NALP3 Forms an IL-1β-Processing Inflammasome with Increased Activity in Muckle-Wells Autoinflammatory Disorder

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Summary

Mutations within the *NALP3/cryopyrin/CIAS1* gene are responsible for three autoinflammatory disorders: Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and CINCA. The NALP3 protein is homologous to NALP1, which is a component of the inflammasome, a molecular platform that activates the proinflammatory caspases-1 and -5. NALP3 (and other members of the NALP family) lacks the C-terminal, CARD-containing sequence of NALP1, and its role in caspase activation is unclear. Here, we report that NALP2 and NALP3 associate with ASC, the CARD-containing protein Cardinal, and caspase-1 (but not caspase-5), thereby forming an inflammasome with high proIL-1β-processing activity. Macrophages from Muckle-Wells patients spontaneously secrete active IL-1β. Increased inflammasome activity is therefore likely to be the molecular basis of the symptoms associated with NALP3-dependent autoinflammatory disorders.

Introduction

Interleukin-1β (IL-1β) functions in the generation of systemic and local responses to infection, injury, and immunological challenges and is the primary cause of chronic and acute inflammation (Dinarello, 1998). IL-1β is produced as an inactive cytoplasmic precursor (proIL-1β, p35) that has to be cleaved by caspase-1 (at Asp116) to generate the mature active form (p17). Caspase-1, like the two other proinflammatory caspases, caspase-4 and caspase-5, possesses an N-terminal CARD protein-protein interaction domain (Thornberry and Lazebnik, 1998).

Although the molecular mechanisms responsible for the activation of proinflammatory caspases remain poorly defined, recent evidence indicates that a CARD-

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containing protein called ASC binds and activates pro-caspase-1 (Martinon et al., 2002; Srinivasula et al., 2002; Wang et al., 2002). ASC contains a C-terminal CARD motif as well as an N-terminal CARD-like Pyrin domain (Bertin and DiStefano, 2000; Martinon et al., 2001; Masumoto et al., 1999; Pawlowski et al., 2001), which associates with the Pyrin domain of NALPs (Martinon et al., 2001). NALPs are recently identified members of the NBS/NACHT family (Tschopp et al., 2003) (Figure 1A), characterized by an N-terminal Pyrin domain and C-terminal leucine-rich repeats (LRR). NALP1 is unique among the NALP family members, as it contains a C-terminal extension composed of two distinct domains, a FIIND domain followed by a CARD domain, which interacts with and activates caspase-5 (Figure 1A) (Tschopp et al., 2003). Thus, a model was proposed in which both caspase-1 and caspase-5 are activated upon assembly with NALP1 and ASC to form the inflammasome responsible for proIL-1β processing (Tschopp et al., 2003).

Such a model, however, cannot explain how other members of the NALP family (NALP2–14, short NALPs; Figure 1A), which lack the capacity to recruit caspase-5 due to the absence of the C-terminal CARD domain, could efficiently process proIL-1β. The human genome contains a gene, described as Cardinal/TUCAN/CARD8 (Bouchier-Hayes et al., 2001; Pathan et al., 2001; Razmara et al., 2002), that encodes a protein whose structural organization is similar to the C terminus of NALP1 and corresponds to the sequence missing in NALP proteins 2–14 (Figure 1A). The CARD domain of Cardinal was reported to interact with caspase-1 (Razmara et al., 2002) and caspase-9 (Pathan et al., 2001).

Results and Discussion

We therefore considered the possibility that Cardinal could be the functional homolog of the NALP1 C terminus containing the FIIND and CARD domain and investigated whether it would associate with the short NALPs. Indeed, when overexpressed in 293T cells with various NALP2 and NALP3 constructs, Cardinal was found in the anti-NALP immunoprecipitates (Figure 1B). Interestingly, full-length NALP2 and NALP3 showed little or no apparent affinity for Cardinal. Removal of either the C-terminal LRR, the N-terminal Pyrin, or both domains was required in order to detect optimal association with Cardinal. No interaction of Cardinal was detectable with the Pyrin domain of NALP2 and NALP3, indicating that (1) Cardinal interaction was mediated through the NACHT domain of NALPs and (2) that the interaction was internally inhibited by the LRR and the Pyrin domains. This domain-based mechanism of autoinhibition is also functional in NALP1 (data not shown) and is similar to the mechanism found in the structurally related Ipaf protein (Poyet et al., 2001). In agreement with previous work (Pathan et al., 2001), Cardinal was found to interact with itself. We further mapped the interaction site of the NALPs on Cardinal and found the FIIND do-

