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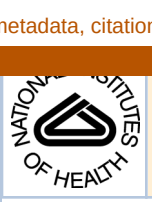

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NLRC5-dependent activation of the inflammasome

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Abstract

The nucleotide-binding domain (NBD) leucine rich repeat (LRR) containing proteins, NLRs, are intracellular sensors of PAMPs and DAMPs. A subgroup of NLRs can form inflammasome complexes, which facilitate the maturation of pro-caspase-1 to caspase-1, leading to IL-1β and IL-18 cleavage and secretion. *NLRC5* is predominantly expressed in hematopoetic cells and has not been studied for inflammasome function. RNAi-mediated knockdown of NLRC5 nearly eliminated caspase-1, IL-1β and IL-18 processing in response to bacterial infection, PAMPs and DAMPs. This was confirmed in primary human monocytic cells. NLRC5 together with procaspase-1, pro-IL-1β and the inflammasome adaptor, ASC, reconstituted inflammasome activity which showed cooperativity with NLPR3. The range of pathogens that activate NLRC5 inflammasome overlaps with those that activate NLRP3. Furthermore, NLRC5 biochemically associates with NLRP3 in an NBD-dependent but LRR-inhibitory fashion. These results invoke a model where NLRC5 interacts with NLRP3 to cooperatively activate the inflammasome.

Introduction

The role of NLRC5 is controversial and unresolved. Five recent publications have offered conflicting and alternating roles of NLRC5 in innate and adaptive immunity (1–5). One view suggests that NLRC5 is a positive regulator of the interferon pathway in HeLa and THP-1 cells and is required for robust levels of interferon secretion(3, 5). However, Benko et. al. demonstrated that NLRC5 is a negative regulator of the interferon, NF-κB and AP-1

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pathways in 293 cells. Furthermore, in mouse monocytic cell line RAW 264.7 cells, Nlrc5 functioned in an inhibitory manner. Cui et. al. provided mechanistic detail by demonstrating that Nlrc5 interacts with IKKα and inhibits its catalytic activity. Therefore in its absence there is a more robust proinflammatory response characterized by increased levels of TNF-α, IL-6 and IL-1β. NLRC5 has also been shown to positively regulate MHC class I gene expression by directly binding to the promoter region of MHC class I and associated genes in 293T cells(4). However, an opposite effect has been described in RAW 264.7 cells, as RNAi-mediated knock down on Nlrc5 induced MHC class I expression(1). Therefore a complex and either cell type or species specific role for NLRC5 is emerging. However, none of the published studies investigated a role of NLRC5 in inflammasome function or formation.

In this study, we delineated a new function for NLRC5 in inflammasome formation in response to pathogens, PAMPs or DAMPs. In the absence of NLRC5, macrophage cell types process proIL–1β and proIL-18 ineffectively and activation of caspase-1 is nearly eliminated in response to NLRP3- specific agonists. Finally, we demonstrate that NLRC5 associates with itself and NLRP3. These data suggest NLRC5 is a novel NLR protein that cooperates with NLRP3 to induce inflammasome formation.

Materials and Methods

Quantitative PCR analysis of NLRC5 expression

Human total RNA Master Panel II (Clontech) and mouse tissue RNA was used for cDNA synthesis using standard procedures. Quantitative PCR was performed with pre-validated *NLRC5* primers (Applied Biosystems). Transcripts were calculated by $\Delta\Delta C_t$ method or relative expression by *Gapdh, Actin* or *18s* rRNA. Microarray data was mined from the Genomics Institute of the Novartis Research Foundation (http://biogps.gnf.org/). Inflammasome related genes were cloned from THP-1 mRNA with primers designed to amplify the ORFs.

Cell culture

The human monocytic cell line THP-1 was transduced with shRNA (supplemental table 1) containing lentivirus. Knock-down efficacies were determined by immunoblot analysis. 2×10^6 cells were transfected by amaxa with 20–200pMoles of siRNA (Ambion) and protocols T008 (THP-1) or V001 (monocytes).

