G809S may be expected to enhance NF-κB activation in the presence of R837. However, G809S did not increase NF-κB activity like as Y859C [6] (Fig. 4b). Since the *NLRP3* LRR domain plays a central role in mediating inflammation induced by another inflammasome activator, MSU crystals, we examined whether G809S affected NF-κB activation in the presence of MSU [14]. Interestingly, G809S and Y859C mutations did not show any NF-κB activity responses by MSU stimulation. In contrast, wild-type, D303N and G755R mutations significantly increased NF-κB activity following MSU stimulation. These data suggest the G809S LRR missense variant, which may diminish the responsiveness to PAMPs as NOD2 LRR variant reported in Crohn's disease, has a pathogenic effect on these pathways [15–17].

Jéru et al. recently identified a pathogenic Y859C mutation in the LRR domain of *NLRP3*, which increased speck formation and pro-caspase 1 processing, but which had no direct effect on *NLRP3* mediated NF-κB signaling. The G809S variant also increased speck formation relative to wild-type *NLRP*3. These results suggest that G809S, as well as Y859C in the LRR domain, may be a gain of function variant. It should be noted that although the assays used in this study are sensitive, our findings may provide limited evidence to prove that the G809S variant is pathogenic. However, these results indicate that the variant alters the function of NLRP3.

The two case studies presented here consistently showed elevated IL-1-related serum cytokines, IL-1ra, during the attack phase. In addition, monocytes from case 1 and 2 secreted high levels of IL-1β, which may indicate a gain of function variant in *NLRP3*, associated with inflammasome activation. Additionally, we previously reported a CINCA/NOMID patient positive for the compound heterozygous gene mutations, E688K and G809S [9]. This patient developed severe a phenotype compared with her mother, who carried a single mutation, E688K. This genotype-phenotype correlation suggests that the G809S variant may act as an additional genetic factor associated with the severity of CAPS.

However, in this study IL-1β was not detectable in the serum of patients, as IL-1β might be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. Furthermore, although elevated serum IL-18, which is activated by caspase-1 as well as IL-1β, and IL-6 levels were observed in CINCA/NOMID patient [9], the serum IL-18 levels were increased in case 2 but not case 1, and serum IL-6 levels in both cases did not increase during the fever episodes. Thus, it may be considered that the differences of cytokine profiles and disease phenotypes between case 1 and 2 and typical CINCA/NOMID patients result not only from their genetic background, but also environmental factors.

Additional mutation analysis of our patients also revealed heterozygous variant haplotype of *MEFV*, a gene involved in the pathogenesis of FMF, in addition to G809S in *NLRP3*. Case 1 was heterozygous for P369S and R408Q in *cis* and case 2 was heterozygous for E148Q, P369S, and R408Q in *cis*. Allele frequencies of P369S and R408Q in the Japanese population are 3.6 % and 4.8 %, respectively, according to the International HapMap Project (http://www.hapmap.org/). These frequent variant haplotypes were found to be in strong linkage disequilibrium in the Japanese population. In addition, P369S and R408Q variant haplotype are associated with a variable phenotype and are infrequently associated with typical FMF symptoms [18–21]. Heterozygous P369S and R408Q variant haplotype are also associated with other inflammatory diseases, such as Behçet's disease [18], and systemic lupus erythematosus [21]. Moreover, heterozygous E148Q-P369S-R408Q variant haplotype is more rare, which is associated with chronic recurrent multifocal osteomyelitis [20]. In this report, case 1 and case 2 showed the similar phenotypes as FMF or TRAPS, respectively. Although detailed clinical features and cytokine profiles of the two cases are various, they exhibited a long duration of recurrent fever episodes compared with typical FMF. Thus, these findings suggest that P369S and R408Q variant haplotype may have effects on several inflammatory diseases, but the functional evidence of these variant haplotype remains unclear.

The *MEFV* gene codes for pyrin, that can interact with ASC to induce ASC oligomerization and the activation of procaspase-1, which promotes IL-1β and IL-18 processing [12, 22]. In contrast, some reports have described that pyrin inhibited NLRP3-mediated NF-κB activation by disrupting the NLRP3-ASC interaction [23, 24]. In accordance with the reports, co-expression of NLRP3 and pyrin in HEK293T cells indicated less ASC-dependent NF-κB activation than expression of NLRP3 only, whereas there was no difference in the inhibitory capacity of NF-κB activity between pyrin variants and the wild-type protein. Interestingly, a recent study using pyrin deficient and mutated pyrin knock-in mice demonstrated a gain of function with pyrin variants located in B-Box domains, which caused autoinflammatory phenotypes [22]. Thus, research using knock-in mouse experiments with *MEFV* exon3 variants into pyrin deficient mice would help clarify the pathogenic effects of the *MEFV* variant.

In general, hereditary periodic fever syndromes have been considered monogenic diseases. On the other hand, recent reports have described patients with heterozygous low penetrance variants in two recurrent fever genes [2, 25, 26]. These indicate that oligogenic inheritance has been related to pathogenesis of autoinflammatory diseases. In some cases, patients presented with specific symptoms of both diseases or with a more severe phenotypes. Although the patients in this study were positive for the *NLRP3* variant, they did not present with typical symptoms of CAPS, such as deafness or cold-induced rash. In addition, variants in *MEFV* have been detected in both cases, but they also lacked typical FMF symptoms. However,

both cases had obviously periodic fever episodes. These suggest the presence of oligogenicity and that variants in *NLRP3* and *MEFV* synergistically modify the symptoms of the atypical autoinflammatory diseases.

There are two important limitations in this study when discussing the pathogenicity of low penetrance rare variants. The first limitation is the limited number of patients in the study. Further study using a large number of patients is necessary to confirm our results. Secondly, we only analyzed a limited number of genes. In this study, we concluded that the presence of an *NLRP3* variant with the co-existence of *MEFV* variants contributed to atypical autoinflammatory disease. However, the patients may have had alternative genetic mutations or other rare variants of inflammasome related genes such as *CARD8* [27] elsewhere in the genome, which are truly disease causing, and the two variants described in these patients may be unrelated.

Conclusions

This study describes the molecular analysis of two cases with heterozygous low penetrance variants in exon5 of *NLRP3* and exon3 of *MEFV*. The findings provide in vivo and in vitro evidence for the effect of an *NLRP3* missense variant. Importantly the mutations are within the same signaling pathway and are associated with inflammasome activation. Our observations suggest that oligogenic inheritance may occur in patients with atypical autoinflammatory syndrome. It is therefore important to consider that the phenotypes could be modified by synergistic effects with plural autoinflammatory-associated gene mutations when the patients have atypical autoinflammatory disease.

Acknowledgments We thank the members of the families who agreed to participate in the study. We thank Dr. Ozaki T for the initial treatment of case 2. We thank K. Kasahara and M. Yamamoto for their technical help. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health and Labour Science Research Grants for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare.

Conflict of Interest The authors have declared no conflicts of interest.

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Familial Mediterranean Fever in Japan

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Abstract: Familial Mediterranean fever (FMF) is a hereditary autoinflammatory disease that is prevalent in Mediterranean populations. While it is considered a rare disease in the rest of world, a significant number of FMF patients have been reported in East Asia, including Japan. Our aim was to determine the prevalence of FMF in Japan and elucidate the clinical and genetic features of Japanese patients. A primary nationwide survey of FMF was conducted between January and December 2009. Hospitals specializing in pediatrics and hospitals with pediatric, internal medicine, and rheumatology/allergy departments were asked to report all patients with FMF during the survey year. The estimated total number of Japanese FMF patients was 292 (95% confidence interval, 187–398 people). We evaluated the clinical and genetic profiles of Japanese patients from the data obtained in a secondary survey of 134 FMF patients. High-grade fever was observed in 95.5%, chest pain (pleuritis symptoms) in 36.9%, abdominal pain (peritonitis symptoms) in 62.7%, and arthritis in 31.3%. Of the patients profiled, 25.4% of patients experienced their first attack before 10 years of age, 37.3% in their teens, and 37.3% after age 20 years. Colchicine was effective in 91.8% of patients at a relatively low dose (mean dose, 0.89 ± 0.45 mg/d). AA amyloidosis was confirmed in 5 patients (3.7%). Of the 126 patients studied, 109 (86.5%) were positive for 1 or more genetic mutations and 17 (13.5%) had no mutation detected. Common Mediterranean fever gene (MEFV) mutations were E148Q/M694I (19.8%) and M694I/normal (12.7%). The differences in the prevalence of peritonitis, pleuritis, and a family history of FMF were statistically significant between FMF patients with MEFV exon 10 mutations compared with those without exon 10 mutations.

In conclusion, a significant number of patients with FMF exist in Japan. Although Japanese patients with FMF are clinically or genetically different from Mediterranean patients, the delay in diagnosis is an issue that should be resolved.

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This work was supported by a grant-in-aid for research on intractable diseases from Ministry of Health, Labour and Welfare of Japan. The authors have no conflicts of interest to disclose.

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ISSN: 0025-7974
DOI: 10.1097/MD.0b013e318277cf75

(*Medicine* 2012;91: 337–343)

Abbreviations: AA = amyloid A, CI = confidence interval, FMF = familial Mediterranean fever, MEFV = Mediterranean fever, PCR = polymerase chain reaction, SAA = serum amyloid A.

INTRODUCTION

Familial Mediterranean Fever (FMF) is an inherited autoinflammatory disease that is observed in Mediterranean populations, such as Armenians, Arabs, non-Ashkenazi Jews, and Turks.[11,47] The disease is characterized by recurrent febrile episodes and inflammation in the form of sterile polyserositis.[5] The gene responsible for FMF, MEFV, encodes a protein called pyrin/marenostrin and is expressed mainly in neutrophils and monocytes.[13,21] To date, 200 mutations or polymorphisms in the MEFV gene have been associated with the FMF phenotype.[28] The prevalence of FMF varies from 1:400-1000 in Turkey,[9,47] 1:1000 (depending on the ethnic group) in Israel,[10] and 1:500 in Armenia.[35] The various manifestations of FMF in different populations could be caused by a diverse repertoire of mutations specific to their ethnic background.[7] For example, patients carrying exon 2 mutations (such as E148Q) present a milder phenotype.[7] In contrast, patients carrying M694V or M694I mutations are prone to more severe disease.[44] Where the disease is common the diagnosis of FMF is principally based on clinical tests, whereas in countries where FMF is rare, a genetic test is useful.[2] The diagnostic power of the colchicine response, where an FMF patient is expected to respond to colchicine, is still important when monitoring atypical FMF cases.[6]

The genetic homogeneity of Japan has been preserved by national geographic borders, and there has been little inward migration since ancient times. FMF was previously thought to affect people mainly from Mediterranean populations, and was considered a rare disease in Japan. However, a significant number of FMF patients with MEFV gene mutations have been reported in Japan since the identification of the MEFV gene.[22,30,36,38,41,42,46] One severe complication of FMF is the development of amyloid A (AA) amyloidosis.[17] The effect of MEFV genotypes, especially when the M694V mutation is homozygous, is evident in FMF patients with AA amyloidosis.[4] In Japanese patients with rheumatoid arthritis, the SAA1.3 allele was shown to be a risk factor of AA amyloidosis.[31] However, there is a strong positive association of the SAA1.1 allele and M694V homozygosity of the MEFV gene as risk factors for AA amyloidosis in white FMF patients.[16]

Since 1972, the Ministry of Health and Welfare of Japan has promoted research to determine the causes of intractable diseases of unknown etiology.[32] To investigate epidemiologic features of disease (prevalence, age distribution, and clinical phenotypes), the Research Committee on the Epidemiology of Intractable Diseases conducted several surveys in cooperation with various disease research committees.[18] In 2009 the Ministry of Health,

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Labour and Welfare of Japan and the Research Committee on the Epidemiology of Intractable Diseases conducted a nationwide survey to elucidate the prevalence of FMF in Japan. We conducted the present study to further estimate the prevalence of FMF and elucidate the clinical features in Japanese patients. Genotype/phenotype correlations were previously reported in Jewish, Turkish, and Armenian patients with FMF.[25,29,48] In the current study we evaluated the genotype/phenotype correlations in Japanese patients with FMF and compared them to those of other ethnic groups.

