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.

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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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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

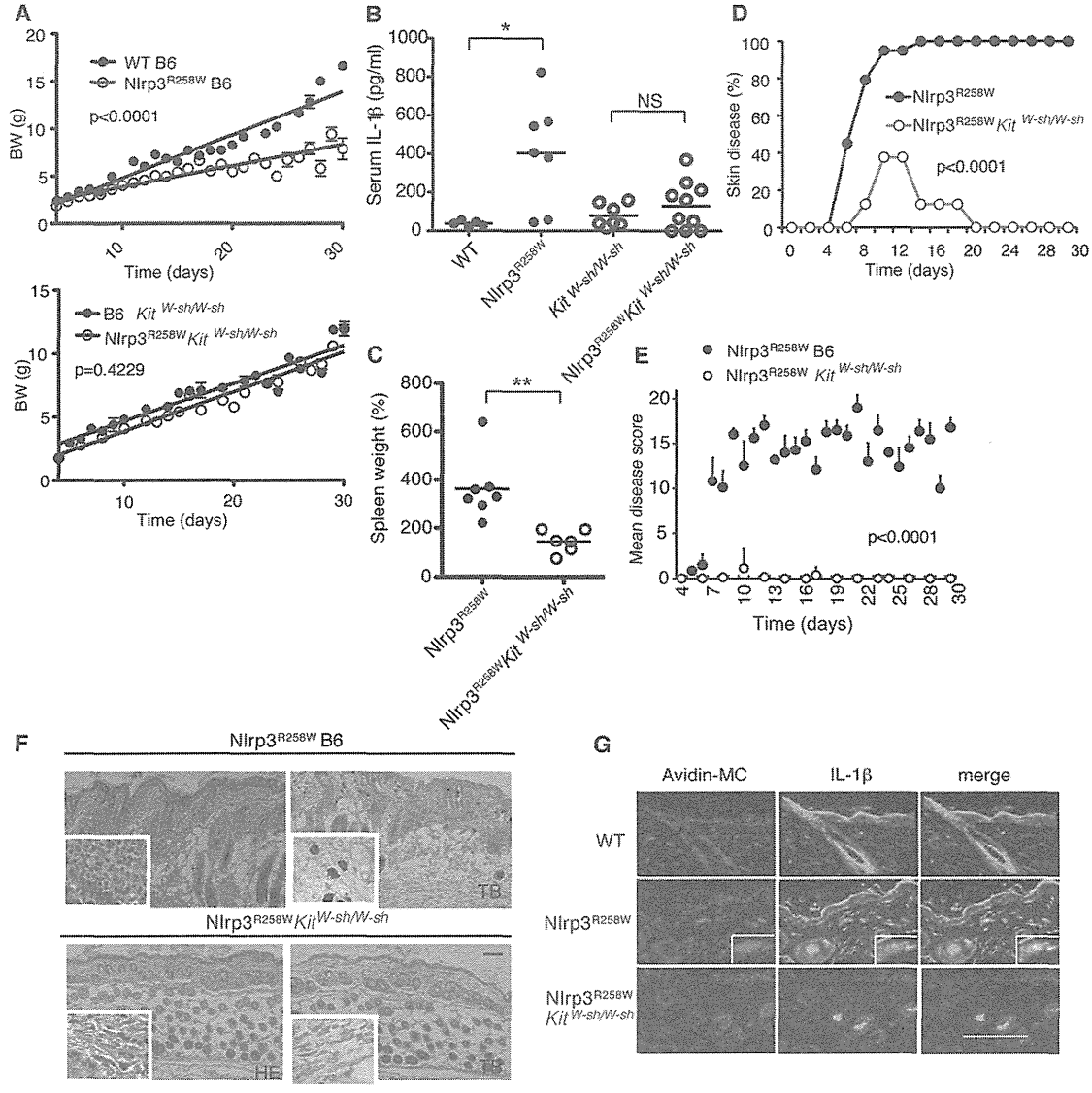

Figure 1. Cutaneous Inflammation in Nlrp3^{R258W} Mice Requires MCs

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

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

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

(D) Percentage of B6 *Kit*$^{W\text{-}sh/W\text{-}sh}$ and Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ 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*$^{W\text{-}sh/W\text{-}sh}$. 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*$^{W\text{-}sh/W\text{-}sh}$.

(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*$^{W\text{-}sh/W\text{-}sh}$. 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

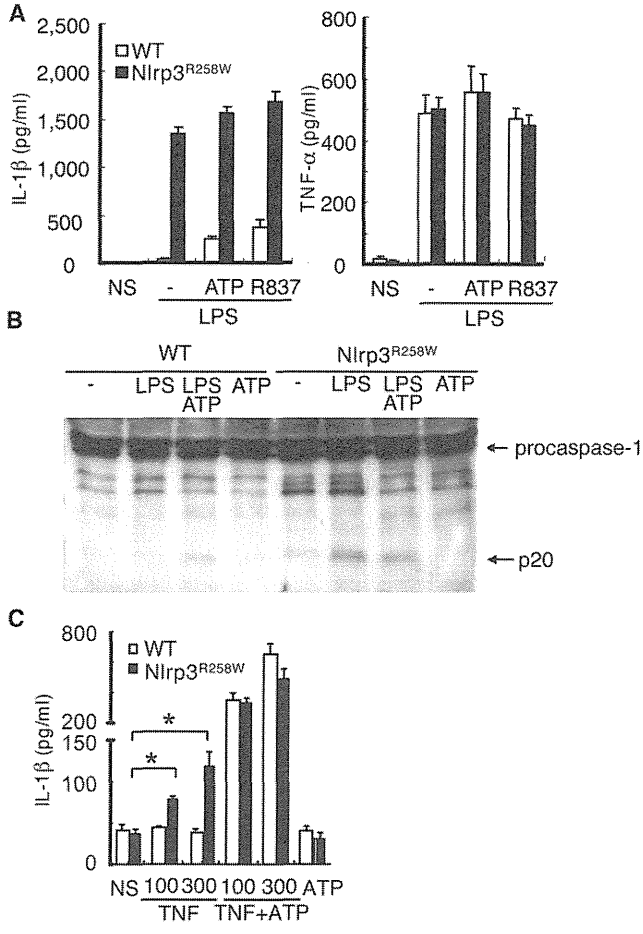

Figure 2. Priming with LPS or TNF-α Is Sufficient to Induce Caspase-1 Activation and IL-1β Secretion in MCs Expressing the Nlrp3^{R258W} Mutation

(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.

