ANIMAL MODELS

ICAM-1 Deficiency Exacerbates Sarcoid-Like Granulomatosis Induced by *Propionibacterium acnes* through Impaired IL-10 Production by Regulatory T Cells

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CME Accreditation Statement: This activity ("ASIP 2013 AJP CME Program in Pathogenesis") has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the American Society for Clinical Pathology (ASCP) and the American Society for Investigative Pathology (ASIP). ASCP is accredited by the ACCME to provide continuing medical education for physicians.

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CME Disclosures: The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.

Accepted for publication
August 15, 2013.

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Propionibacterium acnes has been implicated as one of the suggested causative antigens for sarcoidosis, a systemic granulomatous disease. By injecting heat-killed *P. acnes* into the dorsal skin of C57BL/6J mice on days 1, 3, 5, and 14, sarcoid-like granulomatosis was induced in skin and lungs of these mice on day 28. To clarify the role of cell adhesion molecules in cutaneous sarcoidosis, we induced sarcoid-like granulomatosis in mice deficient of intercellular adhesion molecule (ICAM)-1, L-selectin, P-selectin, or E-selectin via repeated *P. acnes* injection. Histopathologic analysis revealed that granuloma formation was aggravated in the skin and lungs of ICAM-1-deficient mice compared with wild-type mice. Within skin granulomas of ICAM-1-deficient mice, *P. acnes* immunization up-regulated mRNA expression of tumor necrosis factor- α , although it failed to induce IL-10 mRNA expression in contrast to wild-type mice. Infiltration of regulatory T cells into skin granuloma was similar between wild-type mice and ICAM-1-deficient mice. *P. acnes* immunization induced IL-10 production by CD4⁺CD25⁺Foxp3⁺ regulatory T cells in lymph nodes of wild-type mice *in vivo*, which was absent in regulatory T cells of ICAM-1-deficient mice. Our results indicate that ICAM-1 is imperative for inducing regulatory T cells to produce IL-10 *in vivo*, which would prevent granuloma formation. (*Am J Pathol* 2013, 183: 1731–1739; <http://dx.doi.org/10.1016/j.ajpath.2013.08.021>)

Sarcoidosis is a systemic granulomatous disease characterized by a variable clinical presentation and course. Although numerous organs may be involved, lungs, lymph nodes, and skin are most commonly affected. However, the cause of sarcoidosis is unknown.¹ Some of the earliest studies on sarcoidosis reported the involvement of *Chlamydia* or *Borrelia*.^{2,3} Recently, sarcoidosis has been reported to be associated with exposure to various microbial agents and environmental substances.^{4–7} Although it has been indicated that mycobacterial infection is related to sarcoidosis because of their clinical and immunopathologic similarities, their association remains

controversial.^{8–10} *Propionibacterium acnes*, an anaerobic, non-spore-forming, gram-positive rod bacterium, has been implicated as one of the suggested causative antigens for sarcoidosis because this bacterium could be isolated from sarcoid lesions.¹¹ Some studies using quantitative real-time PCR have revealed markedly higher levels of *P. acnes* genomes in the mediastinal or superficial lymph nodes of sarcoid patients than in those of controls, suggesting that there is an intrinsic

Supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

infection of *P. acnes* in patients with sarcoidosis.^{12–14} Nishiwaki et al¹⁵ observed that an immune response against indigenous *P. acnes* already exists in the healthy lung. They also demonstrated 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.¹⁵ In any case, sarcoidosis granuloma involves infiltration of leukocytes, predominantly macrophages.