METHODS

A nationwide survey for FMF was conducted in cooperation with the Japan Research Committee on the Epidemiology of Intractable Diseases in 2009. The target populations were patients with FMF who visited hospitals in 2009. According to the Nationwide Epidemiologic Survey Manual issued by the Research Committee on the Epidemiology of Intractable Disease,[19] we selected 3 types of departments for the targeted survey: pediatrics, internal medicine, and rheumatology/allergy. The hospitals used in the study were selected randomly from a list of all hospitals in Japan. The selection rate was determined according to a stratification based on the number of beds in the hospital. Thus, hospitals with a high number of beds had a greater probability of being selected. The selection rate was 100% for hospitals with 500 beds or more or university hospitals, whereas only 5% of hospitals with fewer than 100 beds were selected at random. In addition, specialized hospitals that had previously reported FMF patients were all selected for the study. After selection, we sent a questionnaire describing the diagnostic criteria for FMF. The primary survey only inquired as to the numbers of patients with FMF who visited the hospital in 2009. Patients were diagnosed clinically according to the modified and simplified diagnostic criteria of Tel-Hashomer,[24] provided with the primary survey. The diagnosis was divided into 1 major criterion: recurrent febrile episodes (3 or more episodes lasting 12 h to 3 d with a fever of 38°C or more) and 8 minor criteria (a febrile attack with 1 of 7 accompanying symptoms including abdominal pain due to peritonitis; chest pain due to pleuritis; monoarthritis of hip, knee, or ankle; pericarditis; scrotum pain due to orchitis; headache due to aseptic meningitis; or a favorable response to colchicine treatment). A diagnosis of FMF was determined if the patient exhibited the major criterion and 1 or more minor criteria. When a suspected FMF patient was identified, a second questionnaire regarding the detailed clinical features for each patient was sent. The present study was approved by the ethical committees of Jichi Medical University (No. 09–20, September 7, 2009).

Using the selection and response rate to the surveys, we estimated the total number of patients with FMF and the 95% confidence intervals (CIs) as described previously.[20,23] The estimate was based on the assumption that department responses were independent of the frequency of patients. The point estimation of prevalence was calculated using the following equation, where SRTk, RRTk, NSk, nk, Nk, and Nki denote the sampling rate, response rate, number of sampling departments, total number of departments, number of responding departments, and the number of departments with i patients in stratum k, respectively.

$$\alpha_k = \frac{1}{SRT_k RRT_k}\Sigma_i iN_{ki} = \frac{1}{\frac{NS_k}{n_k}\frac{N_k}{NS_k}}\Sigma_i iN_{ki} = \frac{nk}{Nk}\Sigma_i iN_{ki}$$

Age and sex distributions of the disease were estimated based on data obtained from the second survey.

Mutation Analysis

Two mL of blood were collected from each subject. Genomic DNA was extracted from whole blood using the Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Mutation analysis was performed by genomic sequencing. Mutations in exon 1–10 of the MEFV gene were tested. Polymerase chain reaction (PCR) was performed using forward and reverse primers for each exon as described previously.[39] PCR products were purified with the ExoSAP-IT (GE Healthcare Japan, Tokyo, Japan) and sequenced directly, using specific primers and BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan). Genetic analysis of the MEFV gene was approved by the Ethics Committee of Nagasaki Medical Center (No. 21003, May 11, 2009).

Determination of SAA1 Alleles

SAA1 genotyping by PCR-RFLP was performed, as previously described.[1] Briefly, a portion specific to SAA1 was amplified using the following primer set: 5′-ATGATGCTGCCAAAA GGGGA-3′ (forward) and 5′-TGGCCAAAGAATCTCTGGAT-3′ (reverse). PCR was carried out with 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The products were digested by Ban I and Bcl I, and genotypes were determined by agarose gel electrophoresis.

Statistical Analyses

Data were analyzed using SPSS (SPSS Inc., Chicago, IL). Results were expressed as the mean ± standard deviation (SD) for continuous variables. For quantitative data, analysis was performed using a Mann–Whitney U rank-sum test for comparison of 2 independent groups. Comparisons for categorical variables were evaluated using the chi-square test. $P < 0.05$ was accepted as significant.

RESULTS

Prevalence

In the primary survey, 2251 hospitals or departments of pediatrics, internal medicine, or rheumatology/allergy were selected. Of them, 1380 (61.3%) responded and 170 patients met the diagnostic criteria for FMF. The results of the questionnaire survey are shown in Table 1. The numbers of patients reported from departments of pediatrics, internal medicine, and rheumatology/allergy were 85 (50.0%), 67 (39.4%) and 18 (10.6%), respectively. The total number of patients was estimated to be 292 (95% CI, 187–398). The estimated numbers from pediatrics, internal medicine, and rheumatology/allergy were 118, 129, and 45, respectively (Table 2).

Demographic Features of Japanese FMF Patients

From 170 FMF patients recruited in the first nationwide survey, detailed clinical data were obtained from 122 patients and from another 12 patients who were diagnosed after the first survey in 2009. We analyzed the clinical and demographic features of these 134 patients. The male to female ratio was 1:1.3. The mean (±SD) age at the time of diagnosis was 28.7 ± 18.5 years, and the mean age at onset of symptoms was 19.6 ± 15.3 years. Thirty-four patients (25.4%) experienced their first attack before the age of 10 years, 50 patients (37.3%) in their teens, and 50 patients (37.3%) after 20 years of age. The mean period from disease onset to diagnosis was 9.1 ± 9.3 years, suggesting a delay in diagnosis.

Clinical Features

Clinical data from 134 patients in the second nationwide survey showed 99 patients (76.2%) with no family history suggestive of FMF. The main clinical findings were present at

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TABLE 1. Response Rates and Reported Numbers of FMF Patients

		No. of Departments	Subjects for 1st Survey (n)	(%)	Response Rates (n)	(%)	No. of Departments Reporting FMF Patients	No. of Patients Reported
Internal Medicine	University hospitals	154	154	100.0	83	53.9	9	28
General hospitals	≧500 beds	210	210	100.0	118	56.2	10	14
	400−499 beds	166	133	80.1	74	55.6	1	1
	300−399 beds	327	131	40.1	74	56.5	3	5
	200−299 beds	426	85	20.0	48	56.5	0	0
	100−199 beds	1112	111	10.0	67	60.4	0	0
	≦99 beds	3199	160	5.0	82	51.3	0	0
	Specialized hospitals	26	26	100.0	18	69.2	10	19
	Total	5620	1010	18.0	564	55.8	10	67
Pediatrics	University hospitals	108	108	100.0	82	75.9	17	62
General hospitals	≧500 beds	195	195	100.0	154	79.0	6	6
	400−499 beds	155	123	79.4	93	75.6	4	5
	300−399 beds	298	120	40.3	87	72.5	2	6
	200−299 beds	329	66	20.1	38	57.6	0	0
	100−199 beds	604	60	9.9	37	61.7	0	0
	≦99 beds	1062	53	5.0	35	66.0	0	0
	Specialized hospitals	17	17	100.0	10	58.8	4	6
	Total	2768	742	26.8	536	72.2	33	85
Rheumatology/ Allergy	University hospitals	96	96	100.0	51	53.1	9	9
General hospitals	≧500 beds	46	46	100.0	27	58.7	0	0
	400−499 beds	34	34	100.0	23	67.6	1	4
	300−399 beds	59	59	100.0	32	54.2	1	1
	200−299 beds	80	80	100.0	45	56.3	1	2
	100−199 beds	213	85	39.9	43	50.6	1	0
	≦99 beds	422	85	20.1	49	57.6	1	2
	Specialized hospitals	14	14	100.0	10	71.4	0	0
	Total	964	499	51.8	280	56.1	14	18
Total		9352	2251	24.1	1380	61.3	80	170

the following frequencies: fever (128 patients, 95.5%), abdominal pain (84, 62.7%), chest pain (48, 35.8%), arthritis (42, 31.3%), erysipelas-like erythema (10, 7.5%) and amyloidosis (5, 3.7%). The remaining minor symptoms were pericarditis (3, 2.2%) and headache (17, 12.7%). Febrile attacks, chest pain, and arthritis were comparable between Japanese FMF patients and Mediterranean patients, while abdominal pain and amyloidosis were less prevalent among Japanese FMF patients (Table 3).[3,37,47] AA amyloidosis was confirmed in 5 patients (3.7%) whose genotypes were M694I/M694I, E148Q/E148Q, E148Q/R202Q/P369S/R408Q, and M694I/E148Q/L110P (2 patients) (Table 4).

Colchicine was administered orally to 132 patients, and a favorable therapeutic effect was seen in 122 patients (91.8%). Treatment efficacy was not obtained from the questionnaire survey of 5 patients, and 2 patients had not yet been treated with colchicine. The mean dose of colchicine required to control attacks (0.89 ± 0.45 mg/d) was lower for Japanese FMF patients compared with previous reports of Mediterranean patients.[5]

Mutational Analysis

Among 134 Japanese FMF patients with detailed clinical data, 126 patients underwent MEFV mutation analysis. The MEFV gene mutation was not identified in 17 of 126 patients (13.5%). Of the remaining 109 patients (86.5%), 14 were homozygotes, 66 were compound heterozygotes or had complex alleles, and 29 were heterozygotes. The distribution of the MEFV genotypes in the study group is presented in Table 5. The major detected mutations were homozygous, heterozygous, and compound heterozygous for E148Q, E148Q-L110P, P369S-R408Q, and/or M694I. The most frequent genotype was M694I/E148Q, followed by M694I/normal and M694I/M694I. In consideration of the allelic frequencies, the most common MEFV mutations and polymorphisms among Japanese FMF patients were M694I (29.4%), E148Q (31.3%), L110P (11.5%), P369S (5.6%), and R408Q (5.6%). Moreover, the rare mutations M680I, G304R, R202Q, and E84K were detected in the heterozygous state. It is noteworthy that no

TABLE 2. Estimated Number of FMF Patients in Japan*

Department	No. of Reported Patients	Estimated Patient Number, SE	(95% CI)†
Internal medicine	33	129 ± 45	(40–218)
Pediatrics	33	118 ± 27	(65–172)
Rheumatology/Allergy	14	45 ± 10	(27–64)
Total	80	292 ± 54	(187–398)

Abbreviations: SE = standard error.

*The estimated total number of patients = number of reported patients/(number of responding hospitals/number of target hospitals).

†Ninety-five percent confidence intervals were calculated with an assumption of multinomial hypergeometric distribution.

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TABLE 3. Main Clinical Features of Japanese and Mediterranean FMF Patients

Feature	Patients From Japan	Patients From Turkey	Patients From Israel	Arab Patients
No. of patients	134	2838	470	175
Fever, (%)	95.5	92	100	100
Abdominal pain (peritonitis), (%)	62.7	93	95	94
Chest pain (pleuritis), (%)	35.8	31	43	32
Arthritis, (%)	31.3	47	75	33
Skin rash (erysipelas-like erythema), (%)	7.5	21	4	3
Amyloidosis, (%)	3.7	13	27	3
Reference	PR	47	37	3

Abbreviations: PR = present report.

homozygous or heterozygous M694V or V726A mutations were observed in Japanese FMF patients. Mutations of the MEFV gene in exon 10 (M694I, M680I) were detected in 67/126 (53.2%) of FMF patients. These patients showed a significantly higher prevalence of chest and abdominal pain and a lower prevalence of arthritis compared with those without mutations in exon 10. In addition, these patients had a more frequent family history of FMF compared to those without mutations (Table 6). Analysis of the frequency of clinical manifestations between FMF patients without MEFV mutations and those with mutations showed no statistical difference: peritonitis (58.8% vs. 63.3%), pleuritis (23.5% vs. 40.4%) and arthritis (52.9% vs. 29.4%), respectively. Similarly, there was no statistical difference in the dose of colchicine (1.02 ± 0.71 mg/d vs. 0.85 ± 0.40 mg/d) and age at onset (17.3 ± 15.9 yr vs. 19.2 ± 14.7 yr) between FMF patients without MEFV mutations and those with mutations.

