Autoinflammatory syndromes cause sterile inflammation in the absence of any signs of autoimmune responses. Familial cold autoinflammatory syndrome (FCAS) is characterized by intermittent episodes of rash, arthralgia, and fever after exposure to cold stimuli. We have identified a missense mutation in the NLRC4 gene in patients with FCAS. NLRC4 has been known as a crucial sensor for several Gram–negative intracellular bacteria. The mutation in NLRC4 in FCAS patients promoted the formation of NLRC4–containing inflammasomes that cleave procaspase-1 and increase production of IL-1β. Transgenic mice that expressed mutant Nlrc4 under the invariant chain promoter developed dermatitis and arthritis. Inflammation within tissues depended on IL-1β–mediated production of IL-17A from neutrophils but not from T cells. Our findings reveal a previously unrecognized link between NLRC4 and a hereditary autoinflammatory disease and highlight the importance of NLRC4 not only in the innate immune response to bacterial infections but also in the genesis of inflammatory diseases.

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is a causative gene for this disease and highlight the crucial roles of NLRC4 not only in the innate immune response to bacterial infections but also in the pathogenesis of human inflammatory diseases.

RESULTS

Linkage and exome analyses of a Japanese family with a history of FCAS revealed a missense mutation in NLRC4

Members of a non-consanguineous Japanese family (Fig. 1 a) experienced episodes of high fever, urticarial-like rash (Fig. 1 b), and arthralgia that began at ~2–3 mo of age. These symptoms have frequently been induced by exposure to cold stimuli, and the urticarial-like rash after cold exposure has not been accompanied by itching. Such patients have not developed splenomegaly or bone erosion. In most cases, the symptoms have been resolved without treatment in this family, although patients have taken nonsteroidal anti-inflammatory drugs to reduce joint pain. The laboratory findings of patient 3, from whom a blood sample was obtained during an episode of urticarial-like rash when he was 7 mo old, are shown in Table 1. We undertook SNP array and linkage analyses to identify the causal gene. Parametric linkage analysis of five affected and two unaffected family members was performed. A maximum LOD score of 1.143 was obtained throughout the whole genome, with a total critical region of 243 Mbp (Table 2), but an LOD score >2.3 was not detected.
The mutation in NLRC4 increased its oligomerization and resulted in hyperactivation of caspase-1

We first asked if the mutation in NLRC4 affected the expression of NLRC4 protein. The 3× Flag-tagged cDNA of wild-type or mutant NLRC4 was transfected into 293T cells and the lysates from those transfected cells were subjected to SDS-PAGE. The expression levels of wild-type and mutant NLRC4 proteins were similar; this finding suggested that the missense mutation did not affect the expression of NLRC4 (Fig. 2 a).

As the activation of NLRC4 induces its oligomerization (Kofoed and Vance, 2011), we wished to determine if the missense mutation increased this process. The 3× Flag-tagged cDNA of wild-type or mutant NLRC4 was transfected into 293T cells and the lysates from those transfected cells were subjected to Blue Native-PAGE. A marked shift of wild-type NLRC4 to an oligomeric complex (≥1,000 kD) was detected in cells transfected with mutant NLRC4, whereas the monomeric form predominated in cells transfected with wild-type NLRC4 (Fig. 2 b).

These data indicated that the missense mutation in NLRC4 facilitated the formation of oligomers.

To determine if the missense mutation in NLRC4 resulted in increased cleavage of procaspase-1, a vector expressing either the wild-type or mutant NLRC4 and a CASP1 expression vector were transfected into 293T cells. Cells transfected with wild-type NLRC4 contained less of the 10 kD species (active form of caspase-1) than those transfected with mutant NLRC4 contained (Fig. 2 c). Because the cleavage of procaspase-1 is required for the production of IL-1β, we measured IL-1β

To identify the critical gene, genomic DNA of the family members (Fig. 1 a; five patients and two healthy family members) was evaluated by whole exome sequencing. A total of 3.7 Gbp of sequences was aligned to the exome target. DNA variants were filtered against the dbSNP build 134, the 1000 genomes project, the NHLBI Exome Sequencing project, and then classified by the predicted function to include all missense, nonsense, frameshift, or splice-site alleles. Exome analysis on linkage analysis–based 243 Mbp candidate regions identified three rare changes in NLRC4, ALK, and DTNB (Table 3). We did not detect any mutations or mosaicism in the exon or exon-intron boundary region of NLRP3. The variation in NLRC4, but not those in ALK and DTNB, was located in a region that is conserved among species and that encodes a functional domain (Table 3). Furthermore, after additional testing of 200 genomes obtained from healthy Japanese donors, we detected the same variations in ALK and DTNB (allele frequency 1/100 in both genes; unpublished data). Those data strongly suggested that NLRC4 was the causative gene for FCAS in this family.