In general, leukocyte recruitment into inflammatory sites is achieved using constitutive or inducible expression of multiple cell adhesion molecules.¹⁶ L-selectin (CD62L), E-selectin (CD62E), and P-selectin (CD62P) primarily mediate leukocyte capture and rolling on the endothelium.¹⁷ L-selectin is constitutively expressed by most leukocytes.¹⁷ Although P-selectin is rapidly mobilized to the surface of activated endothelium or platelets, E-selectin expression is induced within several hours after activation with inflammatory cytokines.¹⁷ Intercellular adhesion molecule (ICAM)-1 (CD54), a member of the immunoglobulin superfamily, is constitutively expressed not only on endothelial cells but also on fibroblasts and epithelial cells.¹⁸ It can be up-regulated transcriptionally by several proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and IL-1.¹⁸ ICAM-1 forms the counter-receptor for the lymphocyte β_2 -integrins, such as lymphocyte function–associated antigen (LFA)-1.¹⁶ The ICAM-1/LFA-1 interaction predominantly mediates firm adhesion and transmigration of leukocytes at sites of inflammation.¹⁶ In addition to its key role in the context of leukocyte adhesion, locomotion, and migration, ICAM-1/LFA-1 interaction also functions as a co-stimulatory signal for T-cell activation.^{19–21} Even a high density of T-cell receptors—major histocompatibility complex–antigen complex could not compensate for the lack of ICAM-1/LFA-1 interaction for CD4⁺ T-cell activation.²²

Although these cell adhesion molecules play important roles in leukocyte transmigration, the way each molecule contributes to cell infiltration is complex. Inhibition or absence of adhesion molecules has been associated with reduction in inflammation and its sequelae, consistent with a central role for leukocytes in promoting inflammation.^{23–29} In contrast, some studies using adhesion molecule–deficient mice have reported exacerbation of leukocyte recruitment and inflammation.^{30–33} In this case, there may be a possibility that the absence of certain key adhesion molecules reduces recruitment of leukocytes that inhibit inflammation, namely, regulatory T cells (Tregs). Moreover, ICAM-1 deficiency or suppression of its function can affect inflammation through impaired activation or differentiation of T cells as a result of a lack of co-stimulatory signaling. Indeed, lack of ICAM-1 on antigen-presenting cells (APCs) leads to poor T-cell activation and proliferation both *in vitro* and *in vivo*, resulting in poor generation of central memory T cells.³⁴

In this study, we examined the role of cell adhesion molecules, such as ICAM-1, L-selectin, P-selectin, and E-selectin, in the *P. acnes*–induced sarcoidosis model, using mice

deficient in these molecules. Remarkably, in *ICAM-1*^{–/–} mice, skin and lung granuloma formation was aggravated without showing a defect in Treg infiltration into the skin. Compared with wild-type mice, *ICAM-1*^{–/–} mice showed reduced IL-10 production by Tregs, indicating the essential role of ICAM-1 in the suppression of skin granuloma formation by Tregs through induction of an anti-inflammatory mediator, IL-10.

Materials and Methods

Mice

L-selectin–deficient (L-selectin^{–/–}) mice were provided by Thomas F. Tedder and were previously described in their article.³⁵ *ICAM-1*^{–/–}, P-selectin^{–/–}, and E-selectin^{–/–} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were backcrossed 10 generations onto the C57BL/6 genetic background. Mice used for experiments were 6 to 10 weeks old. Body size and lung size were similar in mutant and wild-type mice (data not shown). All mice were housed in a pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of University of Tokyo.

Heat-Killed *P. acnes* Injection

P. acnes (ATCC, Manassas, VA) was dissolved in PBS at a concentration of 10⁹/mL and heat-killed at 85°C for 10 minutes. One hundred microliters of heat-killed *P. acnes* or PBS was injected subcutaneously into the shaved back skin of the mice on days 1, 3, 5, and 14 (Supplemental Figure S1).

