

NLRC3 inhibits NALP3 inflammasome- induced inflammation

NLRC3 protein inhibits inflammation by disrupting NALP3 inflammasome assembly via competition with the adaptor protein ASC for pro-caspase-1 binding

Elif Eren^{1,2}, Mesut Berber^{1,2} and Nesrin Özören^{1,2}

¹Department of Molecular Biology and Genetics, Bogazici University, Apoptosis and Cancer Immunology Laboratory (AKIL), Istanbul, Turkey

²Center for Life Sciences and Technologies, Bogazici University, Istanbul, Turkey

Running title: *NLRC3 inhibits NALP3 inflammasome- induced inflammation*

To whom correspondence should be addressed: Prof. Nesrin Özören, Department of Molecular Biology and Genetics, Boğaziçi University, Istanbul, Kuzey Kampüs Kuzey Park Binası, 34342 Etiler, Istanbul, Turkey. Tel: +90 212 359 75 58; Fax: +90 212 287 24 68; E-mail: nesrin.ozoren@boun.edu.tr

Key Words: NLRC3, ASC, Caspase-1, Inflammasome, Sterile Inflammation

ABSTRACT

Inflammasomes are multiprotein complexes that sense pathogen-associated and danger-associated molecular patterns and induce inflammation in cells. The NALP3 inflammasome is tightly regulated by recently discovered control mechanisms, but other modulators still remain to be characterized. NLR family CARD domain containing 3 (NLRC3) protein, a caspase recruitment domain (CARD)-containing member of the nucleotide oligomerization domain (NOD)-like receptor (NLR) family, was found to downregulate the NF- κ B pathway and stimulator of interferon genes (STING)-dependent cytokine secretion. However, the effect of NLRC3 on the NALP3 inflammasome or other inflammasomes is still unknown. We hypothesized that NLRC3 might inhibit NALP3 inflammasome complex assembly. Toward this end, we tested whether NLRC3 overexpression or knockdown influences NALP3 activity in human monocyte and in HEK293FT cells when the complex is ectopically reconstituted. We found that NLRC3 indeed

decreases NALP3-induced IL-1 β maturation and secretion, pro-caspase-1 cleavage, and speck formation by apoptosis-associated speck-like protein containing a CARD (ASC) protein in response to NALP3 activators. We also show that endogenous NLRC3 interacts with both ASC and pro-caspase-1, but not with NALP3, disrupts ASC speck formation through its CARD domain, and impairs the ASC and pro-caspase-1 interaction. Moreover, the NLRC3 CARD domain alone could dampen IL-1 β secretion and ASC speck formation induced by NALP3 mutants associated with auto-inflammatory diseases. In conclusion, we show here that besides its role in the inhibition of the NF- κ B pathway, NLRC3 interferes with the assembly and activity of the NALP3 inflammasome complex by competing with ASC for pro-caspase-1 binding.

Inflammasomes are multi-protein complexes responsible for the sensing of pathogen-associated molecular patterns (PAMPS)

and danger-associated molecular patterns (DAMPs) and the induction of inflammation (1). Different types of inflammasomes have been identified and most of them have in common the adaptor protein ASC, the effector pro-caspase-1 and the downstream inflammatory mediator cytokine pro-IL-1 β (2). The assembly of the inflammasome is mediated by the homotypic interaction of ASC and pro-caspase-1 through their CARD domains (3) and the formation of ASC foci called “specks” (4). This interaction results in the cleavage of pro-caspase-1 and the maturation and the secretion of IL-1 β (4). The NALP3 (also called NLRP3 or Cryopyrin) inflammasome is specifically activated by ionophores such as ATP and Nigericin (5); crystals such as monosulfate urate crystals (MSU) (6) and many others, while the NLRC4 (or IPAF) inflammasome is stimulated by *Pseudomonas aeruginosa* infection (7). A two step-process is required to promote the secretion of IL-1 β by the NALP3 inflammasome: the induction of the NF κ B pathway through Toll-like receptors (TLR, called “the priming step”) (8) and the complex assembly (called “the activation step”) mediated by potassium efflux (9,10), lysosomal rupture (11) and ROS production (12).

Mutations in the *NALP3* gene are associated with auto-inflammatory syndromes known as Cryopyrin Associated Periodic Syndromes (or CAPS) (13) and among these mutations, NALP3 p.R260W and p.D303N mutants enhance the ectopic assembly of ASC specks (14,15) and result in an increase in pro-caspase-1 cleavage (14) and IL-1 β secretion (16,17). IL-1 β blockers such as Anakinra are used to treat CAPS (18,19) and the identification of novel inhibitors of the NALP3 inflammasome that act upstream of IL-1 β , on the NALP3 mRNA level (20), on ASC oligomerization (21) or on caspase-1 inhibition (22) is presently ongoing and these inhibitors may be used in therapy of auto-inflammatory diseases in the future.

Although the most studied NOD-Like Receptor (NLR) family members belong to the pro-inflammatory group, more recently NLR proteins with anti-inflammatory roles have been characterized. Especially, the members of the NLRC subgroup NLRC5, NLRX1 and NLRC3 were shown to be negative regulators of inflammation. NLRX1 inhibited the NF κ B

pathway by binding to the active I κ B Kinase (IKK) in response to LPS stimulation (23,24). Furthermore, an increased IFN- β secretion was observed in immune cells from NLRC5 knockout mice when stimulated with VSV-eGFP or poly(I:C) (24).

NLRC3, a less studied member of the NLR family, contains a C-terminal Leucine Rich Repeat (LRR) domain, a central NACHT domain and a N-terminal CARD domain (25). NLRC3 was shown to inhibit the NF κ B pathway (25,26) through the ubiquitination of TRAF6 (26). NLRC3 also inhibited STING-dependent IFN β , TNF α and IL-6 secretions in response to cytoplasmic DNA stimulation. NLRC3 altered STING and TBK1 interaction by directly binding to these proteins and NLRC3 KO mice responded more efficiently to HSV-1 virus infection since IFN β levels were higher in the sera of these mice compared to WT NLRC3 expressing counterparts (27). We have also previously shown that the overexpressed NLRC3 protein co-localizes with the inflammasome components and decreases the IL-1 β processing and the ASC speck assembly in HEK293FT cells (28).

In this study, the suppressive effect of the NLRC3 protein on inflammasomes is confirmed, and the molecular mechanism of this inhibition is elucidated. We determined that besides its previously described inhibitory role on the NF κ B pathway, NLRC3 also acts on the activation step of the inflammasome by decreasing the efficiency of the complex assembly by competing with ASC for pro-caspase-1 binding via its N-terminal CARD domain. We propose to use the CARD domain of NLRC3 as a novel inhibitor of the NALP3 inflammasome.

RESULTS

The overexpression of NLRC3 inhibits the NALP3-induced IL-1 β secretion—Previous studies showed that overexpression of the inflammasome components was sufficient to induce the activation of the complex and the maturation and secretion of caspase-1 and IL-1 β in a NALP3 and ASC-dependent manner. To assess whether NLRC3 has an effect on the activation of the complex, we ectopically expressed NALP3, ASC, pro-caspase-1 and pro-IL-1 β in HEK293FT cells, in the presence or the absence of NLRC3 and measured the resulting IL-1 β secretion. While negligible levels

of IL-1 β were secreted from ASC, pro-caspase-1 and pro-IL-1 β co-transfected HEK293FT cells, co-expression of the NALP3 with these proteins induced a dose-dependent increase in the IL-1 β secretion (Fig. 1A; 496 \pm 42.7 pg/ml for 250 ng; 1400 \pm 427.5 pg/ml for 500 ng and 2903.7 \pm 229.81 pg/ml for 1000 ng of NALP3 plasmid), thus confirming the specificity of the experimental setting. Interestingly, when NLRC3 was co-expressed with NALP3 and the other inflammasome forming proteins, a significant reduction in the IL-1 β secretion was observed (Fig. 1B; 2903.7 \pm 229.81 pg/ml for 1000 ng of NALP3 plasmid alone vs 1403.5 \pm 48.1 pg/ml for 1000 ng of NALP3 and 1000 ng of NLRC3 plasmids, $p=0.0001$). Moreover, the more NLRC3 was expressed the higher decrease in IL-1 β release was obtained (2925.5 \pm 151.2 pg/ml for 250 ng; 1949.3 \pm 171.9 pg/ml for 500 ng and 1403.5 \pm 48.1 pg/ml for 1000 ng of NLRC3 plasmid co-transfected with 1000 ng of NALP3 plasmid). NLRC3 not only dampened the NALP3-induced IL-1 β secretion (Fig. 1A, B) but also inhibited pro-IL-1 β maturation (Fig. 1C) when ectopically expressed in HEK293FT cells.