The mutation in NLRC4 was heterozygous in all of the patients we studied. It caused an A>C transversion at nucleotide 1589 in exon 4 (Fig. 1 c) and changed the predicted amino acid from a histidine to a proline at position 443 (Fig. 1, c and d). The missense mutation was detected in all affected patients but not in healthy members of the family. Amino acid 443 is located in the nucleotide binding domain (Fig. 1 d) and is conserved among five species (Fig. 1 e).

Table 2. Linkage analysis

| Chromosome | HLOD | Distance (Mb) | Position (start) | Position (end) |
|------------|------|--------------|-----------------|---------------|
| 2          | 1.143| 37,899,000   | 8,247,000       | 46,146,000    |
| 3          | 1.143| 25,141,000   | 0               | 25,141,000    |
| 4          | 1.143| 12,916,000   | 138,293,000     | 151,209,000   |
| 5          | 1.143| 62,984,000   | 54,059,000      | 117,043,000   |
| 6          | 1.143| 5,070,000    | 15,539,000      | 20,609,000    |
| 8          | 1.143| 3,152,000    | 14,762,000      | 17,914,000    |
| 12         | 1.143| 13,013,000   | 89,504,000      | 102,517,000   |
| 12         | 1.143| 8,741,000    | 124,637,000     | 0             |
| 13         | 1.143| 9,413,000    | 98,949,000      | 108,362,000   |
| 14         | 1.143| 14,817,000   | 58,142,000      | 72,959,000    |
| 16         | 1.143| 30,822,000   | 56,372,000      | 87,194,000    |
| **Total 12 loci** |      | **243,041,000** |                |               |

Table 3. Variants by exome analysis

| Gene | Variants | Functional domain | Amino acid | Homology in six species |
|------|----------|-------------------|------------|------------------------|
| ALK  | SNV      | +                 | R510W      | 3/6                    |
| NLRC4| SNV      | +                 | H443P      | 5/5                    |
| DTNB | Insertion| –                 | In-frame at c-terminal | -                     |

SNV: single nucleotide variation.
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patients compared with the healthy donor-derived cells at three different concentrations of PrgI (Fig. 2 e).

The mutation in Nlr4 caused an autoinflammatory syndrome in mice

We next sought to assess if the equivalent of the NLRC4 1589A>C mutation caused FCAS-like inflammatory responses in mice. First, we tested if the equivalent mutation in mouse Nlrc4 also augmented IL-1β secretion. BM-derived macrophages (BMMCs) transfected with wild-type or mutant Nlrc4 were stimulated with LPS and the secretion of IL-1β was measured. BMMCs infected with control retrovirus, retroviruses encoding wild-type or mutant Nlrc4 were stimulated with 10 µg/ml LPS for 1 d in the absence (black) or presence (gray) of a caspase-1 inhibitor. As the control, unstimulated BMMCs were used (white). The concentrations of IL-1β and IL-6 in the supernatant were measured by ELISA. The data shown are means ± SD (*, P < 0.05; **, P < 0.01; n = 5). The data shown in this figure are representative of three independent experiments.

Figure 3. BM-derived macrophages that overexpress mutant Nlr4 secrete IL-1β. BM-derived macrophages (BMMC) infected with control retrovirus, retroviruses encoding wild-type, or mutant Nlr4 were stimulated with 10 µg/ml LPS for 1 d in the absence (black) or presence (gray) of a caspase-1 inhibitor. As the control, unstimulated BMMCs were used (white). The concentrations of IL-1β and IL-6 in the supernatant were measured by ELISA. The data shown are means ± SD (*, P < 0.05; **, P < 0.01; n = 5). The data shown in this figure are representative of three independent experiments.