Histopathologic Assessment of Skin and Lung Granulomas

Morphologic characteristics of skin and lung sections were assessed under a light microscope. All skin and lung samples were taken on day 28 after *P. acnes* or PBS injection. Skin samples were obtained from the nodular region on the dorsal skin into which *P. acnes* or PBS had been injected on day 14. Sections were stained with H&E. The skin granulomas were relatively circumscribed. We measured the thickest part of

Table 1 Grading Scale for Evaluation of Lung Granuloma Formation

Grade of granuloma	Histologic features
0	Normal lung
1	Inflammation without granuloma formation
2	Mild granuloma formation with definite damage to lung structure and formation
3	Moderate granuloma formation with definite damage to lung structure and formation
4	Severe distortion of structure and large granuloma areas

the granuloma vertical to the epidermis in each section (Supplemental Figure S2). The severity of lung granuloma was semiquantitatively assessed according to a grading scale (Table 1), referring to fibrosis assessment as described previously³⁶: the lung granuloma was graded on a scale of 0 to 4 by examining randomly chosen fields of the left middle lobe at a magnification of $\times 100$. Data were obtained from six to eight mice per group. All of the sections were scored independently by two masked investigators.

Immunohistochemical Staining

Paraffinized tissue sections of skin and lung taken from *P. acnes*-injected mice were deparaffinized and stained with rat anti-mouse F4/80 monoclonal antibodies (Abs; Serotec, Oxford, United Kingdom) or rat anti-mouse Foxp3 monoclonal Abs (eBioscience, San Diego, CA) using VECTASTAIN ABC Rat IgG Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer instructions. Foxp3-positive cells in skin were counted in three random grids per mice under magnification of $\times 400$ high-power fields. Data were obtained from six mice per group. Each section was examined independently by 2 masked investigators.

RNA Isolation and Real-Time PCR

Total RNA was isolated from tissue using RNeasy fibrous tissue kit (Qiagen, Crawley, United Kingdom). Granuloma tissue that was confirmed as a nodule macroscopically was obtained for *P. acnes*-injected skin samples. Total RNA from each sample was reverse-transcribed into cDNA using TaqMan Reverse Transcription Reagents (Invitrogen, Carlsbad, CA). Expression of TNF- α , IFN- γ , IL-2, IL-4, IL-10, and IL-12 was analyzed by a StepOnePlus Real Time PCR System (Applied Biosystems, Carlsbad, CA) using a real-time PCR quantification method according to the manufacturer instructions. Sequence-specific primers and probes were designed by TaqMan Gene Expression Assays (Applied Biosystems). All samples were analyzed in parallel with *GAPDH* gene expression as an internal control. The relative change in the levels of the genes of interest was determined by the $2^{-\Delta\Delta CT}$ method.

Flow Cytometry

A single cell suspension of spleen or lymph nodes was obtained by gentle teasing with forceps and 27-gauge needles, followed by filtering through a nylon mesh. Erythrocytes were lysed by treatment with ammonium chloride, and the cells were resuspended in BD PharmingenStain Buffer (BD Biosciences, Franklin Lakes, NJ). The cells were first incubated with the respective monoclonal Abs at 1:20 dilution at 4°C for 20 minutes and then with the respective second monoclonal Abs at 1:40 dilution for the next 30 minutes. The final suspension was made in 500 μ L of BD PharmingenStain Buffer for analysis. Abs used for analysis

via flow cytometry are as follows: APC-conjugated rat anti-mouse CD4 monoclonal Abs (BioLegend, San Diego, CA), phycoerythrin (PE)-conjugated rat anti-mouse CD25 monoclonal Abs (BioLegend), Alexa Fluor 488-conjugated rat anti-mouse Foxp3 monoclonal Abs (BioLegend), PE-Cy7-conjugated rat anti-mouse CD11a Abs (Abcam, Cambridge, MA), and peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated rat anti-mouse IL-10 monoclonal Abs (eBioscience). CD3⁺CD25⁺Foxp3⁺ Tregs in splenocytes were stained using Mouse Treg Flow Kit (BioLegend) according to the manufacturer instructions. Flow cytometry was performed using BD FACSVerse (BD Biosciences).

