5⁺CD4⁺ T cells accumulated in the lungs. These results suggest the blockade of Th1 chemokines to be useful for treatment of sarcoidosis complicated by pulmonary granulomatosis.

Materials and Methods

(Detailed Methods are described in the online supplement.)

Mice and Materials
Eight-week-old female C57BL/6 mice were purchased from Charles River Japan Inc. (Yokohama, Japan), and maintained in the animal facility of the University of Tokushima.[19] The small nonpeptide compound TAK-779 (N, N-dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2H-pyran-4-aminium chloride) was provided by Takeda Pharmaceutical Company (Osaka, Japan).[20] TAK-779 is a selective antagonist of CXCR3 and CCR5.[21]

**Propionibacterium acnes**

*Propionibacterium acnes* (*P. acnes*) was obtained from ATCC (#6919) and grown in GAM broth (Nissui Pharmaceutical Co., Ltd., Tokyo). The suspension was heated by autoclaving, and was kept at -70°C prior to use.

**Mouse bone marrow (BM)-derived dendritic cells (DCs)**

The BM-DCs were generated using rmGM-CSF (Kirin Brewer Inc., Tokyo, Japan) and rmIL-4 (Pepro Tech, London, UK).[22]
Sensitization and challenge of mice with heat-killed \textit{P. acnes}

Mice were subcutaneously immunized twice with heat-killed \textit{P. acnes} with or without incomplete Freund’s adjuvant (IFA) or DCs. Mice were then challenged intravenously with heat-killed \textit{P. acnes} with or without DCs.

**Bronchoalveolar lavage**

Bronchoalveolar lavage was performed as described previously.[23]

**Measurements of CXCR3 ligands, Th1 and Th2 cytokines**

The levels of CXCL9 and CXCL10 were examined by ELISA (R&D System, Minneapolis, MN).[23] Th1 and Th2 cytokines were measured using BD Cytometric Beads Array System mouse Th1/Th2 cytokine kit (Becton Dickinson Company, Franklin Lakes, NJ).

** Trafficking study of injected DCs**
DCs (10^6 cells) were stained with PKH26 (Sigma, St. Louis, MO, USA), and injected from tail vein into mice. Fluorescence images of sections excited at 568 nm wavelength were captured with a confocal laser scanning microscope (Leica TCS NT; Leica, Heidelberg, Germany) equipped with an Ar-Kr laser and a x10 dry objective (Leica Plan Apochromat).

**Isolation of total RNA**

Total RNA was isolated from the lungs of mice using Isogen (Wako K.K., Kyoto, Japan).[24]

**DNA chips**

A description of mouse cytokines and chemokines chips that contained genes of 29 cytokines, 34 cytokine receptors, 33 chemokines and 21 chemokines receptors is given in the online supplement (see Table E1) (Kakengeneqs Co. Ltd., Chiba, Japan).[25]
cDNA preparation and array hybridization

Total RNA samples (100 μg) were converted to double-strand cDNA by using a custom kit (LavelStar Array kit; Qiagen, Valencia, CA) and labeled with cyanine 3-conjugated dUTP.[25] Reference total RNA was labeled with cyanine 5-conjugated dUTP (PerkinElmer, Boston, MA). Array hybridization was performed according to the manufacturer’s instruction.[25]

Microarray Quantification

The fluorescent images of hybridized microarrays were obtained with an array scanner (model 428; Affymetrix, Santa Clara, CA).[25] Raw fluorescence intensity data were used to calculate signal intensities of the spots (DNASIS Array; Hitachi Software Engineering Co., Ltd., Tokyo, Japan). A twofold change in gene expression was used as the cutoff.

Histopathology

The left lungs were fixed in 10% buffered formalin and embedded
in paraffin. Sections were stained with hematoxylin and eosin.[23]

**Immunohistochemistry**

Six-micron sections of the left lungs were stained using R.T.U. VECTASTAIN Universal Quick Kit (Vector Laboratories).[25]

**Administration of TAK-779**

TAK-779 was dissolved in 5% mannitol solution, and was administered subcutaneously into the mice.