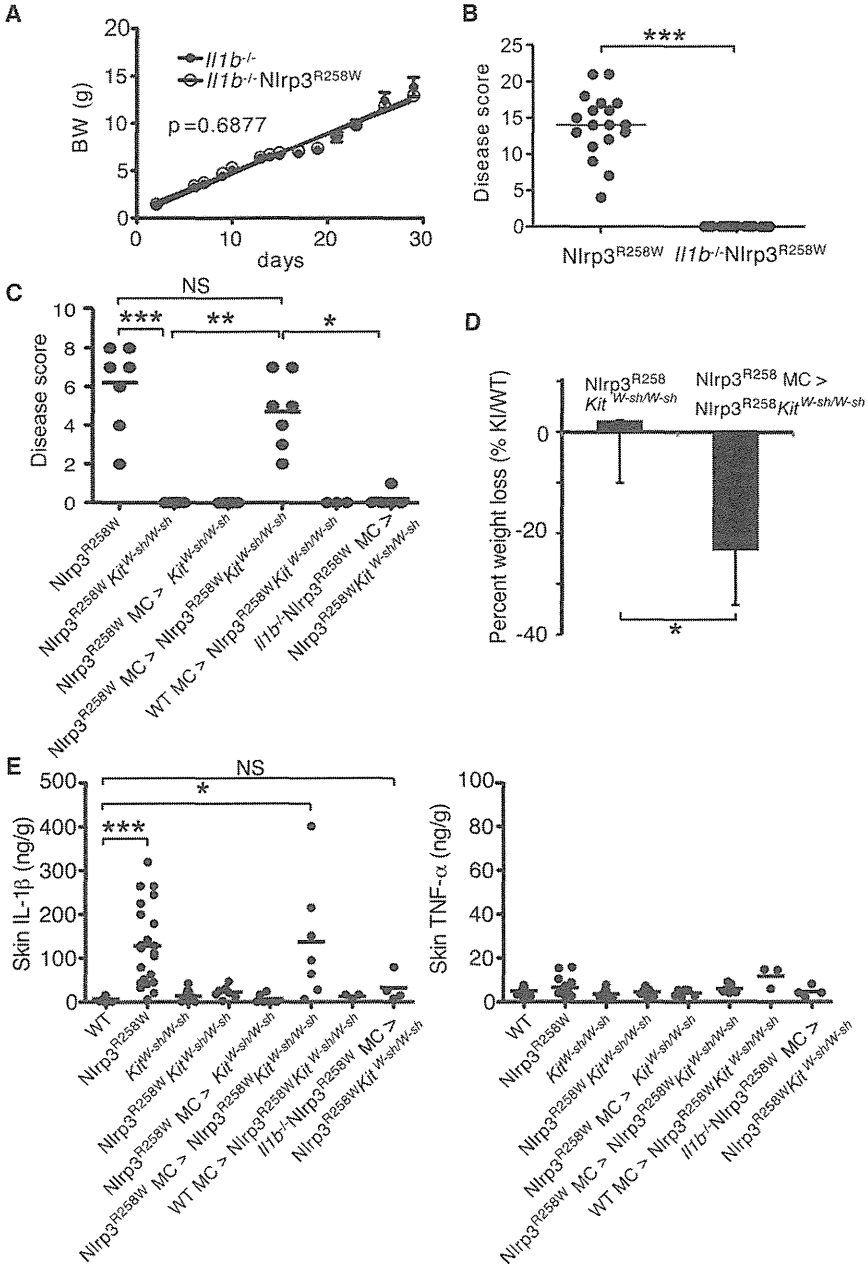

Figure 3. IL-1β Production by MCs Expressing the Nlrp3^{R258W} Mutation Is Important for Skin Disease in Nlrp3^{R258W} Mice