Intracellular IL-10 Expression Analysis in Tregs in Inguinal Lymph Nodes

Inguinal lymph node cells from *P. acnes*- or PBS-injected mice were cultured for 2 days in RPMI 1640 complete medium supplemented with 10% (v/v) fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin at a density of 1×10^6 cell/mL (2 mL per well) in a 24-well culture plate, under stimulation of 5 μ g/mL of plate-bound anti-mouse CD3 Abs NA/LE (BD Biosciences) and 1 μ g/mL of soluble anti-mouse CD28 Abs (BD Biosciences). Cells were fixed, permeabilized, and stained with Abs using a BD Cytotfix/CytoPerm Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer instructions. Briefly, cultured cells were stimulated with a leukocyte activation cocktail and incubated with 2 μ L/mL of BD GolgiPlug (BD Biosciences) for 4 hours. Cells were washed and incubated with APC-conjugated anti-mouse CD4 monoclonal Abs and PE-conjugated anti-mouse CD25 monoclonal Abs at 4°C for 20 minutes. Cells were fixed, permeabilized, and then stained with Alexa Fluor 488-conjugated anti-mouse Foxp3 monoclonal Abs and PerCP-Cy5.5-conjugated anti-mouse IL-10 monoclonal Abs or isotype control. These cells were analyzed by BD FACSVerse.

Statistical Analysis

All data are expressed as means $\pm$ SD or SEM. Statistical analysis was performed using the Wilcoxon rank sum test for comparison between two groups.

Results

Repeated *P. acnes* Injection into the Skin Induces Systemic Granulomatosis

We induced systemic granulomatosis by injecting heat-killed *P. acnes* into the dorsal skin of wild-type mice as described in *Materials and Methods*. As shown in Figure 1, distinct granulomas were formed in both skin and lungs of mice injected with *P. acnes* but not in mice injected with PBS. Immunohistochemical analyses revealed that the

Figure 1 Granuloma formation on day 28 in skin and lungs of wild-type mice (A) and *ICAM-1*^{-/-} mice (B) injected with PBS or *P. acnes*. H&E staining of the skin and lung granuloma is shown. Original magnification: ×100 (A; PBS-injected mice) and ×100, ×200, ×400 (A and B; *P. acnes*-injected mice; left to right).

typical granulomas consisted of F4/80⁺ macrophages (data not shown). Thus, repeated injection into the dorsal skin of mice with *P. acnes* induced not only skin granuloma but also lung granulomatosis, resembling sarcoidosis.

Effect of Cell Adhesion Molecules on Granuloma Formation in Skin and Lungs of *P. acnes*-Injected Mice

We next examined the role of cell adhesion molecules in granuloma formation using mice deficient in adhesion molecules. We injected PBS or *P. acnes* into the dorsal skin of E-selectin^{-/-}, L-selectin^{-/-}, P-selectin^{-/-}, and *ICAM-1*^{-/-} mice on days 1, 3, 5, and 14, as described above. Skin and lung samples were collected on day 28, and histologic sections were made. There were no granulomas in the skin or lungs of PBS-injected mice, whereas granuloma was observed in *P. acnes*-injected mice. As shown in Figure 1, granuloma formation in the skin seemed most exacerbated in *P. acnes*-injected *ICAM-1*^{-/-} mice compared with other mice, showing thicker granuloma formation. Granuloma formation in the lung seemed to be prominent in E-selectin^{-/-}, L-selectin^{-/-}, and *ICAM-1*^{-/-} mice compared with the others.

To quantitatively analyze granuloma formation, we performed histologic analyses. For skin, we measured the thickness of the granuloma, and for lungs, we used a grading scale for histopathologic assessment of granuloma formation (Table 1). *P. acnes*-injected *ICAM-1*^{-/-} mice presented significantly thicker skin granuloma compared with *P. acnes*-injected wild-type mice (Figure 2A). For lungs, the granuloma scores were high in *P. acnes*-injected E-selectin^{-/-}, *P. acnes*-injected L-selectin^{-/-}, and *P. acnes*-injected *ICAM-1*^{-/-} mice compared

with *P. acnes*-injected wild-type mice (Figure 2B). These results demonstrated that granuloma formation was aggravated in both skin and lungs of *P. acnes*-injected *ICAM-1*^{-/-} mice compared with *P. acnes*-injected wild-type mice. Therefore, we focused on examining the role of ICAM-1 in granuloma formation.

