The Inflammasome, an Innate Immunity Guardian, Participates in Skin Urticarial Reactions and Contact Hypersensitivity

Naotomo Kambe¹, Yuumi Nakamura¹,², Megumu Saito³,⁴ and Ryuta Nishikomori³

ABSTRACT
Urticarial rash, one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS), is caused by a mutation in the gene encoding for NLRP3 (nucleotide-binding oligomerization domain, leucine-rich repeats containing family, pyrin domain containing 3). This intracellular pattern recognition receptor and its adaptor protein, called apoptosis associated speck-like protein containing a caspase-recruitment and activating domain (ASC), participate in the formation of a multi-protein complex termed the inflammasome. The inflammasome is responsible for activating caspase-1 in response to microbial and endogenous stimuli. From the analysis of cellular mechanisms of urticarial rash in CAPS, we have traced caspase-1 activated IL-1β in CAPS to a surprising source: mast cells. Recently, two groups have generated gene-targeted mice that harbored Nlrp3 mutations. These mice had very severe phenotypes, with delayed growth and the development of dermatitis, but not urticaria. The reason for the differences in the skin manifestations observed with CAPS and these knock-in mice relates to the findings that the inflammasome also plays a role in contact hypersensitivity, and that IL-18, another cytokine involved with inflammasome-activation of caspase-1, may be a major player in dermatitis development.

KEY WORDS
contact hypersensitivity, IL-1β, inflammasome, NLRP3, urticaria

INTRODUCTION
The skin is the primary interface between the interior of the body and the external environment. The skin functions to retain water, prevent the permeation or loss of other molecules and maintain body temperature. The skin also physically protects us from microbial invasion. Furthermore, it has become established that the skin itself plays a major role in the immune system.¹

Recognition of invading microorganisms is essential for inducing an effective immune response. This process is mediated by germ line-encoded pattern recognition receptors, which can also be found in plants that do not have circulating white blood cells. To date, the most extensively studied pattern recognition receptors have been the Toll-like receptors (TLRs). Using their leucine-rich repeats (LRRs), these transmembrane proteins recognize conserved bacterial constituents, such as lipopolysaccharide (LPS). More recently, another class of pattern recognition receptors, called nucleotide-binding oligomerization domain (NOD)-LRRs containing family (NLRs), have been identified.² While TLRs detect bacterial products at the outer cell surface or in endosomes, intracellular NLRs mediate cytoplasmic recognition of bacterial products.³

Several NLR members participate in the formation of a multi-protein complex termed the inflammasome that is responsible for activating caspase-1 in re-
urticaria, or hives, is a common disease that affects up to 20% of the general population at least once during their lifetimes. This allergic disorder involving the skin is fleeting in nature, as it is characterized by the sudden appearance of wheals but the returning to its normal appearance without pigmentation, usually within 1-24 hours. In its histological aspects, the wheal consistently exhibits localized edema of the dermis with dilatations of the post-capillary venules and lymphatic vessels. However, perivascular infiltrates show variable intensities comprised of neutrophils, and/or eosinophils, macrophages and T cells. These inconsistent histological findings may underline the complex nature of the pathogenesis of urticaria, which has many sources including histamine release by activated mast cells.

Although patients with acute urticaria often complain of an upper airway infection, the eliciting cause is unclear in over 50% of patients. In particular, for chronic urticaria, defined as that persisting longer than 6 weeks, triggers remain unidentifiable in the majority of cases, despite extensive clinical and laboratory investigations. In addition, urticaria is sometimes triggered by cold or heat contact, solar exposure, delayed pressure, mechanical stimuli and vibration. These subtypes of urticaria are classified as physical urticaria. Other types of urticaria are aquagenic, cholinergic, evoked by a contact irritant or exercise-induced. Moreover, 2 or more different subtypes of urticaria can co-exist in any given patient.

Compared with the variety of proposed causes for urticaria, the strategy for treatment is relatively simple. Histamine H1-receptor antagonists are recommended as the first line of treatment because histamine release by cutaneous mast cells plays an important role in the development of urticaria. However, good responses using oral antihistamines have only been recorded for 40% of patients. Antihistamines have been shown not to be effective for physical urticaria, suggesting that in a significant number of individuals, chronic urticaria is mediated via histamine-independent mechanisms.