To further confirm our results, we generated THP-1 stable lines that overexpress the NLRC3 protein (named OvNLRC3) and confirmed its expression both at the mRNA (Fig. 1D) and the protein levels (Fig. 1E). The induction of the NALP3 inflammasome by stimulating stable THP-1 cells with Nigericin (Fig. 1F) or MSU (Fig. 1G) triggered a significantly lower IL-1 β release from THP-1 OvNLRC3 cells compared to their OvControl counterpart (3084.8 \pm 64.1 pg/ml for OvNLRC3 vs 6385.7 \pm 287.2 pg/ml for OvCont when treated with Nigericin, $p=0.0039$), suggesting that NLRC3 acts as an inhibitor of the NALP3-induced IL-1 β secretion in both cell types.

The knockdown of NLRC3 in THP-1 cells results in an increase of IL-1 β secretion and pro-IL-1 β and pro-caspase-1 cleavages—To eliminate possible overexpression artifacts, we used human monocytic THP-1 cells that endogenously express NALP3 and inflammasome components and generated stable NLRC3 knockdown THP-1 cell lines by using three different shRNAs (namely shD6, shD8 and shD9) (Fig. 2A). We obtained a significant decrease in NLRC3 levels with the shD9 and shD6 infected cells, whereas no change was observed with the shD8 cell lines (Fig. 2A).

When stimulated with MSU, the IL-1 β secretion observed correlated with the NLRC3 levels (Fig. 2B; 1118.13 \pm 60.7 pg/ml for shcont vs 1067.11 \pm 110.4 pg/ml for shD8, $p=0.45$; 9991.68 \pm 740.6 pg/ml for shD6, $p=10^{-7}$ and 11591.68 \pm 372.8 pg/ml for shD9, $p=10^{-9}$).

Since the shD9 cell line gave the more significant decrease in NLRC3 level, we used it in the rest of our experiments and named it shNLRC3. Similarly, whereas the basal IL-1 β secretion was not significantly different between shNLRC3 and shcont cells, stimulation of the NALP3 inflammasome with ATP (Fig. 2C; 6376.7 \pm 1473.5 pg/ml for shNLRC3 vs 1746 \pm 71.3 pg/ml for shcont, $p=0.0008$ and Supplementary Fig. 1A) or Nigericin (Fig. 2D; 14253.4 \pm 2239.9 pg/ml for shNLRC3 vs 3760.1 \pm 182.4 pg/ml for shcont, $p=0.022$) resulted in a higher IL-1 β release in the cells where NLRC3 was knocked-down. Moreover, an increase in pro-IL-1 β cleavage in the supernatant (Fig. 2E and Supplementary Fig. 1B) and a higher pro-caspase-1 processing (Fig. 2F) were observed in shNLRC3 lines compared to shcont in response to Nigericin treatment. Moreover, shNLRC3 lines induced higher cell death compared to their control shcont counterpart when stimulated with Nigericin (Fig. 2G; 42.2 \pm 3.3 % vs 18.9 \pm 2.3 % of cell death, $p=10^{-5}$ and Supplementary Fig. 2). Thus, we concluded that the endogenous NLRC3 protein suppresses the activation of the NALP3 inflammasome induced by different types of ligand and the resulting pyroptotic cell death.

NLRC3 is also an inhibitor of the NLRC4 (IPAF) inflammasome—In order to determine if the inhibitory effect of NLRC3 is specific to the NALP3 complex or if could also modulate the activity of other inflammasomes, the NLRC4 (or IPAF) inflammasome was stimulated by live *Pseudomonas aeruginosa* infection. The knockdown of the NLRC3 protein in THP-1 cells significantly increased the IL-1 β secretion in response to *P. aeruginosa* infection compared to NLRC3 expressing cells (Fig. 2H; 9493.9 \pm 1256.8 pg/ml for shNLRC3 vs 3099.96 \pm 1256.7 pg/ml for shcont, $p=0.04$). Because NLRC3 inhibits both the NALP3 and NLRC4 inflammasomes, we concluded that it may be acting downstream of the receptor proteins.

NLRC3 does not regulate the protein levels of the inflammasome components—NLRC3

was shown to inhibit the NF κ B pathway by inducing the ubiquitination of TRAF6 and targeting it to proteasomal degradation (26). We investigated whether NLRC3 exert its inhibitory effect through the induction of the proteasomal degradation of the inflammasome components by co-transfecting constant NALP3, ASC and pro-IL-1 β concentrations and increasing amount of NLRC3. No difference was observed in the transfected NALP3, ASC or pro-IL-1 β protein levels at different NLRC3 concentrations (Fig. 3A,B,C), suggesting that NLRC3 inhibits the inflammasome by a mechanism independent of protein degradation or synthesis.

NLRC3 interacts endogenously with pro-caspase-1 via its CARD domain and with ASC but not with NALP3—We have previously shown that NLRC3 co-localizes and interacts with ASC and caspase-1 when overexpressed in HEK293FT cells (28). Here, we investigated whether NLRC3 could interact with NALP3. When the two proteins were overexpressed in HEK293FT cells, the immunoprecipitation of either NALP3 (Fig. 3D) or NLRC3 (Fig. 3E) did not pull-down NLRC3 or NALP3 respectively. Furthermore, the endogenous NLRC3 and NALP3 proteins did not interact with each other in any of the untreated or Nigericin and MSU treated THP-1 cells (Fig. 3F).

As a next step, to verify that the interactions that were previously seen between NLRC3 and ASC or caspase-1 are also occurring physiologically, the endogenous proteins were immunoprecipitated from THP-1 cells. Endogenous NLRC3 interacted with both endogenous ASC (Fig. 3F,G) and pro-caspase-1 (Fig. 3H). We used in this study a commercial anti-caspase-1 antibody that is directed against the p10 subunit of caspase-1. Whereas the immunoprecipitation with the anti-caspase-1 p10 antibody pulled down both pro-caspase-1 and the mature, CARD-lacking caspase-1 p20 peptide; the anti-NLRC3 antibody immunoprecipitated only the CARD-containing pro-form of caspase-1 (Fig. 3H). Thus, we concluded that NLRC3 interacts with the CARD domain of caspase-1.

NLRC3 prevents the assembly of ASC specks through its CARD and LRR domains—Another informative way of the measurement of the inflammasome activity is to count the number of ASC specks that are formed. To characterize which domain of NLRC3 disrupts the ASC speck

formation, we cloned its NLRC3^{CARD} (aa1-60), NLRC3^{NACHT} (aa61-616) and NLRC3^{LRR} (aa617-1065) domains separately or two of its domains NLRC3^{CARD/NACHT} (aa1-616), and NLRC3^{NACHT/LRR} (aa617-1065) together (Fig. 4A) and verified their expression by Western blotting (Fig. 4B).

Whereas the transfection of NALP3 into HEK293FT-ASC-EGFP cells triggered the ASC speck assembly (Fig. 4 and Fig. 6), full length NLRC3 (NLRC3^{FL}) itself or its domains had no effect on ASC speck formation on their own (data not shown). However, when NALP3 was co-expressed with NLRC3^{FL}, NLRC3^{CARD}, NLRC3^{LRR}, NLRC3^{CARD/NACHT} and NLRC3^{NACHT/LRR}, the number of ASC speck formed was significantly lower than the number of specks formed in the presence of NALP3 alone (Fig. 4C,D). Thus, we concluded that the CARD and LRR domains of NLRC3 were responsible for the disruption of the NALP3-induced complex assembly and the central NACHT domain had no effect.

NLRC3 co-localizes with diffused ASC proteins and competes with ASC for pro-caspase-1 binding—To confirm that NLRC3 co-localizes with ASC endogenously, we stained PMA differentiated and Nigericin treated THP-1 macrophages with anti-NLRC3 and anti-ASC antibodies. We found that NLRC3 and diffuse ASC stainings overlap with each other in the cytosol, however, NLRC3 signal was not observed on ASC specks (Fig. 5A). Furthermore, we could not detect the NLRC3 protein by Western blotting of the lysate containing ASC specks isolated from ASC and NLRC3 co-transfected cells (Fig. 5B). Taken together, these demonstrate that NLRC3 interacts with the diffused ASC proteins but is not embedded into the ASC specks after the inflammasome activation.