Increased TNF- α Expression and Decreased IL-10 Expression in the Skin Granuloma of *P. acnes*-Injected *ICAM-1*^{-/-} Mice Compared with Wild-Type Mice

We next examined the mRNA levels of TNF- α , IFN- γ , IL-2, IL-4, IL-10, and IL-12 in the skin granuloma on day 28 by quantitative real-time PCR analysis. TNF- α mRNA expression was significantly up-regulated in skin granuloma of *P. acnes*-injected wild-type mice compared with PBS-injected wild-type mice (Figure 3A). After *P. acnes* injection, *ICAM-1*^{-/-} mice exhibited significantly augmented TNF- α mRNA expression relative to wild-type mice. In contrast, although IL-10 mRNA expression was significantly up-regulated in skin granuloma of *P. acnes*-injected

Figure 2 Evaluation of granuloma formation in the skin and lungs of wild-type, E-selectin^{-/-}, L-selectin^{-/-}, P-selectin^{-/-}, and *ICAM-1*^{-/-} mice injected with PBS or *P. acnes*. **A:** Thickness of granuloma formation in the skin from *P. acnes*-injected mice. Granuloma formation on day 28 in the skin was assessed by measuring the thickness of the granuloma on the dorsal skin that had been injected with *P. acnes* on day 14. **B:** Grade of granuloma formation in the lung from *P. acnes*-injected mice. The severity of granuloma formation in the lungs was evaluated using a grading scale as described in Materials and Methods. Data were obtained from six to eight mice per group. Values are presented as means $\pm$ SEM. **P* < 0.05, ***P* < 0.01 versus wild-type mice.

Figure 3 Quantitative real-time PCR analysis of TNF- α and IL-10 mRNAs in the skin of wild-type and ICAM-1^{-/-} mice that had been injected with PBS or *P. acnes*. Mice were injected with PBS or *P. acnes*, and total RNA was isolated from skin granuloma on day 28. mRNA levels for TNF- α (A) and IL-10 (B) were determined by quantitative real-time PCR. Data are presented as means $\pm$ SD. ND, not detected. ** $P < 0.01$ versus wild-type mice.

wild-type mice compared with PBS-injected wild-type mice, IL-10 mRNA expression was not detected in skin granuloma of *P. acnes*-injected ICAM-1^{-/-} mice (Figure 3B). IFN- γ , IL-2, and IL-12 mRNA expressions were hardly detected in the skin of PBS- and *P. acnes*-injected wild-type mice and ICAM-1^{-/-} mice (data not shown). IL-4 mRNA expression was not detected in any of the skin samples (data not shown). These results indicated that exacerbated skin granuloma formation in *P. acnes*-injected ICAM-1^{-/-} mice might be due to increased TNF- α expression and decreased IL-10 expression in the skin granuloma of these mice.

Comparable Number of Tregs Infiltrating into the Skin Granuloma Between *P. acnes*-Injected Wild-Type Mice and ICAM-1^{-/-} Mice

Because Tregs are known to be one of the major cell sources of IL-10 during skin inflammation, we first evaluated the number of Foxp3⁺ Tregs infiltrating into the skin granuloma. Cells

positively stained with anti-Foxp3 Ab in the skin granuloma were counted in three random grids per mice under a magnification of $\times 400$ high-power fields (Figure 4A). Skin specimens were isolated from six separate mice, and the mean number was calculated. Although the number of infiltrating Tregs into the skin granuloma of *P. acnes*-injected ICAM-1^{-/-} mice tended to be lower than that in *P. acnes*-injected wild-type mice, the difference was not significant (Figure 4B). Consistent with this, there was no difference in Foxp3 mRNA level in the skin granuloma between these mice (data not shown). Thus, there was no difference in the number of infiltrating Tregs between *P. acnes*-injected ICAM-1^{-/-} mice and *P. acnes*-injected wild-type mice. In the lung, there were only a few Foxp3-positive cells (data not shown), and statistical analysis could not be performed.