**CRYOPYRIN-ASSOCIATED PERIODIC SYNDROME**

An urticarial rash (Fig. 2) that develops in the neonatal or early infant period is one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS). CAPS is caused by a mutation in the gene encoding for NLRP3, previously known as NALP3/C1S/cryopyrin. CAPS includes a spectrum of hereditary periodic fever disorders that comprise 3 phenotypically overlapping, but relatively distinct syndromes: familial cold autoinflammatory syndrome [FCAS, Mendelian inheritance in men number (MIM) #120100], Muckle-Wells syndrome (MWS, MIM #191900) and chronic infantile neurological cutaneous and articular (CINCA) syndrome (MIM #607115), also known as neonatal-onset multisystem inflammatory disease. FCAS and MWS are characterized by periodic attacks of urticarial rash, fever and arthralgia; whereas patients with CINCA syndrome,
the most severe form of CAPS, exhibit chronic urticaria, as well as fever, arthropathy, chronic meningitis, papilledema, growth and mental retardation and hearing loss (Table 1). The urticarial rash observed in CAPS is similar to that associated with common urticaria. However, unlike ordinary urticaria, the rash observed in most CAPS patients is not pruritic and responds to therapy with an IL-1 receptor antagonist rather than antihistamines.

**INFLAMMASOME ACTIVATION IN MAST CELLS**

After development of an anti-IL-1β specific antibody (canakinumab), it was found that patients with CAPS have abnormally high levels of circulating IL-1β, approximately 5 times the normal amount. In addition, treating patients with canakinumab relieved their rashes within a day, suggesting that IL-1β was the sole cytokine responsible for the skin eruptions in CAPS. The disease-responsible gene, *NLRP3*, is predominantly expressed in monocytes, granulocytes and chondrocytes. However, after performing immunohistochemical staining, we traced IL-1β in CAPS to a surprising source: mast cells. Interestingly, mast cells in the skin samples from CAPS patients expressed active IL-1β without any treatment, whereas those from healthy donors only expressed active cytokine when appropriately stimulated.

Primary mast cells derived from mouse bone marrow and human cord blood expressed inflammasome components, including *NLRP3* and its adapter protein ASC. As was the case with macrophages, production of mature IL-1β via the NLRP3-inflammasome in mast cells required 2 signals. Microbial ligands, such as LPS, trigger the synthesis of the IL-1β precursor. A second ATP-triggered signal activates the inflammasome. Although a major function of LPS is to induce pro-IL-1β production, LPS also promotes the expression of *Nlrp3* in mast cells.

In macrophages, ATP-driven stimulation via the purinergic receptor P2X, ligand-gated ion channel 7 (P2RX7) is essential for caspase-1 proteolytic cleavage and IL-1β secretion by LPS-primed cells (Fig. 3). P2RX7 forms a non-selective ion channel upon activation with ATP and, after stimulation, mediates K+ efflux, which may be important for activating the inflammasome. This ion channel mediated by P2RX7 rapidly transforms to a pore-like structure by recruiting a pannexin-1 pore that allows passage of molecules as large as 900 Da. It is possible, as has been proposed for macrophages, that ATP promotes passage of microbial ligands, such as LPS, via pannexin1 to trigger inflammasome activation in mast cells. Consistent with this conjecture, ATP alone did not induce IL-1β secretion by mast cells, even though ATP triggered large-pore formation. IL-1β secretion by mast cells was blocked for cells derived from P2rx7-deficient mice or by incubation in high K+.