Figure 2. A mutation in NLRC4 induces overproduction of IL-1β through activation of caspase-1.

(a) Flag-tagged wild-type, mutant NLRC4 expression vectors or empty vector (EV) were transfected into 293T cells. 24 h later, the cell lysates were subjected to SDS-PAGE. Western blotting was performed with an anti-Flag or anti-actin antibody. Red triangle indicates NLRC4. The data in the figure are representative of three independent experiments. (b) Cell lysates from 293T cells transfected with empty vector (EV), flag-tagged wild-type, or mutant NLRC4 were subjected to Blue Native PAGE and the expression of the tagged proteins or actin was measured by Western blotting with an anti-Flag or actin antibody, respectively. The data in the figure are representative of three independent experiments.

(b) Cell lysates from 293T cells transfected with a CASP1 expression vector together with empty vector (EV), an expression vector for wild-type or mutant NLRC4 24 h later, the expression of procaspase-1, cleaved active caspase-1 (10 kD), and actin was evaluated by Western blotting. Red triangles indicate procaspase-1, cleaved active caspase-1, and actin. The data in the figure are representative of five independent experiments. (c) 293T cells were transfected with an expression vector for CASP1 and IL1B together with empty vector (EV), an expression vector for wild-type or mutant NLRC4. 24 h later, the concentration of IL-1β in the supernatant was evaluated by ELISA. The data are shown as means ± SD (**, P < 0.01; n = 5). The data in the figure are representative of three independent experiments. (e) Peripheral blood mononuclear cells were transfected with three concentrations of PrgI and cultured for 48 h. The concentrations of IL-1β in the supernatants were measured by ELISA. The data shown are means ± SD (**, P < 0.01). The data in the figure are representative of three independent experiments.

NLRC4 is activated in response to bacterial type III secretion systems, including PrgI (Rayamajhi et al., 2013). We transfected PrgI in peripheral blood mononuclear cells from FCAS patient and a healthy donor and evaluated the production of IL-1β. The production of IL-1β was higher in FCAS patients compared with the healthy donor-derived cells at three different concentrations of PrgI (Fig. 2 e).

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that expressed the mutant Nlrc4 produced a greater amount of IL-1β than did those transfected with the wild-type Nlrc4 vector or the control vector. In addition, the production of IL-1β was inhibited by the addition of a caspase-1 inhibitor (Fig. 3). In contrast, the amount of IL-6 secreted by LPS-stimulated BMMCs overexpressing mutant Nlrc4 was similar to that of cells overexpressing wild-type Nlrc4, and the secretion of IL-6 was not inhibited by treatment with a caspase-1 inhibitor (Fig. 3). These data indicated that the mutant NLRC4 affected the secretion of IL-1β and that this activity depended on the cleavage of caspase-1.

We then established transgenic mice (mu-Nlrc4 mice) in which expression of a 3× HA-tagged mutant Nlrc4 cDNA was driven by an invariant chain promoter (van Santen et al., 2000). The transgene was highly expressed in the spleen of mu-Nlr4 mice (Fig. 4 a). The mu-Nlr4 mice exhibited dermatitis and swollen joints starting at 3 wk of age (Fig. 4 b). Histological analysis of mu-Nlr4 mice revealed severe cell infiltration in the skin and joints and erosion of the bone (Fig. 4 c). The mu-Nlr4 mice exhibited splenomegaly and the total cell number in the spleen was approximately fivefold higher in mu-Nlr4 mice than in control mice (Fig. 4 d). Phenotypical analysis of the spleen revealed a 15-fold increase in the number of CD11b+Gr1−F4/80− cells in mu-Nlr4 mice as compared with control mice (Fig. 4 e). Similar phenotypes were detected in six independent transgenic lines. We evaluated the expression levels of total Nlrc4 protein in B cells of wild-type and three mu-Nlr4 lines (line #1 was used in experiments of all figures; Table 4). The expression levels of mutant Nlrc4 was positively correlated with the number of total spleen cells, T cells (CD3+), and neutrophils (CD11b+Gr1−; Table 4).