A

NALP1   PYD — NACHT — NAD — (((LRR))) — FIIND — CARD

NALP2-14   PYD — NACHT — NAD — (((LRR)))
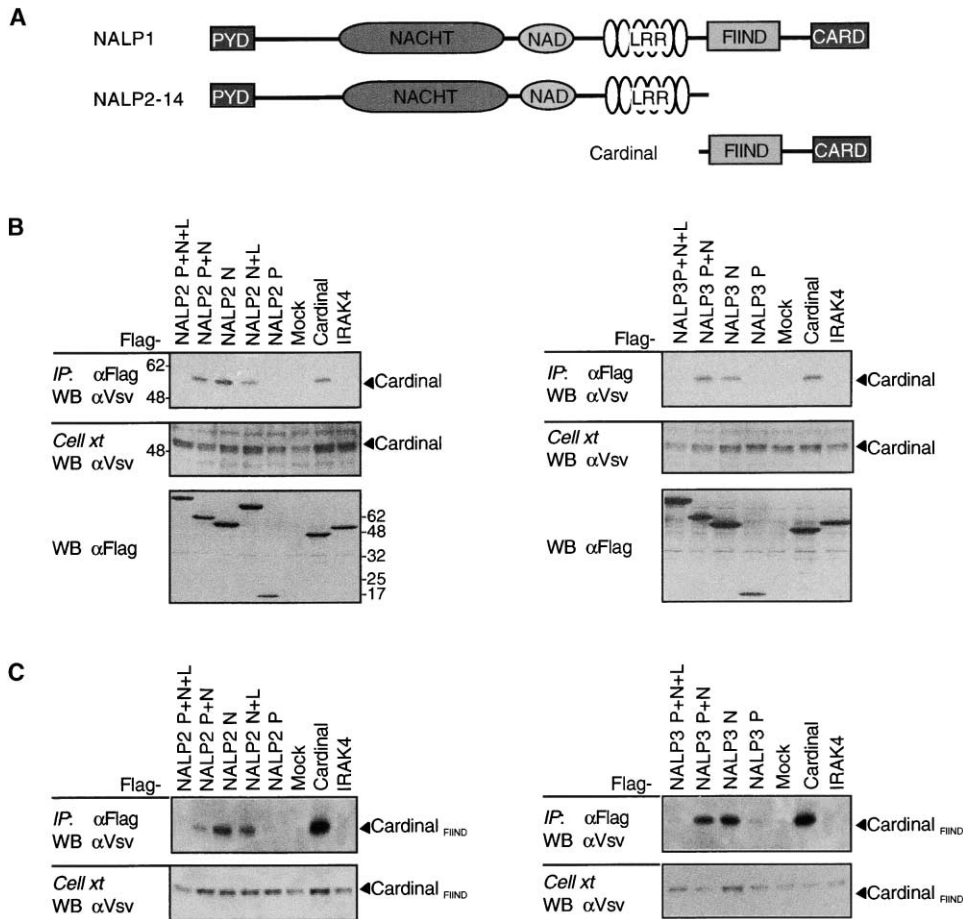
Cardinal   FIIND — CARD

B

C

Figure 1. NALP3 Interacts with Cardinal

(A) Domain structure of NALP1 (long NALP), NALP2 to NALP14 (short NALPs), and Cardinal. Note that the number of LRR repeats in NALP2–14 varies from 0 to 14. Caspase recruitment domain, CARD; Pyrin domain, PYD; leucine-rich repeats, LRR; NALP-associated domain, NAD; domain present in *NA*IP, *C*IITA, *H*ET-E, and *T*P1, NACHT; domain with function to find, FIIND.

(B) NALP2 and NALP3 interact with Cardinal. VSV-tagged Cardinal was coexpressed in 293T cells with the indicated Flag-tagged expression constructs containing the Pyrin (P), NACHT/NAD (N), and LRR (L) domains of NALP2 and NALP3, respectively, or with Flag-tagged Cardinal or IRAK4 as control. Twenty-four hours after transfection, anti-Flag immunoprecipitates (IP) were prepared and analyzed for the presence of Cardinal. Expression of VSV-Cardinal and of the various Flag-tagged proteins was assayed in cell extracts (Cell xt).

(C) As (B), but only the FIIND domain of Cardinal was expressed.

main to be sufficient for this association to occur (Figure 1C and data not shown).

The Pyrin domain of NALP1 and ASC interact with each other (Manji et al., 2002; Martinon et al., 2001), and likewise, we found that the Pyrin domain of both NALP2 and NALP3 interacted with the Pyrin domain of ASC (Figure 2A). This interaction was also detectable with the endogenous ASC, when the FLAG-tagged Pyrin domain of either NALP2 or NALP3 was expressed in 293T cells and the cytoplasmic extracts subsequently mixed with cell extracts from THP-1 cells (Figure 2B).