DISCUSSION

FMF is considered a common hereditary autoinflammatory disease among Mediterranean populations;[2] however, the true prevalence of FMF has not been elucidated in East Asia. To our knowledge, this was the first nationwide survey of the prevalence of FMF in East Asia. The estimated number of Japanese patients with FMF was approximately 300 (95% CI, 187–398 people). In a second survey, we obtained clinical information from 134 FMF patients, currently the largest survey of Japanese FMF patients. Based on these data, we further identified the spectrum of clinical features, MEFV mutations, and genotype/phenotype correlations in Japanese FMF. The age of onset among Japanese FMF patients was significantly different from Mediterranean populations, where 90% of patients developed FMF before 20 years of age compared with 63% of Japanese patients.[4] The incidences of clinical symptoms during febrile attack were relatively similar between Japanese and Mediterranean FMF patients except for peritonitis and amyloidosis. The prevalence of abdominal pain (62.7%) and amyloidosis (3.7%) was lower in Japanese patients than in Mediterranean FMF patients.[3,37] Genetic factors (ethnicity and spectrum of MEFV mutations) and related disease severity could contribute to the differences in the age at onset and incidence of abdominal pain and amyloidosis between Japanese and Mediterranean FMF patients.

Two patients with AA amyloidosis (40%) exclusively carried MEFV gene mutations in exon 2 or 3, which are considered low-risk mutations for FMF-related AA amyloidosis.[43] This supports the concept that the phenotype or genotype of FMF does not necessarily predict the development of amyloidosis.[45] However, recurrent or long-standing subclinical inflammation may have contributed to the association of amyloidosis in these patients. The Turkish FMF Study Group reported that the mean period from disease onset to diagnosis was 6.9 ± 7.7 years.[47] The mean delay from disease onset to diagnosis of Japanese FMF was 9.0 years, thus putting undiagnosed patients at risk of secondary amyloidosis due to recurrent inflammation, which is a significant public health issue. The mainstay of treatment for FMF is daily oral colchicine, which decreases the frequency and intensity of attacks and prevents the development

TABLE 4. Demographic Features of Patients With AA Amyloidosis

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex	M	F	M	M	M
Current age (yr)	46	34	51	50	44
Age at onset (yr)	23	20	20	30	15
Age at diagnosis of FMF (yr)	43	28	51	50	43
Delay in diagnosis of FMF (yr)	20	8	31	20	28
ESRD	(+)	(−)	(−)	(−)	(−)
Current treatment	Colchicine (1 mg/d)	Prednisolone (colchicine was discontinued due to AE)	Colchicine (1 mg/d)	Colchicine (1 mg/d)	Colchicine (1 mg/d)
MEFV genotype	M694I/E148Q/L110P	E148Q/E148Q	E148Q/R202Q/P369S/R408Q	M694I/M694I	M694I/E148Q/L110P
SAA1 genotype	1.3/1.5	NT	1.3/1.5	1.5/1.5	1.1/1.1

Abbreviations: AE = adverse effect, ESRD = end-stage renal disease, NT = not tested.

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TABLE 5. Genotype of 126 Japanese FMF Patients

	Patients	
Mutation	**No.**	**(%)**
M694I/M694I	8	6.3
M694I/normal	16	12.7
M694I/E148Q	25	19.8
M694I/L110P	2	1.6
M694I/E148Q/L110P	14	11.1
M694I/E148Q/E148Q/L110P/L110P	1	0.8
M680I/E148Q/L110P	1	0.8
E148Q/E148Q	1	0.8
E148Q/E148Q/L110P	2	1.6
E148Q/E148Q/P369S/R408Q	2	1.6
E148Q/normal	8	6.3
E148Q/L110P	7	5.6
E148Q/R202Q	2	1.6
E148Q/G304R	1	0.8
E148Q/S503C	1	0.8
E148Q/L110P/R202Q	1	0.8
E148Q/P369S/R408Q	5	4.0
E148Q/R202Q/P369S/R408Q	1	0.8
E148Q/G304R/P369S/R408Q	1	0.8
R202Q/normal	1	0.8
S503C/normal	1	0.8
E84K/normal	3	2.4
P369S/R408Q	5	4.0
(−)	17	13.5
Total	126	

of amyloidosis. The normal adult dose of colchicine is 1.2–1.8 mg/d and leads to clinical improvement in more than 90% of patients.[12] Pras et al[33] reported that 30% of North African Jewish patients required a minimum colchicine dose of 2 mg/d to control symptoms. In the current study, Japanese patients with FMF were treated with a relatively low dose of colchicine (mean, 0.89 ± 0.45 mg/d), which had a therapeutic efficacy of 91.8% of Japanese FMF patients.

In the current study we investigated the spectrum of MEFV mutations and the genotype/phenotype correlation in Japanese FMF patients registered in the first nationwide survey. The mutations associated with the most severe phenotype were located in exon 10 of the MEFV gene. This encodes the C-terminal pyrin domain, B30-2/SPRY, the binding site of caspase-1.[8] The mutations in exon 10 of the MEFV gene, M694V, V726A, and M694I, are predominant in Mediterranean FMF patients.[15] Our data showed heterogeneous MEFV genotypes consisting of M694I, E148Q, L110P/E148Q, and P369S/R408Q in Japanese FMF patients. M694V and V726A are the most common mutations of Mediterranean FMF patients.[45] However, these mutations were not found in Japanese FMF patients. The founder effect is likely to be related to the biased MEFV mutation spectrum of Japanese FMF patients. An intriguing finding in FMF is the differing penetrance associated with certain mutations or polymorphisms. The E148Q mutation has low penetrance and is described as a polymorphism due to its high carrier rate, but demonstrates a lack of phenotype in some homozygous patients.[40] However, in our nationwide survey, patients carrying the E148Q mutation exhibited the typical FMF phenotype. The incomplete penetrance of mutations in exon 2 or exon 3 of the MEFV gene suggests the presence of other genetic factors or environmental factors that could influence the disease expression. Although the classical MEFV mutations in exon 10 are characterized by high penetrance, the carrier rate of MEFV exon 10 mutations among Japanese healthy controls is extremely rare.[38] Therefore, the diagnostic significance of their presence, even when heterozygous, is very pronounced in Japanese patients.

In the present study we also attempted to evaluate the genotype/phenotype correlation in Japanese FMF patients. FMF patients with exon 1, 2, or 3 mutations or no mutations comprised a genetically distinct phenotype compared to those with MEFV exon 10 mutations. These patients were positive for various mutated alleles, such as E84K, E148Q, P369S, R202Q, and were more likely to have nonspecific musculoskeletal symptoms including arthralgia. In contrast, the frequency of serositis (chest pain and abdominal pain) was significantly lower compared to those carrying MEFV exon 10 mutations. MEFV gene mutations located in exon 3 have been shown to be responsible for a variant form of FMF or atypical clinical manifestations of FMF.[6,34] These findings may partly explain the observation that FMF patients with mutations in other exons of the MEFV gene present with diverse clinical manifestations compared to FMF patients with exon 10 mutations. Previous genotype/phenotype correlation studies have suggested that mutations located within exon 10, that is, M694V, are associated with severe disease and the frequent occurrence of amyloidosis.[14,16] In contrast, mutations in exon 2, such as E148Q, were associated with milder disease with no amyloidosis.[43] The frequencies of exon 2 (E148Q, E148Q/L110P) mutations are relatively high in Japanese patients compared to white patients,[27] and may reflect the milder form of phenotype observed. We also demonstrated a relationship between the M694I mutation and a higher prevalence of serositis and familial aggregation in Japanese patients with FMF.

TABLE 6. Genotype-Phenotype Correlation in Japanese FMF Patients

		Patients With *MEFV* Exon 10 Mutations (n = 67)	Patients Without *MEFV* Exon 10 Mutations (n = 59)	
Clinical Feature	**Total (n = 126)**	**No. (%)**	**No. (%)**	**P**
Abdominal pain	79 (62.7)	50 (74.6)	29 (49.2)	0.001
Chest pain	48 (38.1)	40 (59.7)	8 (13.6)	0.0001
Arthritis	41 (32.5)	15 (22.4)	26 (44.1)	0.021
Myalgia	15 (11.9)	7 (10.4)	8 (13.6)	0.641
Amyloidosis	5 (4.0)	3 (4.5)	2 (3.4)	0.560
Age at onset (yr)	19.1 ± 15.1	17.9 ± 11.6	20.6 ± 18.3	0.915
Male/female	53/73	34/33	19/40	0.035
Family history	32 (25.4)	24 (35.8)	8 (13.6)	0.004

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Conclusion

In conclusion, our nationwide survey estimated the prevalence of FMF in Japan and allowed us to establish the spectrum of MEFV gene mutations among Japanese FMF patients. Our data indicated that Japanese FMF patients are clinically or genetically distinct from Mediterranean FMF patients, suggesting a genotype/phenotype relationship. Although Japanese FMF disease may be less severe, patients should be treated earlier to prevent recurrent attacks and subsequent development of AA amyloidosis. Further ethnic-based studies are needed to elucidate the clinical and genetic profiles of FMF in East Asia.

ACKNOWLEDGMENTS

This study could not have been accomplished without the effective and dedicated participation of each of the following contributors: Hideki Kasahara, MD (Sapporo Medical Center NTT EC), Takeshi Ibata, MD (Mino City Hospital), So Oshitani, MD (Kasugai Municipal Hospital), Yuji Yamada, MD (Koseiren Takaoka Hospital), Kei Fujioka, MD, Hiroyuki Morita, MD, Kazuo Kubota, MD, Hidenori Ohnishi, MD (Gifu University Hospital), Kouhei Yamashita, MD (Kyoto University Hospital), Koji Ishii, MD (Oita University), Hideki Ozawa, MD (Tokai University), Kazuhiko Takabe, MD (Tsuchiura Kyodo General Hospital), Kohaku Iguchi, MD (Iguchi Clinic), Ryo Yamagata, MD (Kuroishi General Hospital), Joji Tada, MD (Kagawa prefectural central hospital), Hirokazu Taniguchi, MD (Toyama prefectural central hospital), Makoto Miki, MD (Sendai Red Cross Hospital), Katsuya Fuse, MD (Niigata Prefectural Koide Hospital), Tomochika Kato, MD (Toyokawa City Hospital), Akihisa Sawada, MD (Osaka Medical Center and Research Institute for Maternal and Child Health), Shinichiro Hagiwara, MD (Nakadori General Hospital), Hideto Otoi, MD (Sunagawa City Medical Center), Toshihiko Sinoki, MD (Suzuka Central General Hospital), Hitomi Sano, MD (Sapporo City General Hospital), Shinji Akioka, MD (University Hospital Kyoto Prefectural University of Medicine), Mitsuhiko Nambu, MD (Tenri Hospital), Emi Kadoi, MD, (Gifu Municipal Hospital), Kazushige Nagai, MD (Sapporo Medical University Hospital), Yoshinori Morita, MD, Takayasu Arima, MD, Kotaro Suzuki, MD (Chiba University Hospital), Hirotsugu Kano, MD (The University of Tokyo Hospital), Keiichi Isoyama, MD (Showa University Fujigaoka Hospital), Nobuyuki Yajima, MD (Showa University Hospital), Tomoko Toma, MD (Kanazawa University Hospital), Takayuki Nakazawa, MD (Asama General Hospital), Tadashi Kumamoto, MD (Mie University Hospital), Akihiko Maeda, MD (Kochi Medical School), Koichi Kusuhara, MD (University of Occupational and Environmental Health, Japan), Yasuhito Nerome, MD (Kagoshima University Medical and Dental Hospital), Makoto Kubo, MD (Saiseikai Yamaguchi hospital), Haruyo Iwadate, MD (Fukushima Medical University), Takao Nagashima, MD (Jichi Medical University Hospital), Masato Moriguchi, MD (Saitama Medical Center Jichi Medical Center), Nobuo Negoro, MD (Osaka City University Hospital), Kiyoshi Matsui, MD (Hyogo College of Medicine), Tomohiro Koga, MD, Akitomo Okada, MD (Nagasaki University Hospital), Yuuya Takahashi, MD (NHO Niigata Hospital), Koichiro Saisho, MD (NHO Miyakonojyo Hospital), Shigenori Tamaki, MD (NHO Mie Chuou Medical Center), Hiroshi Tsutani, MD (NHO Awara Hospital), Shunsuke Mori, MD (Kumamoto Saishunso National Hospital), Nariaki Toita, MD (Sapporo-Kosei general Hospital), Akira Hoshioka, MD (Chiba Children's Hospital), Masahiko Azuma, MD (Tokushima University Hospital), Mitsuaki Kimura, MD (Shizuoka Children's Hospital), Hirofumi Taki, MD, (Toyama University Hospital), Kazumi Nagasaka, MD (Tone Chuou Hospital), Eriko Morishita, MD (Minamigaoka Hospital), Sei Samukawa, MD (Yokohama City University Hospital), Yusaku Kanezuka, MD (Hokkaido University Hospital), Takeo Sato, MD (Japanese Red Cross Kitami Hospital), Kenji Kobayashi, MD (Saitama Cardiovascular and Respiratory Center), Makiko Kinoshita, MD (HNO Minami Wakayama Medical Center), Mio Tanaka, MD (Hiroshima University Hospital), Tadashi Nakamura MD (Kumamoto Center for Rheumatology).