**Flow cytometry and Immunofluorescence staining**

Flow cytometric analysis was performed by FACS Calibur (BD Biosciences, Franklin Lakes, NJ).[19,22] Immunofluorescence staining were visualized using a fluorescence microscope (OLIMPUS BX61; OLYMPUS OPTICAL Co. Ltd., Tokyo, Japan).

**Statistical Analysis**
Comparisons among multiple groups were performed using one-way ANOVA with Newman-Keuls post hoc correction (GraphPad Prism, version 3.0). Differences were considered statistically significant if p values were less than 0.05. All data are presented as the mean±SD.

Results

Administration of heat-inactivated *P. acnes* with dendritic cells enhanced the formation of pulmonary granulomas in mice.

We first examined whether immunization with heat-inactivated *P. acnes* could induce granulomas to form in the lungs in mice. C57BL/6 mice were subcutaneously sensitized twice with *P. acnes* and incomplete Freund’s adjuvant (IFA) at a two-week interval. Then two weeks after the second sensitization, mice were challenged with an intravenous injection of heat-killed *P. acnes*. As reported previously,[26] many granulomatous lesions were developed in the liver, but not in the lungs, after the
immunization with heat-inactivated *P. acnes* and IFA (Figure 1A and 1B). When bone marrow-derived dendritic cells (DCs) were co-administered with *P. acnes* in the immunization as well as challenge process, the number and size of pulmonary granulomas were significantly increased (Table 1, Figures 1C and 1D). These granulomas consisted of lymphocytes, epithelioid cells, and the multinucleated giant cells (Figures 1D, E1 and E2), quite similar to those observed in the lungs of patients with sarcoidosis. The size of granuloma was largest on day 7, gradually reduced until day 14 (Figure E2). The immunohistochemical analysis showed CD3- and CD4-positive lymphocytes in the pulmonary granulomas (Figure 1E and 1F). The trafficking study of labeled DCs showed that the injected DCs distributed into the lungs 6 hours after injection, and the granuloma appeared 24 hours later. In the granulomatous lesion, some labeled DCs were detected until 72 hours later after injection. In the granulomatous lesion, some labeled DCs were detected until 72 hours later after injection. These data
demonstrated that the injected DCs migrated into the lungs via blood circulation and induced immune reaction to generate the granulomatous lesions in situ (Figure E5).

Table 1  Pulmonary granulomatosis in mice immunized with heat-killed *P. acnes* and DCs*

<table>
<thead>
<tr>
<th>Method of immunization</th>
<th>Number</th>
<th>Size (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acnes</em> + IFA</td>
<td>5</td>
<td>2,880±1,731</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2,137±1,132</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,800±1,272</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3.6±1.5</td>
<td>2,413±1,426</td>
</tr>
<tr>
<td><em>P. acnes</em></td>
<td>22</td>
<td>5,195±4,172</td>
</tr>
<tr>
<td>+ immature DCs</td>
<td>9</td>
<td>2,025±1,153</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3,318±2,054</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>13±7.8</td>
<td>4,132±3,933</td>
</tr>
<tr>
<td>Mature DCs</td>
<td>32</td>
<td>7,980±8,941</td>
</tr>
<tr>
<td>pulsed with <em>P. acnes</em></td>
<td>21</td>
<td>7,682±6,999</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>8,207±4,693</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>24.7±6.3†</td>
<td>7,960±7,311†</td>
</tr>
</tbody>
</table>

*Definition of abbreviations: *P. acnes* = *Propionibacterium acnes*; DCs = dendritic cells; IFA = incomplete Freund’s adjuvant.

*Mice were subcutaneously immunized twice with 1mg of heat-killed *P. acnes* at a two-week interval. Two weeks after the second immunization, mice were challenged intravenously with heat-killed *P. acnes* (1 mg). The heat-killed *P. acnes* was administered with or without IFA, immature or mature DCs (1x10⁶ cells). Mature DCs were generated by pulsing with heat-killed *P. acnes* (100 μg/ml) for 24 h before their injection into mice. Seven days after the challenge, mice were sacrificed, and Hematoxilin & eosin staining was performed with sections of the lungs. The granulomatosis was evaluated by measuring the number and size of granulomas in the lungs. Data are presented as the mean ± SD. Similar results were obtained in three
separate experiments.