Further support for the NALP 2 and 3 interaction with ASC and Cardinal was obtained when the subcellular localization was analyzed by confocal microscopy (Figure 2C). Granular cytoplasmic staining was obtained with antibodies to Cardinal, NALP2, and ASC, which overlapped when images were merged. We did not observe the speckles previously described for overexpressed ASC (Masumoto et al., 2001; Stehlik et al., 2003), indicating that these prominent structures are only seen

in cells that are stressed due to protein overexpression or treatment with agents that induce cell death (Masumoto et al., 1999).

While in the NALP1-containing inflammasome, ASC is used to recruit caspase-1, the C-terminal CARD of NALP1 recruits, and activates caspase-5. We therefore investigated whether Cardinal preferentially interacts with caspase-5 and monitored its interaction with various CARD-containing caspases. Unlike NALP1, the CARD of Cardinal-bound caspase-1 but not caspase-5 (nor caspase-4 and -9) and, consequently, overexpression of Cardinal led to caspase-1 and not to caspase-5 processing (Figure 2D; data not shown).

Activation of caspase-1 by NALP1 is dependent on the presence of ASC while for caspase-5, ASC is not required. To investigate whether NALP3 and/or Cardinal-induced caspase-1 activation is dependent on the presence of ASC, we developed an assay in which the inflammasome is reconstituted in 293T cells. 293T cells do not express any components of the inflammasome.
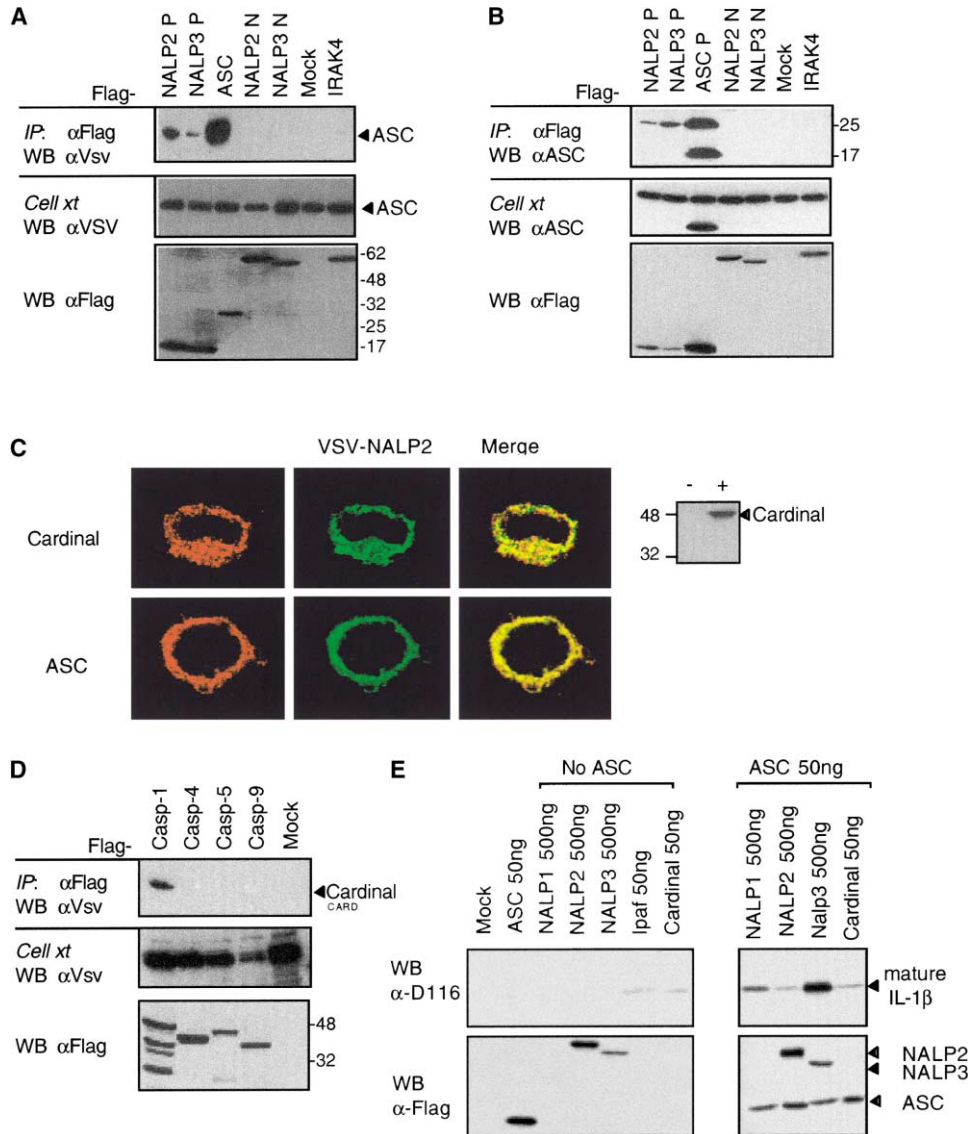
Figure 2. NALP2 and NALP3 Activate Caspase-1 Indirectly via ASC, while Cardinal Binds Directly to Caspase-1

(A) Flag-tagged NALP2 and NALP3 constructs were immunoprecipitated from transfected 293T cells and the associated VSV-tagged ASC detected in Western blots. P, Pyrin domain; N, NACHT and NAD domains; L, LRR.