We next compared the production of cytokines in the spleens of mu-Nlr4 and control mice. Splenocytes of mu-Nlr4 mice produced IL-1β when stimulated with LPS, whereas the production of IL-1β by splenocytes of control mice was limited (Fig. 5 a). The serum levels of IL-1β, IL-17A, and G-CSF

Table 4. Characteristics of three lines of mutant Nlrc4 transgenic mice

| Mouse line | Expression of Nlrc4 (fold increases compared with wild type) | Spleen cells (x10^7) | T cells (x10^7) | CD11b+Gr1+ cells (x10^7) |
|------------|-------------------------------------------------------------|----------------------|----------------|--------------------------|
| Wild type  | 1                                                           | 5.2 ± 0.3            | 0.8 ± 0.1      | 0.05 ± 0.01              |
| Line #1    | 3.1 ± 0.2                                                   | 21.1 ± 1.4           | 3.4 ± 0.4      | 0.59 ± 0.2               |
| Line #2    | 5.2 ± 0.3                                                   | 32.1 ± 3.3           | 5.4 ± 2.2      | 1.2 ± 0.2                |
| Line #3    | 4.2 ± 0.3                                                   | 27.8 ± 4.5           | 4.5 ± 0.4      | 0.89 ± 0.1               |

The data were obtained at the age of 16 wk. The data in line #1 are used in all figures.
were much higher in mu-Nlr4 mice than in control mice (Fig. 5 b). To determine whether the overexpression of wild-type Nlr4 might also up-regulate proinflammatory cytokines, we transplanted BM cells transduced with the wild-type or the mutant Nlr4 gene into irradiated recipient mice. The number of CD11b+Gr1+ cells in the spleen and peritoneal cavity was higher in mutant Nlr4 chimeras than in control chimeric mice (Fig. 5 c). The serum levels of IL-1β, IL-17A, and G-CSF were higher in C57BL/6 mice reconstituted with mutant Nlr4-transduced BM cells than those with wild-type Nlr4 or control virus-transduced BM cells (Fig. 5 d).

**Cold exposure of mu-Nlr4 mice increased autoimmune inflammation**

To determine if mu-Nlr4 mice exposed to cold stimuli would exhibit a heightened inflammatory response, the right feet of mu-Nlr4 mice were cooled for 5 min and then the thickness of the footpads was evaluated. Cooling the footpads of control mice did not change their color or thickness, but the footpads of mu-Nlr4 mice increased in thickness and displayed an exanthema-like color change after being cooled (Fig. 6 a). The foot pad swelled more in C57BL/6 mice reconstituted with mutant Nlr4-transduced BM cells than in those with wild-type Nlr4- or control virus-transduced BM cells (Fig. 6 b). In addition, cooling of the whole body of the mice at 4°C for 1 min increased IL-1β in mu-Nlr4 but not control mice (Fig. 6 c). As for cell level assay, LPS-stimulated MC/9 cells overexpressing mutant Nlr4 or 293T cells overexpressing mutant NLRC4 produced more IL-1β when cultured at 32°C than at 37°C, whereas MC/9 cells overexpressing wild-type Nlr4 or 293T cells overexpressing wild-type NLRC4 did not increase IL-1β in response to 32°C stimulation (Fig. 6 d). Peripheral blood mononuclear cells from an FCAS patient produced IL-1β in response to 32°C stimulation, whereas control cells did not increase IL-1β production in response to 32°C stimulation (Fig. 6 e). The induction of IL-1β by cold stimuli was inhibited by the addition of caspase inhibitor (Fig. 6 e). These data demonstrated that cells harboring mutant Nlr4 activated Nlr4-dependent inflammasomes upon exposure to cold stimuli, and this activation resulted in tissue inflammation.

The increase of neutrophils in mu-Nlr4 mice depended on IL-1β or IL-17A

Th17 cells that produce IL-17A contribute to various inflammatory disorders (Korn et al., 2009). We assessed which circulating cells were responsible for the production of IL-17A. The major IL-17A producers in mu-Nlr4 mice were in the TCR-β-TCR-γδ-B220-CD11c-NK1.1- population, whereas those in control mice were in a lineage-positive fraction (Fig. 7 a). Most of the lineage marker–negative cells that produced IL-17A in mu-Nlr4 mice were Gr1+CD11b+ or Gr1intCD11b+ cells that included neutrophils, monocytes, and macrophages (Fig. 7 a).

