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Sarcoidosis and NOD1 variation with impaired recognition of intracellular *Propionibacterium acnes*

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Abstract

Sarcoidosis is a systemic granulomatous disease of unknown etiology. NOD2 mutations have been shown to predispose to granulomatous diseases, including Crohn's disease, Blau syndrome, and early-onset sarcoidosis, but not to adult sarcoidosis. We found that intracellular *Propionibacterium acnes*, a possible causative agent of sarcoidosis, activated NF-κB in both NOD1- and NOD2-dependent manners. Systematic search for NOD1 gene polymorphisms in Japanese sarcoidosis patients identified two alleles, 796G-haplotype (156C, 483C, 796G, 1722G) and 796A-haplotype (156G, 483T, 796A, 1722A). Allelic discrimination of 73 sarcoidosis patients and 215 healthy individuals showed that the frequency of 796A-type allele was significantly higher in sarcoidosis patients and the ORs were significantly elevated in NOD1-796G/A and 796A/A genotypes (OR [95% CI]=2.250 [1.084, 4.670] and 3.243 [1.402, 7.502], respectively) as compared to G/G genotype, showing an increasing trend across the 3 genotypes ($P=0.006$ for trend). A similar association was found when 52 interstitial pneumonia patients were used as disease controls. Functional studies showed that the NOD1 796A-allele was associated with reduced expression leading to diminished NF-κB activation in response to intracellular *P. acnes*. The results indicate that impaired recognition of intracellular *P. acnes* through NOD1 affects the susceptibility to sarcoidosis in the Japanese population.

Keywords: NOD2; TLR2; Granulomatous disease; NF-κB

1. Introduction

Sarcoidosis, a disease of unknown etiology, may result from exposure of genetically susceptible subjects to a specific environmental agent(s), possibly an infectious one. Polymorphisms of several genes including those encoding the vitamin D receptor [1], CC chemokine receptor [2], RANTES chemokine promoter [3], tumor necrosis factor-beta [4], and IL-18 [5] have been reported to be involved in the susceptibility or disease severity of sarcoidosis.

Propionibacterium acnes is so far the only bacterium to be isolated from sarcoid lesions. Genomes of *P. acnes* have been detected in large numbers of sarcoid lymph nodes by the quantitative polymerase chain reaction [6,7]. By hybridization in situ, *P. acnes* DNA was found in sarcoid granulomas [8]. These results point to an etiological link between *P. acnes* and some cases of sarcoidosis.

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The NOD1 protein consists of an N-terminal caspase-recruitment domain, a centrally located nucleotide-binding oligomerization domain (NOD), and a domain consisting of multiple leucine-rich repeats [9]. NOD1 acts as a pattern recognition receptor for bacterial peptidoglycan containing diaminopimelic acid (iE-DAP) [10]. The iE-DAP structure is present in a subset of bacteria that include Gram-negative bacilli and certain Gram-positive bacteria such as *Listeria monocytogenes* [10]. Activation of NOD1 induces NF-κB activation with subsequent inflammatory cytokine production [11]. Recent studies have revealed that certain NOD1 polymorphisms are associated with asthma, elevated IgE, and inflammatory bowel disease [12,13].

Mutations in the related NOD2 gene have been shown to predispose to granulomatous diseases including Crohn's disease [14], Blau syndrome [15], and early-onset sarcoidosis [16]. Although Blau syndrome and early-onset sarcoidosis were reported to share identical NOD2 mutations, no association has been reported between NOD2 and sarcoidosis [17].

Because NOD1 shares many structural and functional similarities with NOD2, the aim of this study was to investigate the role of NOD1 polymorphisms in determining susceptibility to sarcoidosis, in connection with the ability of NOD1 to recognize intracellular *P. acnes* which is a possible causative agent of this disease.

2. Materials and methods

2.1. Study subjects and DNA extraction

Study subjects included 73 patients with biopsy-proven sarcoidosis, 215 healthy control subjects, and 52 patients with interstitial pneumonia as disease control subjects. The diagnosis was made at the Japanese Red Cross Medical Center. Samples from healthy control subjects were obtained at the health care division of the medical center; samples from people with normal test results were selected. Written informed consent was obtained from all subjects before starting the study, and the ethics committee of the medical center approved the study. Genomic DNA was extracted from peripheral blood from all subjects with QIAamp DNA mini kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instruction.