Induced IL-10 Production in Tregs Is Not Detected in ICAM-1^{-/-} Mice Compared with Wild-Type Mice after *P. acnes* Immunization

Because there was no difference in the number of Tregs infiltrating into skin granuloma between *P. acnes*-injected

Figure 4 Infiltration of Foxp3⁺ Tregs into the skin granuloma of wild-type mice and ICAM-1^{-/-} mice injected with *P. acnes*. A: Skin granuloma sections from *P. acnes*-injected mice were stained with anti-mouse Foxp3 Abs. B: The number of infiltrating Foxp3⁺ Tregs into the skin granuloma. Cells were counted in three random grids per mouse. Cells were counted in three random grids per mouse under magnification of $\times 400$ high-power fields. Data were obtained from six mice per group. Values are presented as means $\pm$ SEM. Original magnification: $\times 600$ (A).

ICAM-1^{-/-} mice and *P. acnes*-injected wild-type mice, we next examined IL-10 production induced by *P. acnes* immunization in these mice. In these experiments, bulk inguinal lymph node cells were stained with anti-CD4, CD25, and Foxp3 Abs, and CD4⁺CD25⁺Foxp3⁺ cells were determined as Tregs. We first confirmed that CD11a, a component of LFA-1, the ligand for ICAM-1, was expressed on Tregs of *ICAM-1*^{-/-} mice at a level similar to wild-type mice (data not shown). To compare the IL-10 production capacity of *ICAM-1*^{-/-} and wild-type mice in the steady state, inguinal lymph node cells were isolated from PBS-injected *ICAM-1*^{-/-} mice and PBS-injected wild-type mice and cultured overnight with CD3 and CD28 ligation. After culture, intracellular IL-10 expression in Tregs was analyzed by flow cytometry. There was no significant difference in IL-10 production capacity between PBS-injected *ICAM-1*^{-/-} and PBS-injected wild-type mice (Figure 5A).

Next, lymph node cells were isolated from *P. acnes*-injected *ICAM-1*^{-/-} mice and *P. acnes*-injected wild-type mice and cultured overnight with CD3 and CD28 ligation. After culture, intracellular IL-10 expression in Tregs was analyzed by flow cytometry. After *P. acnes* injection, Tregs from wild-type mice produced greater amounts of IL-10 compared with those from *ICAM-1*^{-/-} mice (Figure 5B). In Tregs of wild-type mice, *P. acnes* injection induced IL-10 production compared with PBS injection (Figure 5C). In contrast, there was no such induction observed in Tregs of *P. acnes*-injected *ICAM-1*^{-/-} mice (Figure 5D). Our results indicated that although the IL-10-producing capacity of Tregs was the same between wild-type mice and *ICAM-1*^{-/-} mice in the steady state, *P. acnes*, which induced IL-10 production in Tregs of wild-type mice, failed to induce IL-10 production in Tregs of *ICAM-1*^{-/-} mice.

Discussion

In the current study, we induced sarcoid-like granulomatosis in mouse skin and lungs by repeatedly injecting heat-killed *P. acnes* into the skin of wild-type mice. Histopathologic analysis revealed that granuloma formation was aggravated in both skin and lungs of *ICAM-1*^{-/-} mice compared with those of wild-type mice. As for E-selectin^{-/-} and L-selectin^{-/-} mice, granuloma formation was aggravated only in the lungs. P-selectin deficiency did not have any effect on granuloma formation. *P. acnes* immunization up-regulated TNF- α mRNA expression in skin granulomas of *ICAM-1*^{-/-} mice compared with that of wild-type mice, whereas it failed to induce IL-10 mRNA expression in *ICAM-1*^{-/-} mice. These data suggest that ICAM-1 might play a pivotal role in IL-10 induction during granuloma formation. Because Tregs are known as a major cell source of IL-10, we examined the effect of ICAM-1 on Treg recruitment and function. We did not detect any impairment of Treg

recruitment into granuloma of *ICAM-1*^{-/-} mice. However, IL-10 production was induced by *P. acnes* immunization in CD4⁺CD25⁺Foxp3⁺ Tregs of wild-type mice but not in the Tregs of *ICAM-1*^{-/-} mice. Thus, our results indicate that

