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Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria

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Urticarial rash observed in cryopyrin-associated periodic syndrome (CAPS) caused by nucleotide-binding oligomerization domain–leucine-rich repeats containing pyrin domain 3 (NLRP3) mutations is effectively suppressed by anti–interleukin (IL)-1 treatment, suggesting a pathophysiological role of IL-1β in the skin. However, the cellular mechanisms regulating IL-1β production in the skin of CAPS patients remain unclear. We identified mast cells (MCs) as the main cell population responsible for IL-1β production in the skin of CAPS patients. Unlike normal MCs that required stimulation with proinflammatory stimuli for IL-1β production, resident MCs from CAPS patients constitutively produced IL-1β. Primary MCs expressed inflammasome components and secreted IL-1β via NLRP3 and apoptosis-associated speck-like protein containing a caspase recruitment domain when stimulated with microbial stimuli known to activate caspase-1. Furthermore, MCs expressing disease-associated but not wild-type NLRP3 secreted IL-1β and induced neutrophil migration and vascular leakage, the histological hallmarks of urticarial rash, when transplanted into mouse skin. Our findings implicate MCs as IL-1β producers in the skin and mediators of histamine-independent urticaria through the NLRP3 inflammasome.

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Abbreviations used: Ab, antibody; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; BMCMC, bone marrow–derived cultured MC; CAPS, cryopyrin-associated periodic syndrome; FSMC, fetus skin–derived MC; LRR, leucine-rich repeat; MC, mast cell; MIM, Mendelian inheritance in men number; MWS, Muckle-Wells syndrome; NLRP3, NOD-LRRs containing pyrin domain 3; NOD, nucleotide-binding oligomerization domain; P2X7R, purinergic P2X, ligand-gated ion channel 7 receptor; PAMP, pathogen-associated molecular pattern.

Urticaria, or hives, is a common disease that can affect up to 20% of the general population (1). Chronic urticaria, defined as urticaria that persists for >6 wk, occurs in 0.1% of the population (2), and in a significant percentage of cases (~40–80%), there is no identifiable cause (1, 3). H1 antihistamines have remained the first line of treatment because histamine release from cutaneous mast cells (MCs) plays an important role in the pathophysiology of urticaria development. However, only ~55% of patients with chronic urticaria are responsive to antihistamines (4), suggesting that in a significant number of individuals, chronic urticaria is mediated via histamine-independent mechanisms.

An urticarial rash developing in the neonatal or early infantile period is one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS). CAPS consists of a spectrum of hereditary periodic fever disorders that comprise three phenotypically overlapping but relatively distinct syndromes: familial cold autoinflammatory syndrome (Mendelian inheritance in men number [MIM] 120100), Muckle-Wells syndrome (MWS; MIM 191900), and chronic infantile neurological cutaneous and articular syndrome (MIM 607115), which is also known as neonatal-onset multisystem inflammatory disease. Familial cold autoinflammatory syndrome and MWS are characterized by periodic attacks of urticarial rash, fever, and arthralgia, whereas patients with chronic infantile neurological cutaneous and articular syndrome, the most severe form of CAPS, exhibit chronic urticaria as well as fever, arthropathy, chronic meningitis, papilledema, growth and mental retardation, and

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hearing loss (5). The urticarial rash observed in CAPS is similar to that associated with common urticaria. However, unlike the latter disorder, the rash observed in most CAPS patients responds to therapy with IL-1 receptor antagonist rather than antihistamines, suggesting that urticaria in these patients is mediated by IL-1. However, the cellular mechanism responsible for urticaria in CAPS patients remains poorly understood.

The mature form of IL-1β is produced by cleavage of the inactive pro–IL-1β precursor by caspase-1, a protease activated by a large multiprotein complex termed the inflammasome (6). CAPS is caused by missense mutations in the gene, nucleotide-binding oligomerization domain (NOD)–leucine-rich repeats (LRRs) containing pyrin domain 3 (*NLRP3*) (7), whose product is a component of the inflammasome that includes the adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and procaspase-1 (8, 9). NLRP3, a member of the NOD-like receptor family, is an intracellular receptor involved in the recognition of pathogen-associated molecular patterns (PAMPs). Although several microbial activators of NLRP3 have been reported, the precise mechanism by which the NLRP3 inflammasome is activated by PAMPs remains poorly understood. In the presence of ATP or pore-forming molecules, several PAMPs, including LPS, muramyl dipeptide, bacterial mRNA, and the antiviral compound R837, activate the NLRP3 inflammasome (10, 11). In addition to PAMPs, NLRP3 senses endogenous danger signals such as monosodium urate crystals and particulate matter including asbestos, silica (12), and aluminum salts (13, 14). Disease-associated NLRP3 mutations associated with CAPS localize to the centrally located NOD domain and constitutively activate caspase-1 to produce active IL-1β (8, 15). NLRP3 is predominantly expressed in monocytes, granulocytes, and chondrocytes (16, 17), but to date, no reports have investigated the cells in the skin that are involved in the development of urticarial rash associated with CAPS. Our study identifies resident MCs in the skin as a cell population capable of producing IL-1β via the NLRP3 inflammasome and provides evidence that MCs mediate urticarial rash via dysregulated IL-1β production in the skin of CAPS patients.

RESULTS

Resident MCs produce constitutively mature IL-1β in CAPS skin

To identify the source of IL-1β in the human skin, we performed immunolabeling of skin organ cultures with an antibody (Ab) that recognizes p17, the mature form of IL-1β. Expression of mature IL-1β was undetectable in normal skin cells but was induced in resident cells by incubation of skin organ cultures with LPS and R837 (Fig. 1). In contrast, cells in the dermis of two MWS patients harboring the E567K or K355T *NLRP3* mutation and suffering from active disease but not receiving any treatment expressed the mature form of IL-1β constitutively without any stimulation (Fig. 1). Notably, the majority of the mature IL-1β–positive cells colocalized with avidin-FITC (Fig. 1), which specifically labels tryptase-positive MCs, but not with HLA-DR, -DP, -DQ, a marker of DCs and macrophages (Fig. S1), in both normal and CAPS skin.

MCs express inflammasome components and produce IL-1β in response to proinflammatory stimuli

We next analyzed the expression of inflammasome components in MCs using mouse bone marrow–derived cultured MCs (BMCMCs). The purity of cultured MCs, which was >97%, was confirmed by surface expression of CD45 and Kit, as well as FITC-avidin labeling. Exclusion of DCs/macrophages was further supported by the lack of I-A^b expression in the MC population (Fig. S2 A). BMCMCs constitutively expressed *Casp1* and the critical adaptor *Asc*, as determined

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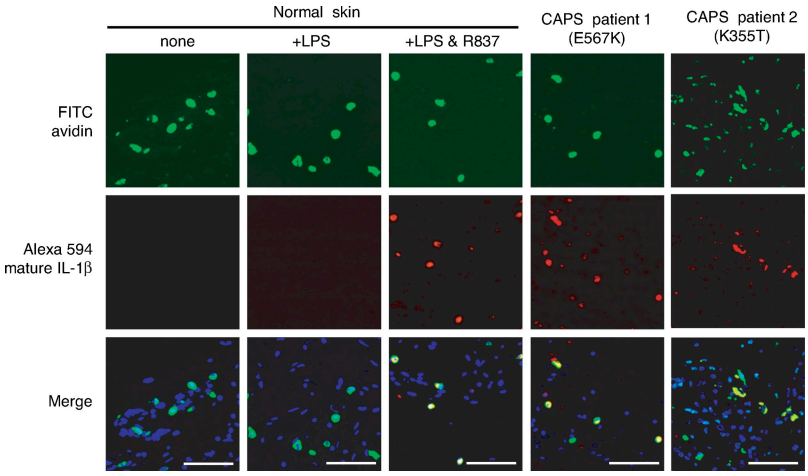

Figure 1. MCs from the skin of CAPS patients expressing disease-associated NLRP3 mutations constitutively express the mature form of IL-1β. Human skin specimens were stained with FITC-avidin to label MCs (top) and Alexa Fluor 594–conjugated anti–IL-1β Ab that recognizes only the mature form of IL-1β (middle). (bottom) Overlay of fluorescence images of the top and middle panels. The nuclei were counterstained with Hoechst 33342 (blue). Normal skin represents organ skin cultures with or without incubation with 100 ng/ml LPS or LPS and 100 μM R837 for 24 h. Results are representative of at least three separate experiments. Bars, 50 μm.