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Critical Role for Mast Cells in Interleukin-1β-Driven Skin Inflammation Associated with an Activating Mutation in the Nlrp3 Protein

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http://dx.doi.org/10.1016/j.immuni.2012.04.013

SUMMARY

Cryopyrin-associated periodic syndromes (CAPS) are caused by aberrant interleukin-1β (IL-1β) production induced by mutations in the NLRP3 protein in humans, but the mechanisms involved remain poorly understood. Using a mouse model, we show a role for the indigenous microbiota and mast cells (MCs) in skin disease associated with mutant Nlrp3 protein. Unlike normal cells, MCs expressing mutant Nlrp3 produced IL-1β in response to lipopolysaccharide or tumor necrosis factor-α (TNF-α). In neonatal mice, the microbiota induced TNF-α and IL-1β and promoted skin disease. MC deficiency greatly reduced disease in Nlrp3 mutant mice, and reconstitution of MC-deficient mice with mutant MCs restored skin disease, which required the expression of IL-1β in MCs. Surprisingly, neutralization of TNF-α abrogated IL-1β production and skin disease in neonatal Nlrp3 mutant mice, but not in affected adult mice. Thus, the microbiota and MCs initiate cellular events leading to dysregulated IL-1β production and skin inflammation in neonatal mice with the CAPS-associated Nlrp3 mutation.

INTRODUCTION

The innate immune system is activated through the engagement of host soluble factors and germline-encoded pattern-recognition receptors (PRRs) by microbial moieties or endogenous molecules generated in the setting of infection or cellular injury (Chen and Nuñez, 2010; Kawai and Akira, 2010). In response to infection, PRR activation initiates signal-transduction pathways that ultimately culminate in host defense responses that eliminate microbial invasion. A major inflammatory pathway is the inflammasome, a multiprotein platform that activates the protease caspase-1 (Franchi et al., 2009a; Schroder and Tschopp, 2010). Once activated, caspase-1 proteolytically processes pro-interleukin-1β (IL-1β) and pro-IL-18, which is important for secretion of the biologically active forms of these cytokines (Chen and Nuñez, 2010). To date, several inflammasomes have been described, of which three, the NLRP1, NLRP3, and NLRC4 inflammasomes, contain a PRR that belongs to the intracellular Nod-like receptor (NLR) family. Activation of the NLRP3 inflammasome is mediated by two signals. The first signal, referred to as priming, is the nuclear-factor-κB-dependent transcription of pro-IL-1β and NLRP3 through the stimulation of PRRs by various Toll-like receptor (TLR) agonists, including lipopolysaccharide (LPS), or certain cytokines such as tumor necrosis factor-α (TNF-α) or IL-1β (Bauernfeind et al., 2009; Franchi et al., 2009b). The second signal activates NLRP3 and is induced by ATP, certain bacterial toxins, or a variety of crystalline and particulate matter, including urate crystals, asbestos fibers, silica, and aluminum salts (Franchi et al., 2010). In response to activating stimuli, NLRP3 recruits the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment and activation domain) and forms an inflammasome that drives caspase-1 activation (Franchi et al., 2009c).

The importance of the NLRP3 inflammasome is underscored by the observation that missense mutations in the *NLRP3* gene are responsible for a spectrum of autoinflammatory syndromes (Aksentijevich et al., 2002; Dowds et al., 2004; Hoffman et al., 2001). These diseases, collectively referred to as cryopyrin-associated periodic syndromes (CAPS), are rare monogenic inherited disorders that are characterized by episodes of fever, urticarial-like skin rash, and other more variable inflammatory manifestations, in the absence of autoimmunity or infection (Aksentijevich et al., 2007; Neven et al., 2004). CAPS include familial cold autoinflammatory syndrome (FCAS [MIM 120100]), Muckle-Wells syndrome (MWS [MIM 191900]), and neonatal-onset multisystem inflammatory disease (NOMID [MIM 607115]). Although CAPS are considered distinct clinical entities, they represent a continuum in disease severity; FACS patients are the least affected, MWS patients are in the middle, and NOMID is the most severe form of disease, causing neurological deficits and deforming arthritis (Neven et al., 2004). These CAPS-associated missense *NLRP3* mutations result in enhanced activation of caspase-1 and secretion of IL-1β by causing

constitutive activation of the NLRP3 inflammasome (Agostini et al., 2004). Notably, treatment of CAPS patients with an IL-1 receptor antagonist or IL-1β-blocking antibody resolves most inflammatory signs and symptoms in CAPS, resulting in life-altering outcomes (Hoffman et al., 2004; Hoffman et al., 2008; Lachmann et al., 2009). Although CAPS are typically dominantly inherited disorders and exhibit high penetrance, there is evidence that environmental factors may play a role in triggering disease. For example, FCAS inflammatory episodes are often induced by systemic exposure to cold, MWS attacks can be induced by several triggers, and NOMID occurs at birth or in early infancy, but the environmental stimuli that induce disease onset remain unknown.

Two groups have recently generated gene-targeted mice harboring *Nlrp3* mutations that mimic the amino-acid substitutions found in CAPS (Brydges et al., 2009; Meng et al., 2009). Brydges et al. generated two disease-associated Nlrp3 mouse strains; one line expressed A350V, corresponding to human A352V associated with MWS, and another line expressed L351P, corresponding to the FCAS-associated L353P mutation (Brydges et al., 2009). Practically all mice from the latter Nlrp3 mutant strains die within 2 weeks after birth due to severe systemic inflammation (Brydges et al., 2009). In contrast, Meng et al. generated Nlrp3 mutant mice that expressed $Nlrp3^{R258W}$, corresponding to the MWS-associated R260W NLRP3 mutation (Meng et al., 2009). Heterozygous $Nlrp3^{R258W}$ mice exhibited delayed growth, reduced body weight, and increased mortality, but the great majority of $Nlrp3^{R258W}$ mice survived after weaning (Meng et al., 2009). Notably, Nlrp3 mutant mice from both groups developed dermatitis rich in neutrophils, which histologically resembles that observed in human CAPS (Brydges et al., 2009; Meng et al., 2009). Consistent with constitutive activation of the inflammasome, priming of macrophages from Nlrp3 mutant mice with TLR agonists alone induced caspase-1 activation and robust IL-1β production (Brydges et al., 2009; Meng et al., 2009). Furthermore, genetic deletion or neutralization of IL-1 signaling induced marked improvement of disease, whereas ablation of Asc abrogated perinatal mortality and cutaneous disease in Nlrp3 mutant mice (Brydges et al., 2009; Meng et al., 2009).

Several hematopoietic cells, including macrophages, dendritic cells, neutrophils, and mast cells (MCs), can produce IL-1β in response to microbial and endogenous stimuli (Franchi et al., 2007; Ghiringhelli et al., 2009; Nakamura et al., 2009). Surprisingly, most of the IL-1β-positive cells in the skin lesions of CAPS patients are MCs (Nakamura et al., 2009). Furthermore, MCs can produce IL-1β via the Nlrp3 inflammasome (Nakamura et al., 2009). However, the role of MCs in skin disease associated with Nlrp3 mutations has not been investigated. Additionally, the environmental and host factors that initiate IL-1β-driven skin inflammation in the context of disease-associated Nlrp3 mutations remain elusive. In the present study, we show that MCs play a critical role in triggering disease in neonatal Nlrp3 mutant mice. Production of TNF-α and IL-1β by MCs was important for IL-1β-driven skin disease in Nlrp3 mutant mice. Furthermore, we provide evidence that the indigenous microbiota promote TNF-α and IL-1β production and contribute to skin disease in Nlrp3 mutant mice.

RESULTS

Cutaneous Inflammation Induced by $Nlrp3^{R258W}$ Mutation Requires MCs

Previous studies showed that the majority of IL-1β-producing cells in the dermis of skin lesions from CAPS patients are MCs (Nakamura et al., 2009). Therefore, we tested whether MCs play a role in skin inflammation of mice expressing an R258W mutation in Nlrp3. To assess this, we crossed heterozygous $Nlrp3^{R258W}$ mice with C57BL6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice to generate littermate mice expressing the $Nlrp3^{R258W}$ mutation in the normal and MC-deficient background. Within the first week after birth, $Nlrp3^{R258W}$ and wild-type littermates were indistinguishable. However, beginning 7 days after birth, $Nlrp3^{R258W}$ mice raised under pathogen-free conditions displayed impaired weight gain (Figure 1A). In contrast, the increase in body weight was comparable in $Nlrp3^{R258W}Kit^{W\text{-}sh/W\text{-}sh}$ and their control B6-$Kit^{W\text{-}sh/W\text{-}sh}$ littermates (Figure 1A). Notably, neonatal $Nlrp3^{R258W}$ mice developed elevated amounts of IL-1β in serum and splenomegaly, which was greatly attenuated or abolished in their $Nlrp3^{R258W}Kit^{W\text{-}sh/W\text{-}sh}$ littermates (Figures 1B and 1C). In addition, 100% of the $Nlrp3^{R258W}$ mice developed skin disease in the posterior collar area and perianal region by 2 weeks after birth (Figures 1D and 1E; Figure S1 available online). Furthermore, the incidence and severity of skin disease in $Nlrp3^{R258W}Kit^{W\text{-}sh/W\text{-}sh}$ mice was greatly reduced compared to their $Nlrp3^{R258W}$ littermates (Figures 1D and 1E; Figure S1). Consistent with these findings, the skin of $Nlrp3^{R258W}$ mice showed hyperkeratosis and marked neutrophil-rich infiltrate, which were absent or highly reduced in the same skin area of $Nlrp3^{R258W}Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 1F). As expected, abundant MCs were observed in the skin of $Nlrp3^{R258W}$ mice, but not in neonatal $Nlrp3^{R258W}Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 1G). Immunohistochemistry revealed that cells labeled with avidin (a marker of MCs) produced IL-1β in the dermis of neonatal $Nlrp3^{R258W}$ mice, but not in their $Nlrp3^{R258W}Kit^{W\text{-}sh/W\text{-}sh}$ or wild-type littermates (Figure 1G). These results indicate that MCs play a critical role in the development of cutaneous inflammation induced by the disease-associated $Nlrp3^{R258W}$ mutation.