(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

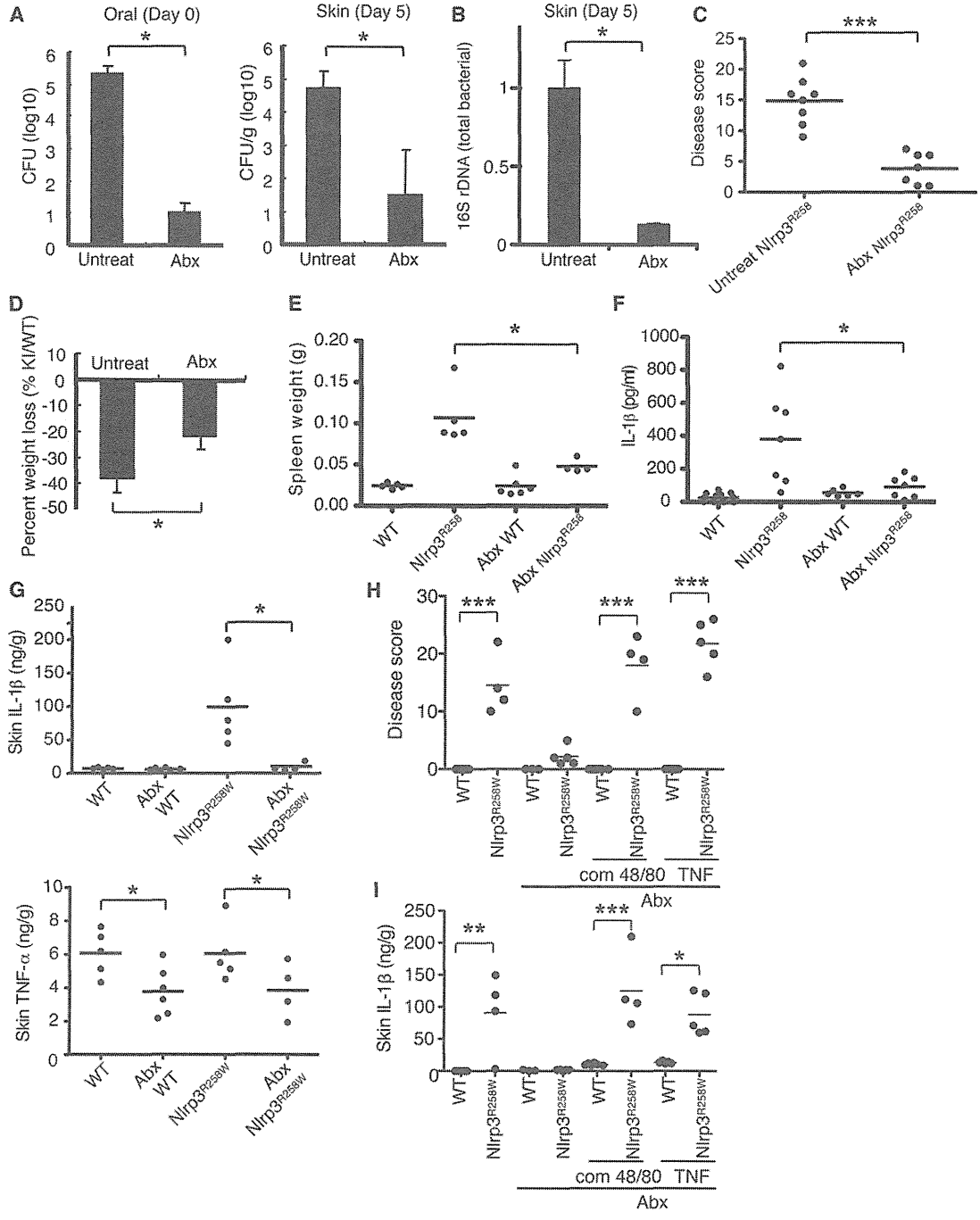

Figure 4. Depletion of the Microbiota Inhibits Disease Development in Nlrp3^{R258W} Mice

(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.

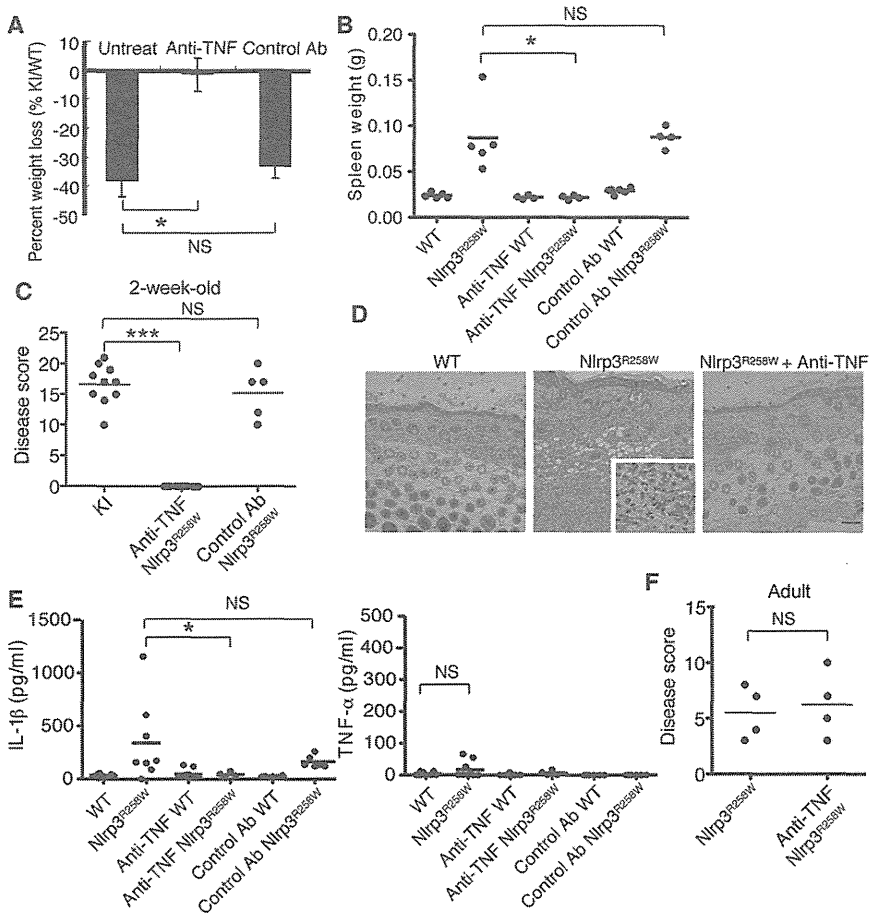

Figure 5. Neutralization of TNF-α Abrogates the Development of Skin Disease in Neonatal Nlrp3^{R258W} Mice

(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,

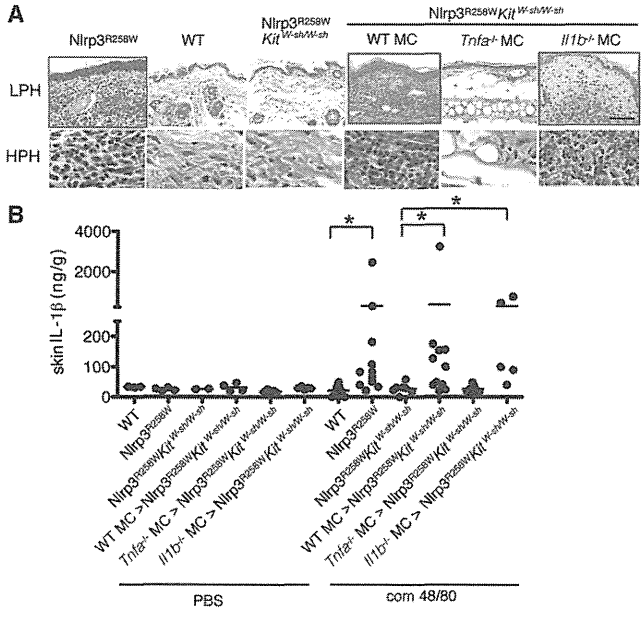

Figure 6. TNF-α Produced by MCs Can Induce Skin Disease in Nlrp3^{R258W} Mice

(A) Adult Nlrp3^{R258W}, B6 (WT), *Kit*$^{W\text{-}sh/W\text{-}sh}$, and Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ 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*$^{W\text{-}sh/W\text{-}sh}$, and Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$ 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.

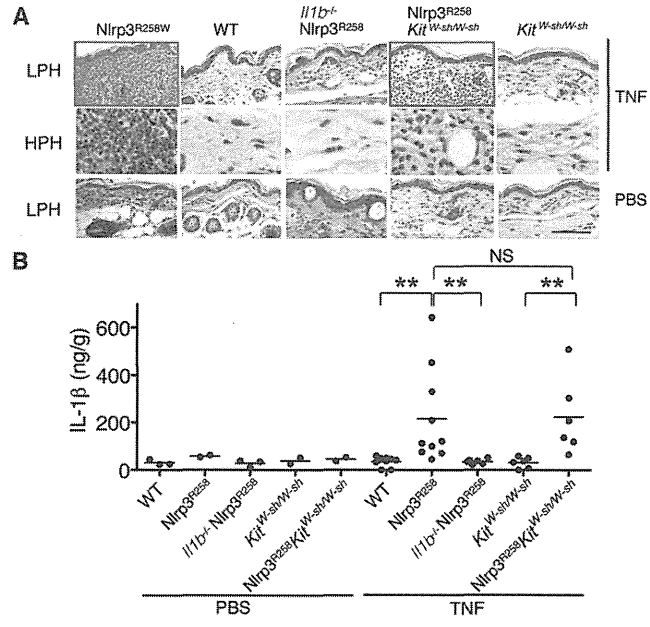

Figure 7. TNF-α Administration Induces IL-1β-Dependent Skin Inflammation in Nlrp3^{R258W} Mice

(A) Adult Nlrp3^{R258W}, B6 (WT), Nlrp3^{R258W}*Il1b*$^{-/-}$, Nlrp3^{R258W}*Kit*$^{W\text{-}sh/W\text{-}sh}$, and B6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ 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.