$^\dagger$P<0.05 vs. \textit{P. acnes} + IFA group

Analysis of bronchoalveolar lavage fluid of mice with pulmonary granulomatosis induced by \textit{P. acnes}. To clarify which type of cell is involved in the granulomatosis caused by \textit{P. acnes}, we analyzed cells in bronchoalveolar lavage fluid (BALF) of mice. As shown in figure 2, neutrophils in BALF increased in number rapidly one day after the challenge with \textit{P. acnes}, but had disappeared by day 14. However, the number of lymphocytes rose gradually and reached a peak 7 days after the challenge, then decreased until day 28. Two peaks in the number of macrophage were found one and 7 days after the \textit{P. acnes} challenge, with a gradual decline from day 7 to day 28. These changes were correlated with the histological findings (data not shown).

Levels of Th1 chemokines were elevated during pulmonary granulomatosis induced by \textit{P. acnes}. Next, we analyzed which
cytokines and chemokines are involved in the pulmonary granulomatosis induced by *P. acnes* using a cDNA Microarray focusing on cytokines and chemokines (Table E1). As shown in figure 3A, the expression of mRNA of the Th1 chemokines CXCL9 and CXCL10 was strongly stimulated. mRNAs of other Th1-related molecules including CCL4, CXCL5, CXCR3 and IFN-γ were also expressed strongly after the *P. acnes* challenge. The mRNA of CCL2 also increased in the granulomatous lungs, consistent with reports describing pulmonary granulomas induced by *P. acnes* in rabbits.[26] The increase in CXCL9 and CXCL10 occurred 3 to 7 days after the challenge with *P. acnes* (figure 3B). On the other hand, levels of the Th2 chemokines CCL17 and CCL22 did not increase during the course of the pulmonary granulomatosis (figure 3C).

To confirm the stimulation of Th1 chemokines and cytokines, we performed an ELISA with BALF. As shown in figure 4, CXCL9, CXCL10 and IFN-γ levels were markedly elevated after the challenge with *P. acnes*. TNF-α also increased in BALF, but the
difference did not reach statistically significant. On the other hand, Th2 cytokines including IL-4 and IL-5 were not detected in the course of pulmonary granulomatosis (data not shown).

**Therapy targeting Th1 chemokines ameliorates the pulmonary granulomatosis caused by *P. acnes***. As we confirmed the elevation in levels of the Th1 chemokines CXCL9, CXCL10 and CCL4 in this model, we hypothesized whether the blockade of Th1 chemokines was effective in inhibiting pulmonary granulomatosis. For this purpose, we used TAK-779, a selective blocker of the Th1 chemokine receptors CXCR3 and CCR5.

Mice were treated every other day with TAK-779 from four days before to six days after the challenge with heat-inactivated *P. acnes* and DCs. TAK-779 significantly reduced the total number of cells as well as numbers of both neutrophils and lymphocytes (figure 5A). The proportion of CXCR3⁺CD4⁺ T cells and CCR5⁺CD4⁺ T cells in BALF determined using flow cytometry was 50–68% and
4-12%, respectively, indicating Th1-skewed immune responses in this model (figure 5B). Treatment with TAK-779 clearly reduced the number of both CXCR3^+CD4^+ and CCR5^+CD4^+ T cells (control vs TAK-779: CXCR3^+CD4^+: 58.54±6.75 vs 38.29±8.24, p=0.016; CCR5^+CD4^+: 7.37±2.52 vs 4.05±1.31, p=0.020). Furthermore, histological examination confirmed major reductions in both the number and size of pulmonary granulomas generated by P. acnes following the administration of TAK-779 (control vs TAK-779: size: 7033.14±1889.6 vs 4233.27±576.57 m^2, p=0.008; number: 25.4±5.89 vs 15.2±4.20, p=0.032) (figure 6).