2.2. Detection of NOD1 polymorphism in sarcoidosis patients

The 11 exons of the NOD1 gene were screened by direct sequencing of DNA from 50 patients with sarcoidosis. PCR for exon amplification was performed in 50 μl of a mixture containing 3 μl of a DNA sample, 5 pmol of each primer in a previous report [18], 10 nmol of each of the four deoxynucleotides, 100 nmol of $MgCl_2$, 1.25 U of Ex Taq, and Ex Taq buffer (1×) (TAKARA Shuzo, Shiga, Japan) to make 50 μl. Amplification was performed with GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with a profile of 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min.

Synthesis of appropriately sized PCR products was confirmed by agarose gel electrophoresis, and PCR products were purified using the DNA Fragment Purification Kit (TOYOBO, Osaka, Japan), according to the manufacturer's protocol. Purified DNA products were sequenced using an ABI PRIME BigDye Terminator Cycle sequencing kit version 3.1 (Applied Biosystems). Primers for the PCR amplification were used as sequence primers. Fragments were analyzed using the ABI PRISM 3100 GENETIC ANALYZER automated DNA sequencing machine (Applied Biosystems). Sequencing data were compared with the published NOD1 sequence (GenBank accession number NM 006092) using BLAST2 sequences software obtained from the website of the National Center for Biotechnology Information (Bethesda, MD; http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

2.3. Genotyping

Genotyping of the NOD1 single nucleotide polymorphism was carried out using a Custom TaqMan® 5′ allelic discrimination assay (Applied Biosystems). Each PCR reaction was carried out using 15 ng of DNA in 25 μl reaction mixture containing 12.5 μl of TaqMan Universal PCR Master mix (2×) (Applied Biosystems), 0.625 μl of Assay Mix (40×) (Applied Biosystems) and 6.875 μl of dH_2O per sample. Primers and TaqMan® probe for this analysis were designed and synthesized by Custom TaqMan® SNP Genotyping assay service (Applied Biosystems). Probes were labeled with VIC or 6-FAM on the 5′-terminal for detecting G-residue or A-residue, respectively, on 796 base-position of NOD1 mRNA. These primers and probes were contained in Assay Mix (40×) with optimal concentration. Thermal cycle conditions were as follows: 95 °C for 5 min and 40 cycles of 92 °C for 15 s and 60 °C for 1 min. All PCR and endpoint fluorescent readings were performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Samples detected with only VIC or 6-FAM signal were judged as 796G/G or 796A/A genotype, respectively. Samples detected with both signals were judged as 796G/A genotype. These results were in complete agreement with the data obtained from the preceding direct sequencing of the genes in 50 patients with sarcoidosis and thus confirmed the reliability of our genotyping method.

2.4. Infection protocol and NF-κB activation assay

P. acnes strains were grown in Gifu-anaerobic-medium broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37 °C under anaerobic conditions for 3 days before use.

HEK293T cells grown to 80% confluence in 12-well plates in DMEM supplemented with 10% fetal calf serum were transfected by the Ca-phosphate method with reporter plasmids (7.3 ng pBxIV-luc and 73 ng pEF-BOS-β-gal/well) and together with pcDNA3-NOD1-FLAG (0.15, 0.3 and 0.6 ng/well), pcDNA3-NOD2 (0.01 ng/well), or pFLAG-CMV1-TLR2 (1.0 ng/well). Eight hours after the transfection, HEK293T cells were infected with bacteria at a multiplicity of infection of 100, or incubated with the respective ligand; iE-DAP (100 ng/ml) for NOD1, muramyldipeptide (10 ng/ml) for NOD2, and N-palmitoyl-S-(2,3-bis[palmitoyloxy]-[2RS]-propyl)-(R)-cysteine-CSK4 (Pam3-CSK4) (10 ng/ml) for TLR2. After incubation for 6 h at 37 °C, extracellular bacteria were removed by washing and the cells were incubated with DMEM containing 300 μg/ml of gentamicin to kill the remaining extracellular bacteria until the luciferase activity assay. To assess the effects by invasiveness of the *P. acnes* strains, bacteria were passively internalized into the cells by the Ca-phosphate transfection method as used in plasmid transfection. Twenty-four hours after transfection of reporter genes, luciferase activity was determined. Results were normalized for transfection efficiency with values obtained with pEF-BOS-β-gal.