(B) Flag-tagged NALP2 and NALP3 proteins were expressed in 293T cells. Cell extracts were subsequently mixed with cell extracts from THP-1 monocytic cells and the interaction of Flag-tagged NALP2, NALP3, and ASC with endogenous ASC of THP-1 cells assayed in anti-Flag immunoprecipitates. Note that ASC also interacts with itself.

(C) The subcellular distribution of NALP2, Cardinal, and ASC was analyzed in THP-1 cells stably transfected with VSV-NALP2 by confocal microscopy. Right panel, antibody specificity control: Western blot of cell extracts of 293T cells transfected (+) or not (−) with Cardinal using the polyclonal anti-Cardinal antibody.

(D) Various Flag-tagged CARD-containing caspases were expressed in 293T cells together with the CARD of Cardinal and their interaction assayed in anti-Flag immunoprecipitates. Activation of caspase-1 is evident in immunoblots from cell extracts by the appearance of the processed enzyme and its capacity to process proIL-1β (see Figure 2E). Caspase-2, another CARD-containing human caspase, did not interact with Cardinal (data not shown).

(E) Activation of Caspase-1 by NALP3 but not by Cardinal is dependent on ASC. 293T cells were transfected with low amounts of caspase-1 and proIL-1β (100 ng of each plasmid) in each case. The left panel shows that transfection of ASC, NALP1, NALP2, and NALP3 does not result in the processing of proIL-1β, while expression of Cardinal or Ipaf induces weak activation. However, in the presence of low amounts of ASC, the NALPs become strong activators of proIL-1β as evidenced by the appearance of the active cleaved form of IL-1β (p17, the α-D116 antibody used specifically recognizes the processed form). Note that the expressed NALP1 is not Flag-tagged and therefore not detectable with α-Flag antibodies.

As shown in Figure 2C, overexpression of neither NALP1 (N-terminal Pyrin domain, NACHT, and LRR), NALP2, nor NALP3 alone led to the activation of caspase-1 and processing of IL-1β. Minute amounts of ASC had to be transfected to see activation of caspase-1 (higher concentrations of ASC led to spontaneous activation),
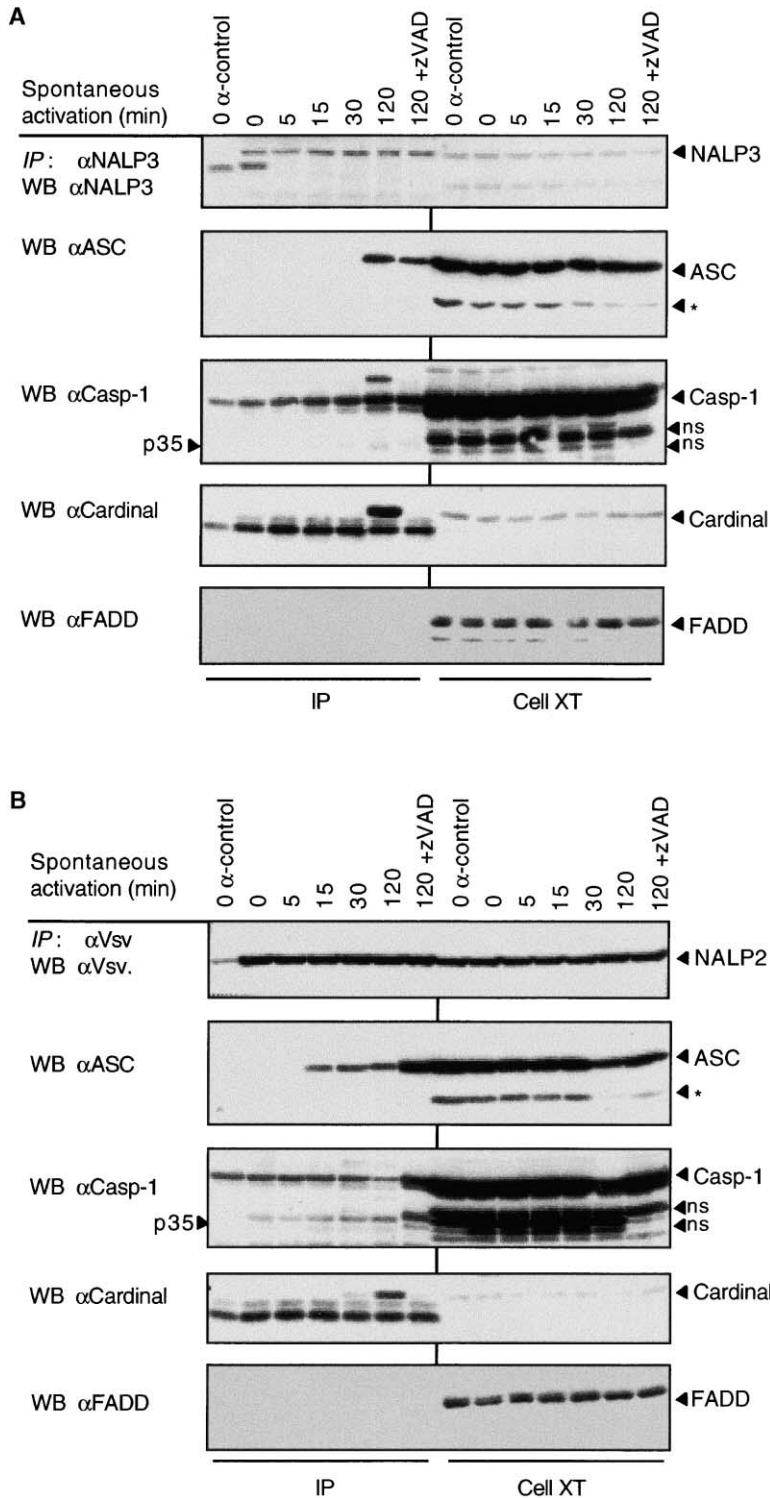
A