Because NLRC3 is interacting with both ASC and pro-caspase-1, we determined whether NLRC3 might affect the interaction between these two proteins. While ASC and pro-caspase-1 interacted with each other when they were overexpressed in HEK293FT cells, the addition of NLRC3 resulted in a weaker interaction between ASC and pro-caspase-1 in both the samples immunoprecipitated with anti-ASC (Fig. 5D) or anti-caspase-1 (Fig. 5E) antibodies. Moreover, increasing the concentration of NLRC3 by keeping a constant amount of ASC and pro-caspase-1

proteins (Fig. 5C) decreased the interaction between ASC and pro-caspase-1 even more. Thus, NLRC3 may exert its inhibitory effect before the complex formation by sequestering the pro-caspase-1 proteins and by blocking the assembly of pro-caspase-1 and ASC required for the inflammasome activity.

The CARD domain of NLRC3 is necessary and sufficient to inhibit the inflammasome activation triggered by the NALP3 mutants associated with auto-inflammatory syndromes—Because mutations in the *NALP3* gene are known to induce the constitutive activation of the NALP3 inflammasome, we tested whether full-length NLRC3 and NLRC3^{CARD} are able to suppress this aberrant inflammatory response. We found that both full length-NLRC3 (269.2 ± 5.8 pg/ml vs 216 ± 13.7 pg/ml for WT NALP3, $p=0.0004$; 841.4 ± 26.6 pg/ml vs 311.2 ± 9.2 pg/ml for p.R260W NALP3, $p=10^{-8}$; and 1150 ± 47.3 pg/ml vs 195.1 ± 32.1 pg/ml for p.D303N NALP3, $p=10^{-7}$) and NLRC3^{CARD} (109.3 ± 21.8 pg/ml for WT NALP3, $p=10^{-6}$; 122 ± 14.8 pg/ml for p.R260W NALP3, $p=10^{-9}$ and 45.2 ± 13.3 pg/ml for p.D303N NALP3, $p=10^{-8}$; all compared to the “no NLRC3” conditions) inhibit the IL-1 β secretion induced by ectopically assembled inflammasome complex in HEK293FT cells and NLRC3^{CARD} had even a more significant effect than the full length protein (Fig. 6A; $p=0.0002$ for WT NALP3; $p=10^{-7}$ for NALP3 p.R260W and $p=0.0001$ for NALP3 p.D303N). Furthermore, NLRC3^{FL} and NLRC3^{CARD} also impaired the ASC speck formation triggered by WT and NALP3 p.R260W proteins (Fig. 6B,C). On the other hand, no effect was observed on the number of ASC specks formed by NALP3 p.D303N proteins probably because the concentration of transfected NLRC3 was not enough to block the excessive effect of NALP3 p.D303N mutant. These data demonstrate that the CARD domain of NLRC3 is able and sufficient to repress the NALP3 inflammasome activity in normal or pathologic conditions.

DISCUSSION

We investigated, in this paper, the potential effect of the NLRC3 protein on the activation of the NALP3 inflammasome by using human THP-1 monocytes and HEK293FT cells when we had to ectopically express an exogenous protein. We found that NLRC3 decreases the

number of ASC speck formed, the maturation of pro-IL-1 β and pro-caspase-1 proteins and the secretion of IL-1 β in both overexpression and endogenous experimental settings. The suppressive effect of NLRC3 on the NALP3 inflammasome in THP-1 cells was stimulant-independent since the complex activation in response to all the ligands used (ATP, Nigericin and MSU) was affected by NLRC3.

Moreover, the CARD domain of NLRC3 was necessary to exert this inhibition. NLRC3 had no effect on the protein levels and its suppressive effect was independent of its role on the NF κ B pathway since with the use of HEK293FT, the overexpression of these proteins bypassed the transcriptional regulation of the inflammasome components. Another advantage of HEK293FT cells that do not express at all the ASC adaptor protein and express the other inflammasome components at negligible basal levels, was the possibility to introduce mutant NALP3 proteins or different NLRC3 domains without a possible interference of the WT NALP3 or full-length NLRC3 proteins respectively.

The recruitment of pro-caspase-1 proteins by ASC adaptor to the foci is a very critical step in the activation of inflammasomes. The CARD/CARD interaction between ASC and pro-caspase-1 was required for the complex formation since the mutagenesis of some residues in these domains abolished ASC/pro-caspase-1 binding and the inflammasome activation (29). Similarly, the self-oligomerization of caspase-1's CARD domains was also shown to prevent its interaction with ASC (30). Here, we showed that the presence of NLRC3, that also contains a CARD domain, decreases the interaction between ASC and pro-caspase-1. The absence of NLRC3 and NALP3 interaction and the ability of NLRC3 to inhibit both NALP3 and NLRC4 inflammasomes and different NALP3 mutants-induced IL-1 β secretion suggest that the NLRC3 acts on ASC/pro-caspase-1 level. A similar phenomenon is observed in the NF κ B pathway activation where ASC protein interferes with RIP2 and caspase-1 assembly through its CARD domain (31). More recently, an antibody generated against the CARD domain of ASC was sufficient to inhibit the inflammasome activation (32) suggesting once more how important the interaction of ASC and caspase-1 through their CARD domain is.

Overall, we propose in this paper that besides its previously defined suppressive role on the NF κ B pathway, NLRC3 is also a novel inhibitor of the activation of the NALP3 and NLRC4 inflammasomes through the disruption of the ASC speck assembly by competing with ASC protein for binding to the CARD domain of pro-caspase-1.

EXPERIMENTAL PROCEDURES

Plasmids that are used in this study—pcDNA3-ASC, pcDNA3-FLAG-pro-caspase-1, pro-IL-1 β -IRES-EGFP, pcDNA3-FLAG-WT_NALP3, pcDNA3-FLAG-R260W_NALP3 and pcDNA3-FLAG-D303N_NALP3 plasmids are kind gifts of Prof. Gabriel Nunez (University of Michigan). Full-length NLRC3 was cloned in our laboratory as previously described (28). NLRC3's domains were amplified from pcDNA3-Myc-NLRC3 template with NLRC3^{CARD} (aa1-60) ForwCARD-XbaI (TAAGTCTAGAAGGAAGC-AAGAGGTGCGG) and RevCARD-Stop-NotI (TATGCGGCCGCTCAGTCATTGCTGCA); NLRC3^{NACHT} (aa61-616) ForwNACHT-NheI (GCGGGCTAGCTCAAGGATACAGAGG) and RevNACHT-Stop-NotI (TAATGCGGCCGCTC-ACTGGGCACAG); NLRC3^{LRR} (aa617-1065) ForwLRR-XbaI (ATGGTCTAGTGAGGCCAAC-CTGTCC) and RevLRR-Stop-NotI (TTGCGGCC-GCTCAATCACATTTCACAG); NLRC3^{CARD/NACHT} (aa1-616) ForwCARD-XbaI and RevNACHT-Stop-NotI, NLRC3^{NACHT/LRR} (aa61-1065) ForwNACHT-NheI and RevLRR-Stop-NotI primers; digested with the indicated enzymes and ligated into XbaI-PspOMI digested and purified empty pcDNA3-Myc vector. To generate pLenti-CMV-Blast-NLRC3 vector, full length NLRC3 was extracted from pET30a-NLRC3 vector by BglII and XhoI digestions and inserted in a 3:1 ratio into BamHI and XhoI digested pure pLenti-CMV-Blast-Empty vector (kindly provided by Assist. Prof. Tolga Emre, Boğaziçi University, Istanbul, Turkey).

Ectopic Reconstruction of the NALP3 Inflammasome in HEK293FT Cells For the Measurement of the IL-1 β Secretion—750 000 HEK293FT cells were transfected using calcium phosphate protocol with 250 ng pcDNA3-ASC, 250 ng pcDNA3-FLAG-pro-caspase-1 and 500 ng pro-IL-1 β -IRES-EGFP plasmids and the indicated amount of pcDNA3-FLAG-WT NALP3 or

pcDNA3-MYC-NLRC3. Equal amount of DNA was transfected in each condition by adding the empty pcDNA3 vector to compensate for differences. Cell supernatants were harvested 24 hours after transfection, spun down to discard cells and the IL-1 β levels in 10% FBS containing DMEM were measured by ELISA as explained later. For the analysis of CAPS mutants, besides the amount of ASC, pro-caspase-1 and pro-IL-1 β indicated above, 1 μ g of WT or p.R260W or p.D303N NALP3 were transfected with 1 μ g empty vector, NLRC3^{FL} or NLRC3^{CARD}.