Discussion

In the present study, we generated the murine model of pulmonary granulomatosis by co-administering P. acnes and DCs. Furthermore, the examination of the expressions of cytokines and chemokines in the lungs demonstrated that levels of Th1-type cytokines and chemokines were strongly elevated in this model.
In addition, the Th1 chemokine receptor blocker TAK-779 inhibited the formation of granulomas in the lungs by reducing the numbers of CXCR3⁺CD4⁺ and CCR5⁺CD4⁺ T cells accumulated in the lungs.

In this study, mice were sensitized twice with systemic administrations of heat-inactivated *P. acnes* and bone marrow-derived DCs to generate pulmonary granulomas. A series of microbiological and molecular investigations have suggested *Propionibacteria spp.* to be etiologically linked to sarcoidosis.[16-18] Using heat-inactivated *P. acnes*, several models of pulmonary granulomatosis have been established.[26, 28] McCaskill et al. showed that the intratracheal administration of heat-killed *P. acnes* induced granulomatosis in the lungs of mice.[28] Since it is likely that the intratracheal administration of heat-killed *P. acnes* induces non-specific inflammation in the lungs, we selected systemic administration as it is more relevant to the condition in humans. We found that the challenge with *P. acnes*-pulsed DCs which have
the phenotype of mature DCs (Figure E3) without heat-inactivated *P. acnes* also could induce many large granulomas in the lungs, indicating that the pulmonary granulomatosis seen in this model was mediated by immune reactions against *P. acnes*. Nishiwaki et al. also reported that repeated immunization with heat-killed *P. acnes* using complete Freund’s adjuvant (CFA) induced pulmonary granulomatosis,[26] although we could not reproducibly generate granulomas using similar methods with IFA. The reason for this discrepancy is not clear, but it is likely due to the difference in the route and dose of immunization with heat-killed *P. acnes* or adjuvant. In particular, CFA may not be suitable for inducing specific immune responses to *P. acnes* since it contains derivatives of *M. tuberculosis*. The granulomas found in previous reports consisted of lymphocytes, macrophages and epithelioid cells without multinucleated cells. However, the granulomas observed in our study contained multinucleated giant cells, being most similar to those in cases of human sarcoidosis. The DCs used
in the immunization might play a critical role because DCs were reported to be key immune cells in initiating granulomatous cell-mediated immunity.[29]

In previous reports, levels of Th1 cytokines including IFN-γ, TNF-α and IL-12 were elevated in models of pulmonary granulomatosis induced by *P. acnes*,[26,29] whereas there is no report analyzing chemokines. Here we showed the significant upregulation of CXCL9 and CXCL10 expressions during pulmonary granulomatosis induced by heat-killed *P. acnes*. Furthermore, these changes were strongest among cytokines and chemokines tested in the cDNA Microarray system. This suggests these chemokines to be ideal molecular targets for regulating Th1-type granulomas in the lungs, because they play critical roles in the trafficking of immune cells, particularly Th1 cells, into the lungs. In our model, CXCR3⁺CD4⁺ T cells probably play more of a role than CCR5⁺CD4⁺T cells since the proportions of these cells were 50-68% and 4-12%, respectively, which is consistent with an analysis in patients with sarcoidosis.[30]
TAK-779 has a unique profile, selectively blocking both CXCR3 and CCR5.[19,20] To test whether the blockade of Th1 chemokines attenuates pulmonary granulomatosis caused by *P. acnes*, we treated the sensitized mice with TAK-779 during the challenge with *P. acnes*. The administration of TAK-779 strongly inhibited pulmonary granulomatosis by preventing the accumulation of CXCR3⁺CD4⁺ and CCR5⁺CD4⁺T cells into the lungs. There are two ways to inhibit the interaction between a ligand and a receptor, neutralize the ligand or block the receptor. With chemokines unlike cytokines, many ligands can bind a single receptor. Consequently, blocking the receptor would be more effective than blocking the ligands. Furthermore, TAK-779 has the advantage of blocking both Th1 chemokine receptors. This characteristic of TAK-779 might lead to the significant reduction in the accumulation of Th1 cells, and inhibit granulomatosis in the lungs. In turn, these observations also indicate that Th1 chemokines are crucial regulators in the formation of pulmonary granulomas by *P. acnes*. Furthermore, we
found the reduced number of neutrophils in BALF of TAK-779-treated mice, which is not surprising because it was reported that the neutrophils can also express the Th1 chemokine receptors CXCR3 and CCR5.\[31,32\] In addition, we could not rule out the possibility that inhibitory effects of TAK-779 was partly mediated by blocking the influx of injected DCs into the lungs since P. acnes-stimulated DCs also express CCR5 and CXCR3 (Figure E4). Unlike human sarcoidosis, the condition in our model is not chronic and improved within 2 weeks, which is consistent with the previous reports, but the limitation of our study.\[26,28\] Recently, Samokhin et al. reported the model of prolonged pulmonary granulomatosis like human sarcoidosis in ApoE-/- mice.\[33\] Further study is required to confirm the efficacy of TAK-779 in chronic models with therapeutic protocols.