### Table 1 Clinical manifestations of cryopyrin-associated periodic syndromes

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FCAS, familial cold auto-inflammatory syndrome; MWS, Muckle-Wells syndrome; CINCA, chronic infantile neurological, cutaneous and articular syndrome.
P2RX7 forms a non-selective ion channel upon activation by ATP. Upon stimulation, P2RX7 mediates K+ efflux and rapidly transforms to a pore-like structure by recruiting a pannexin-1 pore, which allows passage of molecules as large as 900 Da.

extracellular medium. Thus, ATP-driven P2RX7 and K+ efflux are also required for effective IL-1β secretion by mast cells.21

**CAPS-ASSOCIATED NLRP3 INDUCES URTICARIA**

Disease-associated mutations associated with CAPS are localized to the centrally located NOD region in NLRP3. Of note, similar missense mutations in NOD2 have been identified in patients with Blau syndrome, another autosomal-dominant autoinflammatory syndrome, and early-onset sarcoidosis, a set of sporadic granulomatous disorders that phenotypically resemble Blau syndrome.26-28 Interestingly, the amino acids affected by an R260W mutation in NLRP3 and an R334W mutation in NOD2 are at analogous sequence positions, suggesting a common molecular mechanism for their roles in the development of autoimmune disease (Fig. 4). Via molecular interactions of their LRRs with their own NOD regions, NLRP3 and NOD2 are maintained in inactive conformations. This is relieved by ligand recognition via the LRRs.29,30 Disease-associated mutations are thought to mimic active conformational changes that are induced by microbial ligands, and in vitro studies suggest that these mutations exert gain-of-function effects.5,29 In the case of NLRP3, in addition of NF-κB activation, the mutation found in CAPS constitutively activates caspase-1 to produce active IL-1β.

Mouse Nlrp3 mutants, corresponding to those observed in human CAPS, induced constitutive Asc-dependent NF-κB activation and IL-1β secretion.21 Transfer of mast cells expressing an R258W mutant, corresponding to R260W of human CAPS-associated NLRP3, induced perivascular neutrophil-rich inflammation in mouse skin, a histological hallmark of the urticaria observed in CAPS patients. These findings are consistent with a previous report that showed enhanced production of IL-1β in the skin of CAPS patients.18 However, it remains unclear why mast cells with a constitutively activated NLRP3-inflammasome produce mature IL-1β, even in the absence of LPS. One possibility is that CAPS-associated NLRP3 mutants induce pro-IL-1β via constitutive activation of NF-κB induction.31,32 Another possibility is that production of pro-IL-1β is induced by endogenous or environmental cues that operate in the skin independently of NLRP3. Consistent with this latter model, the characteristic skin rash observed in CAPS often develops within the first few weeks of life when the skin is first
exposed to environmental factors. This may include exposure to small amounts of LPS and/or other microbial stimuli. The observation that skin abnormalities in incontinentia pigmenti (MIM #308300) commence at birth is also consistent with this possibility. This disorder is an X-linked dominant inherited disorder caused by a mutation of NEMO, a gene that encodes the regulatory component of the IκB kinase complex responsible for activating the NF-κB signaling pathway.

Collectively, inflammasome activation in mast cells contributes to the pathogenesis of IL-1β-mediated diseases of the skin. Mast cells reside in numerous tissues and also participate in experimental models of arthritis and encephalomyelitis. Thus, it is possible that these cells play a role in disease pathogenesis not only in the skin, but also in the joints and central nervous system, which are also major diseased sites in CAPS patients. Additional studies are needed in order to better understand the contributions of mast cells to IL-1β-mediated diseases associated with NLRP3.

Furthermore, urticaria that is associated with CAPS is usually non-pruritic and unresponsive to antihistamines. This clinical observation is in line with experimental results that NLRP3-inflammasome activation induces IL-1β secretion, but not degranulation, in mast cells. Nonetheless, mast cells that expressed a CAPS-associated NLRP3 mutant promoted vascular permeability, a cellular response critical for wheal formation in vivo. Because many cases of non-CAPS urticaria are unresponsive to histamine H1-receptor antagonists, it is possible that skin rash associated with histamine resistance is mediated via inflammasome activation in mast cells. Thus, understanding the pathophysiology of CAPS may provide critical insights into more common diseases, such as antihistamine-refractory urticaria.

**CAPS AND KNOCK-IN MICE**

Recently, two groups have generated gene-targeted mice harboring Nlrp3 mutations that mimic the amino acid substitutions in NLRP3 that were found to cause disease susceptibility in CAPS. The mice generated in both studies had very severe phenotypes. Newborn mice with an R258W mutation in Nlrp3 exhibited delayed growth, decreased weight gain and increased mortality. Adult animals were infertile and developed dermatitis, but not urticaria, associated with increased sizes of lymphoid organs. The mice with A350V in Nlrp3 died between days 2 and 14 and exhibited profound growth delays, skin abscesses, and hair growth and pigmentation defects.