Generation of the NLRC3 KD and NLRC3 Overexpressing Stable THP-1 Cell Lines—Viruses were produced in HEK293FT cells by transfection of pLKO.1-shNLRC3 and control pLKO.1-shGFP vectors (kindly provided by Assist. Prof. Ömer Yilmaz, the Broad Institute, MIT, Boston, MA) or newly generated pLenti-CMV-Blast-NLRC3 (OvNLRC3) vectors and its control pLenti-CMV-Blast (named OvCont), together with the helper plasmids pMD2G and psPAX2. THP-1 cells were spinoculated 30 minutes at 32°C, incubated with the virus for 4 hours at 37°C, 5% CO₂ and selected with 1 μ g/ml Puromycin or 1 μ g/ml Blasticidin. Four different shRNAs targeting different parts of NLRC3 cloned into pLKO.1 vector, namely shNLRC3-D6 (TRCN0000168401), shNLRC3-D8 (TRCN0000168587), shNLRC3-D9 (TRCN0000168194) and mouse NLRC3-specific shmNLRC3-D7 (TRCN0000168383) were used to confirm the reproducibility of the NLRC3 knockdown phenotype.

Activation of the NALP3 and IPAF/NLRC4 Inflammasomes in THP-1 Cells—10⁶ shcont or shNLRC3; or OvNLRC3 and OvControl THP-1 stable lines were seeded into 6 well-plate and differentiated into macrophages by 3 hours of 0.5 μ M PMA treatment. On the next day, cells were primed for 2 hours or 4 hours with 100 ng/ml LPS and treated with 20 μ M Nigericin, 5 mM ATP or 150 μ g/ml MSU for 4 or 6 hours respectively for NALP3 inflammasome activation and treated with 75 MOI live *Pseudomonas aeruginosa* for 4 hours for IPAF/NLRC4 inflammasome activation. Supernatants were harvested, cleared from cells and the IL-1 β levels were measured by ELISA.

Measurement of the Secreted IL-1 β levels by ELISA—IL-1 β levels were determined in 1 ml of cell supernatant by using R&D human IL-1beta

IL-1F2 kit according to the manufacturer's protocol. Each treatment was performed in duplicate or triplicate and the supernatants were measured in duplicate. Significance between samples was determined by two-tailed Student t-test.

Co-Immunoprecipitation of the Endogenous and Overexpressed Inflammasome Components—Commercially available anti-NLRC3 (Abcam, ab77817), anti-Caspase-1 (Santa Cruz Biotechnology, sc-515), anti-ASC hybridoma supernatant (kind gift of Assist. Prof. Junya Masumoto, Ehime University, Ehime, Japan) and anti-NALP3 (Abcam, ab17267) antibodies were used. For co-immunoprecipitation in the overexpression system, anti-Myc (Cell Signaling, 2272), anti-Flag (Cell Signaling, 2368) antibodies were used.

ASC Speck Formation Assay—HEK293FT-ASC-EFGP stable cell lines generated in our laboratory were transfected with NALP3, full length NLRC3 or NLRC3's different domains in 6 well-plate with 500 ng of NALP3 or 500 ng of NLRC3 or 500 ng of the different domains of NLRC3 (Fig. 4) or 1 µg of WT NALP3, NALP3 pR260W, NALP3 p.D303N and 1 µg of NLRC3 (Fig. 6). Equal amount of DNA was transfected by using the pcDNA3-Myc empty vector. The number of ASC speck was counted 24 hours or 48 hours after transfection under the fluorescent

microscope. Each condition was transfected into two wells and eight randomly chosen fields were counted for each well.

Isolation of the ASC Specks—ASC specks were isolated from four 100 mm plates of 5×10^6 HEK293FT cells each, transfected with 4 µg mCherry-ASC plasmid. 24 hours after the transfection, samples were sonicated, vortexed until filaments were formed and spun down at 200 g for an hour. The pellet was dissolved in 30 ml PBS, passed through 5 µm filter, spun down at 2400 g for an hour. The final pellet containing ASC specks was dissolved in 500 µl PBS. The purity of isolated ASC specks was verified under the microscope and by Western blotting.

Competition Assay for the Binding of NLRC3 to its Interaction Partners— 5×10^6 HEK293FT cells were transfected with constant pcDNA3-ASC (2 µg) and pcDNA3-Flag-pro-caspase-1 (2 µg) and increasing concentrations of pcDNA3-Myc-NLRC3 (1, 2 and 3 µg). Cells were harvested 24 hours after transfection and immunoprecipitation was performed.

LDH Assay—LDH cytotoxicity detection kit from Biovision was used according to manufacturer protocol. The percentage of cell death was determined with the formula $(OD_{\text{Sample}} - OD_{\text{LDH}^-}) * 100 / (OD_{\text{LDH}^+} - OD_{\text{LDH}^-})$. The untreated controls were used as LDH⁺ and the TritonX treated control cells as LDH⁺ samples.

Acknowledgements: We would like to acknowledge the persons cited in the text for generously sharing the indicated materials with us. We would like also to acknowledge Stefan A. Köstler, PhD for taking the confocal images.

Conflict of interest: The authors declare no conflict of interest.

Author contributions: EE designed, performed the experiments and wrote the manuscript. MB helped during the revisions and maintained the cells. NÖ designed the experiments, wrote the manuscript and provided financial resources.

REFERENCES

1. Martinon, F., Burns, K., and Tschopp, J. (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell.* **10**, 417-426
2. Schroder, K., and Tschopp, J. (2010) The inflammasomes. *Cell* **140**, 821-832
3. Srinivasula, S. M., Poyet, J. L., Razmara, M., Datta, P., Zhang, Z., and Alnemri, E. S. (2002) The PYRIN-CARD protein ASC is an activating adaptor for Caspase-1. *J. Biol. Chem.* **277**, 21119-21122
4. Fernandes-Alnemri, T., Wu, J., Yu, J.W., Datta, P., Miller, B., Jankowski, W., Rosenberg, S., Zhang, J., and Alnemri, E. S. (2007) The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ.* **14**, 1590-1604
5. Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., and Dixit, V. M. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440**, 228-232
6. Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237-241
7. Sutterwala, F. S., Mijares, L. A., Li, L., Ogura, Y., Kazmierczak, B. I., and Flavell, R. A. (2007) Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J. Exp. Med.* **204**, 3235-3245
8. Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B. G., Fitzgerald, K. A., Hornung, V., and Latz, E. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* **183**, 787-791
9. Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., and Tschopp, J. (2007) Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* **14**, 1583-1589
10. Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., and Núñez, G. (2013) K⁺ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* **38**, 1142-1153
11. Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., Fitzgerald, K. A., and Latz, E. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat. Immunol.* **9**, 847-856
12. Zhou, R., Yazdi, A. S., Menu, P., and Tschopp, J. (2011) A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221-225
13. Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A., and Kolodner, R. D. (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat. Genet.* **29**, 301-305
14. Yu, J. W., Wu, J., Zhang, Z., Datta, P., Ibrahimi, I., Taniguchi, S., Sagara, J., Fernandes-Alnemri, T., and Alnemri, E. S. (2006) Cryopyrin and pyrin activate caspase-1, but not NF-kB, via ASC oligomerization. *Cell Death Differ.* **13**, 236-249
15. Baroja-Mazo, A., Martín-Sánchez, F., Gomez, A. I., Martínez, C. M., Amores-Iniesta, J., Compan, V., Barberà-Cremades, M., Yagüe, J., Ruiz-Ortiz, E., Antón, J., Buján, S., Couillin, I., Brough, D., Arostegui, J. I., and Pelegrín, P. (2014) The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat. Immunol.* **15**, 738-748
16. Agostini, L., Martinon, F., Burns, K., McDermott, M. F., Hawkins, P. N., and Tschopp, J. (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* **20**, 319-325
17. Dowds, T. A., Masumoto, J., Zhu, L., Inohara, N., and Nunez, G. (2004) Cryopyrin-induced interleukin 1beta secretion in monocytic cells: enhanced activity of disease-associated mutants