Bacteria

S. flexneri (strain 12022), *K. pneumoniae* (strain 43816 serotype 2), *P. gingivalis* (strain A7436) and *L. monocytogenes* (strain 43251) were obtained from ATCC and cultured as directed. *E. coli* (strain LF82) was provided by Dr. R. Sartor. (UNC at Chapel Hill) and *S. aureus* (strain RN6390) from J.A.D.

Infections and stimulations

THP-1 cells were harvested in log phase growth. Bacteria were grown to stationary phase overnight. Cultures were diluted 1:250 and grown for an additional 2hrs then quantitated by optical density. Co-cultures were pulsed with gentamicin at 50μg/ml (Gibco) after 1hr. Infections were harvested after an additional 2hrs. Student T-tests were performed to determine significance. For PAMP stimulation, cells were plated and stimulated with indicated PAMPs (Invivogen) overnight. For monosodium urate (MSU) (Invivogen) and alum (Sigma) cells were primed with 5ng/ml of ultrapure LPS from *E. coli* for 2hrs then pulsed with agonist for an additional 4–6hrs. For nigericin (Invivogen), α-hemolysin (J.A.D) stimulation cells primed as before but harvested after 2hrs.

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Cytokine, immunoblot and cell death analyses

IL-1β, IL-18 and TNF-α were measured by ELISA (BD Biosciences and Binder Life Sciences). Immunoblot were performed as described and probed with anti-IL-1β antibodies from Cell Signaling (#2022 and #2021) and Santa Cruz Biotechnology (sc-52865)(6). Cell-free supernatants were used to quantitate cell death by ToxiLight® bioassay (Lonza)(7).

Immunoprecipitations

HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) or Fugene^{6} (Roche) at a ratio of 3:1 (Lipid:DNA). Cells were lysed in RIPA buffer in the presence of protease inhibitors and Benzoase (Novagen). IPs were washed with 1ml of RIPA buffer 5 times then resolved on 4–12% Bis-Tris gels. Immunoblots were probed with HRP-conjugated M2 (Sigma), anti-HA (Roche), anti-V5 (Invitrogen) antibodies.

Results

Expression, cloning and characterization of NLRC5

NLRC5 is expressed predominantly in the lymphocytic and macrophage/monocytic cell lineages with low expression in non-hematopoetic cells based on public gene profile database (S Fig1A and C). By real-time PCR analysis of human and mouse tissues (S Fig. 1B and D), *NLRC5* is expressed preferentially in immune tissues relative to non-immune tissues. The gene is encoded by 49 exons and its ORF is 5601 nucleotides (46 exons)(8). NLRC5 has an N-terminal CARD-like domain and an extended C-terminus consisting of an expanded LRR, making it the largest member of the NLRs (S Fig. 2A) with an approximate molecular weight of 200kDa compared to the molecular weights of other NLRs ~100kDa each. NLRC5 is well conserved in vertebrate evolution; orthologs can be found in human, chimpanzee, cow, rat and mouse (S Fig. 2B)(9). Thus NLRC5 is a conserved NLR member with preferential expression in hematopoetic cells.

NLRC5-dependent inflammasome activation

We explored the role of NLRC5 in monocytic cells due to their prominent role in innate immunity and inflammation. Its role in inflammasome activation was studied using sh- and siRNA to reduce its expression in the THP-1 monocytic cell line. ShRNA for NLRP3 and ASC served as positive controls. Due to the potential off-target effects of shRNA, a panel of shRNA target sequences to *NLRC5* (shNLRC5) and shRNA controls in lentiviral constructs was tested. ShNLRC5 effectively reduced endogenous levels *NLRC5* transcript (S fig. 3A) and protein (S fig. 3B). The former was determined using three primer sets and the latter was determined by immunoblot using a mAb specific for the N-terminus of NLRC5 (S Fig. 3C–D). *E. coli* infected cells with shNLRC5 produced significantly reduced levels of secreted IL-1β compared to cells with control shRNAs in two different lentivirus vectors (Fig. 1A–B and S Fig. 3E). To avoid potential effects of lentiviral transduction, THP-1 cells were also transiently transfected with siRNA oligonucleotides (siNLRC5 and siNLRP3). Cells with control siRNA (siControl) secreted significantly more IL-1β than cells with either siNLRC5 or siNLRP3 upon *E. coli* infection (Fig. 1C). Importantly, human primary monocytes from two anonymous donors transfected with siRNA oligonucleotides specific for *NLRC5* also failed to mount robust IL-1β in response to *E. coli* (Fig. 1D). IL-1β processing (Fig. 1E) and caspase-1 (Fig. 1F) maturation are also greatly reduced in the absence of NLRC5. Real time PCR analysis demonstrates that in the absence of NLRC5, transcription of *NLRP3* and *ASC* are unaffected (S Fig. 4A–C). Additionally pro-IL-1β levels are comparable (S Fig. 4D). These data suggest that NLRC5 has a role in IL-1β inflammasome formation in transformed and non-transformed human monocytic cells.