At the cellular level, despite the observation that T cells in mutant mice displayed altered polarization profiles favorable to Th17, both groups found that this defect was due to the expression of the mutated Nlrp3 in antigen-presenting cells (APCs), but not in T cells. They isolated bone marrow-derived macrophages or dendritic cells (DCs) and explored their responses to TLR ligands in the presence or absence of ATP. In contrast to a study of CD14-positive peripheral mononuclear cells from a human CAPS patient that secreted IL-1β without any treatment, myeloid cells from mutant mice in both studies did not spontaneously secrete mature IL-1β. Rather, they displayed a considerably higher sensitivity to TLR ligands than cells from wild type (WT) mice. Importantly, for optimal activation, addition of exogenous ATP was not required in order to release IL-1β, supporting the notion that the mutated Nlrp3 was constitutively active and, therefore, did not require additional stimuli for inflammasome activation.

An important outcome from these knock-in mice was that a common feature evoked by CAPS-associated Nlrp3 was the development of severe skin lesions. These included erythema, abscesses, scaling and thickening of both the epidermis and dermis. However, these observations only recapitulated some, but not all, of the urticaria-like skin lesions reported in CAPS patients. Moreover, both studies still failed to address the critical question of whether or not TLR-dependent accumulation of pro-IL-1β was necessary for the burst of IL-1β secretion in CAPS.

**CONTACT HYPERSENSITIVITY**

The reasons for the discrepancies between the skin manifestations of CAPS and gene-targeted mice harboring the Nlrp3 mutations could be directed to findings that the inflammasome also plays a role in contact hypersensitivity. Contact hypersensitivity involves the priming of naïve T cells after sensitizing chemicals penetrate the skin surface (sensitization) and primed T cells are activated upon re-exposure to the antigen (elicitation). Elicitation can be further subdivided into early and late phases. The early phase, characterized by increased vessel permeability and local edema, peaks 8 hours after antigen re-exposure and is believed to be mediated by local release of mediators, including IL-1β and histamine. The late phase develops 12-36 hours after antigen re-exposure and is due to cellular infiltration. During these processes, DC migration, antigen presentation, expansion of specific T cells and recruitment of T cells to the skin depend on the coordinated interactions of inflammatory cytokines, namely IL-1β and IL-18, which are important cytokines for initiating specific T-cell-mediated immune responses.

A role for the inflammasome in contact hypersensitivity was analyzed by Watanabe et al. who found that key components of the inflammasome were present in keratinocytes. Some contact sensitizers, such as trinitrochlorobenzene (TNCB), can induce caspase-1-mediated cleavage and activation of IL-1β and IL-18 in an ASC-dependent manner. Interestingly,
chemical irritants like SDS and physical agents like ultraviolet B could also trigger inflammasome activation in keratinocytes.\textsuperscript{43,44} Subsequently, it was found that mice lacking \textit{Nlrp3} and adaptor protein \textit{Asc}-deficient mice had impaired early phase reactions during the challenges. Thus, the inflammasome can modulate the early effector phase of T cell-mediated immune responses.

These findings, however, are in contrast with a report by Sutterwala et al.\textsuperscript{45} who found that impaired responses in \textit{Nlrp3}- and \textit{Asc}-deficient mice were seen in late-phase. Interestingly, in this report, \textit{Nlrp3}-deficient mice that received cells from sensitized WT animals showed almost normal ear swelling, whereas WT mice that received cells from sensitized donors lacking \textit{Nlrp3} failed to develop ear swelling. This suggests that \textit{Nlrp3} is necessary for inducing antigen-specific T-cell responses. Indeed, Langerhans cell migration and an optimal contact hypersensitivity response require functional caspase-1,\textsuperscript{46} even though recent data suggest that important cross-presenting APCs in the skin are not Langerhans cells, but are langerin\textsuperscript{+}CD103\textsuperscript{-} DCs, most likely of dermal origin.\textsuperscript{47,48}