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REVIEW ARTICLE

Review Series: Primary Immunodeficiency and Related Diseases

Nakajo-Nishimura Syndrome: An Autoinflammatory Disorder Showing Pernio-Like Rashes and Progressive Partial Lipodystrophy

Nobuo Kanazawa[1]

ABSTRACT

Nakajo-Nishimura syndrome (ORPHA2615; also registered as Nakajo syndrome in OMIM#256040) is a distinct inherited inflammatory and wasting disease, originally reported from Japan. This disease usually begins in early infancy with a pernio-like rash, especially in winter. The patients develop periodic high fever and nodular erythema-like eruptions, and gradually progress lipomuscular atrophy in the upper body, mainly the face and the upper extremities, to show the characteristic thin facial appearance and long clubbed fingers with joint contractures. So far about 30 cases have been reported from Kansai, especially Wakayama and Osaka, Tohoku and Kanto areas. At present, about 10 cases are confirmed to be alive only in the Kansai area, including one infant case in Wakayama. However, more cases are expected to be added in the near future. Although cause of the disease has long been undefined, a homozygous mutation of the *PSMB8* gene, which encodes the β5i subunit of immunoproteasome, has been identified to be responsible in 2011. By analyses of the patients-derived cells and tissues, it has been suggested that accumulation of ubiquitinated and oxidated proteins due to immunoproteasome dysfunction causes hyperactivation of p38 mitogen-activated protein kinase and interleukin-6 overproduction. Since similar diseases with *PSMB8* mutations have recently been reported from Europe and the United States, it is becoming clear that Nakajo-Nishimura syndrome and related disorders form proteasome disability syndromes, a new category of autoinflammatory diseases distributed globally.

KEY WORDS

immunoproteasome, Nakajo-Nishimura syndrome, partial lipodystrophy, pernio, PSMB8

DEFINITION OF THE DISEASE

Nakajo-Nishimura syndrome (NNS) was first reported as "secondary hypertrophic osteoperiostosis with pernio" in 1939 by Dr. Nakajo, a medical staff of Tohoku University Department of Dermatology and Urology.[1] He described a brother and a sister cases of a consanguineous family showing pernio and clubbed fingers accompanied with periosteal thickening and suspected that peripheral circulatory failure due to cardiac insufficiency was the disease cause. In 1950, Dr. Nishimura, the first professor of Wakayama Medical University Department of Dermatology and Urology, and colleagues further reported three cases of two consanguineous families showing the similar phenotype and pointed out a possibility that this disease was a primary inherited disease.[2] Several cases were subsequently reported by dermatological groups mainly in Kansai area and, in 1985, Dr. Kitano and colleagues of Osaka University Department of Dermatology summarized 12 cases of 8 families including their own 4 cases and reported them in Archived of Dermatology as a novel "syndrome with nodular erythema, elongated and thickened fingers, and emaciation".[3] According to this report, this disease has been registered in Online Mendelian Inheri-

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Table 1 Tentative criteria for the clinical diagnosis of NNS

A clinical diagnosis of NNS can be made if at least 5 of the following 8 features are positive.

1. Autosomal recessive inheritance (parental consanguinity and/or familial occurrence)
2. Pernio-like purplish rash in hands and feet (appearing in winter since infancy)
3. Haunting nodular erythema with infiltration and induration (sometimes circumscribed)
4. Repetitive spiking fever (periodic, not necessarily)
5. Long clubbed fingers and toes with joint contractures
6. Progressive partial lipomuscular atrophy and emaciation (marked in the upper part of body)
7. Hepatosplenomegaly
8. Basal ganglia calcification

tance in Man (OMIM), an international online database of human genes and genetic diseases, as Nakajo syndrome (OMIM#256040) and in ORPHANET, an European website collecting rare diseases and orphan drugs, as Nakajo syndrome (ORPHA1953: nodular erythema-digital changes) and Nakajo-Nishimura syndrome (ORPHA2615: amyotrophy-fat tissue anomaly).

In the field of internal medicine, a sporadic case of "collagen disease-like disease with skin eruption, muscular atrophy, splenomegaly, hyper γ-globulinemia and decreased IgA" was reported as an atypical dermatomyositis by a group of Nihon University in 1971 and raised physicians' attention.[4] The report of this case was later published as "a case of partial lipodystrophy with erythema, dactylic deformities, calcification of the basal ganglia, immunological disorders and low IQ level.[5]" Three cases of two consanguineous families with a similar disease were further reported as a particular lipodystrophy by groups of Akita University Department of Internal Medicine and Niigata University Department of Neurology.[6,7] After it was pointed out that the disease of these cases seemed to be the same as the one formerly reported by dermatologists, they were unified as "hereditary lipo-muscular atrophy with joint contracture, skin eruptions and hyper-γ-globulinemia" and reported in Japan Medical Journal in 1991 and in Internal Medicine in 1993.[8,9]

On the other hand, from the field of pediatrics, the first reported case was an adult case of a consanguineous family, originally described as lupus profundus in 1985 and revised as a lipodystrophy-like hereditary disease with basal ganglia calcification in 1989, by a group of Kochi University.[10,11] However, a series of child cases have only been reported in a meeting by Dr. Sugino and colleagues of Wakayama Medical University in 1986.[12] They reported four child cases, three of whom were born in consanguineous parents, of a hereditary disorder showing partial lipodystrophy-like appearance, pernio-like eruptions, long clubbed fingers, basal ganglia calcification and positive inflammatory reactions, and proposed a new disease entity. In 2006, they focused on the characteristic periodic fever and limited localization of the disease and proposed the designation "familial Japanese fever", in contrast to the major autoinflammatory disorder, familial Mediterranean fever.[13]