- and requirement for ASC. *J. Biol. Chem.* **279**, 21924-21928
18. Hoffman, H. M., Rosengren, S., Boyle, D. L., Cho, J. Y., Nayar, J., Mueller, J. L., Anderson, J. P., Wanderer, A. A., and Firestein, G. S. (2004) Prevention of cold- associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* **364**, 1779-1785
19. Hawkins, P. N., Lachmann, H. J., and McDermott, M. F. (2003) Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. *N. Engl. J. Med.* **348**, 2583-2584
20. Bauernfeind, F., Rieger, A., Schildberg, F. A., Knolle, P. A., Schmid-Burgk, J. L., and Hornung, V. (2012) NLRP3 inflammasome activity is negatively controlled by miR-223. *J. Immunol.* **189**, 4175-4181
21. Coll, R. C., Robertson, A. A., Chae, J. J., Higgins, S. C., Munoz-Planillo, R., Inserra, M. C., Vetter, I., Dungan, L. S., Monks, B. G., Stutz, A., Croker, D. E., Butler, M. S., Haneklaus, M., Sutton, C. E., Núñez, G., Latz, E., Kastner, D. L., Mills, K. H., Masters, S. L., Schroder, K., Cooper, M. A., and O'Neill, L. A. (2015) A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat. Med.* **21**, 248-255
22. Stack, J. H., Beaumont, K., Larsen, P. D., Straley, K. S., Henkel, G. W., Randle, J. C., and Hoffman, H. M. (2005) IL-converting enzyme/caspase-1 inhibitor VX-765 blocks the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients. *J. Immunol.* **175**, 2630-2634
23. Xia, X., Cui, J., Wang, H. Y., Zhu, L., Matsueda, S., Wang, Q., Yang, X., Hong, J., Songyang, Z., Chen, Z. J., and Wang, R. F. (2011) NLRX1 negatively regulates TLR-induced NF- κ B signaling by targeting TRAF6 and IKK. *Immunity* **34**, 843-853
24. Tong, Y., Cui, J., Li, Q., Zou, J., Wang, H. Y., and Wang, R. F. (2012) Enhanced TLR-induced NF- κ B signaling and type I interferon responses in NLRC5 deficient mice. *Cell Res.* **22**, 822-835
25. Conti, B. J., Davis, B. K., Zhang, J., O'connor, W. Jr., Williams, K. L., and Ting, J. P. (2005) CATERPILLER 16.2 (CLR16.2), a novel NBD/LRR family member that negatively regulates T cell function. *J. Biol. Chem.* **280**, 18375-18385
26. Schneider, M., Zimmermann, A. G., Roberts, R. A., Zhang, L., Swanson, K. V., Wen, H., Davis, B. K., Allen, I. C., Holl, E. K., Ye, Z., Rahman, A. H., Conti, B. J., Eitas, T. K., Koller, B. H., and Ting, J. P. (2012) The innate immune sensor NLRC3 attenuates Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-kappaB. *Nat. Immunol.* **13**, 823-831
27. Zhang, L., Mo, J., Swanson, K. V., Wen, H., Petrucelli, A., Gregory, S. M., Zhang, Z., Schneider, M., Jiang, Y., Fitzgerald, K. A., Ouyang, S., Liu, Z. J., Damania, B., Shu, H. B., Duncan, J. A., and Ting, J. P. (2014) NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. *Immunity* **40**, 329-341
28. Gültekin, Y., Eren, E., and Özören, N. (2015) Overexpressed NLRC3 acts as an anti-inflammatory cytosolic protein. *J. Innate Immun.* **7**, 25-36
29. Proell, M., Gerlic, M., Mace, P. D., Reed, J. C., and Riedl, S. J. (2013) The CARD plays a critical role in ASC foci formation and inflammasome signaling. *Biochem. J.* **449**, 613-621
30. Narayanan, K. B., and Park, H. H. (2015) Purification and analysis of the interactions of caspase-1 and ASC for assembly of the inflammasome. *Appl. Biochem. Biotechnol.* **175**, 2883-2894
31. Sarkar, A., Duncan, M., Hart, J., Hertlein, E., Guttridge, D. C., and Wewers, M. D. (2006) ASC directs NF- κ B activation by regulating receptor interacting protein-2 (RIP2) Caspase-1 interactions. *J. Immunol.* **176**, 4979-4986
32. Schmidt, F. I., Lu, A., Chen, J. W., Ruan, J., Tang, C., Wu, H., and Ploegh, H. L. (2016) A single domain antibody fragment that recognizes the adaptor ASC defines the role of ASC domains in inflammasome assembly. *J. Exp. Med.* **213**, 771-790

FOOTNOTES

This work was supported in whole or in part by EMBO-SDIG 1468 and Boğaziçi University's Research Fund projects BAP 6526 (11B01D10) and BAP 7360 (13B01M3), granted to NÖ.

FIGURE LEGENDS

Figure 1. The overexpression of NLRC3 inhibits the NALP3-induced IL-1 β secretion. **A, B.** IL-1 β secretion in ectopically assembled NALP3 inflammasome in HEK293FT cells were transfected with 500 ng pro-IL-1 β , 250 ng ASC and 250 ng pro-caspase-1 plasmids and the indicated amount of NALP3 (**A**), or 1 μ g NALP3 and the indicated amount of NLRC3 (**B**). Each condition was duplicated. The secreted IL-1 β was quantified 24h after transfection in cell-free supernatants by ELISA. **C.** IL-1 β cleavage in HEK293FT cells with ectopically assembled NALP3 inflammasome. **D.** NLRC3 mRNA levels in THP-1 OvCont and OvNLRC3 cell lines. qPCR results of relative mRNA of NLRC3/Beta-Actin are given. NLRC3/Beta-Actin ratio of OvCont cells was assigned as 1 a.u. **E.** NLRC3 protein levels in THP-1 OvControl and OvNLRC3 stable lines. **F, G.** IL-1 β secretion in THP-1 OvNLRC3 and THP-1 OvControl stable lines in response to Nigericin (**F**) and MSU (**G**) treatments.

Figure 2. The knockdown of NLRC3 in THP-1 cells results in an increase of IL-1 β secretion and pro-IL-1 β and pro-caspase-1 cleavages. **A.** NLRC3 protein levels in three different stable THP-1 NLRC3 KD cell lines (shD6, shD8, shD9) and their control line (shcont). **B.** IL-1 β secretion in shD6, shD8, shD9 and shcont THP-1 stable cell lines in response to MSU stimulation (representative result of four independent sets of experiments). **C, D.** IL-1 β secretion in shNLRC3 THP-1 cell lines in response to ATP (**C**) (representative result of three independent sets of experiments) and Nigericin (**D**) (combined results of two independent sets of experiments) stimulations. **E.** Pro-IL-1 β cleavage in the cell lysate and the supernatant of shcont and shNLRC3 cell lines in response to Nigericin stimulation. Control samples represent the cells untreated by Nigericin. Two independent samples were treated by Nigericin (N1 and N2). **F.** Pro-caspase-1 cleavage in the supernatant of Nigericin treated shcont and shNLRC3 cells. **G.** LDH results of Nigericin treated shcontrol and shNLRC3 THP-1 cells. Representative result of three sets of independent experiment. **H.** IL-1 β secretion in response to *Pseudomonas aeruginosa* infection of shcont and shNLRC3 cell lines. Two-tailed Student's t-test was performed.

Figure 3. NLRC3 interacts endogenously with pro-caspase-1 via its CARD domain and with ASC but not with NALP3. **A, B, C.** NLRC3 does not regulate the protein levels of the inflammasome components. Equal amount of the NALP3 (**A**), ASC (**B**) and pro-IL-1 β (**C**) proteins were transfected into HEK293FT cells in the presence of increasing concentration of NLRC3. Protein levels were analyzed by Western blotting. **D, E.** 5x 10⁶ HEK293FT were transfected with 6 μ g of ASC, Myc-NLRC3 and Flag-NALP3 plasmids. Irrelevant anti-HA antibody was used as negative IP control. WCL: whole cell lysates, Rem: remaining, flow through fraction after IP, * IgG heavy chains (55 kDa). Results from anti-Flag (**D**) and anti-Myc (**E**) are shown. **F.** Interaction of endogenous NLRC3 with endogenous NALP3 and endogeneous ASC. IP was performed with anti-NLRC3 antibody in PMA-differentiated THP-1 cells and Nigericin and MSU treated THP-1 cells. **G.** Interaction of endogenous NLRC3 with endogenous ASC. IP was performed with anti-ASC antibody in control and Nigericin treated THP-1 cells. **H.** Interaction of endogenous NLRC3 with endogenous Caspase-1 in THP-1 cells. +: IP with anti-NLRC3 or anti-caspase-1 antibody. -: IP with anti-rabbit IgG antibody.

Figure 4. NLRC3 prevents the assembly of ASC specks through its CARD and LRR domains. **A.** NLRC3 protein's structure. Different domains and amino acid numbers are shown. **B.** Western blot result showing the expression of Myc-tagged full length NLRC3 and separate domains. Anti-Myc antibody was used. FL: Full-Length; C/N: CARD/NACHT; N/L: NACHT/LRR. Predicted molecular weights: FL: 117

kDa, NACHT: 63 kDa, LRR: 49 kDa, C/N: 69 kDa and N/L: 110 kDa. **C.** Representative pictures of three sets of independent experiments of the effect of NLRC3's domains on ASC speck formation induced by NALP3. **D.** Quantification of ASC specks formed in the presence of NLRC3's domains. (p-values shown are the indicated condition compared to the NALP3+ Myc only condition, Student's t-test).