Figure 5 IL-10 production induced in CD4⁺CD25⁺Foxp3⁺ Tregs after *P. acnes* injection is not detected in *ICAM-1*^{-/-} mice compared with wild-type (WT) mice. Inguinal lymph nodes of mice injected with PBS or *P. acnes* were collected on day 28. Bulk lymph node cells were cultured for 2 days with CD3 and CD28 ligation. Cells were harvested, fixed, permeabilized, and stained with anti-IL-10 Abs or isotype control Abs. These cells were analyzed by flow cytometry. Comparison of intracellular IL-10 staining intensity between WT mice and *ICAM-1*^{-/-} mice among PBS-injected mice (A) and *P. acnes*-injected mice (B). Comparison of intracellular IL-10 staining intensity between PBS-injected mice and *P. acnes*-injected mice among WT mice (C) or *ICAM-1*^{-/-} mice (D). The results shown are representative of five duplicated individual experiments. Mean fluorescence intensities (MFI) of anti-IL-10 mAb/isotype control stained Tregs were determined, and relative percentage (means $\pm$ SD) was calculated in respective experiments according to the following formula and shown as a bar graph (E): (MFI of anti-IL-10 mAb-stained Tregs from each group of mice – MFI of isotype control Ab-stained Tregs from each group of mice)/(MFI of anti-IL-10 mAb-stained Tregs from PBS-injected WT mice – MFI of isotype control Ab-stained Tregs from PBS-injected WT mice). Significant differences between samples are indicated (** $P < 0.01$).

ICAM-1 is imperative for inducing the production of IL-10 in Tregs during granuloma formation *in vivo*, using the sarcoidosis mouse model.

The fact that granuloma formation was not confined to the skin injection site led us to consider that the skin granulomas observed in our study were not merely foreign-body granulomas. Nishiwaki et al¹⁵ have already made a murine pulmonary sarcoidosis model by injecting heat-killed *P. acnes* into the footpad every 2 weeks for a total of three or four times. In their experiments, immunohistochemical analyses revealed that the CD4⁺ T cells in granulomas expressed IFN- γ but not IL-4, which are the Th1 cells,¹⁵ which is consistent with sarcoidosis.^{1,37} However, there was no verified murine model of cutaneous sarcoidosis yet. We induced skin granuloma that resembled sarcoidosis within 28 days by simply injecting heat-killed *P. acnes* into the mouse dorsal skin four times.

In sarcoidosis, it has been reported that activated CD4⁺ cells differentiate into Th1-like cells that secrete predominantly IL-2 and IFN- γ , augment TNF- α production by macrophages, and thereby amplify the local cellular immune response.³⁸ Indeed, TNF- α mRNA expression was up-regulated in skin granuloma of *P. acnes*-injected wild-type mice compared with PBS-injected wild-type mice. Furthermore, TNF- α up-regulation was augmented in *P. acnes*-injected *ICAM-1*^{-/-} mice, supporting the histopathologic findings that granuloma formation was aggravated in both skin and lungs of *P. acnes*-injected *ICAM-1*^{-/-} mice compared with *P. acnes*-injected wild-type mice.