Construction of the plasmids pcDNA3-NOD1-FLAG and pcDNA3-NOD2 has been previously described [9,19]. The plasmid expressing TLR2 was generously provided by Dr. R. Medzhitov (University of Yale). iE-DAP was synthesized as described previously [10]. Synthetic muramyldipeptide was purchased from Peptide Institute, Inc. (Osaka, Japan) and Pam3-CSK4 was purchased from Invitrogen (Carlsbad, CA, USA).

796A-NOD1 was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and used for the NF-κB activation assay described above as well as for protein expression assay. To compare the protein expression level, HEK293T cells were transfected with 30 ng or 60 ng of either p796G-NOD1-FLAG or p796A-NOD1-FLAG together with 30 ng of plasmid encoding a Myc-tagged control protein. Twenty-four hours after transfection, the cells were lysed in 0.2% Nonidet P-40 lysis buffer and resolved by 10% SDS-PAGE. The expressed protein was detected by immunoblotting with anti-FLAG (Sigma-Aldrich, Tokyo, Japan) and anti-Myc (Roche, Tokyo, Japan) monoclonal antibodies.

2.5. Statistical analysis

Statistical comparisons were performed using SAS version 9.1 (SAS institute, Cary, NC, USA). χ^2 tests and ANOVA were used to compare qualitative and quantitative variables between groups, respectively, followed by Bonferroni correction for multiple comparisons. Student's *t*-test was used to

compare the luciferase activity indices of two NOD1 variants for each stimulant. A *P*-value less than 0.05 was considered significant. Odds ratios (ORs) and confidence intervals (CIs) were calculated with the use of a logistic regression model.

3. Results

3.1. P. acnes strains isolated from sarcoidosis patients invade epithelial cells

We initially studied a *P. acnes* strain isolated in our laboratory from a subcutaneous lesion of a 25-year-old woman with sarcoidosis and a type strain of *P. acnes* (ATCC 6919). No difference was found between the clinical strain and the type strain by the bacteriological method or by polymerase chain reaction with 16 s rRNA. Notably, infection of epithelial cell lines (A549 and HEK293T) with the clinical strain led to localization of the bacteria inside the cells whereas that with the type strain did not (Fig. 1). We then examined the cellular localization of other *P. acnes* strains isolated from sarcoid lymph nodes and found strains with invasive phenotype localized intracellularly and strains with non-invasive phenotype localized extracellularly. Ten strains of *P. acnes* including five with invasive and five with non-invasive phenotypes were used to determine intracellular recognition as described below.

3.2. Recognition of intracellular P. acnes by NOD1 and NOD2

We performed overexpression assays in HEK293T cells to explore a possible function of NOD proteins in host recognition of *P. acnes*. HEK293T cells were selected to facilitate the analysis of bacterial recognition because they are highly transfectable and lack endogenous Toll-like receptors. Infection of HEK293T cells with the clinical strain of *P. acnes* enhanced the reporter gene activity in NOD1-, NOD2-, and TLR2-transfected cells (Fig. 2A), indicating that both TLR2 and NOD family proteins can activate NF-κB in response to *P. acnes*. In contrast, NOD1- and NOD2-dependent NF-κB activation by the type strain of *P. acnes* was not detectable, indicating the importance of intracellular invasiveness of *P. acnes* for NOD signaling. Both strains of *P. acnes* activated NF-κB in a TLR2-dependent manner. Notably, NOD1 and NOD2 conferred responsiveness to the type strain after passive internalization of the bacteria into the cells (Fig. 2B). To confirm these results, we examined NOD1 responses to ten additional *P. acnes* isolates from sarcoid lymph nodes including five with invasive and five with non-invasive phenotypes (Fig. 2C). Although all five invasive isolates activated NOD1, all five non-invasive isolates stimulated NOD1 only after passive internalization. Taken together,these data indicate that NOD1 and NOD2 are involved in the recognition of intracellular *P. acnes*.