Priming with LPS or TNF-α Is Sufficient to Induce Caspase-1 Activation and IL-1β Secretion in MCs Expressing the $Nlrp3^{R258W}$ Mutation

To begin to understand how MCs contribute to inflammatory skin disease, we prepared bone marrow-derived MCs (BMCMCs) from wild-type and $Nlrp3^{R258W}$ mice and determined their ability to produce IL-1β through the Nlrp3 inflammasome. BMCMCs from $Nlrp3^{R258W}$ and wild-type mice exhibited comparable morphology, expression of CD117, and the ability to release β-hexosaminidase in response to several stimuli (Figure S2). As previously reported (Nakamura et al., 2009), IL-1β secretion by MCs from wild-type mice required both priming with LPS and stimulation with ATP or the RNA-like molecule R837 (Figure 2A). In contrast, stimulation with LPS was sufficient to induce robust IL-1β secretion in MCs from $Nlrp3^{R258W}$ mice (Figure 2A). This differential response was specific in that MCs from wild-type and $Nlrp3^{R258W}$ mice produced comparable amounts of TNF-α in response to the same stimuli (Figure 2A). Whereas stimulation with both LPS and ATP was required to induce processing of

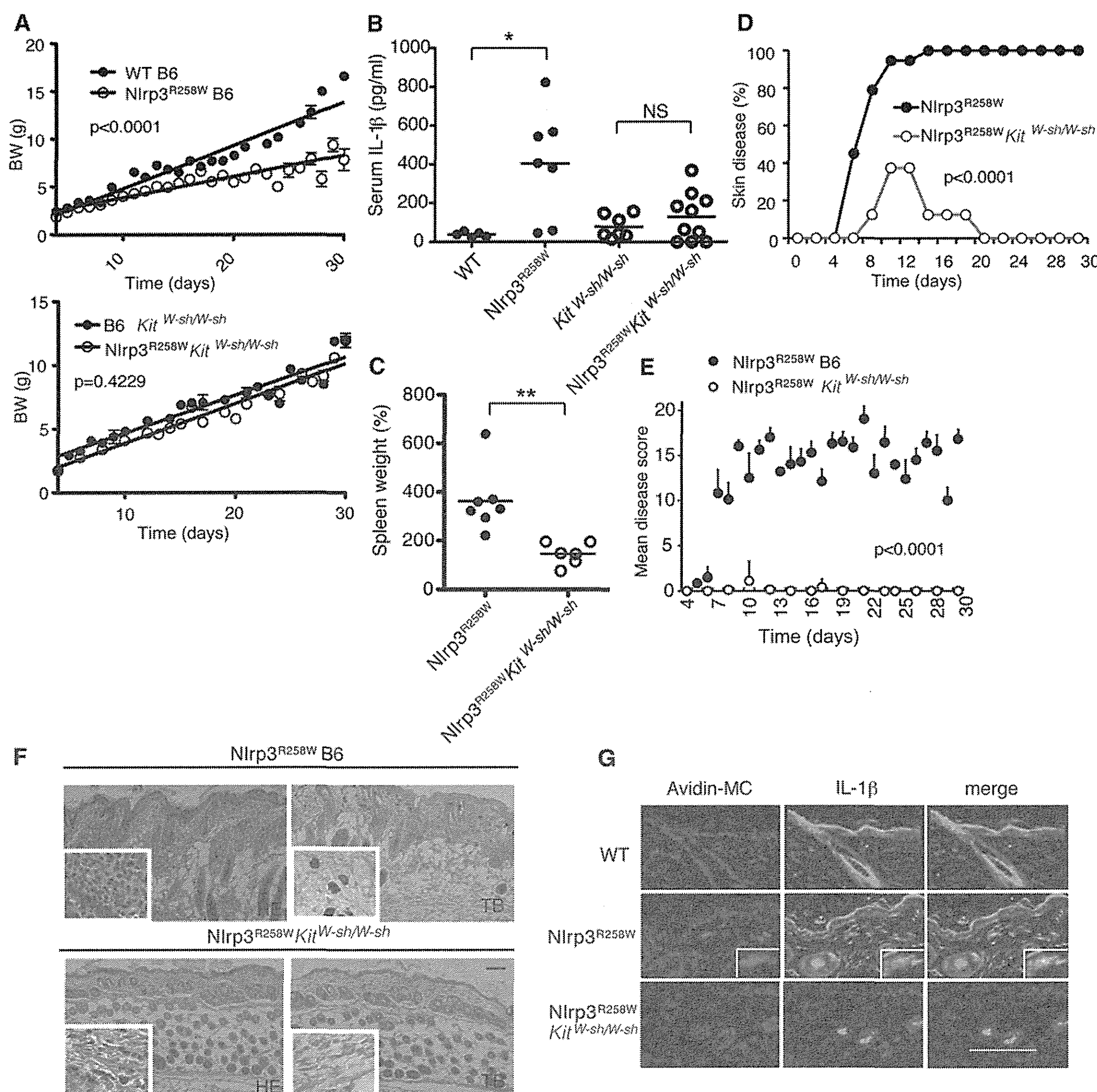

Figure 1. Cutaneous Inflammation in Nlrp3^{R258W} Mice Requires MCs

(A) Linear body weight (BW) curves of wild-type (WT, n = 13) and Nlrp3^{R258W} (n = 20) mice (left panel) and B6 *Kit*$^{W-sh/W-sh}$ (n = 10) and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$ (n = 10) mice (right panel). Error bars represent mean ± SEM.

(B) Amounts of IL-1β in serum of WT, Nlrp3^{R258W}, B6 *Kit*$^{W-sh/W-sh}$, and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$ mice.

(C) Spleen weight of WT, Nlrp3^{R258W}, B6 *Kit*$^{W-sh/W-sh}$, and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$ mice.

(D) Percentage of B6 *Kit*$^{W-sh/W-sh}$ and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$ mice that developed skin disease in the neonatal period. Results are derived from mice depicted in (A).

(E) Skin-disease score in Nlrp3^{R258W} and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$. Results are derived from mice depicted in (A). Error bar indicates mean ± SEM.

(F) H&E (HE) and toluidine blue (TB) staining of involved skin from Nlrp3^{R258W} and comparable region of Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$.

(G) Immunofluorescence staining of avidin-positive MCs (red) and IL-1β (green) of involved skin from Nlrp3^{R258W} and comparable region from WT B6 and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$. Merged images are also shown. Inset represents high magnification of avidin-positive MC producing IL-1β (yellow-orange color). Notice autofluorescence of epidermis and hair follicle in WT and Nlrp3^{R258W} mice. Scale bar represents 100 μm.

pro-caspase-1 into its active p20 subunit in wild-type MCs, stimulation with LPS was sufficient to activate caspase-1 in MCs from Nlrp3^{R258W} mice (Figure 2B). In addition, treatment with TNF-α alone induced IL-1β secretion in MCs from Nlrp3^{R258W} mice, whereas both TNF-α and ATP were required to induce IL-1β secretion in wild-type MCs (Figure 2C). Collectively, these results indicate that the Nlrp3^{R258W} protein is constitutively active in MCs in that IL-1β production only requires a priming step.

IL-1β Production by MCs Expressing the Nlrp3^{R258W} Mutation Is Important for Disease Development in Neonatal Nlrp3^{R258W} Mice

To determine whether IL-1β production is important for disease development, we crossed heterozygous Nlrp3^{R258W} mice with *Il1b*$^{-/-}$ mice to generate mice expressing the Nlrp3^{R258W} mutation in the presence and absence of IL-1β. Nlrp3^{R258W} mice lacking IL-1β developed normally after birth and, unlike their Nlrp3

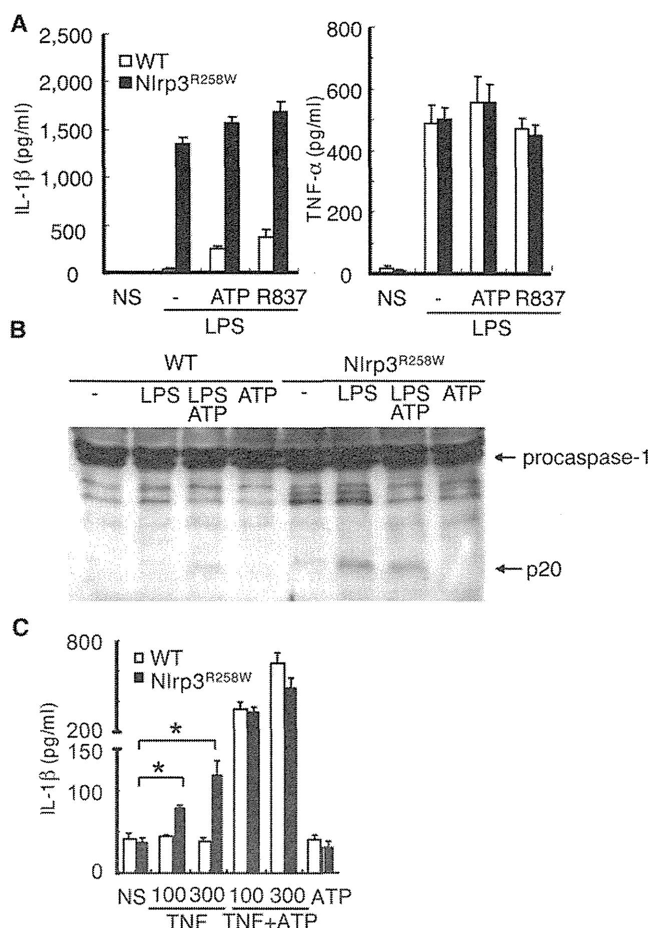

Figure 2. Priming with LPS or TNF-α Is Sufficient to Induce Caspase-1 Activation and IL-1β Secretion in MCs Expressing the Nlrp3^{R258W} Mutation

(A) BMCMCs were incubated with LPS (100 ng/ml) for 15 hr and stimulated with ATP (5 mM) or R837 (100 μM) for 30 min. IL-1β (left) and TNF-α (right) in culture supernatants was measured by ELISA.

(B) Immunoblot analysis of extracts of cells together with cell supernatant of BMCMCs from Nlrp3^{R258W} and wild-type (WT) mice. Cells were incubated with LPS (100 ng/ml) for 4 hr and then stimulated by ATP (5 mM) or left untreated.

(C) BMCMCs were pretreated with TNF-α (100 ng/ml or 300ng/ml) for 24 hr and stimulated with ATP (5 mM) for 30 min. IL-1β in culture supernatants were measured by ELISA. Error bars represent mean ± SD. Significance was determined by two-tailed Student's t test; $^{*}p < 0.05$. Data shown are representative of three independent experiments.

mutant littermates, they gained weight and did not develop skin disease (Figures 3A and 3B). To determine whether IL-1β production by MCs is important for disease development, we adoptively transferred BMCMCs from wild-type, Nlrp3^{R258W}, or Nlrp3^{R258W}*Il1b*$^{-/-}$ mice into the skin of B6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ or Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice at postneonatal day 1 (PND 1). MC-deficient recipient mice were reconstituted with wild-type or mutant MCs in the skin of the posterior collar area, where disease develops in 100% of neonatal Nlrp3^{R258W} mice. Neonatal B6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with MCs from wild-type, Nlrp3^{R258W}, or Nlrp3^{R258W}*Il1b*$^{-/-}$ mice contained 3- to 4-fold more MCs than their B6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ littermates and ~40% of conventional-type MCs present in the same skin area of age-matched wild-type or Nlrp3^{R258W} mice (Figure S3). Notably, Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ recipient mice adoptively transferred with MCs from Nlrp3^{R258W} mice, but not from Nlrp3^{R258W}*Il1b*$^{-/-}$ or wild-type mice, developed inflammatory skin disease at the site of MC reconstitution that was comparable to that observed in nonmanipulated Nlrp3^{R258W} mice (Figure 3C; Figure S3E). Disease development was associated with reduced body weight (Figure 3D) and production of IL-1β in the skin (Figure 3E). Notably, adoptive transfer of MCs from Nlrp3^{R258W} mice into B6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice did not induce skin disease (Figures 3C and 3E). These results indicate that MCs expressing the Nlrp3^{R258W} mutation and IL-1β are important for disease, but also suggest that another cell harboring the Nlrp3^{R258W} mutation is also required for disease development.

Depletion of the Microbiota Inhibits Disease Development in Nlrp3^{R258W} Mice

The skin lesions observed in newborn Nlrp3^{R258W} mice developed in limited areas such as the posterior collar area and perianal region (Figure S1), suggesting that environmental stimuli may play a role in triggering cutaneous disease. Because microbial TLR ligands such as LPS are necessary and sufficient to induce robust IL-1β production in MCs expressing the Nlrp3^{R258W} mutation (Figure 2A), we examined whether depletion of commensal bacteria with antibiotics could alter the development of skin disease. Newborn mice are colonized soon after birth by commensal bacteria derived from their mother (Hasegawa et al., 2010). Therefore, we treated pregnant female Nlrp3^{R258W} mice with a cocktail of antibiotics in the drinking water for 2 weeks, beginning 2 days prior to giving birth. This antibiotic treatment resulted in ~4-log depletion of culturable bacteria in the oral cavity of nursing mothers and ~3-log depletion in the skin of newborn mice (Figure 4A). Furthermore, antibiotic treatment resulted in robust reduction of skin bacteria in neonatal mice as assessed by quantitative PCR of eubacteria 16S ribosomal RNA (rRNA) DNA (Figure 4B) and did not alter the number of MCs in the skin (Figure S3). Importantly, treatment with antibiotics led to reduced disease incidence and score compared to untreated Nlrp3^{R258W} mice (Figure 4C; Figure S4). Furthermore, antibiotic-treated Nlrp3^{R258W} mice had less weight loss (Figure 4D) and splenomegaly (Figure 4E) than their untreated littermates. Furthermore, administration of antibiotics led to reduced amounts of IL-1β in the serum and skin of Nlrp3^{R258W} mice compared to untreated Nlrp3^{R258W} mice (Figures 4F and 4G). Notably, antibiotic treatment also reduced the amounts of TNF-α in the skin of both wild-type and Nlrp3^{R258W} littermates (Figure 4G). However, the production of TNF-α was comparable in the skin of untreated wild-type and Nlrp3^{R258W} littermates (Figure 4G). If the microbiota induces TNF-α to trigger skin disease in neonatal Nlrp3^{R258W} mice, administration of TNF-α or induced release of endogenous TNF-α release would be expected to trigger skin disease in antibiotic-treated Nlrp3^{R258W} mice. Notably, intradermal administration of compound 48/80, a molecule that induces the release of TNF-α, and other molecules from connective-tissue-type MC secretory granules (Mousli et al., 1990) or intradermal injection of recombinant (r) TNF-α induced skin disease (Figure 4H) and IL-1β production (Figure 4I) in antibiotic-treated Nlrp3^{R258W} mice, but not in wild-type littermates.