Although IL-10 mRNA expression was up-regulated in skin granuloma of *P. acnes*-injected wild-type mice compared with PBS-injected wild-type mice, IL-10 mRNA expression was not detected in skin granuloma of *P. acnes*-injected *ICAM-1*^{-/-} mice. Indeed, several reports indicated increased IL-10 expression in bronchoalveolar lavage fluid of patients with sarcoidosis.^{39,40} IL-10 inhibits the synthesis of various inflammatory cytokines, such as TNF- α , and plays a major role in the regulation of immune responses as an inhibitor of T-cell proliferation and macrophage activation.⁴¹⁻⁴⁶ Therefore, decreased IL-10 mRNA expression could account for the increased TNF- α mRNA expression in skin granuloma of *P. acnes*-injected *ICAM-1*^{-/-} mice. One of the possible cell sources of IL-10 is the Treg, which suppresses T-cell proliferation and inflammation in the skin.^{47,48} Tregs are reported to be increased in patients with active granulomatous sarcoidosis lung.⁴⁹ It has been suggested that despite extensive local inflammation, anergy in active sarcoidosis, as indicated by suppression of the immune response to tuberculin, is accounted for by expansion of Tregs, which abolishes IL-2 production and strongly inhibits T-cell proliferation. Together with our results, these reports led us to study the function of Tregs in *ICAM-1*^{-/-} mice.

LFA-1 (CD11a/CD18) is a component of the receptor for ICAM-1 and is expressed on leukocytes, including Tregs.

LFA-1 binds to ICAM-1 expressed on endothelial cells, which enables LFA-1-positive cells to transmigrate into tissues.⁵⁰⁻⁵² We first confirmed that absence of ICAM-1 did not affect CD11a expression on Tregs (data not shown). We also confirmed that ICAM-1 deficiency did not affect Treg infiltration into skin granuloma by comparing the number of Foxp3-positive cells and the expression level of Foxp3 mRNA in granuloma because the *Foxp3* gene has been identified as the master transcriptional factor of Tregs.⁵³ These results suggest that ICAM-1 is not necessary for Tregs to infiltrate into the skin granuloma. This finding was consistent with the previous report that E- and P-selectin ligands expressed on Tregs are adhesion molecules necessary for their infiltration into the skin.⁵⁴ Using a contact sensitivity model, Deane et al⁵⁵ previously reported that endogenous Tregs adhered in inflamed dermal vessels via ICAM-1 were able to control adhesion of other proinflammatory leukocytes *in vivo*. Different from their observation within acute inflammation, Treg infiltration was not impaired in our chronic inflammatory sarcoidosis model using *ICAM-1*^{-/-} mice. However, it is still possible that during the acute phase of granuloma formation, there might have been a difference in Treg infiltration.

Sarcoidosis represents an unresolved immunologic paradox: affected organs are the staging ground for an intense immune response, yet at the same time, a state of anergy is established as indicated by suppression of immune response to tuberculin in sarcoidosis patients. Regarding this paradox, it was previously found that although Tregs accumulating at the periphery of sarcoid granuloma and in the peripheral blood exhibit powerful antiproliferative activity that may account for the state of anergy, these Tregs do not completely inhibit TNF- α production.⁵⁶ Thus, our result indicates that ICAM-1 expression might be involved in the insufficiency of Tregs to control local inflammation in sarcoidosis.

In our study, the capacity of Tregs to produce IL-10 in the steady state was comparable between wild-type and *ICAM-1*^{-/-} mice. However, when these mice were immunized with *P. acnes*, although IL-10 production was induced in Tregs of wild-type mice, IL-10 production was not induced in Tregs of *ICAM-1*^{-/-} mice. Consistent with our result, Tran et al⁵⁷ previously found that interaction between LFA-1 on Tregs and ICAM-1 on dendritic cells is critical for the suppressor function of Tregs. They first demonstrated that suppression of T-cell activation across species is highly efficient *in vitro* and is cell contact dependent. Then, by using responder CD4⁺ T cells from *ICAM-1*^{-/-} mice, they demonstrated that human LFA-1 on Tregs specifically interacts with ICAM-1 on mouse dendritic cells rather than on the responder T cells and that this interaction is sufficient for suppression of T cell activation *in vitro*.⁵⁷ Our study found, for the first time, that ICAM-1 is imperative for inducing Tregs to produce IL-10 *in vivo*, which would prevent chronic granuloma formation.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.08.021>.

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