In summary, we demonstrated that production of Th1 chemokines is strongly stimulated during pulmonary granulomatosis caused by P. acnes with DCs, and targeted
inhibition of Th1 chemokines with receptor blockers might be useful for inhibiting Th1-biased granulomatous diseases including sarcoidosis.

Acknowledgements

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References


proteins 1, 3, and 4; and eotaxin in \( \text{T}_{\text{H}}1 \)- and \( \text{T}_{\text{H}}2 \)-mediated lung disease. \textit{J Allergy Clin Immunol} 2001;\textbf{107}:664-70.


17) Ishige I, Usui Y, Takemura T, et al. Quantitative PCR of


development of sarcoid-like pulmonary granulomatosis in mice.


Figure legends

Figure 1  Histological findings in the lungs and livers of mice administered with heat-inactivated *P. acnes*. Mice were subcutaneously immunized twice with 1mg of heat-killed *P. acnes* at a two-week interval with or without incomplete Freund’s adjuvant (IFA), and immature or mature DCs (1x10^6 cells). Two weeks after the second immunization, mice were challenged intravenously with heat-killed *P.acnes* (1 mg) and DCs. To obtain the best results, immature DCs were matured by treatment with heat-killed *P. acnes* (100 μg/ml) for 24 h before their injection into the mice. Seven days after the challenge, mice were sacrificed, and Hematoxilin & eosin staining was performed with sections of the lungs and liver. A, B: Histological examination of the lungs (A) and liver (B) in mice immunized with *P. acnes* and IFA. Arrows: granulomas in the liver. C, D: Histological examination of the lungs in mice immunized with *P. acnes* and mature DCs. C: x40 (Arrows: granulomas in the lungs), D: x200. E, F: Immunostaining with anti-CD3 (E) and anti-CD4 (F)
antibodies. Original magnification: x200. G, H: Trafficking study of injected DCs. DCs stimulated with heat-killed *P. acnes* were labeled with PKH26, and injected into mice from tail vein. 48 hours later, the lung section was analyzed with a confocal laser scanning microscope equipped with an Ar-Kr laser. G: Fluorescence image (x100) (Arrows: labeled and injected DC in granulomas in the lungs), H: Hematoxylin staining (x100). The labeled DCs were detected in granulomatous lesion. (Arrows: granulomas in the lungs). Bars = 100 μm.

**Figure 2  Analysis of bronchoalveolar lavage fluid of mice with pulmonary granulomatosis induced by *P. acnes* and DCs.** Mice were subcutaneously immunized twice with 1mg of heat-killed *P. acnes* and mature DCs at a two-week interval. Two weeks after the second immunization, mice were challenged intravenously with heat-killed *P.acnes* (1 mg) and mature DCs (1x10⁶ cells). On days 0, 1, 2, 3, 7, 14 and 28 after the challenge, bronchoalveolar lavage was performed as described in the Methods. Data are
presented as the mean ± SD for the group of 4 mice. Similar
results were obtained in three separate experiments. \( ^{\dagger} P<0.001 \)
vs. the data on day 0.