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NLRC5-dependent response to pathogens

To determine the specificity of signals that activate the NLRC5-dependent inflammasome, THP-1 cell lines stably expressing shNLRC5 or control shRNA were infected with a panel of bacteria. Candidate pathogens *E. coli, S. flexneri* and *S. aureus* require NLRP3 and ASC to activate the inflammasome (Fig. 2A–C) and they also induced significantly less IL-1β secretion in cells expressing shNLRC5 relative to cells expressing shControl. The decrease in IL-1β and IL-18 with different bacteria was observed over various concentrations (S Fig. 5A–D) except at high concentrations of *S. aureus*, IL-1β/IL-18 secretion is independent of NLRC5 (S Fig. 5B, D). These data suggest that NLRC5 modulates inflammasome activation to a wide array of bacterial pathogens.

NLRC5 is required for inflammasome activation by PAMPs, DAMPs and toxins

To further dissect possible agonists that activate the NLRC5 inflammasome, THP-1 cells with shNLRC5 or control shRNA were stimulated with pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and pore-forming toxins, all of which can activate the NLRP3 inflammasome, possibly via the release of endogenous secondary signals such as ATP or reactive oxygen species . The PAMPs induce significantly less IL-1β in the absence of NLRC5 (Fig. 2D). Treatment with MSU or alum crystals also induces IL-1β secretion in an NLRC5-dependent fashion (Fig. 2D). Nigericin from *Streptomyces hygroscopicus* and α-hemolysin from *S. aureus* activate the NLRP3 inflammasome by pore-formation(6, 10, 11). Interestingly, IL-1β secretion in response to these toxins is independent of NLRC5 (Fig. 2E). These data revealed that NLRC5 is not required for inflammasome activation by the pore-forming toxins tested. The fact that purified *S. aureus* α-hemolysin induces NLRC5-independent IL-1β production is compatible with earlier results showing that high MOIs of *S. aureus* causes NLRC5-independent inflammasome activation and is consistent with α-hemolysin being a synergistic but not sole mechanism for robust IL-1β secretion(6, 11). These findings suggest that inflammasome activation by bacterial PAMPs and crystals, but not pore-forming toxins, requires NLRC5.

NLRC5-independent regulation of TNF-α and cell death

To assess the specificity of NLRC5 function, we assayed supernatants from bacteria-infected cells for TNF-α. TNF-α levels from shNLRC5-containing cells are not significantly different from control cell lines after infection with bacteria (Fig. 2F). Pathogen-mediated activation of caspase-1 and/or NLRs can induce several cell death pathways that are broadly characterized as proinflammatory since they result in the further release of inflammatory molecules(12, 13). Interestingly, pathogen-induced cell death that are documented to require NLRP3 in THP-1 cells is not affected by the absence of NLRC5 (Fig. 2G), suggesting that the function of NLRC5 is more restricted to inflammasome activation.

Interactions of NLRC5 with NLRP3 and ASC but not other inflammasome components

The overlapping biologic functions and pathogen-specificity of NLRC5 with NLRP3 suggests that these proteins might act in a cooperative manner during the inflammasome assembly of ASC, NLRP3, and caspase-1. To test if NLRC5 interacts with inflammasome components we performed co-immunoprecipitation experiments with NLRC5 and itself, NLRP3, AIM2, ASC, procaspase-1 and proIL-1β. Epitope-tagged NLRC5 interacts with itself, NLRP3 and ASC (Fig. 3A and S Fig 6A). In contrast, it did not interact with the other inflammasome components AIM2, procaspase-1, proIL-1β nor additional cytosolic proteins such as TRAF2, JNK (S Fig. 6B–C). To determine if the interactions of NLRC5 are specific