Thus, although this disease was considered as an autosomal recessively-inherited disease uniquely reported in Japan, its causative gene has long been unidentified. In 2006, Dr. Ida of Nagasaki University Department of Internal Medicine and Kanazawa of Wakayama Medical University Department of Dermatology, who are expertized at autoinflammatory syndromes, found this disease and started a genetic analysis. By a collaborative research with Dr. Yoshiura of Nagasaki University Department of Human Genetics, a homozygous missense mutation of the *PSMB8* gene encoding the β5i subunit of immunoproteasome (IP) has finally been identified in 2009, and the proteasome disability has proven to be associated with this disease.[14] At the same time, Dr. Yasutomo and colleagues of Tokushima University have independently identified the same mutation in cases of Akita and Niigata.[15] Furthermore, phenotypically similar cases with joint contractures, muscular atrophy, microcytic anemia and panniculitis-associated lipodystrophy (JMP) syndrome (formally registered in OMIM as 613732) and chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome were reported in 2010 from groups of the United States and Spain, respectively.[16,17] Since other mutations in *PSMB8* reducing the IP activity have been identified in both syndromes, NNS and these diseases are now considered to form proteasome disability syndromes, a new category of autoinflammatory diseases, distributed globally.[18,19]

EPIDEMIOLOGY

Following the acceptance of this disease under the designation of Nakajo-Nishimura syndrome as one of the 177 diseases listed for the promoting division of Research Project to Overcome Intractable Diseases among grants from the Japan Ministry of Health, Labor and Welfare in 2009, national surveillance was performed.[20] To this aim, tentative criteria for clinical diagnosis of NNS were determined as shown in Table 1. At first, eight common characteristic features were

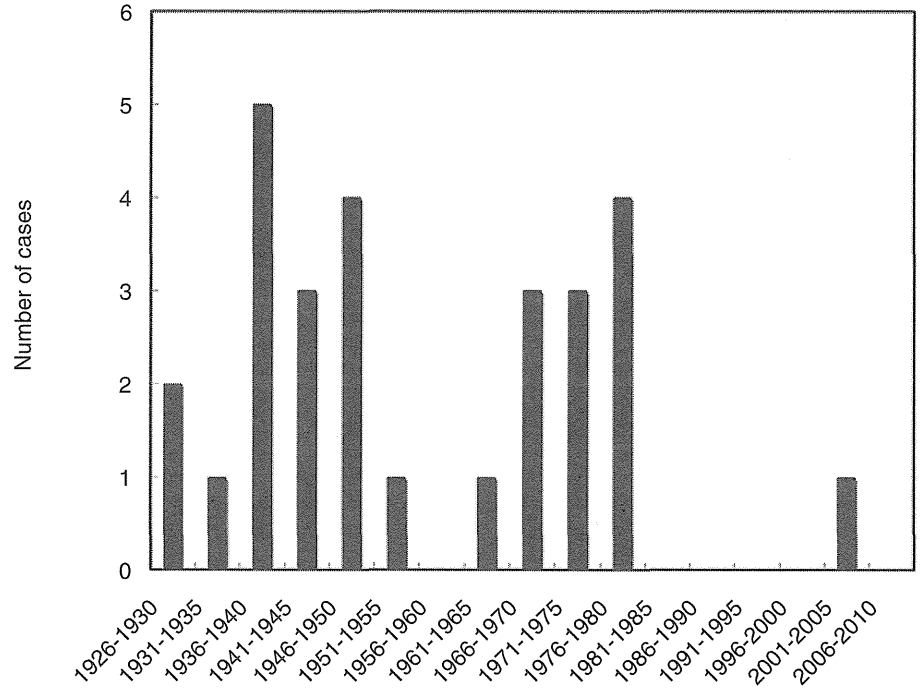

Fig. 1 Years at birth of the identified 28 NNS cases.

selected for the list of criteria, according to the summary of 11 NNS cases in Wakayama; parental consanguinity and/or familial occurrence, pernio-like purplish rash in hands and feet, haunting nodular erythema with infiltration and induration, repetitive spiking fever, long clubbed fingers and toes with joint contractures, progressive partial lipomuscular atrophy and emaciation, hepatosplenomegaly, and basal ganglia calcification. Then, it was proposed that, among these features, cases showing at least five are definite, while cases showing more than two are suspected, to be NNS. A questionnaire asking experience of such cases in the last five years was sent to Departments of Metabolic Medicine, Endocrinology, Rheumatology, Neurology, Dermatology, Pediatrics and Orthopedics in all University Hospitals (623 Departments) and General Hospitals equipped with more than five hundreds beds (1193 Departments). Although answers were returned from 371 Departments of University Hospitals and 433 Departments of General Hospitals, none of new cases, definite or suspected, have been informed other than previously reported cases or those who were already referred to us. Already reported and known cases include 7 cases of 5 families in Tohoku and Kanto (Miyagi, Akita, Niigata and Tokyo) and 20 cases of 17 families in Kansai area (Wakayama, Osaka and Nara). Many of them have not been followed and still followed were only 10 cases in Kansai.

At the same time, a 5-year-old boy followed since 3 years before in Departments of Dermatology and Pediatrics of Wakayama Rosai Hospital with three hundreds beds has been revealed to be a new case of NNS which appeared at 20 years' interval after the last case, because he satisfied its diagnostic criteria and actually harbored the homozygous *PSMB8* mutation. Accordingly, surveillance was further performed by sending the same questionnaire to the Departments in General Hospitals with more than three hundreds beds in Tohoku and Kansai areas (761 Departments), but no other novel cases have been discovered. As shown in Figure 1, years at birth of these 28 cases are concentrated in 1930/40's and 1960/70's. If most cases were actually born at 30 years' interval, new cases born in 1990/2000's might appear in the near future.

CLINICAL FEATURES AND LABORATORY FINDINGS

28 cases include 19 male and 9 female and therefore male cases are twice more than female ones. Consanguinity or familial history is observed in about seventy percent of the affected families. Clinical features of a female case born in consanguineous parents are shown in Figure 2.[21] All cases except for one case onset in infancy at the age from 2 months to 8 years old and most of them show pernio-like rash as the first symptom (Fig. 2a). Typically, severe pernio appears in the first winter after birth and repeats every year, and therefore in some cases the underlying disease was not recognized by either of the patient or the doctor. As a skin manifestation, so-called nodular erythema-like eruptions which can be palped as red, slightly swollen, well-defined, hard nodules or infiltra-