Figure 5. NLRC3 co-localizes with diffused ASC proteins and competes with ASC for pro-caspase-1 binding. **A.** Endogenous ASC (green) and endogenous NLRC3 (red) co-localization in MSU treated THP-1 cells. White arrows show ASC specks. **B.** NLRC3 expression in ASC specks isolated from mCherry-ASC transfected HEK293FT. 12 μ l of pure ASC specks were loaded on gel. Left panel: ASC mono- and oligomers. Right panel: NLRC3 and Actin blot. ***: position where NLRC3 band is expected to be (117 kDa). **C, D, E.** Transfection of ASC and FLAG-Pro-caspase-1 with increasing concentrations of Myc-NLRC3. Protein expression in whole cell lysate (**C**), anti-ASC IP (**D**) and anti-caspase-1 IP (**E**) lysates.

Figure 6. The CARD domain of NLRC3 is necessary and sufficient to inhibit the inflammasome activation triggered by the NALP3 mutants associated with auto-inflammatory syndromes. **A.** IL-1 β secretion induced by WT NALP3 or NALP3 p.R260W and p.D303N mutants in the absence (black) or the presence of full-length NLRC3 (gray) and NLRC3^{CARD} (light gray). **B, C.** ASC speck formation induced by WT NALP3 or NALP3 p.R260W and NALP3 p.D303N mutants in the presence or absence of NLRC3 and NLRC3^{CARD}. Quantification of ASC specks (**B**) and representative images of three sets of independent experiments (**C**). Samples were compared to the Student's t-test.

Figure 1. The overexpression of NLRC3 inhibits the NALP3-induced IL-1 β secretion.

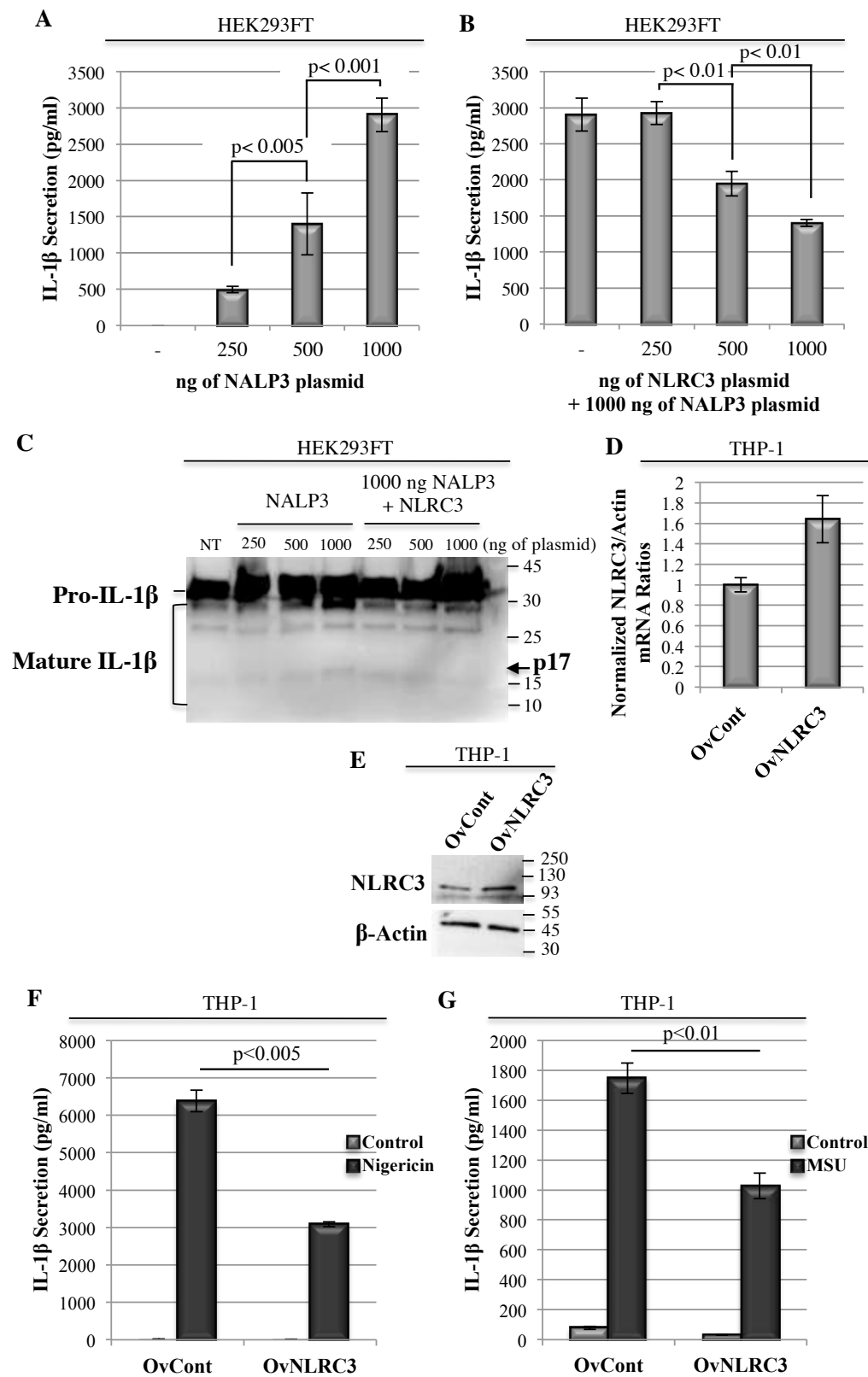


Figure 2. The knockdown of NLRC3 in THP-1 cells results in an increase of IL-1 β secretion and pro-IL-1 β and pro-caspase-1 cleavages.

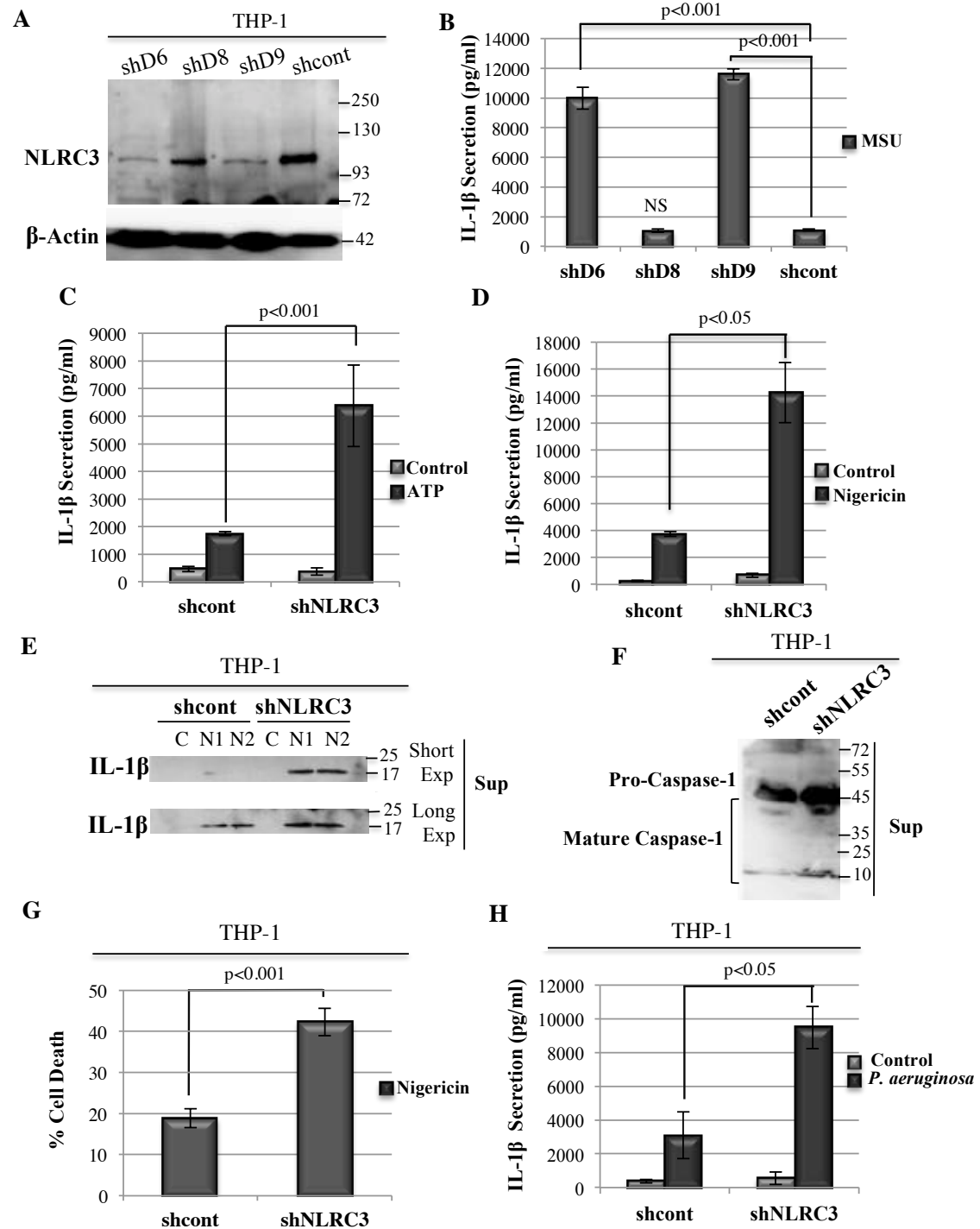


Figure 3. NLRC3 interacts endogenously with pro-caspase-1 via its CARD domain and with ASC but not with NALP3.