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by RT-PCR analysis (Fig. 2, A and B). In contrast, there was no or little expression of *Nlrp3* and *Il1b* in MCs, but both genes were induced by stimulation with LPS (Fig. 2, A and B), which is consistent with what was reported in human monocytes (18). Induction of *Nlrp3* and *Il1b* by LPS was reduced by treatment with MG-132, a broad proteasome inhibitor that can affect several signaling pathways including NF-κB (Fig. 2 A), suggesting that induction of *Nlrp3* and *Il1b* may require NF-κB activation.

We next determined the ability of MCs to produce IL-1β in response to proinflammatory stimuli known to activate caspase-1. Stimulation of MCs with LPS alone induced little IL-1β secretion, whereas treatment with R837, a small antiviral synthetic compound that activates the NLRP3 inflammasome (11), induced potent IL-1β secretion in MCs pretreated with LPS (Fig. 2, C and D). In accordance with these results, R837 effectively induced intracellular processing of pro–IL-1β into p17 in LPS-primed MCs, whereas secretion of mature IL-1β into the culture supernatant was more potently induced by ATP than by R837 (Fig. 2 D). BMCMCs did not express *Tlr7* mRNA as assessed by real-time PCR (not depicted) and Tlr7 protein by immunoblotting (Fig. S2 B), suggesting that R837 promotes caspase-1 activation independently of Tlr7 in MCs. In contrast, incubation of MCs with LPS alone was sufficient to induce secretion of TNF-α and IL-6 (Fig. 2, E and F). Notably, co-stimulation with LPS and R837 did not induce MC degranulation as determined by the release of β-hexosaminidase (Fig. 2 G) and histamine (Fig. 3 A, right), indicating that the mechanisms involved in IL-1β secretion and degranulation are differentially regulated in MCs.

ATP stimulation through the purinergic P2X, ligand-gated ion channel 7 receptor (P2X7R) is essential for the activation of the NLRP3 inflammasome in macrophages (10, 19). We found that in addition to R837, ATP induced secretion of IL-1β in MCs stimulated with LPS (Fig. 2 D; and Fig. 3 A, left), which is consistent with the expression of the P2x7r in MCs (20). K^+ efflux induced by ATP is important for caspase-1 activation via P2x7r (21), and this ion channel rapidly transitions to a pore-like structure that allows passage of molecules as large as 900 D (19). Notably, IL-1β secretion induced by ATP or R837 was completely abrogated when the MCs were incubated in medium containing a high K^+ concentration (Fig. 3 B). Furthermore,

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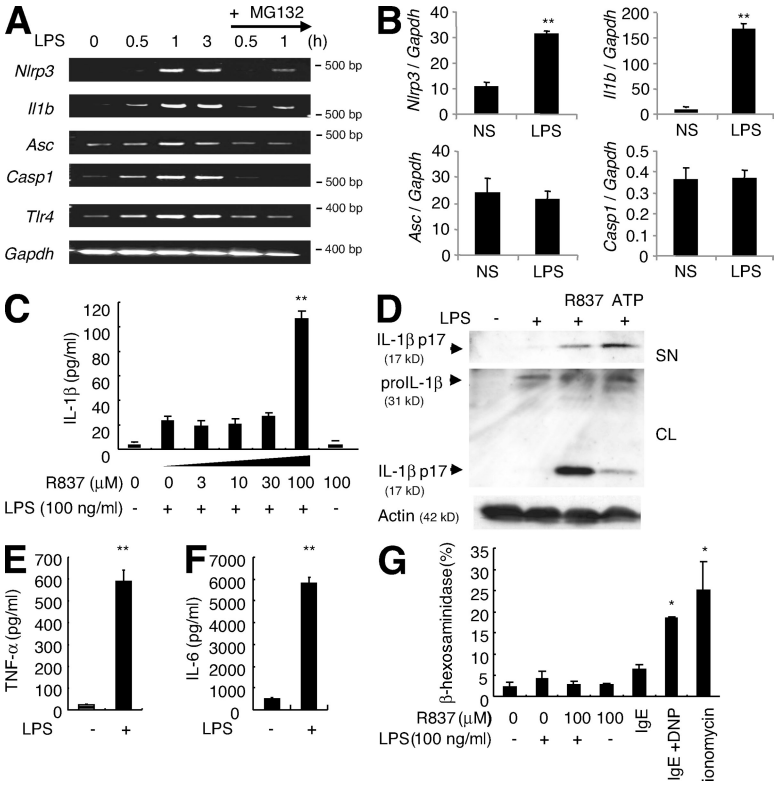

Figure 2. Activation of inflammasome with LPS and R837 induces IL-1β secretion but not degranulation in MCs. (A) Semiquantitative RT-PCR analysis of *Nlrp3*, *Il1b*, *Asc*, *Casp1*, and *Tlr4* in mouse BMCMCs stimulated with 100 ng/ml LPS. 5 μM MG132 was added 30 min before LPS stimulation. (B) Expression of specific mRNAs after stimulation for 3 h with 100 ng/ml LPS in BMCMCs was quantified by real-time PCR and normalized to *Gapdh* expression ($\times 10^4$). (C) BMCMCs were pretreated with LPS for 15 h and stimulated by R837 for 30 min. IL-1β levels in supernatants were measured by ELISA. (D) Immunoblot analysis of cultured supernatant (SN) and cell lysate (CL) from BMCMCs incubated with the indicated stimuli. (E and F) BMCMCs were stimulated with 100 ng/ml LPS for 15 h, and the amounts of TNF-α and IL-6 in culture supernatants were measured by ELISA. (G) Degranulation of BMCMCs was assessed by the release of β-hexosaminidase. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. *, $P < 0.005$; and **, $P < 0.001$ compared with untreated BMCMCs. NS, not stimulated.

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ATP induced large pore formation in MCs as determined by YoPro-1 staining (Fig. 3 C, left), which was not observed in P2x7r-deficient MCs (Fig. 3 C, right). Unlike ATP, R837 did not induce large pore formation (Fig. 3 C, left) even though R837 is structurally related to ATP. However, secretion of IL-1β induced by ATP required expression of P2x7r in LPS-stimulated MCs as well as that elicited by R837 was also dependent, at least in part, on P2x7r (Fig. 3 D). These results suggest that the P2x7r and K^+ efflux are important for both ATP- and R837-induced secretion of IL-1β in MCs.