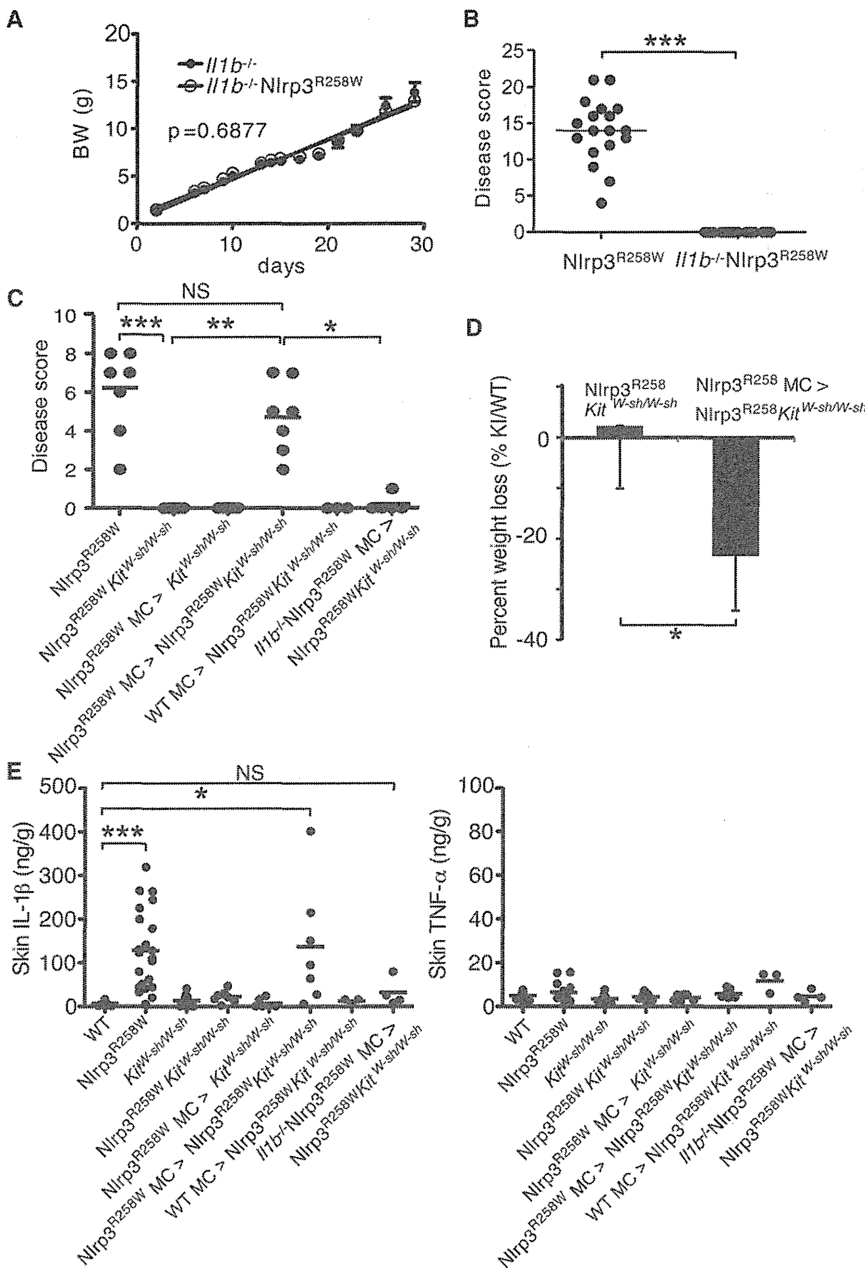

Figure 3. IL-1β Production by MCs Expressing the Nlrp3^{R258W} Mutation Is Important for Skin Disease in Nlrp3^{R258W} Mice

(A) Linear growth curve of Nlrp3^{R258W}*Il1b*$^{-/-}$ (n = 6) and *Il1b*$^{-/-}$ littermates (n = 8).

(B) Skin-disease score from 2-week-old Nlrp3^{R258W} and Nlrp3^{R258W}*Il1b*$^{-/-}$ mice. Dots represent individual mice. Bar represents mean values. ***$p < 0.001$.

(C) Skin-disease score in Nlrp3^{R258W}, Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$, and indicated recipient mice reconstituted in the skin with MCs from indicated mice at PND 1. Dots represent individual mice. Bar represents mean values.

(D) Percentage of weight loss of Nlrp3^{R258W} *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with MCs from Nlrp3^{R258W} mice at PND 1. Results shown are from 2-week-old mice and normalized to weight of control B6 *Kit*$^{W\text{-}sh/W\text{-}sh}$ littermates (WT). *$p < 0.05$. KI, knockin.

(E) IL-1β (left panel) and TNF-α (right panel) levels in skin of Nlrp3^{R258W}, Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$, and indicated recipient mice reconstituted in the skin with MCs from indicated mice at PND 1. Results are from 2-week-old mice. Bars represent mean values.

Significance in (D) and (E) was determined by one-way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS, not significant. Data shown are representative of two independent experiments (C)–(E).

These results indicate that the indigenous microbiota contribute to TNF-α and IL-1β production and the development of skin disease in newborn Nlrp3^{R258W} mice.

Neutralization of TNF-α Abrogates the Development of Skin Disease in Neonatal Nlrp3^{R258W} Mice

Because treatment with antibiotics reduced the amounts of TNF-α in the skin, and TNF-α alone can induce IL-1β secretion in MCs from Nlrp3^{R258W} mice (Figures 4G and 2C), we tested whether TNF-α plays a role in the development of skin disease in newborn Nlrp3^{R258W} mice. To assess this, we treated Nlrp3^{R258W} mice at PND 1 with TNF-α-blocking monoclonal antibody or isotype-matched control immunoglobulin G (IgG) intraperitoneally. Neutralization of TNF-α abrogated the loss of body weight, the development of skin disease, and splenomegaly in newborn Nlrp3^{R258W} mice (Figures 5A, 5B and 5C; Figure S5A). Histological analysis revealed that administration of TNF-α antibody prevented *inflammatory* skin disease *in newborn* Nlrp3^{R258W} mice (Figure 5D). Notably, treatment with TNF-α antibody inhibited the production of IL-1β in Nlrp3^{R258W} mice (Figure 5E). After weaning, cutaneous disease gradually disappeared in Nlrp3^{R258W} mice, but as previously reported (Meng et al., 2009), a significant number of adult Nlrp3^{R258W} mice relapsed at older age (>12 weeks) and developed skin disease affecting the ears, the top of the head, and the tail base region (Figure S5B). In contrast to neonatal mice, treatment with TNF-α antibody did not inhibit ongoing skin disease in adult Nlrp3^{R258W} mice (Figure 5F). Collectively, these results indicate that TNF-α is critical for the development of disease in neonatal mice, but its inhibition does not improve disease in adult Nlrp3^{R258W} mice.

MC-Intrinsic TNF-α, but Not IL-1β, Is Critical for Skin Disease Induced by Compound 48/40 in Adult Nlrp3^{R258W} Mice

TNF-α is a key cytokine that is stored in the granules of MCs (Echtenacher et al., 1996; Gordon and Galli, 1990; Maurer

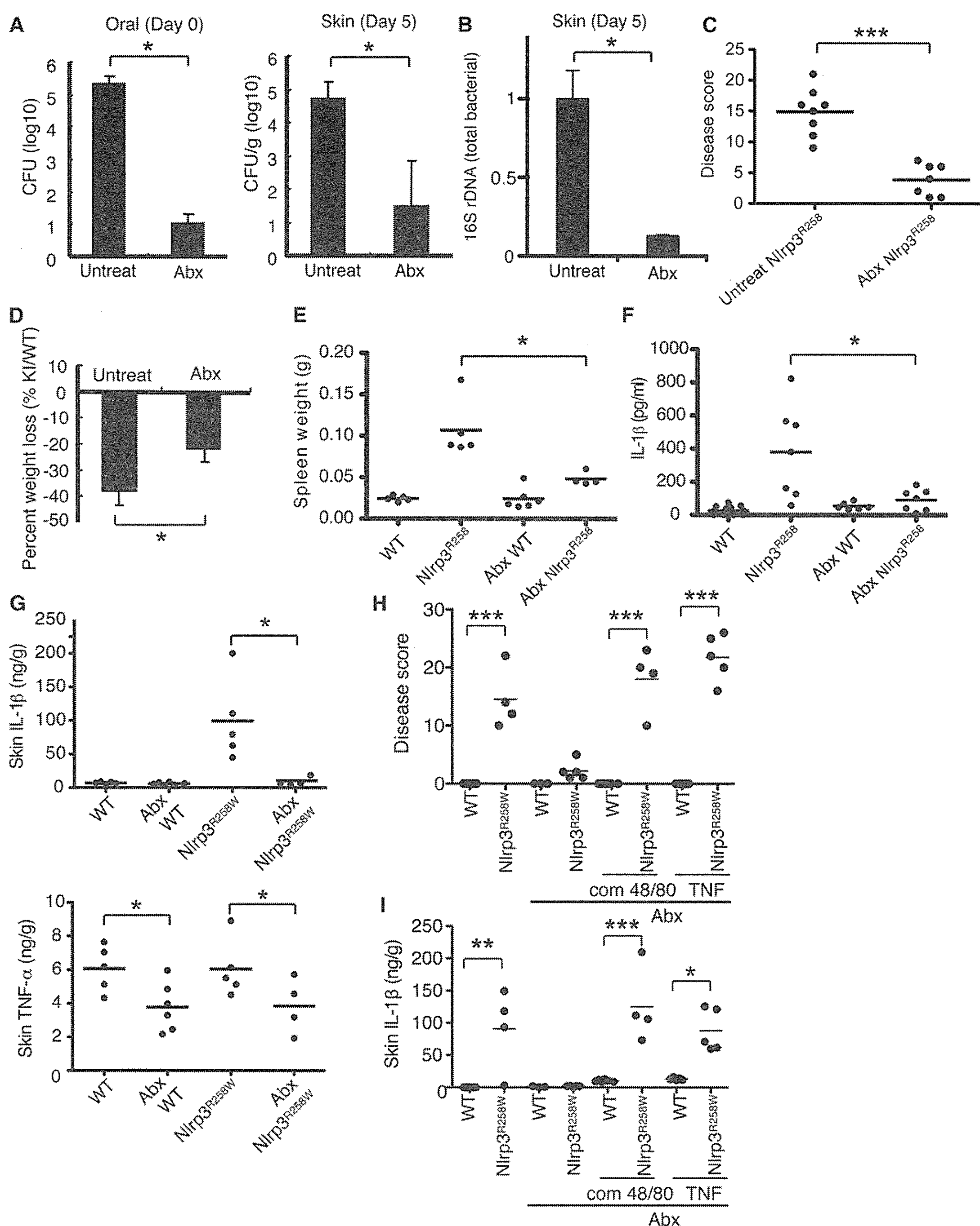

Figure 4. Depletion of the Microbiota Inhibits Disease Development in Nlrp3^{R258W} Mice

(A) Number of culturable bacteria in the oral cavity of nursing female mice (left panel) and skin (posterior collar region) of newborn mice (right panel) in untreated (Untreat) and antibiotic-treated (Abx) mice. Results are mean ± SD (n = 5).

(B) Normalized 16S rRNA gene analysis of the skin from untreated and Abx-treated mice.

(C) Skin-disease score in untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice. Dots represent individual mice. Bar represents mean values.

(D) Percentage of weight loss in untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice. Results shown are normalized to weight of control B6 littermates (WT). *$p < 0.05$. KI, knockin.