3.3. NOD1 polymorphism and genotyping

We carried out a systematic search for the presence of polymorphic variants by the direct sequencing of the 11 exons of NOD1 from 50 sarcoidosis patients (Table 1). Analysis revealed one nucleotide change in exon 1 (156C>G) and four in exon 3 (483C>T, 796G>A, 1722G>A, 2108C>A) (Table 1). Two (796G>A and 2108C>A) of the five detected NOD1 variants resulted in changed amino acid residues (E266K and P703Q) located in the nucleotide-binding domain and the leucine-rich repeat domain, respectively. As the 2108C>A variant was observed only monoallelically in a single patient, subsequent studies focused on the other four NOD1 variants. Because all of the four nucleotide polymorphisms occurred together without exception, the NOD1 alleles were classified into two haplotypes, 796G-haplotype (156C, 483C, 796G, 1722G) and 796A-haplotype (156G, 483T, 796A, 1722A).

Genotype results in 73 patients with sarcoidosis, 52 patients with interstitial pneumonia, and 215 healthy subjects are shown in Tables 2 and 3. A comparison of the sarcoidosis and healthy control groups showed that the ORs were significantly elevated in both NOD1-796G/A and 796A/A genotypes (OR [95% CI]=2.250 [1.084, 4.670] and 3.243 [1.402, 7.502], respectively) as compared to G/G genotype, showing an increasing trend across the 3 genotypes (OR per +1 allele [95% CI]=1.769 [1.182, 2.647], $P=0.006$). A similar association was found in the analysis using interstitial pneumonia patients as disease controls although some results

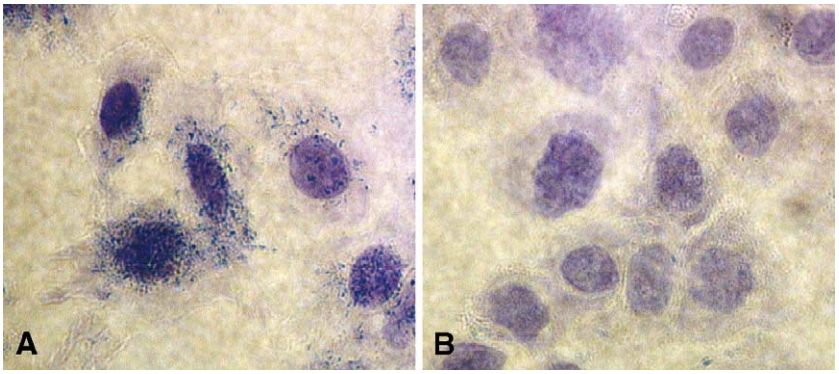

Fig. 1. Invasiveness of *P. acnes* into epithelial cells. A549 cells were infected with a clinical strain (A) or a type strain (B) of *P. acnes*. After incubation for 6 h at 37 °C, extracellular bacteria were removed by washing and by incubation for 2 h with DMEM containing 300 μg/ml of gentamicin to kill the remaining extracellular bacteria. Intracellular *P. acnes* was found by Giemsa stain in only the clinical strain.