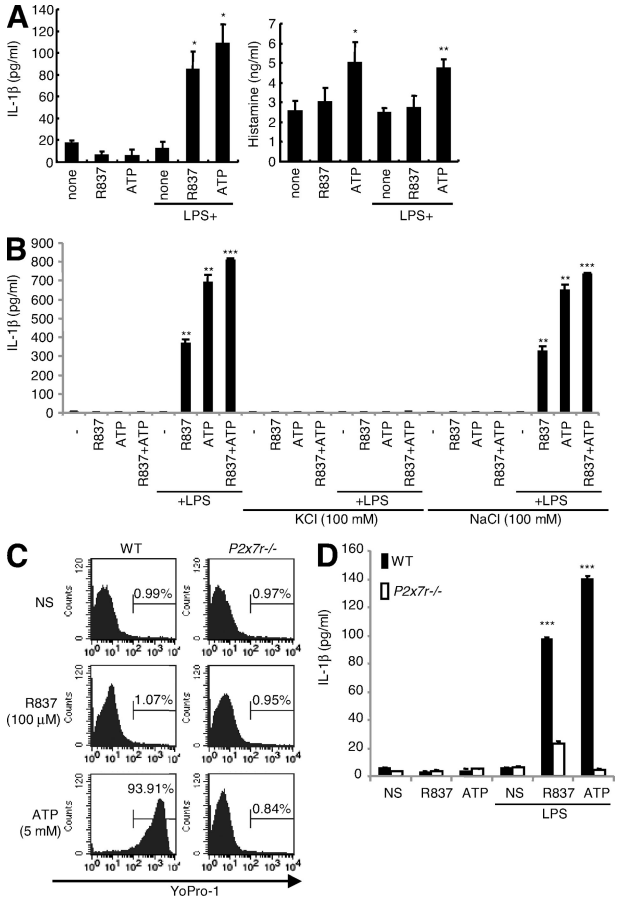

Figure 3. IL-1β secretion induced by R837 and ATP is mediated via P2x7r and K^+ efflux in MCs. (A, left) IL-1β secretion from BMCMCs. BMCMCs were cultured with or without LPS for 15 h and then stimulated with 5 mM ATP or 100 μM R837. (right) Histamine release from BMCMCs was analyzed by ELISA (same samples as in the left panel). (B) BMCMCs were cultured with or without LPS for 15 h. Medium containing 100 mM KCl or 100 mM NaCl was added for the last 30 min of culture at the same time as ATP or R837. IL-1β was measured in cell-free supernatants. (C) 2 mM YoPro-1 was added 10 min before BMCMCs were left untreated (NS) or stimulated with 5 mM ATP or 100 μM R837 for 30 min, and fluorescence intensity was analyzed by FACS. (D) BMCMCs were incubated with the indicated stimuli, and IL-1β was measured in cell-free supernatants. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. *, $P < 0.005$; **, $P < 0.001$; and ***, $P < 0.0001$ compared with untreated BMCMCs or between WT and mutant MCs. NS, not stimulated.

IL-1β secretion from MCs depends on the Nlrp3 inflammasome

To determine if the inflammasome is required for IL-1β secretion in MCs, we prepared MCs from WT and mutant mice deficient in Nlrp3, Nlrc4, or Asc. Stimulation of WT and mutant MCs lacking Nlrc4, a NOD-like receptor family member involved in inflammasome activation in response to flagellin (22, 23), induced processing of procaspase-1 into the mature p20 fragment (Fig. 4 A, top). In contrast, production of the processed p20 caspase-1 subunit was impaired in MCs deficient in Nlrp3 or Asc (Fig. 4 A, middle and bottom). Consistently, IL-1β secretion was not detected in MCs from Nlrp3- or Asc-deficient mice in response to LPS plus ATP or R837 (Fig. 4 B, top), whereas that produced by MCs lacking Nlrc4 was unimpaired when compared with WT MCs (Fig. S3, top). The lack of IL-1β secretion in MCs deficient in Nlrp3 or Asc was specific in that production of TNF-α and IL-6 was maintained in WT and mutant MCs (Fig. 4 B, middle and bottom; and Fig. S3, middle and bottom), although the amounts of both cytokines was reduced in MCs lacking Nlrp3 (Fig. 4 B, middle and bottom).

We next assessed the expression of inflammasome components in other populations of MCs. In mice, MCs are often divided into connective tissue– and mucosal-type MCs. Mouse fetus skin–derived MCs (FSMCs) are often used as a model of connective tissue–type MCs (24) that are distributed

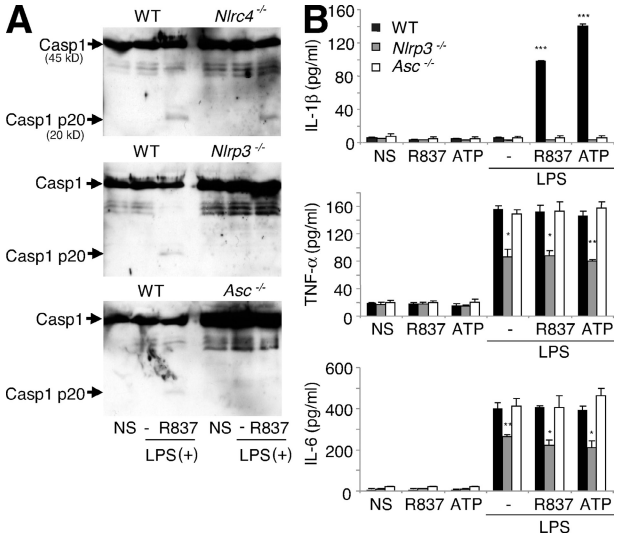

Figure 4. Activation of caspase-1 and IL-1β secretion requires the Nlrp3 inflammasome in MCs. (A) Immunoblot analysis of total cell extracts and supernatant from BMCMCs. Processing of procaspase-1 induced by R837 in LPS-primed BMCMCs from WT, Nlrc4-deficient (*Nlrc4*$^{-/-}$), Nlrp3-deficient (*Nlrp3*$^{-/-}$), and Asc-deficient (*Asc*$^{-/-}$) mice. Cells were pretreated with 100 ng/ml LPS and then stimulated with 100 μM R837. Casp1, procaspase-1; Casp1 p20, cleaved product of caspase-1. (B) Secretion of IL-1β, TNF-α, and IL-6 by LPS-stimulated BMCMCs from the indicated mice in response to R837 or ATP. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. *, $P < 0.005$; **, $P < 0.001$; and ***, $P < 0.0001$ compared between WT and mutant MCs. NS, not stimulated.

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in the dermis. We found that the expression of *Nlrp3*, *Asc*, and *Il1b* was low in unstimulated FSMCs but enhanced after LPS treatment, as it was shown in BMCMCs (Fig. S4 A). Similarly, the pattern of IL-1β secretion and MC degranulation was comparable in BMCMCs and FSMCs (Fig. S4, B and C). Likewise, human MCs derived from cord blood progenitors cultured with stem cell factor expressed *ASC*, *NLRP3*, and *IL1B* after LPS stimulation (Fig. S5 A). Similar to that reported in human monocytes (25), incubation of human MCs with LPS alone was sufficient to induce the processing of pro–IL-1β into the mature form of IL-1β (p17), which was enhanced by R837 or ATP (Fig. S5, B and C).

Constitutive activation of the inflammasome by disease-associated Nlrp3 mutants in MCs

CAPS-associated NLRP3 mutations exhibit constitutive ASC-dependent NF-κB activation when expressed in tumor cell lines (17, 26). To assess the function of mutant NLRP3, we generated mouse Nlrp3 mutants (R258W, D301N, and Y570C) corresponding to the major CAPS-associated mutations (R260W, D303N, and Y570C, respectively) and tested their ability to induce NF-κB activation by luciferase reporter assay. In agreement with human studies, all three Nlrp3 mouse mutants as well as the Nlrp3 mutant lacking the LRR exhibited constitutive Asc-dependent NF-κB activation in HEK293 cells (Fig. 5 A). To further assess the function of disease-associated mutants, we introduced WT and mutant Nlrp3 into BMCMCs using a retroviral expression system that simultaneously coexpresses GFP. After normalization for the number of GFP-expressing cells, we found that the secretion of IL-1β was significantly higher in MCs producing disease-associated Nlrp3 mutants than in cells expressing WT protein after stimulation with LPS (Fig. 5 B). Notably, the increased production of IL-1β induced by Nlrp3 mutants R258W and Y570C was abolished when their pyrin domain was deleted (ΔPYD_R258W and ΔPYD_Y570C; Fig. 5 B), which is consistent with a critical role for the pyrin domain in the interaction with caspase-1 through the adaptor Asc.