**NECROSIS AND NLRP3**

How can we explain the discrepancies in these two reports on contact hypersensitivity in mice? One possibility is that TNCB is very potent when causing direct tissue damage, and that the signaling involved might be strong enough to overcome the requirement of inflammasome activation for T-cell priming.\textsuperscript{49}

Of potential interest is a recent report showing that necrosis directly activates the NLRP3 inflammasome.\textsuperscript{50,51} By treating human monocytic THP-1 cells with indirubin oxime derivative 7-bromoindirubin-3'-oxime, Li et al.\textsuperscript{52} showed that the NLRP3 inflammasome was activated in cells undergoing necrosis, resulting in the production of mature IL-1\textbeta and IL-18. It is remarkable that inflammasome activation and the release of these caspase-1 targeting cytokines did not require LPS priming or other pro-inflammatory stimuli.

Along these lines, the activation of NLRP3 itself mediates a form of necrosis termed pyronecrosis.\textsuperscript{52,53} We previously showed that the expression of a disease-associated mutation of \textit{NLRP3} resulted in a caspase-1 independent, but cathepsin B-dependent form of cell death.\textsuperscript{52} This characteristic cell death can also be observed in monocytes derived from CAPS patients. NLRP3-mutant monocytes rapidly and selectively underwent necrosis-like programmed cell death after treatment with LPS accompanied by the induction of \textit{Nlrp3} expression. This unique NLRP3 phenotype enabled us to differentiate NLRP3-mutated cells from WT cells in CAPS patients who had disease-associated mutant \textit{NLRP3} as a latent mosaicism.\textsuperscript{54}

Pyronecrosis is caspase-independent; neither the activating cleavage of effector caspase-3 nor its substrate poly-(adenosine diphosphate ribose) polymerase (PARP) occurs during cell death. Pyronecrosis proceeds in the presence of caspase-1-specific inhibitors, and even pan-caspase inhibitors. However, cell death is abrogated in the presence of CA074-Me, an inhibitor of the lysosomal protease cathepsin B, implicating the contribution of lysosome activity in the pathway. Pyronecrotic cells do not demonstrate DNA fragmentation or a loss of mitochondrial membrane potential. By electron microscopy, the morphological changes characteristic of pyronecrosis are consistent with necrosis and include membrane degradation and uncondensed chromatin. Similar to classic necrosis, pyronecrosis is accompanied by the release of the immune modulator high-mobility group box 1 (HMGB1). Pyronecrosis induced by NLRP3 activation suggests an exciting connection between cell death and inflammation in response to cellular insult to injury.

However, we should note that necrosis does not always induce inflammasome activation. Treatment with hydrogen peroxide or paclitaxel, which induce caspase-independent necrosis, fails to induce inflammasome activation. Similarly, induction of necroptosis through TNF-\alpha in the presence of caspase-3 and caspase-9 inhibitors\textsuperscript{55} did not result in inflammasome activation. If necrosis was induced too rapidly by repeated cycles of freeze-thaw or excessive osmotic shock, inflammasome activation was greatly reduced. Based on these findings, during activation of the NLRP3 inflammasome, the dissolution of the cellular architecture that occurs during some specific forms of necrosis may be required for lysosome destabilization.\textsuperscript{56}

Another impressive point to remember from the study by Li et al.\textsuperscript{50} is that it remains unclear how pro-IL-1\textbeta is induced in a sterile environment without bacterial components like LPS. Several endogenous danger signals released by necrotic cells have been shown to stimulate TLRs and other pattern recognition receptors and, therefore, have the potential to induce pro-IL-1\textbeta, although this has not been definitively demonstrated. Of note, endogenous molecules can serve a priming role for NLRP3-inflammasome activation.\textsuperscript{51} Biglycan and hyaluronic acid, components of the extracellular matrix, were capable of priming macrophages for Nlrp3-inflammasome activation in response to pressure-disrupted necrotic cells. Hence, extracellular matrix components that accumulate in non-physiological sites or amounts can function as signal for the induction of pro-IL-1\textbeta accumulation, suggesting that inflammasome activation can occur \textit{in vivo} in sterile settings without microbes.