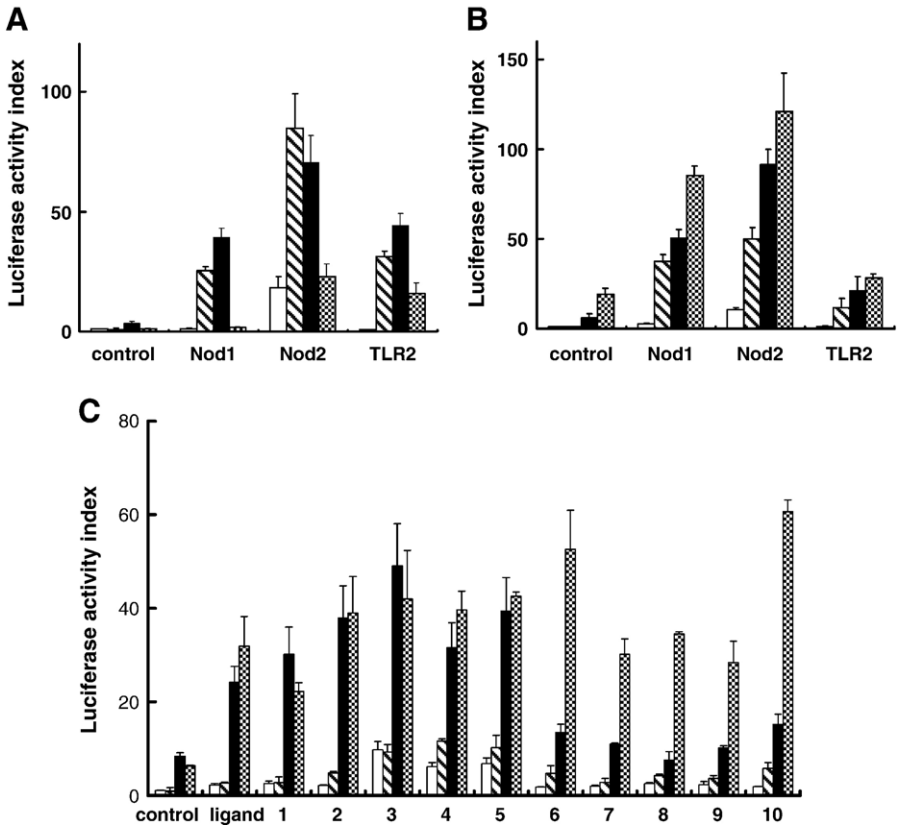

Fig. 2. NOD1 and NOD2 mediate NF-κB activation by intracellular *P. acnes*. HEK293T cells were transiently transfected with reporter genes alone (control), or together with NOD1, NOD2, or TLR2 expression plasmids. A. The transfected cells were incubated in the absence (□) or presence of each ligand (▨), or infected with the clinical strain (■) or the type strain (▩) of *P. acnes*. B. NOD1, NOD2 and TLR2 stimulation by *P. acnes* strains or microbial ligands passively internalized into HEK293T cells by the Ca-phosphate transfection method. C. NOD1 response to ten additional isolates of *P. acnes*. HEK293T cells were incubated with (□) or passively internalized with (▨) each isolate. The NOD1-transfected HEK293T cells were also incubated with (■) or passively internalized with (▩) each isolate. Control means absence of stimulator and ligand means iE-DAP. 1 to 5 represent invasive *P. acnes* strains and 6 to 10 non-invasive strains. Values represent the mean of normalized data ± SD of triplicate cultures.

were not statistically significant. No significant difference was seen between disease controls and healthy controls (data not shown).

Frequencies of 796G- and 796A-haplotypes are summarized in Table 4. A comparison of data obtained from sarcoidosis patients and both healthy and disease controls showed that the 796A-type allele was also associated with sarcoidosis (OR [95% CI]=1.681 [1.151, 2.454] and 1.747 [1.051, 2.904], respectively).

Table 1
Sequence variations of NOD1 gene in 50 Japanese patients with sarcoidosis

Location	Nucleotide change	Amino acid change	Protein domain	Allele frequency	Heterozygous/ homozygous
Exon 1	156 C>G[a]	–	CARD	0.55	25/15
Exon 3	483 C>T[a]	–	–	0.55	25/15
Exon 3	796 G>A[a]	E266K	NOD	0.55	25/15
Exon 3	1722 G>A[a]	–	–	0.55	25/15
Exon 3	2108 C>A	P703Q	LRR	0.01	1/0

CARD, caspase activation and recruitment domain; NOD, nucleotide-binding oligomerization domain; LRR, leucine-rich repeat domain.

[a] These four mutations occurred together forming two haplotypes in all cases analyzed.