To further study the function of disease-associated Nlrp3 mutant in MCs, we stably expressed WT and the common R258W mutant in the MC line MC/9 by retroviral infection (Fig. S6). MC/9 cells constitutively expressed *Nlrp3*, *Asc*, *Casp1*, and *Tlr4* but little or no *Il1b* in the absence of LPS (Fig. 5 C). Expression of *Il1b* was induced by LPS (Fig. 5 C). Importantly, unlike MC/9 cells expressing the CAPS-associated R258W mutant, cells transduced with control GFP vector or producing WT-Nlrp3 required stimulation with both LPS and ATP or R837, two stimuli that activate the Nlrp3

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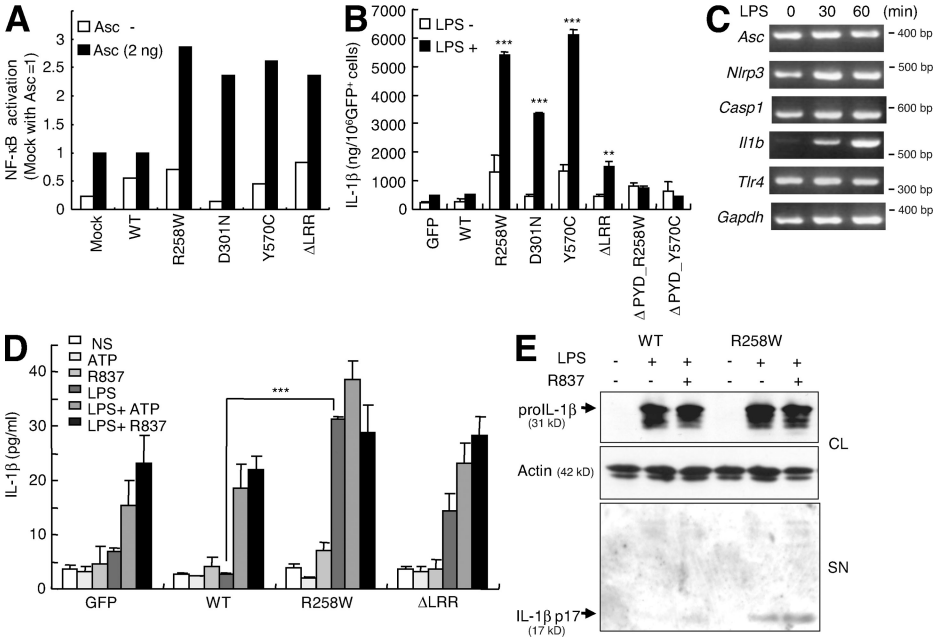

Figure 5. Expression of disease-associated Nlrp3 mutants induce constitutive activation of the inflammasome in MCs. (A) Constructs expressing WT, mutants (R258W, D301N, and Y570C), or Nlrp3 lacking LRR (ΔLRR) were transfected in the presence and absence of Asc plasmid into HEK293 cells. NF-κB activation was assessed by a dual luciferase reporter assay. Values represent the fold increase over that observed by transfection with control or Asc plasmid alone, which was considered as 1. (B) BMCMCs expressing GFP, WT, mutants, and mutants lacking the pyrin (ΔPYD_R258W and ΔPYD_Y570C) by retroviral infection were stimulated with LPS or left untreated. IL-1β was measured by ELISA and normalized to the number of GFP⁺ cells, as previously described (reference 47). **, $P < 0.001$; and ***, $P < 0.0001$ versus LPS-stimulated BMCMCs transfected with WT plasmid. (C) RT-PCR for gene expression in MC/9 cells stimulated with LPS. (D) MC/9 cells stably expressing GFP, WT, mutant Nlrp3, or ΔLRR were stimulated with LPS for 15 h, and then stimulated with ATP or R837. IL-1β secretions were measured by ELISA. ***, $P < 0.0001$ versus LPS-stimulated MC/9 cells expressing WT plasmid. Error bars in B and D represent means ± SD of triplicates. (E) Immunoblot analysis of culture supernatant (SN) and cell lysate (CL) from MC/9 cells stably expressing WT or R258W, and incubated with the indicated stimuli. All results are representative of at least three separate experiments.

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inflammasome, for IL-1β secretion (Fig. 5 D). Although stimulation with LPS alone induced the production of pro–IL-1β in MC/9 cells expressing either WT or mutant Nlrp3, the processed p17 form of mature IL-1β could be detected only in the culture supernatant of MC/9 cells expressing the R258W mutant (Fig. 5 E). In contrast and consistent with results presented in Figs. 2–4, the secretion of mature IL-1β in MC/9 cells expressing WT-Nlrp3 required stimulation with both LPS and R837, which was enhanced in cells producing the CAPS-associated Nlrp3 mutant (Fig. 5 E).

To assess the effect of disease-associated Nlrp3 in vivo, we injected MC/9 cells expressing either WT or mutant Nlrp3 i.p. into mice and assessed the recruitment of neutrophils in the i.p. cavity. FACS analysis revealed that MC/9 cells expressing mutant R258W but not WT-Nlrp3 constitutively produced intracellular IL-1β after injection into the peritoneal cavity (Fig. 6 A). At 36 h after injection, the number of Gr-1$^+$ neutrophils was significantly increased in the peritoneal cavity of mice injected with MC/9 cells expressing mutant R258W-Nlrp3 compared with that found in mice injected with cells expressing WT-Nlrp3 (Fig. 6 B). In contrast, administration of ionomycin, but not MCs expressing WT or mutant Nlrp3, increased the levels of histamine in the peritoneal cavity (Fig. 6 C). Collectively, these results indicate that disease-associated Nlrp3 mutants induce constitutive production of IL-1β but not histamine release, and promote neutrophil recruitment when expressed in MCs.

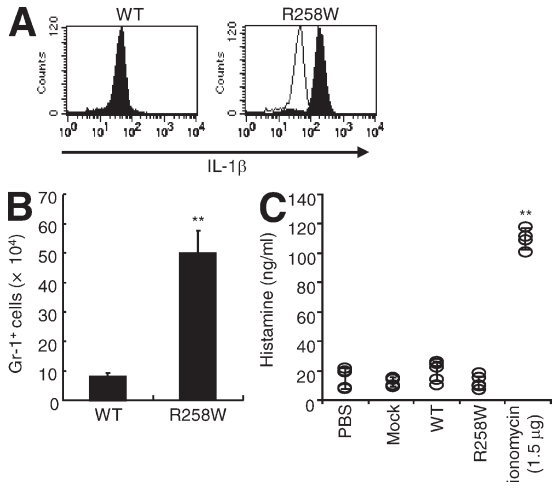

Figure 6. MCs expressing disease-associated Nlrp3 mutant promote neutrophil recruitment but not histamine release in the mouse peritoneal cavity. MC/9 cells stably expressing WT or R258W-Nlrp3 were injected into the mouse peritoneal cavity. Results were obtained 36 h after injection. (A) Shaded histograms represent intracellular IL-1β expression in MC/9 cells. The open histogram corresponds to labeling with isotype-matched control Ab. (B) Gr-1$^+$ cells in the peritoneal fluid. (C) The amounts of histamine in the peritoneal cavity 36 h after the injection of MC/9 cells. As a positive control, 1.5 μg ionomycin was injected 15 min before sample collection. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. **, $P < 0.001$ ($n = 5$ mice per group).