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to inflammasome-forming NLRs, we show that NLRC5 does not interact with another NLR, CIITA (S Fig. 6D)

To determine the domain of NLRC5 that is necessary and sufficient for NLRP3 binding, truncation mutants were used in co-immunoprecipitation experiments (Fig. 3B). The NBD domain appears to be necessary and sufficient to bind NLRP3, as it co-immunoprecipitates with NLRP3. The N-terminal CARD (N-term) or C-terminal LRR (LRR) of NLRC5 failed to co-immunoprecipitate with NLRP3. The presence of the N-terminus to the NBD domain in the N-NBD construct did not influence the binding capacity of the NBD. However the presence of LRR in the NBD-LRR construct reduced interaction with NLRP3. This result agrees with general concept that the LRR is inhibitory in function. To note, we have consistently observed decreased levels of NLRP3 in the presence of the LRR of NLRC5 and are currently investigating this phenomenon.

Others have demonstrated that reconstitution of inflammasome components in epithelial cells can lead to spontaneous or PAMP-induced IL-1β processing(14). To test whether NLRC5 can facilitate inflammasome function, we transfected these inflammasome components into HEK293T cells and measured IL-1β processing. Transient transfection with NLRC5, procaspase-1, proIL-1β and ASC results in IL-1β processing that was dependent on the inflammasome component expression as measured by secreted IL-1β (Fig. 3C). The level of IL-1β induced by the non-inflammasome NLR protein NOD1 is significantly less than the amount seen with NLRC5. When NLRC5 was co-transfected with increasing amounts of NLRP3 or vice versa, a synergistic increase in IL-1β secretion is observed (Fig. 3D). These data suggest that NLRC5 is a component of the inflammasome in a reconstituted and ectopic system either by itself or in conjunction with NLRP3.

Discussion

Inflammasome activation represents a major function of some NLR proteins(15) although the function of a majority of NLR proteins remains unclear. *NLRC5* is primarily expressed in adaptive and innate immune system. The conserved expression pattern of *NLRC5* orthologs suggests a critical biological role in immunity. As its expression in monocytic cells might be indicative of a role in innate immunity to pathogens, we examined the role of NLRC5 in inflammasome function. We now describe NLRC5 as a critical component of inflammasome-dependent IL-1β secretion in response to a repertoire of stimuli that also activate NLRP3. This function is supported by the interactions of NLRC5 with NLRP3 and ASC as well as its ability to form inflammasome complexes in an ectopic system. These interactions might greatly increase the repertoire of biological moieties that can be sensed by NLR inflammasomes as has been previously predicted(9).

In THP-1 cells infected with higher concentrations of *S. aureus* or stimulated with purified α-hemolysin, secretion of IL-1β is independent of NLRC5. *S. aureus* is known to elaborate many pore-forming and immune-modulating toxins. Thus at high concentrations it is possible that these toxins activate an NLRC5-independent but NLRP3-dependent inflammasome. Interestingly, many of these toxins disrupt the cell membrane and ultimately induce cell death. In our studies NLRC5 appears to be dispensable in pathogen-induced cell death, while NLRP3(7) is essential. This divergence of function may signify segregation between multiple pathways.

The mechanisms that govern pathogen-induced inflammasome activation remain poorly characterized and the contribution of individual NLRs is controversial. For example, early studies demonstrated an absolute role for Nlrc4 in *Salmonella*-dependent IL-1β secretion(10); however recent studies have challenged the notion that IL-1β secretion relies

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exclusively upon Nlrc4(16). Similarly, Miao et al. has recently demonstrated Nlrc4-dependent inflammasome activation in response to components of the type 3 secretion system in a broad panel of Gram-negative bacteria. Likewise, there is controversy over the role of Nlrp3- and Nlrc4-dependent inflammasome activation in response to *S. flexneri* infection(7, 17). These differences might reflect differences in bacterial strains, host species, infected cell types or duration and multiplicities of infection. Notwithstanding, the mounting evidence suggests that during bacterial infections multiple NLR inflammasomes have the potential to become activated.