3.4. Recognition of intracellular P. acnes by the 796A-NOD1 variant

To examine whether 796G>A change affects the expression and/or function of the NOD1 gene, we constructed a NOD1 cDNA containing the G796A substitution and compared the expressed protein level of the two variants by immunoblotting. The analysis revealed that transfection of the NOD1 cDNA bearing 796A results in reduced expression in comparison with cDNA bearing 796G (Fig. 3A). By densitometric analysis, the expression of 796A-NOD1 was found to be decreased by approximately 50% compared to that of 796G-NOD1 (Fig. 3A, top). Control experiments showed that the reduced level of

Table 2
Genotype frequencies of NOD1 polymorphism in sarcoidosis and controls

Group	*n*	The number (%) of each genotype		
		G/G	G/A	A/A
Sarcoidosis	73	11 (15)	42 (58)	20 (27)
Interstitial pneumonia	52	14 (27)	32 (62)	6 (12)
Healthy individuals	215	66 (31)	112 (52)	37 (17)

Table 3
Results of logistic regression analysis for the association of NOD1 genotype with sarcoidosis

Genotype	Odds ratio (95% CI) for sarcoidosis	
	vs. healthy control	vs. disease control
Dominant model		
G/G	1	1
G/A or A/A	2.497 (1.235, 5.046), P=0.011	2.077 (0.855, 5.041), P=0.106
Recessive model		
G/G or G/A	1	1
A/A	1.816 (0.973, 3.391), P=0.061	2.893 (1.071, 7.818), P=0.036
Per allele		
G/G	1	1
G/A	2.250 (1.084, 4.670)	1.670 (0.670, 4.166)
A/A	3.243 (1.402, 7.502)	4.242 (1.269, 14.179)
Per allele	1.769 (1.182, 2.647), P=0.006	2.027 (1.122, 3.661), P=0.019

CI, confidence interval.

expression of 796A NOD1 was not due to unequal transfection efficiency, because co-transfection with a plasmid encoding a control Myc-tagged protein showed similar levels of expression for 796G and A (Fig. 3A, bottom).

We then evaluated the functional difference of 796A- and 796G-NOD1 by analyzing the NF-κB activity in response to iE-DAP in HEK293T cells transfected with various amounts of the vectors encoding the two variants. Consistent with reduced expression of the NOD1 protein, the ability of 796A-NOD1 to activate NF-κB was reduced by approximately 50% compared with that of 796G-NOD1 (Fig. 3B). The activity of 796A-NOD1 produced by transfection with 0.3 ng or 0.15 ng of the expression vector were almost comparable with that of 796G-NOD1 transfected with 0.15 ng and 0.3 ng of expression vector, respectively. In response to intracellular *P. acnes*, the 796A-NOD1 variant exhibited reduced ability to activate NF-κB compared with 796G-NOD1 (Fig. 3C). Thus, the reduced activity of the 796A-NOD1 variant in response to iE-DAP and *P. acnes* can be explained by reduced expression of the NOD1 protein.

4. Discussion

In the presented study, we examined the role of NOD1 and NOD2 proteins in host recognition of *P. acnes* and the association of NOD1 polymorphisms with sarcoidosis. We show for the first time that both NOD proteins recognize intracellular *P. acnes* and that the 796A-NOD1 variant that exhibits impaired response to *P. acnes* is associated with sarcoidosis. These findings warrant further studies on NOD1 polymorphisms to clarify the functional effects of NOD1 variants and their relevance in the context of sarcoidosis.

It is well accepted that sarcoidosis arises through a combination of genetic and environmental factors, and Th1 polarization and persistent accumulation of inflammatory cells are regarded as pathophysiologic characteristics. We previously reported the involvement of *P. acnes* in the etiology of sarcoidosis by showing the accumulation of *P. acnes* genomes in sarcoid granulomas [6–8]. The Kveim test phenomenon implicates that host factors play an important role in sarcoidosis. Recombinant trigger-factor protein from *P. acnes* has been reported to cause a cellular immune response in some patients with sarcoidosis, but not in subjects without sarcoidosis [20]. *P. acnes* trigger factor protein has also been shown to cause pulmonary granulomas in mice sensitized with the protein and adjuvant [21]. Especially considering that *P. acnes* is the most common commensal bacterium in the lungs and mediastinal lymph nodes [22,23], local proliferation of this endogenous bacterium may trigger sarcoid granulomatous inflammation in genetically predisposed individuals [24].