(E) Spleen weight of untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice and WT mice.

(F) and (G) IL-1β in serum (F) and skin (G, top panel) and TNF-α in skin (G, bottom panel) of untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice and wild-type littermates. Each dot represents an individual mouse. Horizontal bars indicate mean values.

(H) and (I) Skin-disease score (H) and skin IL-1β (I) in untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice induced by compound 48/80 or TNF-α intradermal injection. Dots represent individual mice. Bar represents mean values. (I) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data shown are representative of two independent experiments.

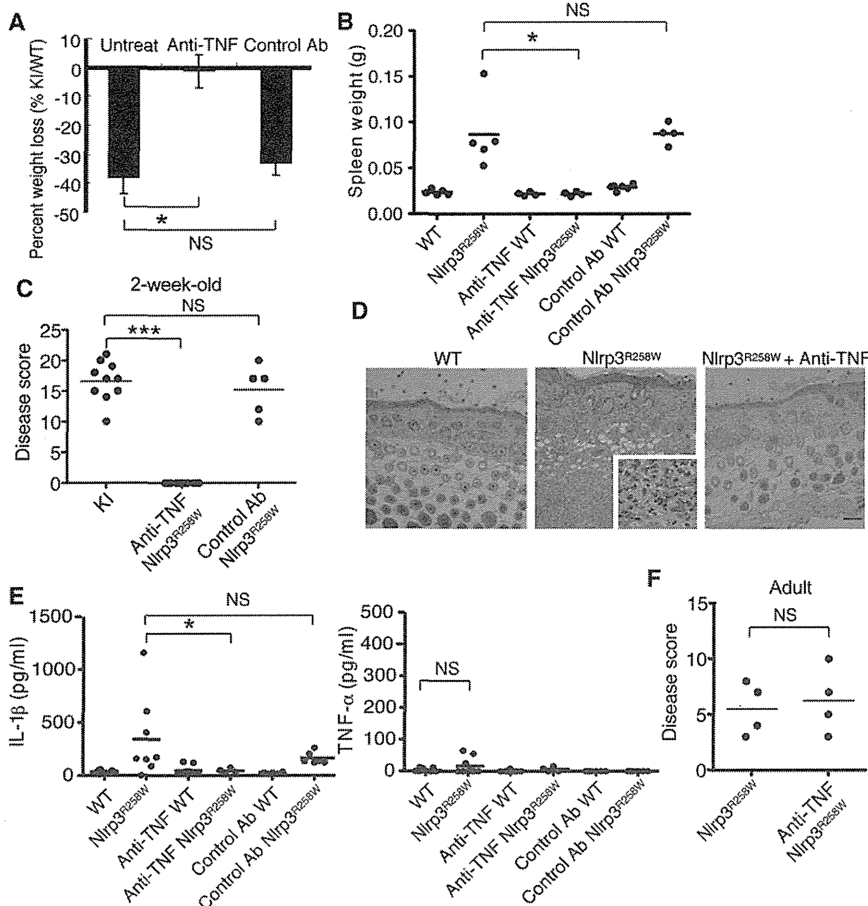

Figure 5. Neutralization of TNF-α Abrogates the Development of Skin Disease in Neonatal Nlrp3^{R258W} Mice

(A) Percentage of weight loss in untreated and Nlrp3^{R258W} mice treated with TNF-α antibody or control antibody at PND 1. Results shown are from 2-week-old mice normalized to the weight of control B6 littermates (WT). $^{*}p < 0.05$. KI, knockin.

(B) Spleen weight of 2-week-old untreated mice and antibody-treated Nlrp3^{R258W} and WT littermates. Mice were treated with control or TNF-α antibody at PND 1. $^{*}p < 0.05$. NS, not significant.

(C) Skin-disease score in 2-week-old untreated and antibody-treated Nlrp3^{R258W} littermates. Mice were treated with control or TNF-α antibody at PND 1. Dots represent individual mice. Bar represents mean values. $^{***}p < 0.001$. NS, not significant.

(D) Representative H&E staining of skin from 2-week-old untreated WT and Nlrp3^{R258W} littermates and a littermate treated with TNF-α antibody.

(E) Serum IL-1β (left panel) and TNF-α (right panel) levels in 2-week-old untreated WT and Nlrp3^{R258W} mice and antibody-treated littermates. Mice were treated with control or TNF-α antibody at PND 1.

(F) Skin-disease score in adult untreated and antibody-treated Nlrp3^{R258W} mice. Mice were treated with TNF-α antibody twice weekly. Horizontal bars indicate mean values. Dots represent individual mice. NS, not significant. Data shown are representative of three (A–D) and two (E and F) independent experiments.

et al., 2006; Suto et al., 2006). Therefore, we hypothesized that granule-associated TNF-α within MCs may be important in triggering skin disease in Nlrp3^{R258W} mice. To test this, we injected compound 48/80 into the uninvolved skin of wild-type, Nlrp3^{R258W}, and Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ adult mice and assessed disease and IL-1β production in the skin. Administration of compound 48/80 induced marked neutrophilic inflammation in the skin and IL-1β cytokine production at the injection site in Nlrp3^{R258W} mice, but not in wild-type or Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figures 6A and 6B; Figure S6). To determine whether TNF-α and/or IL-1β produced by MCs is important for inducing skin inflammation in Nlrp3^{R258W} mice, we adoptively transferred BMCMCs from wild-type, *Tnfa*$^{-/-}$, and *Il1b*$^{-/-}$ mice into the skin of adult Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. MC-deficient Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with MCs from wild-type, but not *Tnfa*$^{-/-}$ mice, developed neutrophilic skin inflammation and increased IL-1β production upon administration of compound 48/80 at the site of injection (Figures 6A and 6B). Notably, reconstitution with BMCMCs from *Il1b*$^{-/-}$ mice induced inflammation and IL-1β production (Figures 6A and 6B). These results indicate that TNF-α from MCs can induce IL-1β production and inflammatory skin disease in Nlrp3^{R258W} mice. Furthermore, MC-intrinsic IL-1β is not required for 48/80-induced skin disease and IL-1β production in adult Nlrp3^{R258W} mice.

Administration of TNF-α Induces IL-1β-Dependent Skin Inflammation in Adult Nlrp3^{R258W} Mice

We next asked whether TNF-α is sufficient to induce skin inflammation via IL-1β in adult Nlrp3^{R258W} mice. To test this, we first injected rTNF-α into the skin of the ear pinna of disease-free adult Nlrp3^{R258W} and wild-type mice. Administration of rTNF-α induced marked thickening and redness in the skin of *Nlrp3*^{R258W} B6 mice, but not wild-type mice (Figure S7). Furthermore, skin disease induced by rTNF-α was reduced in Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ and abrogated in Nlrp3^{R258W} mice lacking IL-1β (Figure S7). Histological examination revealed marked neutrophil infiltration in the dermis of Nlrp3^{R258W} mice injected with rTNF-α, which was abolished in Nlrp3^{R258W}*Il1b*$^{-/-}$ mice (Figure 7A). Consistent with these findings, the amounts of IL-1β in the skin of Nlrp3^{R258W} increased at the site of TNF-α administration, and this was also observed in Nlrp3^{R258W} *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figure 7B). Taken together, these results indicate that TNF-α can trigger skin inflammation via IL-1β in adult Nlrp3^{R258W} mice and that this is largely independent of MCs.

DISCUSSION

CAPS-associated missense *NLRP3* mutations result in enhanced activation of caspase-1 and secretion of IL-1β, leading to inflammatory disease in the skin and other organs. However,

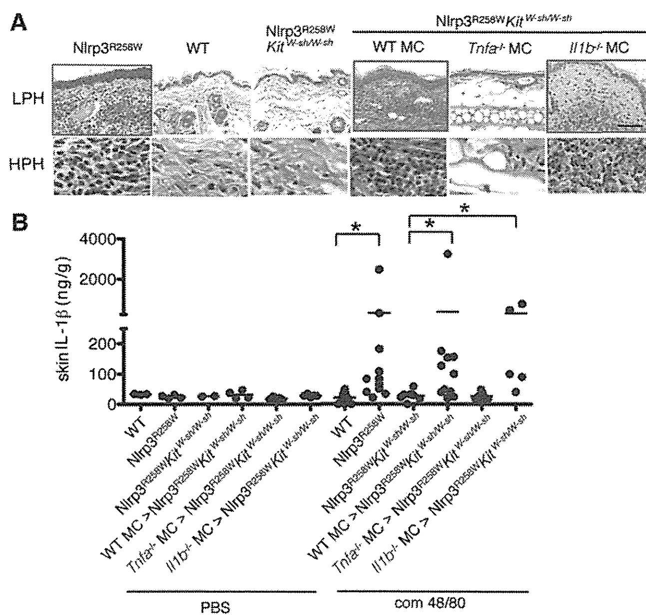

Figure 6. TNF-α Produced by MCs Can Induce Skin Disease in Nlrp3^{R258W} Mice

(A) Adult Nlrp3^{R258W}, B6 (WT), *Kit*$^{W-sh/W-sh}$, and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$ mice reconstituted with MCs from WT, *Tnfa*$^{-/-}$, and *Il1b*$^{-/-}$ mice. Mice were intradermally injected with compound 48/80 at the site of reconstitution. Representative images of low power histology (LPH) and high power histology (HPH) of H&E stained skin are shown. Bar indicates 100 μm.

(B) IL-1β levels in skin of wild-type (WT), Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$, and Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$ mice reconstituted with MCs from WT, *Tnfa*$^{-/-}$, and *Il1b*$^{-/-}$ mice. Mice were injected intradermally with compound 48/80 at the site of MC reconstitution. Bars represent mean values. Significance was determined by one-way ANOVA; *p < 0.05. NS, not significant. Data shown are representative of two independent experiments.

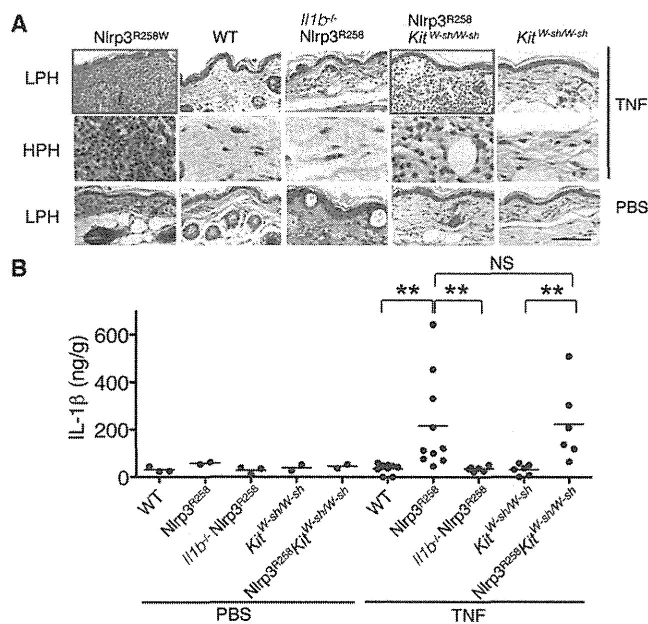

Figure 7. TNF-α Administration Induces IL-1β-Dependent Skin Inflammation in Nlrp3^{R258W} Mice

(A) Adult Nlrp3^{R258W}, B6 (WT), Nlrp3^{R258W}*Il1b*$^{-/-}$, Nlrp3^{R258W}*Kit*$^{W-sh/W-sh}$, and B6-*Kit*$^{W-sh/W-sh}$ mice were injected intradermally with TNF-α or PBS in the ear pinna. Representative images of low power histology (LPH) and high power histology (HPH) of H&E stained skin 48 hr after injection are shown. Bar indicates 100 μm.