Induction of neutrophil-rich inflammation and vascular leakage by MCs expressing disease-associated Nlrp3 mutant

Urticarial rash in CAPS skin is characterized histologically by the presence of neutrophils and edema in the dermis (27). To study the role of IL-1β produced by MCs in vivo, we injected MC/9 cells expressing WT or mutant Nlrp3 (R258W) into the mouse skin. Histopathological analysis revealed the presence of neutrophils and edema in the dermis of the mouse ear at the site of injection, with MC/9 cells expressing R258W-Nlrp3 in a pattern that was similar to that observed in the involved skin of human CAPS (Fig. 7 A). In contrast, injection with MC/9 cells expressing WT-Nlrp3 induced minimal or no neutrophilic infiltrate or edema in the dermis of the mouse ear (Fig. 7 A). Furthermore, there was enhanced expression of IL-1β in the skin of the ear injected with MC/9 cells expressing R258W when compared with that observed with injection of MC/9 cells producing WT-Nlrp3 (Fig. 7 B). To determine whether the R258W-Nlrp3 mutation promotes vascular leakage, a hallmark of urticaria, we injected mice with MC/9 cells expressing WT and mutant Nlrp3 (R258W) in the ear skin and measured vascular leakage by Evans blue dye. Vascular leakage was significantly higher in the skin of mice injected with MC/9 cells expressing R258W-Nlrp3 than with cells expressing GFP alone or WT-Nlrp3 (Fig. 8, A and B). These results indicate that MCs expressing disease-associated Nlrp3 promote neutrophil recruitment and vascular leakage in the skin, two histological hallmarks of urticarial rash associated with CAPS.

DISCUSSION

MCs are widely distributed throughout vascularized tissues, where they are located near epithelial surfaces that are exposed to environmental cues, including the skin, airways, and gastrointestinal tract (28). MCs are known to promote inflammation and tissue remodeling in IgE-associated allergic disorders as well as to produce multiple cytokines, including IL-1β, in response to microbial stimuli, although the mechanisms involved remained unknown (29, 30). In this paper, we provide evidence that MCs express components of the inflammasome, and these factors are critical for the activation of caspase-1 and IL-1β secretion.

Our analysis revealed that Nlrp3 mutations associated with CAPS induce constitutive activation of the inflammasome in MCs, leading to dysregulated IL-1β production in the skin. These studies are consistent with a previous report that showed enhanced production of IL-1β in the skin of CAPS patients (31), although the cellular source of IL-1β was not investigated by the authors. We showed that transfer of MCs expressing the CAPS-associated Nlrp3 mutant induces perivascular neutrophil-rich inflammation in the mouse skin, which is the histological hallmark of the urticarial rash observed in CAPS patients. Collectively, these studies implicate MCs in inflammasome activation and the pathogenesis of IL-1β–mediated disease in the skin. Because MCs reside in multiples tissues and also participate in experimental models of arthritis (32) and encephalomyelitis (33–35), it is possible that these cells play a role in disease pathogenesis not only in

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the skin but also in the joints and central nervous system, which are also major disease targets in CAPS patients (36). Further studies are needed to better understand the contribution of MCs to IL-1β–mediated disease in autoinflammatory syndromes associated with NLRP3 mutations.

The mechanism by which NLRP3 mutations cause inflammatory diseases is still poorly understood. Studies in vitro suggested that these mutations exert a gain-of-function effect, probably through the loss of a regulatory step associated with NLRP3 activation (6, 8). Consistently, mouse Nlrp3 mutants, corresponding to those observed in human CAPS, induced constitutive Asc-dependent NF-κB activation and IL-1β secretion. Notably, the increased production of IL-1β induced by CAPS-associated Nlrp3 mutants was abolished when their pyrin domain was deleted, which is in accordance with a critical role for that module in the interaction with caspase-1 and assembly of the inflammasome (6, 8).

As is the case with macrophages, production of mature IL-1β via the Nlrp3/Asc inflammasome in mouse MCs required two signals, LPS and ATP or R837. Although a main function of LPS is to induce pro–IL-1β production, LPS also promoted expression of *Nlrp3* in MCs, suggesting that LPS regulates inflammasome activation via several mechanisms in MCs. In macrophages, ATP-driven stimulation through the P2x7r is essential for caspase-1 proteolytic cleavage and IL-1β secretion via Nlrp3 (10, 19). The P2x7r forms a nonselective ion channel upon activation with ATP (19) and upon stimulation mediates K^+ efflux, which may be important for Nlrp3 activation (21). This ion channel mediated by P2x7r rapidly transitions to a pore-like structure by recruiting the pannexin-1 pore, which allows passage of molecules as large as 900 D (19). Therefore, it is possible, as it has been proposed for macrophages (37), that ATP promotes passage of microbial ligands such as LPS via pannexin-1 to trigger inflammasome activation in MCs. Consistently, ATP did not induce caspase-1 activation alone and triggered large pore formation in MCs, and this activity was blocked in P2x7r-deficient MCs. Unlike ATP, however, R837 did not induce large pore formation but elicited IL-1β secretion, at least in part, through P2x7r. The role of P2x7r in mediating IL-1β secretion in response to R837 stimulation is consistent with the observation that high K^+ extracellular medium blocked IL-1β secretion in MCs. Collectively, these results suggest that ATP and R837 may promote inflammasome activation via different mechanisms in MCs, although both stimuli require P2x7r and K^+ efflux for effective IL-1β secretion in MCs.

We observed a reduction in the amounts of TNF-α and IL-6 produced by MCs lacking *Nlrp3* compared with those from WT or Asc-deficient cells. The reason for this different regulation of cytokine secretion by Nlrp3 and Asc is unclear. One possibility is that Nlrp3 contributes to MC maturation in an Asc-independent manner. *Nlrp3* was first reported as an MC maturation–inducible gene (38) whose expression was

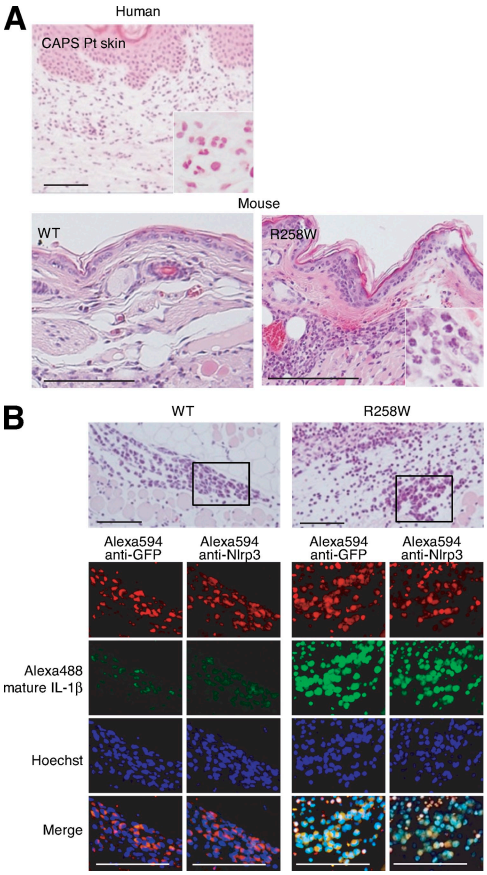

Figure 7. MCs expressing disease-associated Nlrp3 mutant induce neutrophil-rich inflammation in the mouse skin. 10^5 MC/9 cells expressing WT or mutant Nlrp3 (R258W) were injected into the skin of mice. (A, top) Representative images of the involved skin from a CAPS patient harboring an E567K *NLRP3* mutation. (bottom) Cross sections of mouse ears 36 h after injection with MC/9 cells. Tissue sections were stained with hematoxylin and eosin. Insets show a higher magnification demonstrating the presence of numerous neutrophils. Bars, 100 µm. (B) IL-1β expression in GFP^+ and $Nlrp3^+$ MC/9 cells. (bottom) Immunofluorescence images of mouse skin injected with MC/9 cells expressing WT or R258W-Nlrp3. Images were derived from tissues boxed in the top panels. All results are representative of at least three separate experiments. Bars, 50 µm.