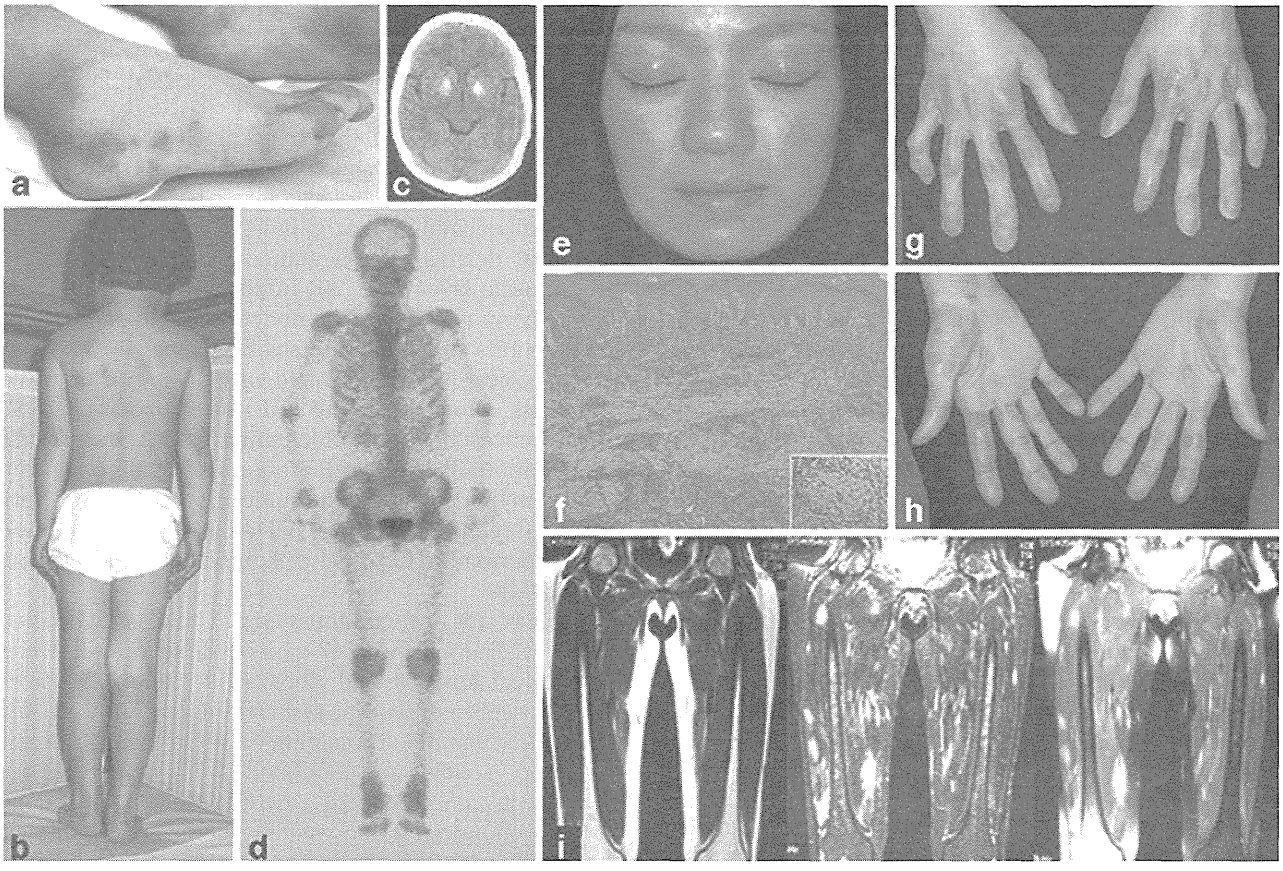

Fig. 2 Clinical features of an NNS case. **a**) Pernio-like purplish rashes on feet at 5 years of age. **b**) Equinus position caused by myositis in lower extremities. **c**) Basal ganglia calcification on cranial computed tomography at 24 years of age. **d**) Technetium uptake in multiple joints shown by bone scintigram. **e**) Angular facial appearance with emaciation and helliotrope-like periorbital rash at 27 years of age. **f**) Dense infiltration of inflammatory cells throughout dermis and vasculopathy with endotheial proliferation. **g**) Long clubbed fingers. **h**) Nodular erythema-like eruptions on hands and wrists. **i**) Multiple focal myositis revealed by magnetic resonance imaging. T1, T2, and T1 with gadlinium enhancement, from left to right.

tive rashes, seem to be rather characteristic and are actually found in all NNS cases (Fig. 2h). Notably, as these eruptions are worsened in winter and reportedly induced by cold stimulation test, Dr. Nishimura and colleagues reported them as pernio-like eruptions.[2] Indeed, both of pernio-like and nodular erythema-like skin eruptions seem to be caused by the common mechanism of inflammatory changes due to vasculopathy. However, dark-purplish edematous rashes, which appear in winter on fingertips and earlobes and resemble typical pernio, have been defined as pernio-like rashes distinct from nodular erythema-like eruptions, as they can be the first symptom but are easily overlooked. Periodic fever is not necessarily observed in all cases, but description of various types of fever can be found in most of the previous reports. Some cases were even accompanied with lymphadenopathy. Pertussis might be a trigger of the disease onset in one case reported by Nakajo, and also reported were a case which onset after middle otitis and a case which onset together with cytomegalovirus infection.[1] Long clubbed fingers and partial lipomuscular atrophy mainly in the upper body are the most characteristic for NNS and observed in almost all the cases (Fig. 2e, g). These features usually become apparent with age, but can also be the first symptom in some cases and thus attention should be paid from the early stage. In contrast, mental retardation was detected in only 8 cases, and therefore it is unlikely to be caused by NNS. Other characteristic symptoms observed in some cases include heliotrope-like rash in eyelids, myositis, short statue, hyperhidrosis in hands and feet, and severe tyrosis on feet.

In laboratory findings, erythrocyte sedimentation rate is elevated in almost all cases. Anemia, which is microcytic and usually accompanied with iron deficiency, is unresponsive to iron preparation and possibly caused by chronic inflammation and splenomegaly. Thrombocytopenia is also seen in some cases. Increased serum creatine phosphokinase level is due to myositis but is not necessarily correlated

with muscular atrophy, although a neurogenic examination shows myogenic changes in many cases. Hyper-γ-globulinemia, which is also observed in almost all cases, is considered to be a result of chronic inflammation. Although serum IgG level is always elevated, some cases with low serum IgA level or with abnormally high IgE level were reported. Furthermore, although no cases show positive serum autoantibodies at the disease onset, anti-nuclear antibody and some other specific autoantibodies such as anti-double stranded DNA antibody become detectable in the serum of not a few cases during the course of the disease. In contrast, for estimation of cellular immunity, lymphocyte blastoid formation with mitogen shows normal reaction, while tuberculin test shows negative in not so many but all examined cases. Regarding natural killer (NK) activity, remarkable impairment was detected in three cases, while remarkable increase of the NK cell number with normal or even high activity was reportedly observed in one case, and therefore further examination should be required. As the visceral involvement, hepatosplenomegaly and calcification of basal ganglia are recognized in most cases. Especially, the latter symptom is considered highly specific for NNS and cranial computed tomography should be checked if NNS is suspected (Fig. 2c). Various levels of conduction block and ischemic changes were frequently detected with electrocardiogram and considered a cause of precmature or sudden death. Although no abnormalities are usually found by an endocrinological analysis, growth hormone was administered for short stature in one case.