**Figure 3** Change in gene expression in mice with pulmonary
granulomatosis induced by *P. acnes* and DCs. Total RNA was
isolated from the lungs of untreated mice as well as *P. acnes*-immunized mice before or 3, 7 and 14 days after the
intravenous administration of *P. acnes* with DCs. Briefly, the
lungs were quickly perfused with 5 ml of cold PBS(-) through
the right ventriculum. The lungs were then homogenized in 1 ml
of Isogen (Wako K.K., Kyoto, Japan), total RNA was extracted,
and a cDNA Microarray analysis was performed as described in
the Online Only Repository. Data are presented as the mean ±
SD. A: Major upregulated genes in the lungs of mice 3 days after
the *P. acnes* challenge. B: Time course of the gene expression
of CXCL9, CXCL10 and CXCR3 in the lungs of mice after the *P.
acnes* challenge. C: Time course of the gene expression of CCL17
and CCL22 in the lungs of mice after the *P. acnes* challenge.

**Figure 4  Elevation in levels of Th1 cytokines and chemokines in BALF of mice with pulmonary granulomatosis generated by *P. acnes***. Mice were anesthetized and a soft cannula (23G) was inserted into the trachea. Bronchoalveolar lavage (BAL) was performed five times with the instillation and withdrawal of 1ml of saline at various time points. BAL fluid (BALF) was stored at -80°C until used. The frozen BALF was quickly thawed and used to examine concentrations of cytokines and chemokines. Th1 and Th2 cytokines (IL-2, IL-4, IL-5, IFN-γ (A) and TNF-α (B)) were measured using the BD Cytometric Beads Array System. Mouse CXCL9 (C) and CXCL10 (D) levels were measured by ELISA. Data are presented as the mean ± SD for the group of 4 mice. Similar results were obtained in three separate experiments.

**Figure 5  Administration of TAK-779 blocks the trafficking of Th1 lymphocytes into the lungs.** TAK-779 (150 μg/day) was
subcutaneously administered from four days before to six days after the challenge with heat-inactivated *P. acnes* and mature DCs every other day. The BAL analysis was performed seven days after the challenge. Mice were anesthetized and a soft cannula (23G) was inserted into the trachea. Bronchoalveolar lavage (BAL) was performed five times with the instillation and withdrawal of 1ml of saline at various time points. The total cell count of the BAL fluid (BALF) was determined using Turk staining solution. BALF was centrifuged, and the cell pellets were re-suspended into saline and cytospun onto glass slides. These cells were stained with Diff-Quick staining solution (Baxter, Miami, FL), and 200 cells were counted for cell classification. A: The numbers of all cells, macrophages, neutrophils and lymphocytes in BALF of mice treated with control (5% mannitol solution) or TAK-779. Data are presented as the mean ± SD for each group of four or five mice. B: The percentages of CXCR3⁺CD4⁺ and CCR5⁺CD4⁺ T cells in BALF of mice treated with control (5% mannitol solution) or TAK-779. BAL cells were
analyzed by flow cytometry. CXCR3+CD4+ and CCR5+CD4+ T cells were determined with two color fluorescence staining. Data are presented as the mean ± SD for the group of 5 mice. Data are representative of three separate experiments.

**Figure 6 Administration of TAK-779 ameliorates the formation of granulomas in the lungs.** TAK-779 (150 μg/day) was subcutaneously administered from four days before to six days after the challenge with heat-inactivated *P. acnes* and mature DCs every other day. The histological analysis was performed seven days after the challenge. A: The histology of the lungs treated with control (5% mannitol solution) or TAK-779. Arrows: granulomas in the lungs. Bars = 100 μm. B: The quantitative analysis of pulmonary granulomatosis. The granulomatosis was evaluated by measuring the number and size of granulomas in the lungs. Data are presented as the mean ± SD of 5 mice in the group. Similar results were obtained in three separate experiments.