**Figure 5. Cytokine production in mu-Nlr4 mice or mice reconstituted with mutant Nlr4 transduced BM cells.** (a) Splenocytes from wild-type or mu-Nlr4 mice were stimulated with LPS for 24 h, and the concentration of IL-1β in each supernatant was evaluated by ELISA. The data shown are means ± SD (**, P < 0.01), N.D.: not detected. The data in the figure are representative of three independent experiments. (b) The concentrations of IL-1β, IL-17A, and G-CSF in the serum of wild-type (white) and mu-Nlr4 (black) mice at the age of 8 wk were evaluated by ELISA. For all panels, the data shown are means ± SD (**, P < 0.01; n = 5), N.D.: not detected. The data in the figure are representative of three independent experiments. (c) Intrapitoneal cells or splenocytes from C57BL/6 mice transplanted with BM cells infected with control virus (white), wild-type (black), or mutant Nlr4 (gray)-encoding virus were evaluated for the number of Gr1+CD11b+ cells 2 mo after the transplantation. The data shown are means ± SD (**, P < 0.01; n = 5 in each group). The data in the figure are representative of three independent experiments. (d) Irradiated C57BL/6 mice were reconstituted with BM cells infected with control virus (white), wild-type (black), or mutant Nlr4 (gray)-encoding virus. Serum IL-1β, IL-17A, and G-CSF were evaluated by ELISA 6 wk after BM transplantation. For all panels, the data shown are means ± SD (**, P < 0.01; n = 5), N.D.: not detected. The data in the figure are representative of two independent experiments.
To determine which cells were responsible for IL-17A production in mu–Nlrc4 mice, we depleted CD4+, CD8+, Thy1.2+, or Gr1+ cells. The depletion of CD4+, CD8+, or Thy1.2+ cells did not affect the IL-17A level (Fig. 7 b), although the depletion of CD8−TCR−β+, CD4−TCR−β+, or CD4+CD8−TCR−β+ cells was efficient (Fig. 7 c). However depletion of Gr1+ cells and suppression of anti–IL-1β reduced the IL-17A level (Fig. 7 b), suggesting that IL-1β was involved in neutrophil accumulation. The number of CD11b+Gr1+ cells in mu-Nlrc4 mice was decreased by treating the mice with anti–IL-1β or anti–IL-17A (Fig. 7 d). The combination of anti–IL-1β and anti–IL-17A further decreased the number of CD11b+Gr1+ cells (Fig. 7 d).

To evaluate which cells or cytokines contributed to inflammation in mu–Nlrc4 mice, the animals were treated with intraperitoneal injections of anti–CD4+, anti–CD8+, anti–Thy1.2+, or anti–Gr1+ antibodies. Alternatively, intraperitoneal injections contained anti–IL-1β, anti–IL-17A, or anti–IL-1β plus anti–IL-17A antibodies. Footpad swelling after antibody treatment was subsequently measured (Fig. 8 a). The depletion of CD4+, CD8+, or Thy1.2+ cells did not affect swelling. In contrast, depletion of Gr1+ cells suppressed the footpad swelling (Fig. 8 a). Footpad swelling in mu–Nlrc4 mice was inhibited by treating the mice with anti–IL-1β or anti–IL-17A, and the combination of anti–IL-1β plus anti–IL-17A further decreased the number of CD11b+Gr1+ cells (Fig. 8 a). The treatment of mu–Nlrc4 mice with anti–IL-6 or TNF did not inhibit footpad swelling (Fig. 8 b). These data demonstrated that the accumulation of neutrophil was mediated by IL-1β, IL-17A, and neutrophils.