Hyperperiostosis, which was considered characteristic for NNS soon after the first report, has never been detected in most of the following reports. In contrast to the characteristic appearance of long clubbed fingers, neither of lytic bone lesions or narrowing of interphalangeal joints are detected by roentgenogram and serum matrix metalloprotease-3 level is within normal range. Cold stimulation test was originally described by Nakajo, by which perniolike erythematous nodules are induced on the forearm along the superficial vessels within several hours after soaking hands for 15 minutes in cold water of 4 degree centigrade.[1] Even though positivity of the test is not high, it is considered important for investigating the pathogenesis of NNS. By an analysis of serum cytokine levels, interleukin (IL)-6 and interferon (IFN) γ-induced protein-10 (IP-10) levels are commonly and significantly higher in NNS patients compared with healthy controls, while IL-1β and tumor necrosis factor (TNF)α are not.[14] Serum lipid levels were analyzed in relation to lipodystrophy to reveal high triglyceride level in many cases, while total cholesterol level was not stable. Gallium scintigram, bone scintigram, positron-emission tomography and magnetic resonance imaging are beneficial for systemic search of inflammatory lesions in bones, joints and muscle (Fig. 2d, i).

HISTOPATHOLOGY

On biopsy specimen of pernio-like or nodular erythema-like eruptions, focal dense infiltration of inflammatory cells in perivascular or periadnexal areas, especially around sweat glands, is observed from superficial dermis to subcutaneous fat, sometimes to the muscle layer (Fig. 2f). Infiltrating cells mainly include lymphocytes and histiocytes, and in some cases neutrophils and eosinophils with nuclear dusts. However, typical leukocytoclastic vasculitis with fibrinoid necrosis cannot be found but rather observed is an obstructive change by thickening of the vessel walls with endothelial cell proliferation and hyaline deposit. Slight atypia is detected in infiltrating cells but a variety of cells including CD4, CD8, CD68 and myeloperoxidase-positive cells infiltrate without monoclonality.

As a result of the autopsy of a NNS case who died with cardiac failure at 47 years old, severe, discrete, multifocal atrophy and fibrosis of skeletal muscles which replaced several primary fascicules were observed.[22] In the remaining muscle fibers, many rimmed vacuoles and lobulated fibers were revealed. On electron microscopy, myofibrillary necrosis, intramitochondrial paracrystalline bodies and cytoplasmic and myeloid bodies were revealed, whereas intramuscular peripheral nerves and neuromuscular junctions of the remaining muscle fibers looked well preserved. No evident regenerating fibers, central nuclei, myophagia or inflammatory cell infiltration were observed. Fundamentally identical but less severe lesions were observed in the tongue and the heart.

Although restricted to the very severely atrophic fascicules of the affected muscles, peculiar morphological changes were observed in blood vessels. Arteries and veins showed hyperplasia of the media with obstruction of the lumen, while most small vessels showed hypertrophy of endothelial cells with luminal obstruction. By electron microscopy, arterioles showed hyperplasia of smooth muscle cells containing centrioles and hypertrophied or degenerating endothelial cells. Terminal arterioles frequently showed centriols in endothelial and smooth muscle cells and narrowing of the lumen by debris of necrotic endothelial cells. Increased Weibel-Palade bodies and filaments were obvious in endothelial cells, whereas no evident increase of collagen, discontinuity of internal elastic lamina, fibrinous deposition, atherosclerotic changes, viral infection bodies or inflammatory cell infiltration were observed.

In the brain, no evident pathological changes were observed except for ferocalcium deposition in the small vessels of the globus pallidus and centrum semiovale. Myocardial hypertrophy with patchy fibrosis was observed in the heart. The aorta and large ar-

teries showed patchy calcification of the media adjacent to the internal elastic lamina, whereas no atheroma or intimal fibrosis were present around these lesions. Central fatty degeneration with acute cell necrosis was observed in the liver. The spleen was congested and the pancreas showed chronic pancreatitis. Subcutaneous fat was reduced and the fat around the visceral organs was increased, whereas the fat cells did not show remarkable pathological changes ultrastructurally.

DIFFERENT DIAGNOSIS

The characteristic angular facial appearance was described as gargoil-like in some reports and congenital metabolic diseases such as mucopolysaccharidosis might be suspected. This characteristic facial appearance and long clubbed fingers due to lipoatrophy are really the partial lipodystrophy in itself. Partial lipodystrophy includes the familial type with a mutation in either gene of *LMNA*, *PPARγ*, *AKT2*, *CIDEC* or *ZMPSTE24*, and the second type, mostly with hypocomplementemia and rarely with autoimmune diseases such as systemic lupus erythematosus (SLE), dermatomyositis and Sjogren's disease.[23,24] Actually, some NNS cases were first diagnosed as dermatomyositis, lupus profundus and SLE. Notably, a case of NNS, who had originally been diagnosed as SLE, further developed myositis with muscle weakness and was then diagnosed as inclusion body myositis by the histological findings of muscle biopsy. As previously discussed, histological differentiation of inclusion body myositis from NNS seems to be quite difficult. As a disease showing both pernio and basal ganglia calcification since early infancy, Aicardi-Goutieres syndrome caused by a mutation of the endonuclease gene such as *TREX1* should be considered for differentiation.[25] Progeria such as Werner syndrome is also suspected by progressive lipoatrophy with severe clavus, however, early-onset cataract and gray hair are not observed in NNS.

Some NNS cases were diagnosed as Weber-Christian disease (WCD), which shows relapsing febrile attacks with erythematous nodules caused by lobular panniculitis with lipophagy and leaves local collapses after healing of nodules. Defect of α1-antitrypsin or α1-antichymotrypsin also shows nodular erythema with lobular panniculitis. Lipoatrophy in NNS shows systemic loss of fat from periphery and thus can be distinguished from collection of collapses in WCD and related diseases. However, since exclusion of other diseases is required for the diagnosis of WCD, active diagnosis of NNS is fundamentally required. The tentative criteria for clinical diagnosis of NNS satisfy more than 5 features of the listed 8 ones in all the recent 23 cases. However, further improvement would be necessary to enable the early diagnosis of NNS before development of the full features and to exclude other diseases showing pseudopositive.