Spontaneous activation (min)

IP: αNALP3 WB αNALP3 ◄ NALP3

WB αASC ◄ ASC
◄ *

WB αCasp-1 ◄ Casp-1
◄ ns
p35 ► ◄ ns

WB αCardinal ◄ Cardinal

WB αFADD ◄ FADD

IP   Cell XT

Figure 3. NALP3 and NALP2, Caspase-1, ASC, and Cardinal Form Short NALP Inflammasomes

(A) Cell extracts of THP-1 cells were incubated at 30°C for increasing periods of time in the presence and absence of z-VAD-fmk, and association of ASC, caspase-1, and Cardinal with endogenous NALP3 was monitored in NALP3 immunoprecipitates in the presence and absence of z-VAD-fmk. Note that FADD is not incorporated into the complex. Control lane: immunoprecipitate using an irrelevant control antibody of the same isotype. Note that the polyclonal antibody against caspase-1 used in this experiment crossreacts with other proteins present in the cell extracts as indicated (ns). Moreover, caspase-1 appears to stick nonspecifically to beads, as even in the isotype control some caspase-1 is already detectable.

(B) As (A), but NALP2 inflammasome assembly was followed in THP-1 cells stably expressing VSV-NALP2. NALP2 inflammasome assembly was followed using anti-VSV antibodies. The asterisk points to a putative short form of ASC. Interestingly, caspase-1 that is recruited to the inflammasome is modified.

B

Spontaneous activation (min)