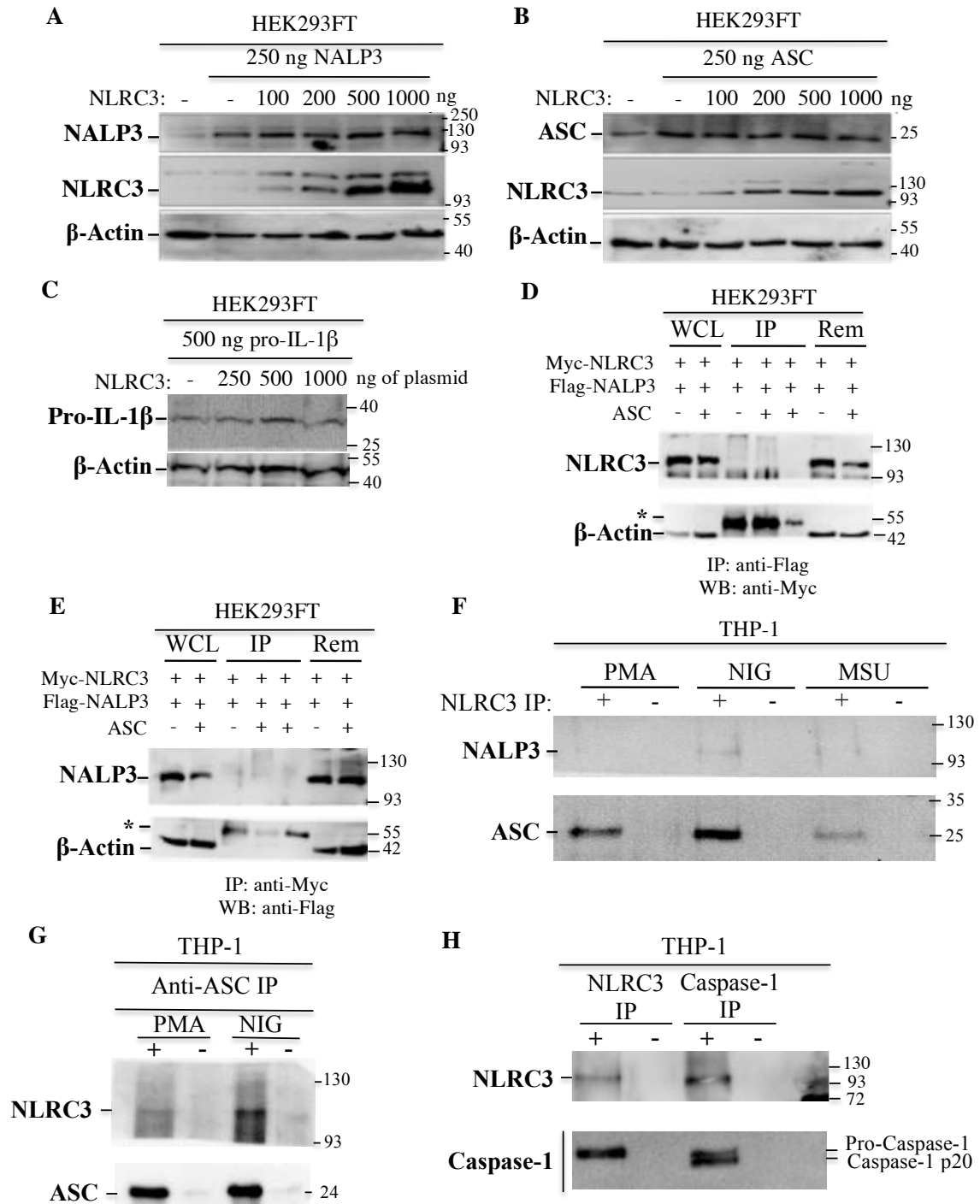


Figure 4. NLRC3 prevents the assembly of ASC specks through its CARD and LRR domains.

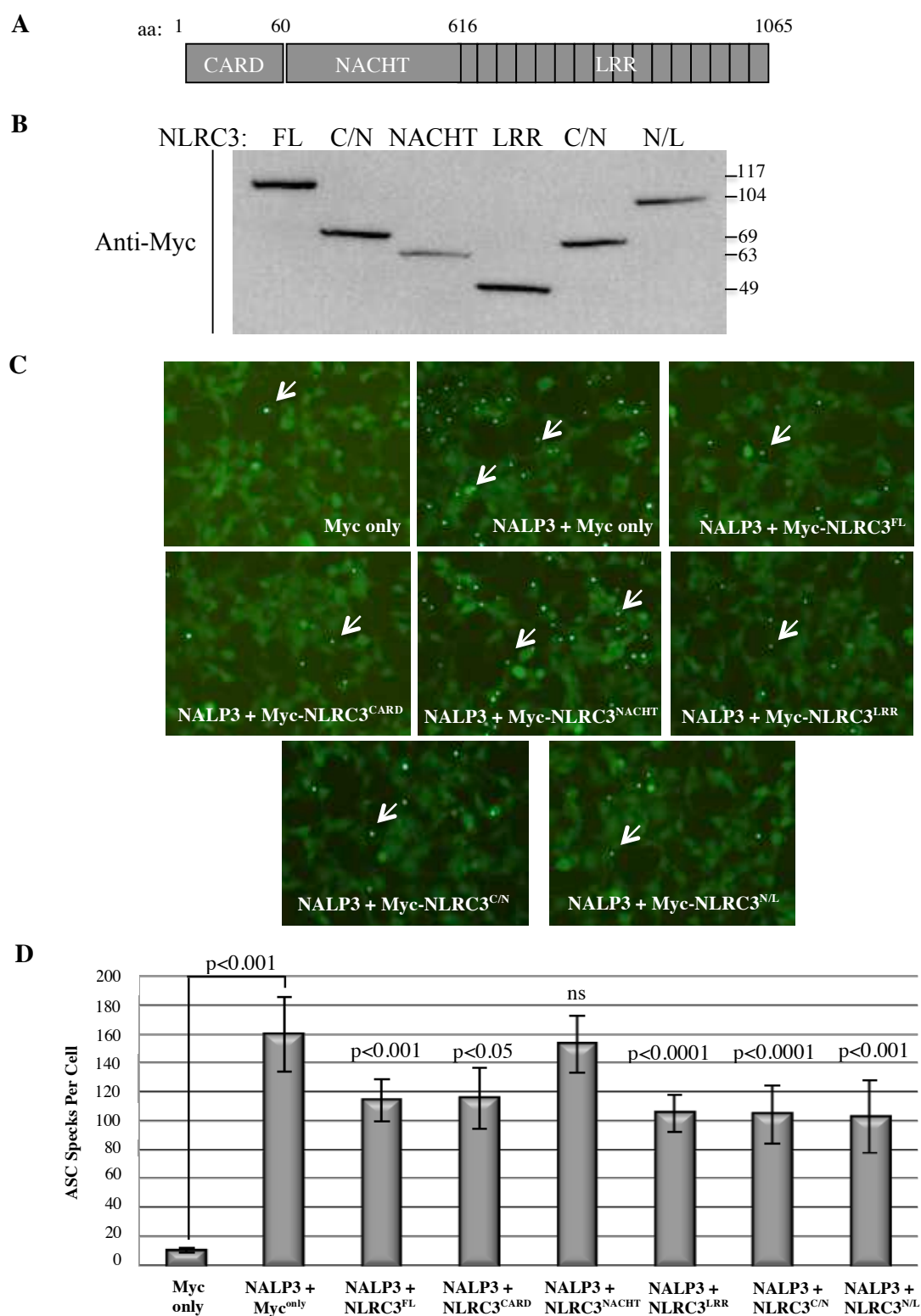


Figure 5. NLRC3 co-localizes with diffused ASC proteins and competes with ASC for pro-caspase-1 binding.