**DISCUSSION**

Previous studies have identified mutations in **NLRP3** and **NLRP12** in patients with FCAS (Hoffman et al., 2001; Jéru et al., 2008). However, not all patients with FCAS carry mutations in these two genes (Aksentijevich et al., 2007). In the current study, we used linkage and exome resequencing to analyze genetic variations in patients with FCAS in one Japanese family and discovered a missense mutation in **NLRC4**.
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stimuli in addition to increased production of IL-1β from peripheral blood mononuclear cells from FCAS patients than those from control in response to bacterial product. An interesting question is whether intrinsic ligands are required for the oligomerization of mutant NLRC4 or whether there are intrinsic ligands that are able to activate wild-type NLRC4. These questions could be at least partly addressed by comparing the abilities of wild-type and mutant NLRC4 proteins to self-assemble in a cell-free system. In addition, a recent study has revealed the structure of NLRC4 (Hu et al., 2013). Thus, a comparison of the structures of the wild-type and mutant NLRC4 proteins might help reveal the activation mechanisms for mutant NLRC4 as well as wild-type NLRC4.

The mu-Nlrc4 mice developed severe arthritis with bone erosion, dermatitis, and splenomegaly together with an increased number of CD11b+Gr1+ neutrophil and activated T cells. Although mu-Nlrc4 mice developed an urticarial-like dermatitis upon exposure to a cold stimulus, it is important to note that these mice spontaneously developed an inflammatory syndrome even without exposure to cold stimuli. Previous studies have reported that mice harboring a mutation in NLRP3 also spontaneously develop an inflammatory disorder (Meng et al., 2009). Therefore, these data suggest that a cold environment is one of the factors that trigger the appearance of the syndrome but also that it is not required to cause the disease. As for the mechanisms of autoinflammation in mu-Nlrc4 mice, neutrophil infiltration and footpad inflammation were suppressed by blocking the function of IL-1β and IL-17A or depleting neutrophils but not CD4+ or CD8+ or Thy1.2+ cells. Furthermore, IL-17A production was inhibited by suppressing IL-1β or depleting neutrophils but not CD4+ or CD8+ or Thy1.2+ cells. Collectively, these data suggest that increased production of IL-1β by the mutation in NLRC4 would up-regulate the production of IL-17A from neutrophils, which would further recruit neutrophils leading to progressive inflammation.

The mice harboring a mutation in NLRP3 spontaneously develop an inflammatory disorder (Meng et al., 2009) that depends on Th17. Therefore, mutations in NLRP3 and NLRC4 likely lead to production of IL-17A through different molecular networks. Previous studies have not discovered mutations in NLRC4 in American patients with CAPS who do not have mutations in NLRP3 (Aksentijevich et al., 2007). Our discovery of a new mutation in NLRC4 in one Japanese family will facilitate the screening of NLRC4 in patients with CAPS. Furthermore, the finding that expression of the mutant Nlrc4 in mice gives rise to spontaneous and severe autoinflammatory symptoms, including splenomegaly and infiltration of neutrophils into the joints, suggests that NLRC4 may be involved in non-FCAS types of autoinflammatory syndromes that are currently not associated with known genetic mutations. In addition, two recent papers reported that the heterozygous mutation in NLRC4 causes macrophage activation syndrome or neonatal-onset enterocolitis, periodic fever, and fatal or near-fatal episodes of autoinflammation (Canna et al., 2014; Romberg et al., 2014). Furthermore, it has been reported that polymorphisms of NLRC4 are associated with atopic dermatitis (Macaluso et al., 2007). Therefore, it will be important to characterize the NLRC4 genotypes/phenotypes association in patients with an autoinflammatory syndrome as well as in patients with inflammatory disorders. In conclusion, our findings reveal a previously unrecognized link between NLRC4 and FCAS, and they highlight the importance of NLRC4 not only in the innate immune response to bacterial infections but also in the genesis of inflammatory diseases.

**MATERIALS AND METHODS**

**Patients.** Genomic DNA was extracted from peripheral blood after the Ethical Committee of the University of Tokushima approved the human genome research protocol and informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki. The cell number or serum levels of enzymes or cytokines in...
SNP microarray and linkage analysis. SNP genotyping was conducted with an Illumina Human 660 W Quad system (Illumina). DNA was prepared in accordance with the manufacturer’s instructions and hybridized onto the array. Parametric linkage analysis, which used a fully penetrant dominant model, was conducted with the Merlin software package (Abecasis et al., 2002).