IP: αVsv WB αVsv. ◄ NALP2

WB αASC ◄ ASC
◄ *

WB αCasp-1 ◄ Casp-1
◄ ns
p35 ► ◄ ns

WB αCardinal ◄ Cardinal

WB αFADD ◄ FADD

IP   Cell XT

indicating that NALP1, 2, and 3 are strictly dependent on ASC to exert their role as activators of caspase-1. Of particular note, NALP3 appeared to be more potent than NALP1, at least in this reconstitution system. In contrast to the NALPs, Cardinal- and Ipaf- (Poyet et al., 2001) induced caspase-1 activation proceeded in the absence of ASC and, if compared to NALPs, were weak activators. As expected from this low activity, NALP3-induced caspase-1 activation was not detectably increased by coexpression of Cardinal and NALP3 or NALP2 (data not shown). Taken together, these results show that an inflammasome with short NALPs can be assembled that contains ASC, Cardinal, and caspase-1 and that leads to the potent processing of caspase-1. Despite the capacity to activate caspase-1, Cardinal appears to play a minor role in the processing of IL-1β. Preliminary results suggest that it is important for inflammasome association with the cytoskeleton.
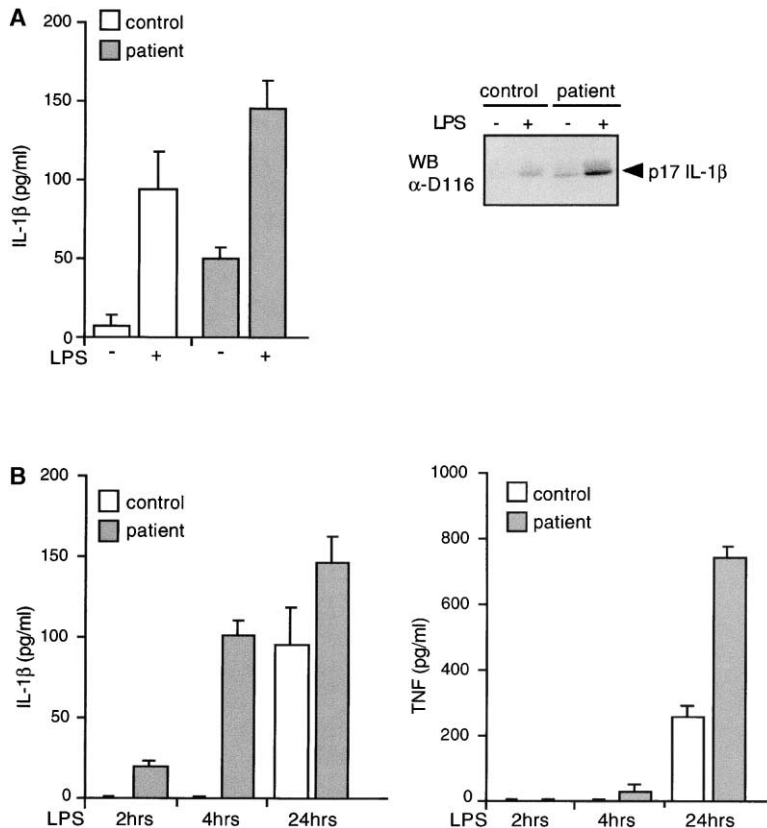
Figure 4. Increased IL-1β Processing in Macrophages Isolated from a Muckle-Wells Patient Carrying the R260W Mutation

Purified monocytes from a patient or a nonaffected donor were allowed to adhere overnight and subsequently stimulated or not with LPS for 24 hr. Il-1β present in the supernatant was measured by an ELISA ([A], left panel) or by Western blotting ([A], right panel) using an antibody specifically directed against the active fragment (p17) of proIL-1β (D116). (B) Kinetics of secretion of TNF and active, processed IL-1β.

Since the above conclusion is based on overexpression studies, it was essential to corroborate assembly with endogenous proteins. We have previously found that LPS-mediated assembly of the NALP1 inflammasome is difficult to follow as the complex is immediately secreted upon its formation (Martinon et al., 2002). We therefore took advantage of the same system that has been successfully used to study assembly of the apoptosome (Li et al., 1997) and the NALP1 inflammasome (Martinon et al., 2002). Cell extracts of THP-1 cells were incubated at 30°C in order to promote the spontaneous activation of the inflammasome. Although inflammasome assembly is most conveniently followed by immunoprecipitating ASC (Martinon et al., 2002), we developed a panel of monoclonal antibodies against NALP3 in order to specifically study the NALP3 inflammasome and avoid coprecipitation of the NALP1 inflammasome. Upon activation, assembly of the NALP3 inflammasome consisting of a complex of NALP3, ASC, caspase-1, and Cardinal was detectable after 30–60 min and reached a maximum after 2 hr (Figure 3A and data not shown). These kinetics of assembly were similar to the one previously found for the NALP1 inflammasome (Martinon et al., 2002). The complex contained ASC, Cardinal, and caspase-1 but no detectable caspase-5. A small but significant portion of caspase-1 was converted into the processed form, giving rise to the p35 fragment. Caspase-1 processing was higher when assembly of the NALP2 inflammasome was studied in THP-1 cells stably expressing VSV-tagged NALP2 (Figure 3B). Interestingly, the recruitment of Cardinal was abolished in the presence of the pan-caspase inhibitor z-VAF-fmk, indicating that assembly of the short NALPs inflammasomes are themselves dependent on the activation of caspases.

Considering the potent caspase-1/proIL-1β activity of the NALP3 inflammasome, the simplest mechanism to explain the autoinflammatory disorders associated with NALP3 mutations would be the spontaneous activation of the inflammasome, ultimately leading to increased caspase-1 activation and IL-1β release. To test this hypothesis, monocytes were isolated from a Muckle-Wells patient with a NALP3 R260W mutation (Aganna et al., 2002) and stimulated with LPS. As shown in Figure 4A, processed IL-1β was detectable in the supernatant even without prior stimulation of the cells. Following a 24 hr LPS stimulation, IL-1β levels further increased. In contrast, IL-1β found in the supernatant of macrophages from a normal donor was only detectable upon stimulation, reaching levels that were only slightly higher than those found with nonstimulated patient macrophages. In agreement with a previous report (Aksentijevich et al., 2002), cytokine levels were generally upregulated in the patient's monocytes. For example, TNF levels were significantly higher after LPS stimulation (Figure 4B). TNF release, which is not dependent on caspase processing, was found to be a late event as significant levels were only detectable 24 hr poststimulation. Unlike the rapid release of processed IL-1β, which was noticeable already 2 hr after LPS treatment, TNF secretion was slow and not detectable after 4 hr. Thus, it is likely that the increased general cytokine production observed is a consequence of increased IL-1β release.