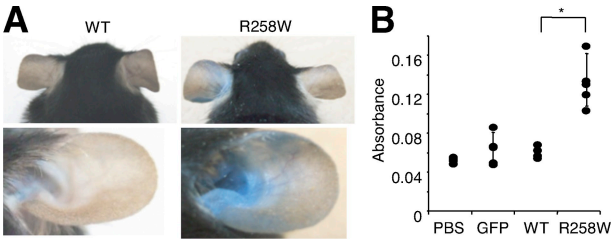

Figure 8. MCs expressing disease-associated Nlrp3 mutant induce vascular leakage in the mouse skin. Vascular leakage was analyzed by intravenous injection of 1% Evans blue solution 36 h after injection of mice with MC/9 cells expressing WT and mutant Nlrp3. (A, left) Mouse ears 30 min after dye injection. (B) Ears were collected, and vascular leakage was determined by absorbance at 620 nm in dye tissue extracts. Error bars represent means ± SD of results that are representative of at least three separate experiments. *, $P < 0.005$ between WT and R258W (n = 4 or 5 mice per group).

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increased after the maturation into MCs with a connective tissue phenotype. Our BMCMCs were cultured with IL-3 and stem cell factor, which partially induce connective tissue–type maturation compared with the cells cultured with IL-3 alone. This possibility may explain, at least in part, why CAPS patients suffered from skin eruption but not asthma reaction or rhinitis, even though MCs in these tissues are likely to express the constitutively activated disease-associated NLRP3 mutation. However, no morphological and functional differences were observed between WT and Nlrp3-deficient MCs. Alternatively, Nlrp3 may directly or indirectly regulate cytokine production independently of the inflammasome in MCs.

CAPS-associated NLRP3 mutants constitutively produced IL-1β in MCs of the skin of patients and when injected into mouse tissues. However, it remains unclear why MCs with constitutive activation of the NLRP3 inflammasome produce mature IL-1β in the absence of LPS in patients given that microbial stimulation appears to be required for pro–IL-1β production. One possibility is that CAPS-associated NLRP3 mutants induce pro–IL-1β via constitutive activation of NF-κB induction, which is consistent with results found in the current work and also reported by others (26). Another possibility is that production of pro–IL-1β is induced by endogenous or environmental cues operating in the skin independently of NLRP3. Consistent with this model, the characteristic skin rash observed in CAPS and systemic inflammatory syndromes often develops within the first few weeks of life when the skin is first exposed to environmental factors. These may include exposure to small amounts of LPS and/or other microbial stimuli after birth. The observation that skin abnormalities in incontinentia pigmenti (MIM 308300), an X-linked dominant inherited disorder caused by the mutation of *NEMO,* a gene that encodes the regulatory component of the IκB kinase complex responsible for the activation of the NF-κB signaling pathway, commence at birth (39, 40) is also consistent with this possibility.

Urticarial rash associated with CAPS is usually nonpruritic and unresponsive to antihistamines. This clinical observation is in accordance with our observation that NLRP3 inflammasome activation induces IL-1β secretion but not degranulation in MCs. Nonetheless, MCs expressing CAPS-associated NLRP3 mutant promoted vascular permeability, a cellular response also induced by histamine release, which is critical for wheal formation in vivo. Because many cases of non-CAPS urticaria are unresponsive to histamine receptor antagonists, it is possible that skin rash associated with histamine resistance is mediated via inflammasome activation in MCs. Thus, understanding the pathophysiology of CAPS may provide critical insight into more common diseases such as antihistamine-refractory urticaria.

MATERIALS AND METHODS

Chemicals and reagents. Ultrapure LPS (*Escherichia coli* O55:B5), ATP, and MG-132 (Z-Leu-Leu-Leu-al) were purchased from Sigma-Aldrich. R837 (tlrl-imq) was purchased from InvivoGen. Abs for human cleaved IL-1β and IL-1β were purchased from Cell Signaling Technology, and human HLA-DR, -DP, -DQ (TAL.1B5) was purchased from Dako. Anti–human tryptase (G3) was provided by L. Schwartz (Virginia Commonwealth University, Richmond, VA). Anti–mouse IL-1β was purchased from R&D Systems. Abs for actin and cryopyrin were purchased from Santa Cruz Biotechnology, Inc., Tlr7 was purchased from Imgenex, and I-A^b (clone AF6-120.1) was purchased from BD. Anti-GFP was purchased from MBL International, and FITC- or Texas red–conjugated avidin was purchased from Invitrogen. Alexa Fluor 647–labeled anti–mouse IL-1β, Alexa Fluor 647–labeled anti–Ly-6G, and PE-labeled anti-CD45 were purchased from eBioscience.

Animals. *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Nlrc4*$^{-/-}$, and *P2x7r* $^{-/-}$ mice backcrossed at least eight times to C57BL6/J6 mice have been previously described (11, 41, 42). C57BL6/J6 mice were purchased from Crea Japan and housed in a pathogen-free facility. The animal studies were conducted under approved protocols by the Committee on the Use and Care of Animals of the University of Michigan and Chiba University.

Cultured MCs. The preparations of BMCMCs and FSMCs were previously described (24). The purity of MCs was confirmed by surface expression of CD45 and Kit, FITC-avidin labeling, and negative I-A^b expression (Fig. S2 A). Degranulation of MCs was assessed by β-hexosaminidase assay as previously described (24). Human MCs from cord blood were cultured as previously described (43).

Plasmid construction and retrovirus production. Plasmids to express mouse Nlrp3 have been previously described (31). Mutant Nlrp3 constructs corresponding to a human disease-associated mutation in the pEF-BOS vector were generated as previously described (18, 44). The ability of each construct to induce NF-κB activation was assessed with mouse Asc plasmid as previously described (18, 44). The retrovirus vector pMC-IRES-GFP (provided from T. Kitamura, University of Tokyo, Tokyo, Japan) containing Nlrp3 and its mutants was produced and introduced into Plat-E packaging cells with FuGENE6 (Roche). Immature BMCMCs cultured for 2 wk were incubated with retroviral supernatants for 15 h with 10 μg/ml polybrene (Sigma-Aldrich) (45). GFP-Nlrp3 fusion proteins were cloned into the pMX-IP vector and transfected into Plat-E cells. MC/9 cells were incubated with virus-containing supernatants and selected with 1.5 mg/ml puromycin.

Immunohistochemistry. Human and mouse skin biopsy samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin staining. Immunohistochemical staining was performed with primary Abs at the recommended concentrations followed by incubation with fluorescent dye–conjugated secondary Abs. Written informed consents were obtained from patients, according to the protocol of the institutional review board of Kyoto University Hospital and in accordance with the Declaration of Helsinki.