Exome resequencing and DNA sequencing. A paired-end library was prepared from genomic DNA and hybridized to biotinylated cRNA oligonucleotide baits from the SureSelect Human All Exon kit (Agilent Technologies). The library was sequenced with paired-end, 75 bp reads on one lane of an Illumina Hiseq2000 sequencing systems. The sequence reads were aligned to the human reference sequence of the UCSC Genome Browser (hg19). The entire targeted exome covered by >10 reads was 90%. DNA variants located in the candidate region were filtered against the databases of dbSNP.

Flow cytometry and antibodies. Cells were resuspended in staining buffer at a density of 2 × 10^6 cells/ml. The cells were incubated with mAb anti-CD11b (M1/70), CD11c (N418), CD44 (IM7), CD62L (MEL-14), TCR-β (H57-597), B220 (RA3-6B2), F4/80 (MKS), NK1.1 (PK136), TCR-γδ (GL3), or Gr1 (RB6-8C5; BioLegend). After gating out cells that were positive for 7-AAD, the fluorescence intensity of 10^5 cells was measured with a FACS Canto II flow cytometer (BD) and analyzed with the FACS Diva (BD) or Flowjo (Tree Star) software programs. For intracellular staining, cells were fixed with an intracellular staining kit (eBioscience), permeabilized, and stained with PE-conjugated anti-IL-17A antibody (BioLegend).

Western blotting and Blue Native PAGE. Cell pellets were lysed in cold RIPA buffer (Wako Pure Chemical Industries) and protease inhibitor (Roche). The lysates were boiled in SDS loading dye. The samples were resolved by SDS-PAGE and the blots were incubated with anti-Flag (F1804; Sigma-Aldrich), anti-HA (3724; Cell Signaling Technology), or anti–caspase-1 (Santa Cruz Biotechnology, Inc.) antibodies, followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Thermo Fisher Scientific) or goat anti-rabbit IgG (Bio-Rad Laboratories) antibodies. As a control, membranes were probed with polyclonal anti-actin (Sigma-Aldrich) and HRP-conjugated goat anti-rabbit IgG antibodies (Bio-Rad Laboratories). In some experiments, membranes were blocked with anti-Flag and anti-actin, followed by HRP-conjugated anti-mouse and rabbit IgG. The bands were detected with ECL Prime Chemiluminescent Substrate (GE Healthcare) and with the Peroxidase-conjugated goat anti–mouse IgG (Thermo Fisher Scientific) or anti–caspase-1 (Santa Cruz Biotechnology, Inc.) antibodies, followed by incubation with peroxidase-conjugated goat anti–rabbit IgG antibodies (Bio-Rad Laboratories).

Vector and cDNA. The cDNAs of human NLRC4 (provided by Y. Yanagi and T. Ichinohe (Kyushu University, Fukuoka, Japan)) and S. Ishido (Showa Pharmaceutical University, Tokyo, Japan)) were inserted by PCR-based mutagenesis and ×3HA tagged cDNA was cloned into pDOI6 (van Santen et al., 2000; provided by D. Mathis, C. Benoist (Harvard Medical School, Cambridge, MA), and S. Ishido (Showa Pharmaceutical University, Tokyo, Japan)) and microinjected into fertilized eggs from C57BL/6N mice at the RIKEN CDB facility in Kobe, Japan (LARGE, 2014, accession no. CDB0496T).

Antibody treatment of mice. The mu-Nlrc4 mice were intraperitoneally injected with rat IgG, anti-CD4 (CD1.5; 200 µg/dose), anti-CD8 (3395; 67.2; 150 µg/dose), anti-Gr1 (RB6-8C5; 150 µg/dose), or anti–IL-17A (17F3; 150 µg/dose), or anti–IL-6 (MP5-20F3; 200 µg/dose), or anti–IL-1β (B122; 150 µg/dose), or anti–IL-17A (17F3; 150 µg/dose), or anti–TNF (XT3.11; 200 µg/dose) antibody five times at 3-d intervals.