(B) IL-1β levels in the skin of indicated mice injected intradermally with PBS or TNF-α in the ear pinna. Results shown are from 48 hr after injection. Bars represent mean values. Significance was determined by one-way ANOVA; **p < 0.01. NS, not significant. Data shown are representative of two independent experiments.

the cells and the mechanism that orchestrate NLRP3-dependent IL-1β-driven inflammatory disease in vivo remain unclear. We show here that MCs play a critical role in the initiation of skin inflammation and systemic disease in neonatal Nlrp3^{R258W} mice. Reconstitution experiments revealed that production of IL-1β by MCs expressing the *Nlrp3* mutation was important for skin disease and delayed growth in neonatal mutant mice. Our studies suggest that colonization of newborn mice by the microbiota induces local production of TNF-α, which primes MCs to elicit dysregulated production of IL-1β, causing skin and systemic disease. This model is consistent with the observation that MCs expressing the Nlrp3^{R258W} mutation require stimulation with TNF-α or LPS to secrete IL-1β. Unlike in normal cells, this priming step is sufficient to trigger caspase-1 activation and IL-1β secretion by inducing the expression of Nlrp3 and pro-IL-1β (Bauernfeind et al., 2009; Franchi et al., 2009b). In our Nlrp3^{R258W} model, the microbiota are critical to induce IL-1β-driven disease in neonatal mice. There is evidence that environmental factors may play a role in triggering CAPS in humans. For example, inflammatory episodes in FCAS are often induced by exposure to cold, whereas NOMID patients are born healthy, but they develop disease in early infancy. Thus, it is possible that exposure to cold, which induces cytokines or other proinflammatory molecules (Theoharides et al., 2004; Zhu et al., 1996), can prime MCs to elicit IL-1β-driven disease. Similarly, colonization by microbes could trigger inflammatory disease in NOMID patients. Clearly, further work is needed to understand how environmental factors contribute to the pathogenesis of CAPS in humans.

MCs are widely distributed throughout the body; they are located near epithelial surfaces such as the skin and airways that are normally exposed to environmental cues (Galli et al., 2008). Unlike macrophages, MCs can secrete a wide array of granule-associated, preformed biologically active products, including TNF-α, in response to a variety of stimuli, including microbes (Gordon and Galli, 1990). Hence, exposure to microbial products from commensal organisms could induce exocytosis of MCs and secretion of TNF-α to prime mutant MCs for IL-1β production in an autocrine and paracrine manner. Alternatively, newly synthesized TNF-α derived from stimulation of MCs by microbial molecules could mediate priming of MCs. Consistent with the former model, administration of 48/80, a compound that induces MC exocytosis, triggered IL-1β production and neutrophilic inflammation in the skin of Nlrp3^{R258W} mice, but not in Nlrp3^{R258W} mice deficient in MCs or in wild-type mice. Furthermore, reconstitution of MC-deficient Nlrp3^{R258W} mice with MCs from wild-type mice, but not *Tnfa*$^{-/-}$ mice, induced IL-1β production and skin inflammation at the site of compound

48/80 administration. Other key findings also support an important role for TNF-α in initiating skin disease in Nlrp3^{R258W} mice. First, neutralization of endogenous TNF-α abrogated the development of skin disease. Second, intradermal injection of TNF-α triggered local IL-1β production and inflammatory skin disease, which was blocked in the absence of IL-1β, specifically in adult Nlrp3^{R258W} mice. In adult Nlrp3^{R258W} mice, IL-1β production induced by administration of TNF-α was undisturbed in the absence of MCs. Similarly, MC-intrinsic IL-1β production was dispensable for skin disease triggered by intradermal administration of compound 48/80 into adult Nlrp3^{R258W} mice. Thus, IL-1β from a cell source other than MCs is important for skin disease induced by 48/80 or rTNF administration in Nlrp3^{R258W} mice. In these adult-mouse models, MC-derived TNF-α is likely to act on an intermediary myeloid cell-type that is the source of IL-1β to cause disease. A role for cells other than MCs in skin disease is also supported by the finding that adoptive transfer of MCs expressing the Nlrp3^{R258W} mutation induced skin disease in MC-deficient Nlrp3^{R258W} *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, but not in B6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Collectively, the results indicate that resident MCs expressing CAPS-associated NLRP3 mutations produce TNF-α locally upon exposure to commensals, leading to IL-1β secretion, which in turn primes additional innate immune cells expressing the NLRP3 mutation to amplify local IL-1β production and inflammatory disease. Although production of both TNF-α and IL-1β by MCs is important for inducing disease in the neonatal model, MC-independent production of IL-1β appears to be critical for disease in adult Nlrp3^{R258W} mice. Although additional studies are needed to identify the cell(s) involved, it is likely that macrophages, dendritic cells, or neutrophils expressing mutant Nlrp3 could contribute to IL-1β production and the development of disease.

Activation of the Nlrp3 inflammasome leads not only to IL-1β secretion, but also to production of IL-18, whose maturation is mediated by caspase-1 (Arend et al., 2008). In addition, monocytes from CAPS patients carrying disease-associated *NLRP3* mutations exhibit enhanced pyroptosis after stimulation with LPS (Fujisawa et al., 2007; Willingham et al., 2007). Therefore, it is possible that activities other than those induced via IL-1β contribute to inflammatory disease in CAPS patients. Consistent with this possibility, genetic deletion of IL-1 signaling was associated with marked improvement, but it did not abrogate disease in mice harboring the disease-associated A350V or L351P Nlrp3 mutations (Brydges et al., 2009). In contrast, we found that deletion of IL-1β in Nlrp3^{R258W} mice fully rescued the mutant mice from disease. In mice, the A350V or L351P Nlrp3 mutations confer a more severe phenotype than the R258W mutation, and this could explain, at least in part, the difference in results (Brydges et al., 2009; Meng et al., 2009). In the last decade, biological inhibitors of IL-1 signaling or IL-1β antibodies have become the most effective treatments for CAPS patients (Goldbach-Mansky et al., 2006; Hoffman et al., 2008; Lachmann et al., 2009). In contrast, anti-TNF-α treatment had some positive effects; however, its efficacy was very limited compared to anti-IL-1β therapies (Ebrahimi-Fakhari et al., 2010; Federico et al., 2003; Gunduz et al., 2008; Kallinich et al., 2005; Matsubara et al., 2006). In accordance with the human studies, neutralization of IL-1β with antibody was very effective in reducing inflammatory disease in adult Nlrp3^{R258W} mice (Meng et al., 2009). In contrast, TNF-α-blocking antibody abrogated the development of disease in neonatal Nlrp3^{R258W} mice, whereas it was ineffective in reducing ongoing disease in adult Nlrp3^{R258W} mice, which is consistent with studies in CAPS patients. These results suggest that TNF-α is important in the initiation phase of the disease, but it plays a minimal or no role at the later phases of the disease. Although further work is needed to understand the role of TNF-α in the pathogenesis of CAPS, one possibility is that TNF-α is critical for the initial induction of pathogenic IL-1β. Once IL-1β is produced, MCs and other IL-1β-producing cells expressing mutant Nlrp3 can rely on IL-1β for priming and eliciting IL-1β-driven inflammatory disease. The latter could explain why therapies targeting IL-1β are very effective in treating CAPS patients with disease, whereas anti-TNF-α blockade is not. In addition to CAPS, another IL-1β-driven autoinflammatory disease, familial Mediterranean fever (FMF), is associated with excess production of IL-1β through NLRP3-independent activation of caspase-1 (Chae et al., 2006; Papin et al., 2007). A recent study demonstrated that FMF-associated pyrin mutant mice also developed skin inflammation at as early as 1 week of age and suffered from severe inflammation in multiple tissues (Chae et al., 2011). Because the time course and disease phenotype of pyrin mutant mice are similar to those of Nlrp3 mutant mice, it is possible that MC-mediated mechanisms observed in Nlrp3^{R258W} mice also contribute to the development of disease in pyrin mutant mice and related IL-1β-driven diseases in humans.

EXPERIMENTAL PROCEDURES

Mice

Nlrp3^{R258W} mice have been described (Meng et al., 2009). C57BL/6, C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (B6.CG-*Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ), and *Tnfa*$^{-/-}$ (B6.129S6-*Tnf*^{tm1Gkl}/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). B6 *Il1b*$^{-/-}$ mice were originally obtained from Y. Iwakura (University of Tokyo, Tokyo). All strains were housed under pathogen-free conditions. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

Preparation and Adoptive Transfer of Mast Cells

The preparation of BMCMCs was previously described (Yamada et al., 2003). The purity of MCs was > 95% based on toluidine blue staining and surface expression of CD117 and FcεR.

Adoptive transfer of MCs into MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was performed as described (Grimbaldeston et al., 2005). Briefly, BMCMCs were obtained after a 4–5 week culture of bone marrow cells in medium containing 20 ng/ml recombinant mouse IL-3 (R&D Systems). For MC-reconstitution studies, BMCMCs were adoptively transferred via intradermal injection of 2×10^6 cells in 40 μl PBS into the ear pinna (at 4–8 weeks old) or posterior collar area (at PND 1) of MC-deficient recipient mice. Mice were evaluated at 2–3 weeks (neonatal mice) or 4–5 weeks (adult mice) after intradermal transfer of BMCMCs.

Administration of TNF-α Antibody and Antibiotics

Anti-TNF-α-blocking IgG (rat clone; MPG-XT3) was a gift from T. Moore (University of Michigan). Newborn mice were given 60 μg in 30 μl of TNF-α antibody or isotype-matched control rat IgG (Sigma-Aldrich), intraperitoneally. Adult Nlrp3^{R258W} mice were treated with 500 μg of TNF-α antibody intraperitoneally, weekly for 2 weeks. For antibiotic treatment, pregnant mice received an antibiotic cocktail (1 g/L of ampicillin, 0.5 g/L of vancomycin, 1 g/L of metronidazole, and 1 g/L of neomycin) in the drinking water 2 days before delivery, and the treatment continued for 2 weeks. Newborn mice were given 50 μg in 40 μl of rTNF-α at PND 3 or 1 μg in 40 μl of compound 48/80 at PND 3 and PND 6,

intradermally. Bacterial depletion was assessed in the mothers' oral cavity and newborns' skin through collection and homogenization of tissue in sterile PBS. The number of bacteria was determined by serial dilution of tissue samples, followed by serial plating on brain-heart infusion broth for 48 hr at 37°C under aerobic conditions.

Skin-Disease Score

The severity of skin disease was evaluated using a modified skin-index scoring system (Matsuda et al., 1997). Briefly, skin inflammation was evaluated every other day as a cumulative score. The total disease-severity score from three areas (head and neck, body, and perianal region) was defined as the sum of the individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of four disease signs (erythema, alopecia, scales, and erosions). The maximum score was 36.

Intradermal Injection

Adult mice (6–12 weeks old) were given compound 48/80 (1 μg in 20 μl per ear pinna; Sigma-Aldrich), rTNF-α (500 ng in 20 μl per ear pinna; PeproTech), or control PBS via intradermal injection. Tissue samples were collected 48 hr after injection for analysis.

Histology

Skin tissue was formalin fixed, paraffin embedded, and sectioned for hematoxylin and eosin (H&E), toluidine blue, and safranin O staning. For immunofluorescence staining, sections were subjected to labeling with anti-IL-1β (Armenian Hamster clone B122; Leinco Technologies) followed by fluorescein isothiocyanate-conjugated secondary antibodies and Texas red-Avidin staining.

Immunoblotting

Cells were lysed together with the cell supernatant by the addition of 1% NP-40 complete protease inhibitor cocktail (Roche) and 2 mM dithiothreitol. Clarified lysates were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes by electroblotting. The rabbit anti-mouse caspase-1 was a kind gift from P. Vandanabeele (Ghent University, Belgium).

Preparation of Skin Extracts

Skin tissue (5 × 5 mm area) was removed and homogenized as illustrated in Figure S2A. The skin homogenates were centrifuged and supernatants were collected for cytokine measurements by ELISA.

Cytokine Levels

Chemokines and cytokines were measured with ELISA kits (R&D Systems).

Statistical Analysis

Most of the data were compared by two-tailed t test with unequal variance (GraphPad Prism). Analysis of data involving multiple comparisons was performed by one-way ANOVA. The slope of two regression lines was compared using GraphPad Prism. Differences were considered significant when p values were less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.04.013.

ACKNOWLEDGMENTS

We thank S. Koonse for animal husbandry, J. Whitfield for ELISAs, and Y. Iwakura for the generous gift of mice. This work was supported by NIH grants R01AR059688 and R01AI06331, and funds to the Michigan Comprehensive Cancer Center Immunology Monitoring Core from the University of Michigan's Cancer Center Support Grant. Y.N. was supported by fellowships from Chiba University Global COE Program, the Cell Science Research Foundation, and the Kanae Foundation for the Promotion of Medical Science.

Received: December 26, 2011
Revised: March 20, 2012
Accepted: April 17, 2012
Published online: July 19, 2012

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