RT-PCR. Total RNA extracted from MCs (2×10^6) with TRIzol reagent (Invitrogen) was reverse transcribed and analyzed. The primers were as follows: *Nlrp3*, 5′-CACTTGGATCTAGCCACATC-3′ and 5′-AGCTCCAGCTTAAGGGAACTC-3′; *Asc*, 5′-ACTTGTCAGGGGATGAACTC-3′ and 5′-TGGTACTGTCCTTCAGTCAG-3′; *Casp1*, 5′-TACCTGGCAGGAATTCTGGA-3′ and 5′-ATGATCACCTTGGGCTTGTC-3′; *Il1b*, 5′-GCTTCCAAACCTTTGACCTG-3′ and 5′-CTGTTGTTTCCCAGGAAGAC-3′; *Tlr4*, 5′-CTGGCATCATCTTCATTGTCC-3′ and 5′-GCTTAGCAGCCATGTGTGTTCC-3′; *Tlr7*, 5′-CCACCAGACCTCTTGATTCC-3′ and 5′-TCCAGATGGTTCAGCCTACG-3′; *Gapdh*, 5′-CGGGAAGCTTGTCATCAATGG-3′ and 5′-GGCAGTGTGATGGCATGGATCTG-3′; *NLRP3*, 5′-AACAGCCACCTCACTTCCAG-3′ and 5′-GACGTAAGGCCAGAATTCAC-3′; *ASC*, 5′-TGGTCAGCTTCTACCTGGAG-3′ and 5′-TCCAGGCTGGTGTGAAACTG-3′; *CASP1*, 5′-GTACTTTCTTCCTTTCCAGCTC-3′ and 5′-TTCACATCTACGCTGTACCC-3′; and *IL1B*, 5′-TTCACATCTACGCTGTACCC-3′ and 5′-GTACTTTCTTCCTTTCCACCTC-3′. For real-time analysis, the mixture with Power SYBR Green PCR Master Mix (Applied Biosystems)

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was amplified and analyzed with 7300 Real-Time PCR systems (Applied Biosystems).

Immunoblotting. Cell extracts were prepared in M-PER reagent (Thermo Fisher Scientific) in the presence of protease inhibitor cocktail (Thermo Fisher Scientific). Samples were denatured in 2× Laemmli sample buffer (Bio-Rad Laboratories) with 1 μl 2-ME. Lysates were separated by SDS-PAGE and transferred onto membranes (GE Healthcare). Membranes were incubated with an Ab against caspase-1, IL-1β, GFP, Tlr7, and actin, followed by horseradish peroxidase–conjugated Abs. Immunoreactive proteins were visualized with ECL detection reagents (GE Healthcare).

Cytokine ELISAs. Supernatants collected from 10^6 MCs/ml were measured for IL-1β (eBioscience), histamine (Invitrogen), TNF-α, and IL-6 (R&D Systems) by ELISA.

Neutrophil migration assay and histamine release. MC/9 cells stably expressing WT or mutant Nlrp3 (R258W) were suspended in PBS (10^7 cells/ml) and administrated i.p. (0.5 ml/mouse). 36 h after the injection, mice were sacrificed and the peritoneal cavity was lavaged with 2 ml PBS. After gentle massage, 1 ml of the peritoneal fluid was collected and centrifuged at 500 *g* for 5 min. Cells were stained for FACS analysis and the supernatant was used for histamine assay.

Evans blue dye injection assay. We modified the passive cutaneous anaphylaxis assay in mice as previously described (46). Right ears were injected intradermally with 10^5 cells in 0.1 ml saline and left ears were injected with saline as a control; 36 h later, mice were challenged with 0.5 ml saline containing 5 mg/ml Evans blue dye. Extravasation of Evans blue dye was monitored for 15 min, and ear biopsies were incubated at 63°C overnight in 700 μl formamide. Quantitative analysis of extracts was determined by measuring the absorbance at 620 nm.

Statistical analyses. All data are expressed as means ± SD. We accumulated the data for each condition from at least three independent experiments. We evaluated statistical significance with the Student's *t* test for comparisons between two mean values.

Online supplemental material. Fig. S1 shows the immunochemical staining of a CAPS patient's skin where tryptase-positive MCs, but not HLA-DR, -DP, -DQ–positive DCs or macrophages, were labeled with avidin and produced mature IL-1β. Fig. S2 shows the purity of BMCMCs and negative protein expression of Tlr7 on BMCMCs. Fig. S3 shows IL-1β, TNF-α, and IL-6 secretion from MCs in WT and Nlrc4-deficient mice. Fig. S4 shows a PCR analysis of the inflammasome components in mouse FSMCs that produced IL-1β, but did not show degranulation, in response to Nlrp3 activator. Fig. S5 shows a PCR analysis of the inflammasome components in human MCs that produced IL-1β in response to Nlrp3 activator. Fig. S6 shows the schematic structures of plasmids in pMX-IP retrovirus vector and the purity of MC/9 stably Nlrp3-GFP–expressing cells that did not show degranulation in response to Nlrp3 activator. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082179/DC1.

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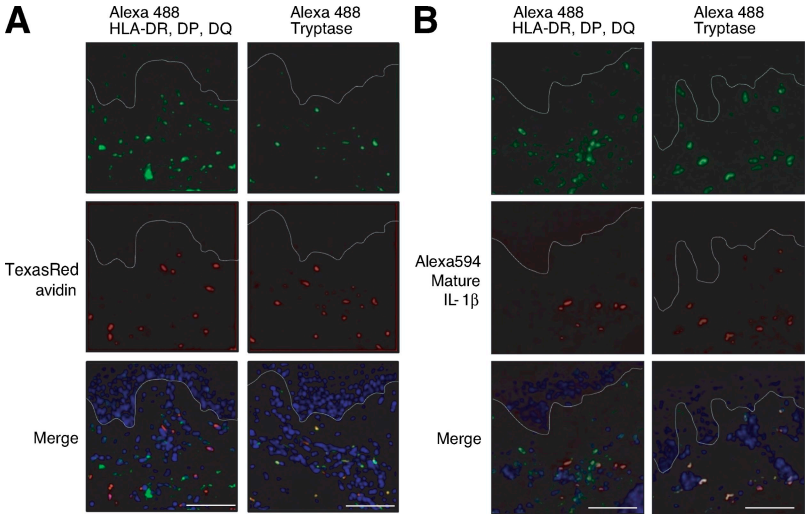

Figure S1. Tryptase-positive MCs, but not HLA-DR, -DP, -DQ–positive DCs or macrophages, are labeled with avidin and produce mature IL-1β in the skin of CAPS patient. Skin specimens from a CAPS patient with the E567K *NLRP3* mutation were stained with Alexa Fluor 488–conjugated secondary Ab to identify HLA-DR, -DP, -DQ expression, a marker of dermal DCs, Langerhans cells, and macrophages, or MC-specific tryptase and colabeled with (A) Texas red–avidin or mature (B) IL-1β. The bottom panels show overlays of fluorescence images from the top and middle panels. The nuclei were counterstained with Hoechst 33342 (blue). Dotted lines show epidermal–dermal junctions. Results are representative of at least three separate experiments. Bars, 100 μm.

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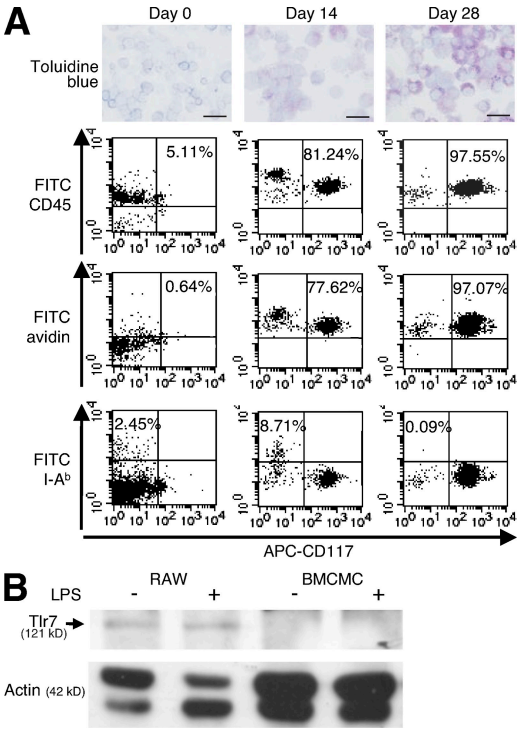

Figure S2. Expression of CD45, CD117, I-A^b, and Tlr7 in cultured BMCMCs. (A, top) Cultured MCs were collected at the indicated days and stained with toluidine blue. (bottom) Surface expression of CD45, CD117 or I-A^b, and FITC-avidin was determined by FACS. Bars, 30 µm. (B) Immunoblot analysis of total cell lysate of Tlr7 protein expression in mouse BMCMCs stimulated with 100 ng/ml LPS. The mouse monocytic cell line RAW264.7 was used as positive control. Results are representative of at least three separate experiments. APC, allophycocyanin.