Since the publication of the initial report demonstrating association of mutations in the *NALP3* gene with Muckle-Wells, familial cold autoinflammatory disorder

(FCAS), or with chronic infantile neurologic cutaneous and articular (CINCA) autoinflammatory diseases (Hoffman et al., 2001), more than 100 patients with those inflammatory diseases have been diagnosed as carrier of the mutated NALP3 gene (Hull et al., 2003). The role of NALP3 and the biological consequences of such mutations, however, remained unexplained. This study now provides evidence that NALP3 and another short NALP, NALP2, form an inflammasome with Cardinal and ASC resulting in caspase-1 activation and strong processing of proIL1β. Unlike the NALP1 inflammasome, the NALP3 inflammasome does not simultaneously activate caspase-5. Whether this difference implies different substrate preferences, such as IL-1β versus IL-18, remains to be investigated.

To date, 23 distinct mutations have been described in the *NALP3* gene, and they are all localized within the NACHT domain (McDermott, 2002). The most frequent R260W mutation is found in patients with MWS and FCAS. We found that monocytes from these patients display spontaneous processing and secretion of IL-1β, which is detectable in supernatants even without stimulation. This increased activity could be explained by enhanced propensity for inflammasome assembly as the NACHT domain is known to be responsible for oligomerization. Alternatively, the mutations may block binding of a putative inhibitor of inflammasome assembly.

The observation that macrophages from a patient with MWS secrete IL-1β even in the absence of a stimulus predicts a rational treatment, namely the inhibition of the proinflammatory activity of IL-1β. Indeed, the administration of the IL-1β antagonist IL-1Ra (Arend and Guthridge, 2000) to patients with Muckle-Wells syndrome proves to be highly successful. In all patients treated, the inflammatory symptoms cease within hours after the first injection of IL-Ra (Hawkins et al., 2003, 2004), further supporting the concept that excessive inflammasome activation is the causative mechanism in these autoinflammatory diseases.

Experimental Procedures

Antibodies
Polyclonal antibodies against human Cardinal were generated in rabbits (Eurogentec, Belgium) by injecting GST-cardinal FIIND (AL228). The polyclonal serum was purified on an HiTrap protein G HP column (Amersham). Monoclonal antibodies against NALP3 were generated from GST-NALP3-immunized mice. Four monoclonal antibodies were purified (Nalpy3a, Nalpy3b, Nalpy3c, Nalpy3d). Other antibodies were purchased from the following companies: cleaved IL-1β D116 (Cell Signaling); anti-Flag antibody (M2, Sigma); anti-VSV antibody (P5D4, Sigma); mAb anti-ASC and FADD (MBL); and polyclonal anti-ASC (AL177, Apotech). Peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were from Jackson IR and isotype-specific anti-IgG1 from Southern Biotech. Secondary antibodies for immunofluorescence and Alexa-conjugated mouse and rabbit antibodies were purchased from Molecular Probes.

Plasmids
Cloning of NALP1 and ASC was described previously (Martinon et al., 2002). NALP1 Cter (amino acids 1030–1430, covering FIIND and CARD domains), NALP1 P + N (amino acids 1–665, covering the pyrin and NACHT domains), NALP1 Nter (amino acids 1–1079, covering the pyrin, NACHT, and LRR domains), and NALP1 P (amino acids 1–121, covering the pyrin domain) were amplified by PCR and subcloned into the mammalian expression vectors pCR3 in frame with the indicated tags. The caspase-1 expression construct contains a C-terminal Flag.