Isolation, culture, and transfection of BM-derived macrophages. Mouse BM cells were recovered by flushing the dissected femurs and tibias with RPMI 1640 medium. After lysing of the RBCs with NH4Cl, the cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM l-glutamine, 40 U/ml penicillin, 40 µg/ml streptomycin, 55 µM 2-mercaptoethanol, and 20% culture supernatant from 10 ng/ml M-CSF (R&D Systems) for 7 d. Gene knockout (EMD Millipore) was used to generate retroviruses by transfecting each vector encoding GFP into Plat-E cells (provided by T. Kitamura; Morita et al., 2000). Retroviruses carrying wild-type or mutant Nlrc4 and control viruses were used to infect MC/9 cells by incubating MC/9 cells with virus-containing medium for 24 h. After expansion of cells, GFP-positive MC/9 cells were enriched with a cell sorter (FACSAnAllIE; BD). Peripheral blood mononuclear cells were transfected with different concentration of PrgI by Nucleofector (Lonza) according to the manufacturer’s protocol.

Animal husbandry and generation of transgenic mice. Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu). The mutant mouse Nlrc4 cDNA tagged with ×3HA was cloned into pDOE6 (van Santen et al., 2000; provided by D. Mathis, C. Benoist (Harvard Medical School, Cambridge, MA), and S. Ishido (Showa Pharmaceutical University, Tokyo, Japan)). The linearized transgene construct, generated from the plasmid by BanI digestion, was microinjected into fertilized eggs from C57BL/6N mice at the RIKEN CDB facility in Kobe, Japan (LARGE, 2014, accession no. CDB0496T). The genotypes of the transgenic mice were determined by PCR (forward primer: 5’-GCATGCTCATCTTTACATCGATGTCCTG-3’, reverse primer: 5’-TAAAGCAGCTACAGTTTTAAGTGCCCCTTTAATTGGC-3’). All mice were maintained under specific pathogen-free conditions in the animal research center of the University of Tokushima and all experiments were performed in accordance with our institution’s guidelines for animal care and use.

Exposure of mice or cells to cold stimuli. Either the foot or the whole body of mice was exposed to 4°C for 5 min or 4°C for 1 h, followed by room temperature (24–25°C) for 3 min, respectively. The size of the footprint was measured before and after exposure. As for cold stimuli for cells, the cultured cells were kept at 32°C for 48 h and cytokines in the supernatant was measured.

Cell culture. 293T cells were maintained in DMEM supplemented with 10% FBS. MC/9 cells were provided by N. Kanibe (Chiba University, Chiba, Japan) and maintained in DMEM supplemented with 10% FBS and 10 ng/ml. The 293T cells were transfected with human NLRC4 or mutant Nlrc4 using Genejuice (Millipore) according to the manufacturer’s protocol. Genejuice was used to generate retroviruses by transfecting each vector encoding GFP into Plat-E cells (provided by T. Kitamura, University of Tokyo, Tokyo, Japan; Morita et al., 2000). Retroviruses carrying wild-type or mutant Nlrc4 and control viruses were used to infect MC/9 cells by incubating MC/9 cells with virus-containing medium for 24 h. After expansion of cells, GFP-positive MC/9 cells were enriched with a cell sorter (FACSAnAllIE; BD). Peripheral blood mononuclear cells were transfected with different concentration of PrgI by Nucleofector (Lonza) according to the manufacturer’s protocol.

ELISA. The concentration of IL-1β and IL-6 was measured with the mouse IL-1β or IL-6 ELISA Ready-Set-Go kit, respectively (eBioscience). The concentration of IL-17 was measured with a biotin-conjugated anti–mouse
IL-17A antibody and a purified anti-mouse IL-17A antibody (BioLegend). The concentration of G-CSF was measured with the Mouse G-CSF Quantikine ELISA kit (R&D Systems).

**BM transplantation.** Recipient female C57BL/6 mice were irradiated with 9.5 Gy when they were 8 wk old. Lineage-negative BM cells from C57BL/6 mice were purified with a mouse Lineage Cell Depletion kit (Miltenyi Biotec). After purification, the cells were infected 3× with control viruses or with viruses carrying either the wild-type or mutant Nlrc4 cDNA, as described previously (Morita et al., 2000). Approximately 5–7 × 10^5 BM cells were injected into irradiated mice 1 d after they were irradiated.

**Histological analysis.** The tissues were fixed in 10% formalin, embedded in paraffin, and sectioned. The tissue sections were stained with hematoxylin and eosin.

**Statistical analysis.** The statistical significance of between-group differences was evaluated by an unpaired, two-tailed t test. A p-value of <0.05 was considered significant.

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