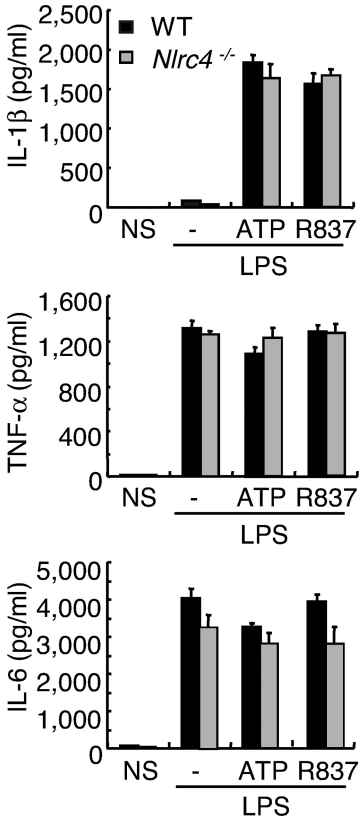

Figure S3. Cytokine secretion from MCs was maintained in WT and Nlrc4-deficient mice. Secretion of IL-1β, TNF-α, and IL-6 by LPS-primed BMCMCs from WT and Nlrc4-deficient (*Nlrc4*$^{-/-}$) mice in response to 100 µM R837 or 5 mM ATP. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. NS, not stimulated.

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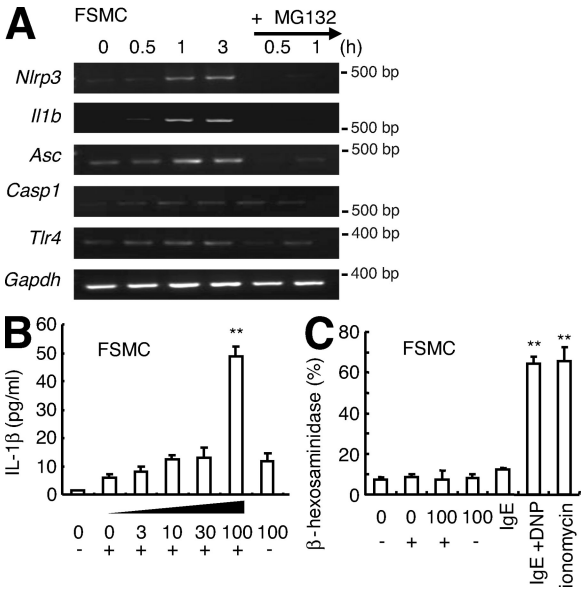

Figure S4. Mouse connective tissue–type FSMCs produce IL-1β in response to activators of the Nlrp3 inflammasome. (A) Semiquantitative RT-PCR analysis of *Nlrp3*, *Il1b*, *Asc*, *Casp1* and *Tlr4* in mouse FSMCs stimulated with 100 ng/ml LPS. 5 μM MG132 was added 30 min before LPS treatment. (B) FSMCs were pretreated with LPS for 15 h and then stimulated by R837 for 30 min. IL-1β was measured in culture supernatants by ELISA. **, $P < 0.001$ versus untreated FSMCs. (C) Degranulation of FSMCs was assessed by the release of β-hexosaminidase. **, $P < 0.001$ versus untreated FSMCs. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments.

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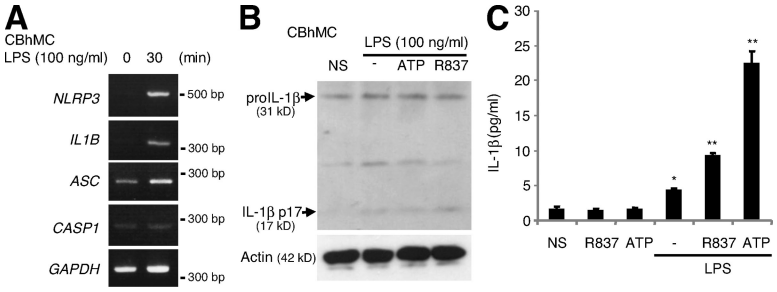

Figure S5. Human MCs produce IL-1β in response to activators of the Nlrp3 inflammasome. (A) Semiquantitative RT-PCR analysis of the inflammasome components *NLRP3*, *IL1B*, *ASC*, and *CASP1* in MCs derived from the incubation of human cord blood progenitors (CBhMCs) with stem cell factor. CBhMCs were stimulated with LPS. (B) Immunoblot analysis of total cell lysates from CBhMCs. Cells were cultured with or without 100 ng/ml LPS for 15 h and then stimulated with 500 μM ATP or 100 μM R837. Cell extracts were immunoblotted with Abs for IL-1β and β-actin. (C) CBhMCs were pretreated with LPS for 15 h and then stimulated with ATP or R837 for 30 min. IL-1β levels in supernatants were measured by ELISA. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. *, $P < 0.005$; and **, $P < 0.001$. NS, not stimulated.

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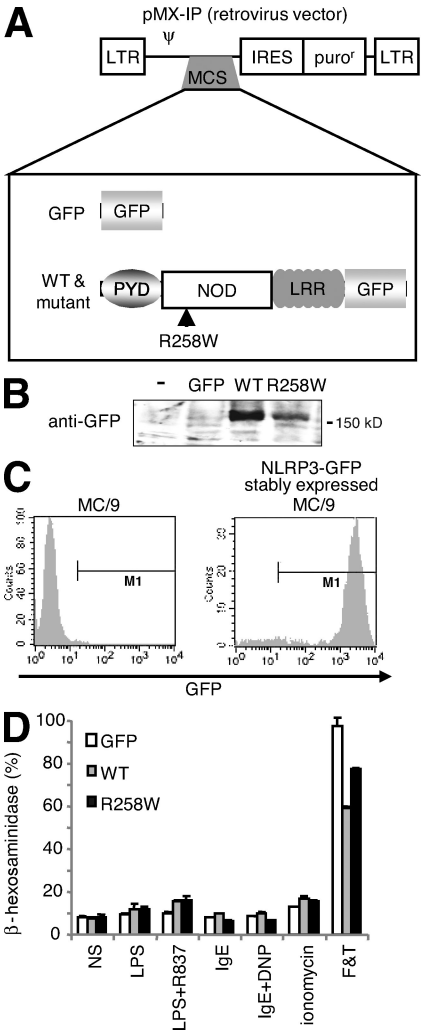

Figure S6. Nlrp3 WT and mutant (R258W) stably expressed MC/9 cells. (A) The schematic structures of GFP and mouse Nlrp3 WT and its mutant (R258W) in the pMX-IP retrovirus vector. (B) Nlrp3 and GFP fusion protein expression in MC/9 cells were analyzed by immunoblot analysis. (C) The purity of MC/9 cells stably expressing Nlrp3-GFP fusion protein. After the selection with puromycin, >98% of the cells expressed Nlrp3-GFP fusion protein as determined by the percentage of GFP$^+$ cells by FACS analysis. (D) Degranulation of MC/9 cells stably expressing Nlrp3 as assessed by the release of β-hexosaminidase. Error bars represent means ± SD of triplicates. All results are representative of at least three separate experiments. F&T, freeze and thaw; LTR, long terminal repeat; MCS, multicloning site; NS, not stimulated; Ψ, packaging signal; Puro(r), puromycin-resistant gene; PYD, pyrin domain.

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