We have shown in the present study that the clinical isolate of invasive *P. acnes* stimulates NOD1 and NOD2 as well as TLR2, suggesting a possible association between pathogenicity and NOD response. *P. acnes* has been studied for its role in immunomodulation with the conclusion that TLR2, TLR4 and TLR9 mediated the effects of *P. acnes* infection [25,26]. However, these studies were only concerned with non-invasive *P. acnes*. TLRs are likely to serve as the first line receptors for *P. acnes*, but NOD proteins might play a major role in a subsequent phase of intracellular infection.

As we have shown in this study, both invasive and non-invasive *P. acnes* can be isolated from sarcoid lymph nodes. This difference in invasiveness and the subsequent response by NOD proteins might explain, at least in part, the previously reported diversity of the clinical isolates of *P. acnes* in their ability to modulate the antimicrobial peptide and chemokine expression [27].

Although the exact mechanism that regulates the invasiveness of *P. acnes* has not been determined, a recent report has demonstrated a critical dependency on the interaction of bound fibronectin with integrins and involvement of TGF-β1 in the cellular invasion of *Group A Streptococcus* [28]. Further studies are needed to examine the involvement of these factors in the invasion of *P. acnes* and their role in the pathogenesis of sarcoidosis.

Table 4
Haplotype frequencies of NOD1 polymorphism in sarcoidosis and controls

Group G	Haplotype		Odds ratio (95% CI)	
	G type	A type		
Sarcoidosis	0.44	0.56	1.681 (1.151, 2.454), P = 0.007	1.747 (1.051, 2.904), P = (0.031)
Interstitial pneumonia	0.58	0.42	0.962 (0.624, 1.484), P = 0.861	
Healthy individuals	0.57	0.43		

CI, confidence interval.