Cloning of NALP2, NALP3, and Cardinal
NALP2 FL was cloned from the EST accession number AK000517, kindly provided by the NEDO human cDNA sequencing project (Japan). NALP2 FL (amino acids 1–1062), NALP2 P + N (amino acids 1–589, covering the pyrin and the NACHT domains), NALP2 N (amino acids 89–589, covering the NACHT domain), NALP2 N + L (amino acids 89–1062, covering the NACHT and LRR domains), and NALP2 P (amino acids 1–112, covering the pyrin domain) were amplified by PCR and subcloned into the mammalian expression vectors pCR3 in frame with the indicated tags. NALP3s was cloned from the EST accession number AK027194 provided by the NEDO human cDNA sequencing project. NALP3s (amino acids 1–717, covering the pyrin, the NACHT domain, and the intermediate domain), NALP3 P + N (amino acids 1–544, covering the pyrin and NACHT domain), NALP3 N (amino acids 67–544, covering the NACHT domain), and NALP3 P (amino acid 1–93, covering the pyrin domain) were amplified by PCR and subcloned into the mammalian expression vectors pCR3 in frame with the indicated tags. Cardinal was cloned from the EST (KIAA0955) accession number AB023172 provided by the Kazusa DNA Research Institute (Japan). Cardinal FL (amino acids 1–431, covering the FIIND and the CARD domain), Cardinal FIIND (amino acids 1–346), Cardinal CARD (amino acids 320–431), and ASC P (amino acids 1–125, covering the pyrin domain) were amplified by PCR and subcloned into the mammalian expression vectors pCR3 in frame with the indicated tags. The construct VSV-NALP2 FL (amino acids 1–1062) was cloned in pMSCV puromycin. Expression constructs for caspase-1, -4, and -9 have been described elsewhere (Thome et al., 1998). The plasmid encoding caspase-5 was a kind gift of Christoph Froelich.

Cell Lines
THP-1 and 293T were cultured in RPMI 1640 and DMEM, respectively, supplemented with 10% fetal bovine serum (FCS), 50 mM β-mercaptoethanol, and penicillin/streptomycin (100 μg/ml of each). THP-1 stably expressing VSV-NALP2 were cultured with medium supplemented with 5 μg/ml puromycin.

Transient Transfection and Immunoprecipitation
Transient transfection of 293T cells was performed as described previously (Thome et al., 1998). For coimmunoprecipitation experiments, transfected 293T cells (1 × 10^6) were suspended in 200 μl of lysis buffer (50 mM Tris [pH 7.8], 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA) and lysed by three freeze/thaw cycles. Note that overexpression of large amounts of Pyrin domain-containing proteins was avoided as these proteins have a strong tendency to aggregate and precipitate in insoluble fractions. The lysates were incubated with 3 μg of anti-Flag agarose (Sigma), 3 μg of anti-VSV bound to protein G, or 3 μg of anti-ASC (AL177) bound to protein G at 4°C overnight. The agarose beads were washed five times with lysis buffer, and the precipitated proteins were then fractionated on 10% and 12% SDS-PAGE and analyzed by Western blotting. For coimmunoprecipitation with endogenous ASC, transfected 293T lysates were mixed with THP-1 lysate and were immunoprecipitated with AL177 as described before.

ASC Activity Assay
Transient transfection of 293T cells was performed using optimal amounts of each plasmid. Twenty-four hours later, cells were harvested and the pellets were sonicated in SDS-PAGE buffer with DTT. The samples were analyzed by SDS-PAGE 15% and by Western blotting.

Confocal Microscopy
THP-1 cells were grown on coverslips coated with polylysine. Slides were fixed with paraformaldehyde 4% for 20 min at room temperature. Slides were blocked (30 min) and immunostained for 1 hr with the first antibody, anti-VSV, and AL228 in PBS, 2% normal goat serum, 0.1% saponin for 1 hr at RT. After washing the cells with PBS, the cells were stained with the secondary antibody Alexa mouse 488 and rabbit Cy5, respectively. Cells were analyzed with a Zeiss LSM 510 confocal laser scanning microscope through a 63× objective.

Immunoprecipitation of the Inflammasome
Immunoprecipitation of endogenous caspase-1/NALP3 or NALP2/ASC/Cardinal interaction were performed using 5×10^8 THP-1 cells/time point, as described (Martinon et al., 2002). Cell extracts prepared in buffer W (Martinon et al., 2002) were incubated with 3 μg of the indicated antibodies (anti-VSV, anti-NALP3) conjugated to protein G at 4°C for overnight incubation. The immunoprecipitates were recovered by centrifugation and washed six times with buffer W before SDS-PAGE and Western analysis.

Determination of IL-1β Secretion
Using an Optiprep (axis-shield) 1.074 g/ml density barrier solution as described (Graziani-Bowering et al., 1997), 50 ml of fresh blood was fractionated from a patient diagnosed as carrier of the R260W mutation (Aganna et al., 2002) by density. The patient had been treated with anakinra (IL1ra) and was asymptomatic at the time blood was taken.

The monocyte-containing fraction was plated on 12 wells plate (500,000 cells/well) for 12 hr. The medium was replaced with Optimem, and the stimulation was done with LPS (10 g/ml) for various time periods. The supernatants and the pellets were prepared as described (Martinon et al., 2002). The concentration of IL1-β and TNF was determined using a kit purchased from Pharmingen and Boehringer, respectively.

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