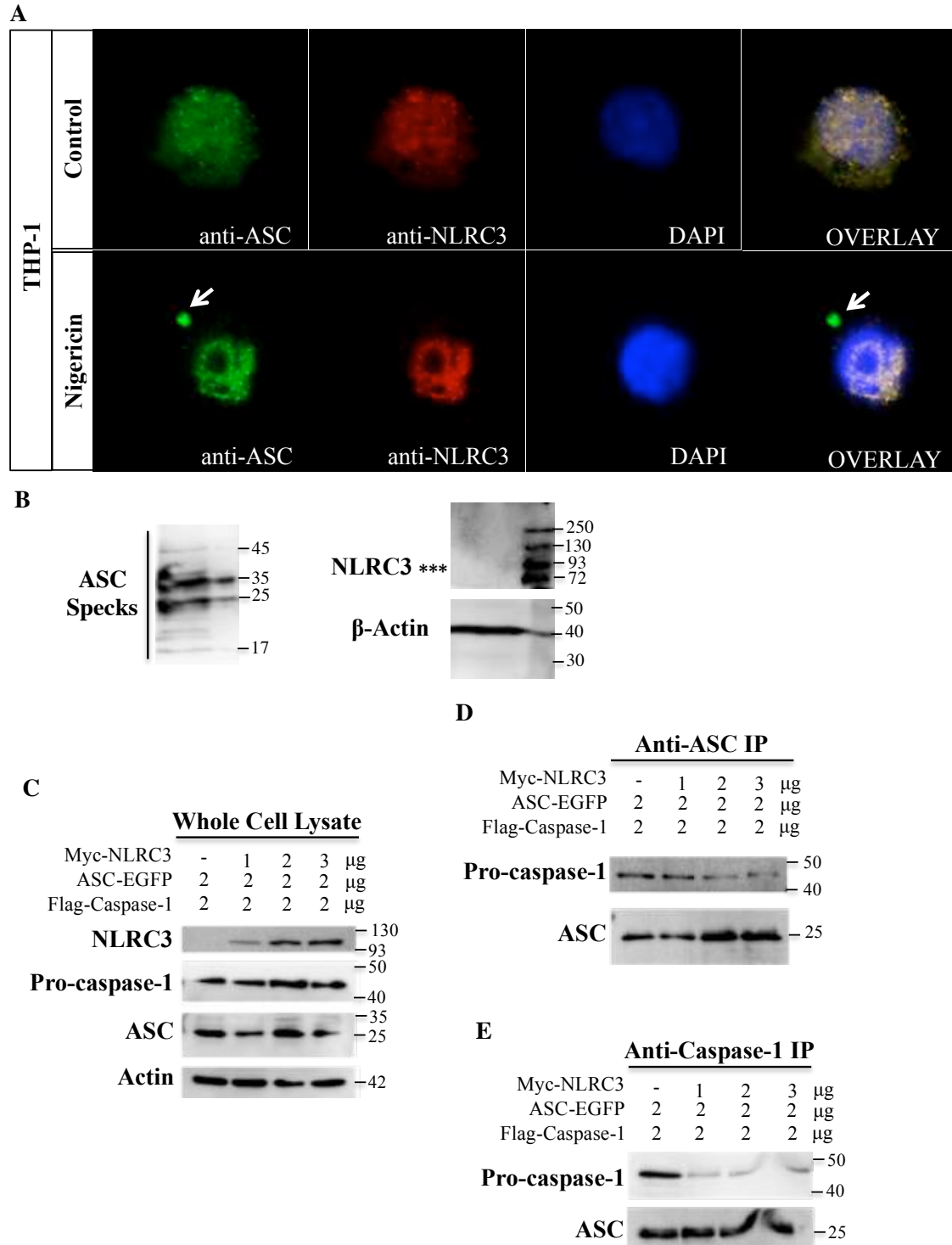
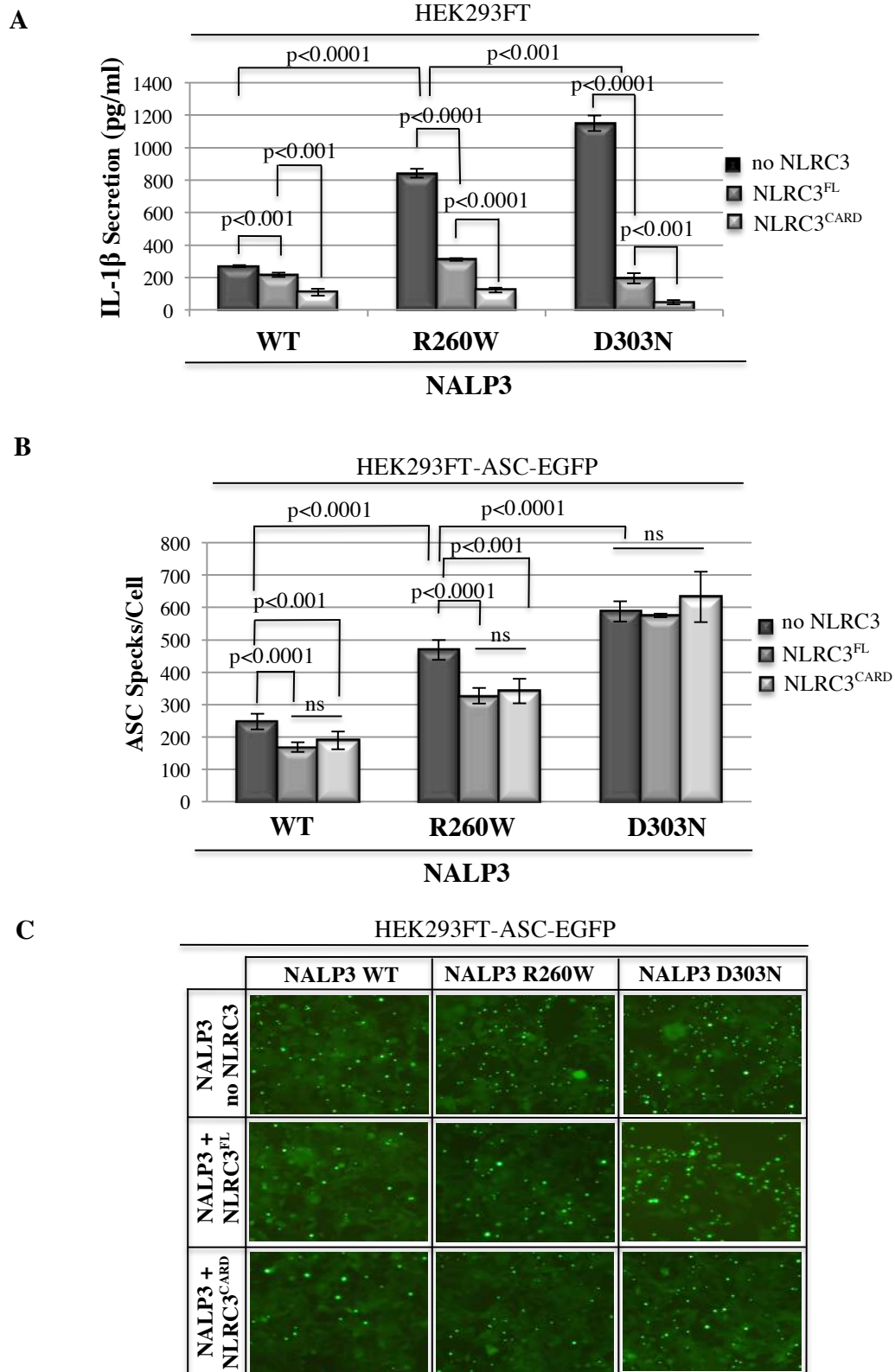


Figure 6. The CARD domain of NLRC3 is necessary and sufficient to inhibit the inflammasome activation triggered by the NALP3 mutants associated with auto-inflammatory syndromes.



NLRC3 protein inhibits inflammation by disrupting NALP3 inflammasome assembly via competition with the adaptor protein ASC for pro-caspase-1 binding

Elif Eren, Mesut Berber and Nesrin Özören

J. Biol. Chem. published online June 5, 2017

Access the most updated version of this article at doi: [10.1074/jbc.M116.769695](https://doi.org/10.1074/jbc.M116.769695)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

Supplemental material:

<http://www.jbc.org/content/suppl/2017/06/05/M116.769695.DC1>

This article cites 0 references, 0 of which can be accessed free at

<http://www.jbc.org/content/early/2017/06/05/jbc.M116.769695.full.html#ref-list-1>