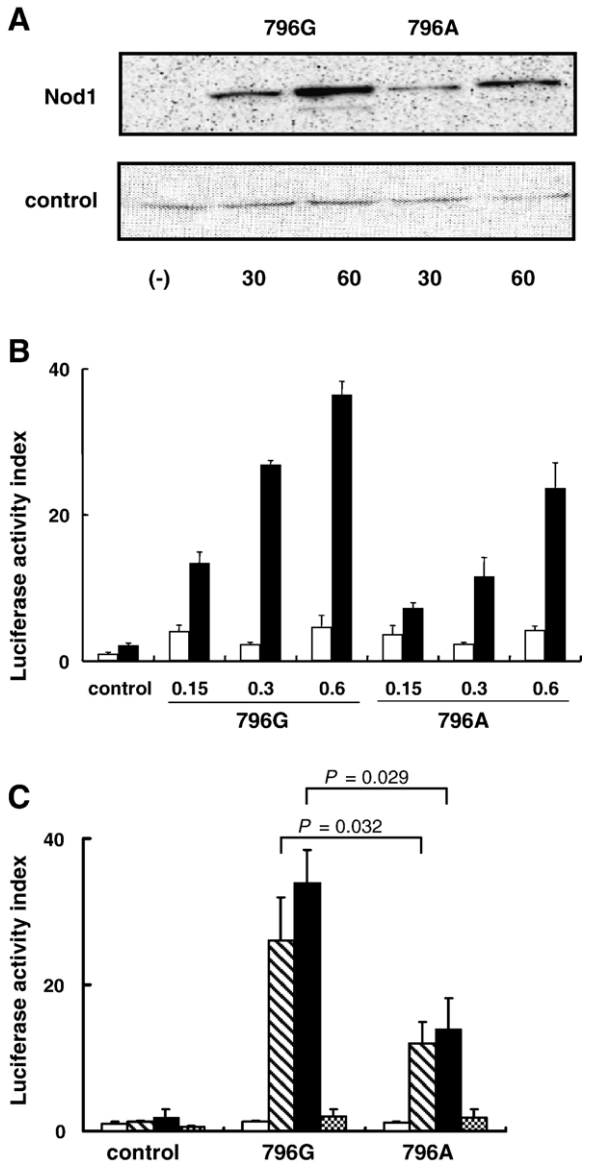

Fig. 3. Reduced expression and impaired response to *P. acnes* of the 796A-NOD1 variant. A. HEK293T cells were transfected with 0, 30, or 60 ng of either 796G-NOD1 or 796A-NOD1 together with 30 ng of control plasmid encoding. Myc-tagged protein. The cell lysates were separated by 10% SDS–PAGE, and immunoblotted with anti-FLAG or anti-Myc antibody. Representative results are shown. B. HEK293T cells were transiently transfected with reporter genes alone (control), or together with 0.15, 0.3, or 0.6 ng of either 796G- or 796A-NOD1 expression plasmid. The transfected cells were incubated in the absence (□) or presence of iE-DAP (■). C. HEK293T cells were transiently transfected with reporter genes alone (control), or together with 796G- or 796A-NOD1 expression plasmid. The transfected cells were incubated in the absence (□) or presence of iE-DAP (▨), or infected with the clinical strain (■) or the type strain (▩) of *P. acnes*. Values represent the mean of normalized data ± SD of triplicate cultures.

No link has been found between NOD2 and sarcoidosis [17], but the present study has identified genetic variation in NOD1 that is associated with sarcoidosis. The two NOD proteins have distinctive distribution patterns. NOD1 is widely distributed [9], whereas the expression of NOD2 is mainly restricted to monocytes and macrophages [19]. In a series of studies [29], we found intracellular *P. acnes* not only in the lymphoid tissues (lymph nodes, spleen, etc.) but also in many parenchymal organs including the lungs, liver, heart, skeletal muscles, and kidneys. Whereas, in the lymphoid tissues, *P. acnes* infects macrophages or dendritic cells which express both NOD1 and NOD2, in parenchymal organs, the bacterium infects non-phagocytic parenchymal cells which express NOD1 but not NOD2. These observations lead us to speculate that the difference in tissue distribution might explain, at least in part, the association of NOD1, but not NOD2, with sarcoidosis susceptibility especially when systemic organs other than lymphoid tissues are involved.

Functional studies demonstrated similar levels of activity between the wild-type and mutant NOD1 under conditions with comparable expression levels, leading us to conclude that impaired response of the NOD1 variant to *P. acnes* is due to reduced expression rather than reduced intrinsic activity of the NOD1 mutant protein. Our conclusion suggests that the E266K substitution reduces the stability of NOD1 protein.

Impaired function of NOD2 is associated with Crohn's disease whereas increased constitutive activity of NOD2 causes Blau syndrome [30]. Although the mechanism by which impaired NF-κB activation associated with NOD2 mutations leads to Crohn's disease remains poorly understood, it has been suggested that loss of production of antimicrobial molecules such as alpha-defensins may impair the local response to control bacterial infection thereby inducing NF-κB overexpression via NOD2-independent pathways and aberrant inflammation at intestinal sites [31,32,33]. A similar mechanism may link the E266K change in NOD1 to sarcoidosis, although the suspected causative agents of the two diseases are different [34].

NOD1 has recently been reported to be a critical regulator of beta-defensin-2 during *Helicobacter pylori* infection [35]. It is possible that impaired expression of beta-defensin-2 through 796-A NOD1 due to reduced ability to induce NF-κB enables *P. acnes* to survive and persist intracellularly, leading to the pathogenesis of sarcoidosis.

Haplotype frequencies for the E266K-NOD1 variant were 0.42 and 0.43 in the disease controls and healthy controls, respectively, with no significant difference from the value (0.45) obtained from the NCBI database (rs2075820) where 1228 Japanese subjects were examined. Although the frequency of the E266K-NOD1 variant was increased significantly in sarcoidosis patients ($P=0.046$) even compared with the NCBI SNP database, our data suggest that in addition to NOD1, other genetic or environmental factors contribute to the development of sarcoidosis. Similarly, in Crohn's disease, only a subset of patients with NOD2-associated mutations develop the disease [36].

In conclusion, our data indicate that NOD1 polymorphisms with haplotypic combinations are important determinants of sarcoidosis susceptibility. This suggests that altered recognition of intracellular *P. acnes* may be one of the key events in the pathogenesis of sarcoidosis.

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