Another possibility is that inflammasome activation during necrosis can lead to the release of mature IL-18, even in the absence of microbial stimuli, as IL-18...
is constitutively expressed by several cell types. IL-18, in turn, via activation of the MyD88-dependent pathway, leads to the transcription of pro-inflammatory cytokine genes, including IL-1β, and amplifies inflammation. Thus, IL-18 may be one of the earliest mediators of the sterile inflammatory response that is triggered by necrosis or tissue damage.

**IL-18 AND DERMATITIS**

In a report by Watanabe et al., IL-1 receptor deficiency resulted in a significant decrease in the intensity of ear swelling. However, it did not totally abrogate ear swelling after elicitation with TNCB, suggesting that other cytokines/signals are likely to modulate the early phases of contact hypersensitivity. This may be due, at least in part, to the presence of IL-18, which is also activated by caspase-1. Keratinocytes constitutively produce both pro-IL-1β and pro-IL-18, but lack endogenous caspase-1 activity under normal conditions. Using established keratinocyte-specific caspase1-transgenic mice with a human keratin 14 promoter that specifically expressed the targeted gene at the basal layer of keratinocytes, Yamanaka et al. showed that the mice spontaneously suffered from chronic dermatitis under specific pathogen-free (SPF) conditions, which was accompanied by abnormally elevated serum levels of IL-18 and IL-1β. Another transgenic mouse model in which epidermal cells over secreted IL-18 also spontaneously developed atopic dermatitis-like skin eruption under SPF conditions, and a deletion of Il18 protected against the development of skin eruptions. This finding suggests that excessive cutaneous IL-18 release is a causative factor for the development of dermatitis. Of potential interest is that the phenotypes in the skin of these caspase-1 and IL-18 knock-out mice closely resembled those in disease-associated Nlrp3 knock-in mice.

Furthermore, Terada et al. developed an intrinsic atopic dermatitis mouse model with daily applications of protein A, a surface molecule and virulence factor of *Staphylococcus aureus*, which resulted in destruction of the skin barrier with a subclinical dose of SDS. In this model, neutralizing anti-IL-18 antibodies and Il18-deficient mice could completely protect against SDS plus *S. aureus*-derived protein A-induced dermatitis, suggesting the importance of IL-18 for atopic dermatitis-like skin eruptions. This is also interesting when we consider the recent findings that hemo-lysins and bacterial lipoproteins in *S. aureus* can induce activation of the NLRP3 inflammasome. Thus, another target inflammasome-activating cytokine, IL-18, may be a major player for dermatitis development.

**LESSONS FROM CAPS**

During the past decade, our understanding of the cellular and molecular mechanisms by which innate immune system molecules sense specific molecular patterns of components of invading organisms has increased tremendously. The NLRs, together with the TLRs, are now appreciated as parts of this important sensing system that allows the host to generate effective immune responses. NLRP3 also detects various endogenous, sterile danger signals in the absence of microbial infection. Progress in understanding the roles of NLRs will improve our knowledge to answer questions, such as how to transfer insights from mouse model systems to translational research focusing on human pathology, especially from the rare genetic disorder CAPS, associated with an NLRP3 mutation, to more common diseases, such as ordinary urticaria and dermatitis. Interestingly, two NLRP3 single nucleotide polymorphisms (SNP, rs4612666 and rs10754558) were recently reported to be significantly associated with susceptibility to food-induced anaphylaxis as well as aspirin-induced asthma. Functional analysis of the rs4612666 SNP located in intron 7 of NLRP3 showed 1.2-fold higher transcriptional enhancer activity than the other constructs containing the T allele, whereas rs10754558 in the 3′ untranslated region affected the stability of the NLRP3 mRNA. Thus, NLRP3 polymorphisms may increase the risk of the hypersensitive phenotype of allergy.

However, we still cannot answer all of the questions for the roles of the inflammasome in the skin. IL-18, as well as IL-33, are target molecules for inflammasome-activated caspase-1. Nevertheless, why does the urticarial rash in CAPS depend solely on IL-1β? Why can’t we induce hives in mice, even if activated Nlrp3 is induced? We should still look carefully at the clinical manifestations of CAPS in order to determine what happens in humans when the inflammasome is activated.

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