Among autoinflammatory diseases with periodic fever, cryopyrin-associated periodic syndrome (CAPS) seems to be the most similar to NNS, because both diseases are induced or worsened by cold stimuli and have common features of unstable pattern of febrile attacks and characteristic angular facial appearance.[26] However, arthritis and IL-1β overproduction are not revealed in NNS. As there is a case of tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), which was originally reported as WCD, genetic analysis might be required for the definite diagnosis.[27]

In 2010, a group of Spain, France and the United States and a group of Israel reported 5 cases with CANDLE syndrome, which satisfies 7 features (familial occurrence, nodular erythema-like eruptions, periodic fever, long clubbed fingers, partial lipodystrophy, hepatosplenomegaly and basal ganglia calcification) of the diagnostic criteria and resembles NNS very well.[17,28] Although neutrophilic infiltration is remarkable on histology and neutrophilic dermatosis is used for the designation of the disease, the main infiltrating cells include atypical histiocytes with large nuclei and thus such histopathology is fundamentally the same as that of NNS. Furthermore, another group of the United States, Mexico and Portugal reported 3 cases with JMP syndrome.[16] They pointed out the similarity between this disease and NNS but finally differentiated them, because seizures and anemia are specifically detected in JMP while mental retardation is specifically reported in NNS. JMP syndrome does not show fever and looks severer than NNS, because of the systemic lipodystrophy and severe contracture of wrist and phalangeal joints, but satisfies 6 features (familial occurrence, nodular erythema-like eruptions, long clubbed fingers with joint contractures, partial lipodystrophy, hepatosplenomegaly and basal ganglia calcification) of the diagnostic criteria for NNS. Comparison of these three syndromes is summarized in Table 2.

THERAPIES AND PROGNOSIS

Skin lesions disappear by administration of systemic steroid but can reappear after tapering. Furthermore, systemic steroid is ineffective for lipodystrophy but rather worsens the central obesity. Long-term administration of systemic steroid since infancy can cause severe side effects including growth retardation and glaucoma, and therefore, adaptation of this medication should be carefully determined. It was reported that kallikrein and DDS (dapson) were effective for NNS, however, the effect should be temporal. Administration of biological drugs such as anti-TNFα and anti-IL-1β antibodies might be effective, but has not been applied yet.

In cases with CANDLE syndrome, various antirheumatic and immunosuppressant drugs, including

Table 2 Comparison of the three proteasome disability syndromes

	Nakajo-Nishimura syndrome	JMP syndrome	CANDLE syndrome
Parental consanguinity	-/+	-	-
Family history	-/+	-/+	-/+
Age at onset of pernio	2 m-5 y	-	-/1 m?
Eruptions in trunk	-/++	+	+
Age at onset of fever	-/3 m-8 y	-	1m-1y
Long clubbed fingers	+	+	+
Joint contractures	-/+++	+++	-
Hyperhidrosis	-/+	-	-
Partial lipoatrophy	+-/+++	++	+
Loss of muscle power	-/+	+	-
Dyspnea	-/+	-	-
Hepatosplenomegaly	-/+	+	+
Microcytic anemia	-/+	++	+
Basal ganglia calcification	+	+	+
Seizures	-	+	-
Electrocardiogram	np/LVH, LAE, CRBBB	?	np
PSMB8 mutation	p.G201V	p.T75M	p.T75M, p.C135X, none

m, month(s); y, year(s); np, nothing particular; LVH, left ventricular hypertrophy; LAE, left atrial enlargement; CRBBB, complete right bundle branch block.

methotrexate, hydroxychloroquine, azathioprine, cyclosporine, tacrolimus, infliximab, adalimumab, etanercept, anakinra, tocilizumab, rituximab, were administered but satisfying effect has never been obtained, especially on progressive lipodystrophy.[19]

IDENTIFICATION OF THE RESPONSIBLE GENE

After informed consents were obtained, typing of single nucleotide polymorphisms (SNPs) in the whole genomic DNA extracted from peripheral blood of five NNS patients and unaffected three siblings was performed using Affimetrix GeneChip Human Mapping 500k array set.[14] As a result of homozygosity mapping using Partek Genomics Suite v6.4, an 1.1 Mb region on chromosome 6p21.31-32 was identified for the candidate locus, where homozygous SNPs were continuously present commonly in the patients whereas not continuously in unaffected siblings. By direct sequencing of all exons with exon-intron boundaries of 53 genes located on this locus, one missense mutation has been identified in exon 5 of the *PSMB8* gene, which is homozygously present only in the patients but is absent in 272 healthy controls. This homozygous c.602 G > T mutation causing p.G201V transition of the *PSMB8* gene, encoding the β5i subunit of IP, was actually observed in all examined NNS patients. Furthermore, all SNPs located in the genomic region between 15 kb before and 15 kb after the mutation were all homozygous in all examined NNS patients, and thus the strong founder effect has been revealed. Surprisingly, at the same time, a group of Tokushima University has identified the same mutation by homozygosity mapping of three patients in Akita and Niigata.[15]

By homozygosity mapping of three cases with JMP syndrome, another homozygous missense mutation of the *PSMB8* gene, c.224C > T in exon 2 causing p.T75M transition was identified to be responsible for all cases.[18] Homozygosity mapping was further performed on five cases with CANDLE syndrome and the homozygous p.T75M transition of the *PSMB8* gene was also detected in four cases and another homozygous c.405C > A in exon 3 causing p.C135X nonsense mutation was detected in one case of an Ashkenazi-Jewish origin.[19] Interestingly, no mutation was identified in one case and only the heterozygous p.T75M transition without any second mutation was detected in two cases, including one case who had not shown the similar homozygosity with other 4 cases at the original mapping.

PATHOGENESIS

Proteasome is the intracellular protease complex specialized for degradation of polyubiquitinated proteins.[29] The full complex called 26S is formed by one 20S core and two 19S regulatory units. The 20S core unit is composed of 2 sets of 2 rings with 7 α and 7 β subunits, including catalytic β1, β2 and β5 subunits with caspase-like, trypsin-like and chymotrypsin-like activities, respectively. The ubiquitin-proteasome system not only degrades unnecessary proteins and controls the protein quality, but also works on various cellular functions, including cell cycle regulation, gene repair and signal transduction such as nuclear factor (NF)-κB activation. Immunoproteasome, in