NLRs have been shown to have different roles in separate cell types(18). A prime example is NOD2, which was initially described as an epithelial cell and/or macrophage sensor of MDP that induces NF-κB activation(19, 20). Others have shown that NOD2 functions as an inflammasome component that facilitates NLRP1-dependent activation in monocytic cells(21). The multiple functions of NLRs are also illustrated in the analysis of NLRC5. NLRC5 is reported to have a positive role in antiviral immunity in a human epithelial cell lines and primary fibroblasts(3, 5), and as a negative regulator of both type I interferon and NF-κB-dependent cytokines via interactions in epithelial and monocytic cells(1, 2), and as a positive regulator of MHC class I gene transcription in human cells(4) but not mouse cell lines(1). Our data demonstrate a separate function of NLRC5 in monocytic cells. Whether these diverse functions relate to the unique structure of NLRC5 or the biological systems used remains to be seen. The generation of mice deficient in *Nlrc5* might shed light on the *in vivo* role of this gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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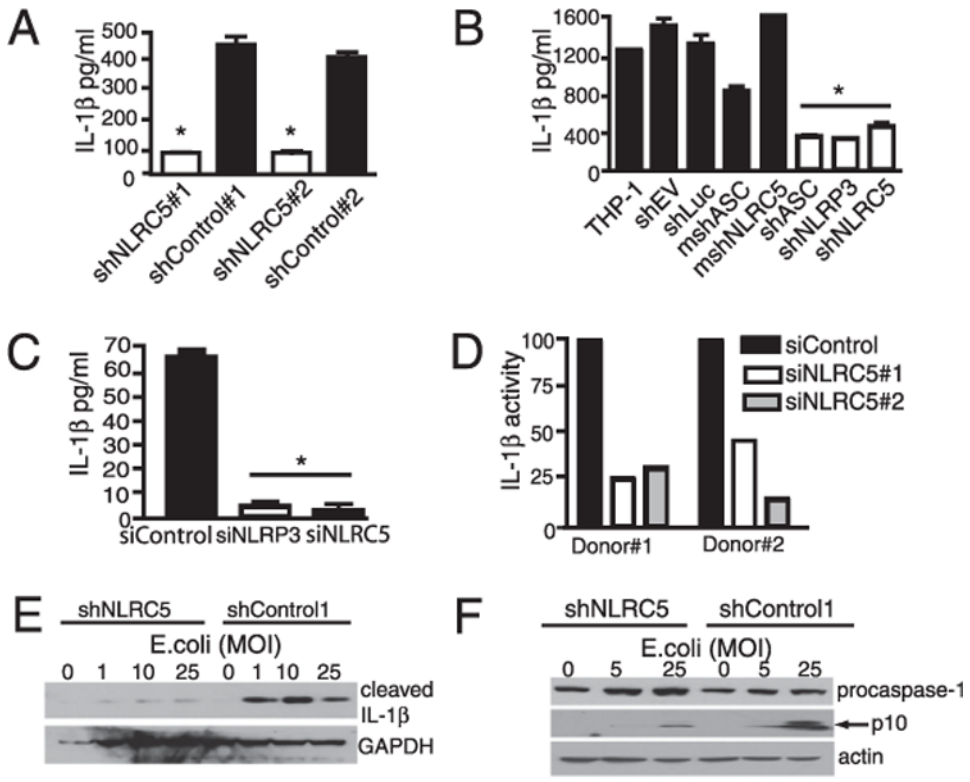

Figure 1. NLRC5 is necessary for inflammasome activation in response to *E. coli*

A) Lentivirus transduced THP-1 cells were stimulated with *E. coli*. Cell supernants were assayed for IL-1β by ELISA. Two shNLRC5 and their control shRNA with sequence specific mutations (shControl1 and shControl2) were analyzed. B) ShRNA containing Control cell lines were assayed for IL-1β secretion in response to *E. coli*: shEV = empty vector; shLuc = shRNA to luciferase; mshASC = mutant ASC; mshNLRC5 = mutant shNLRC5#1; shASC, shNLRP3, shNLRC5 = sequence specific shRNA. C) THP-1 cells were nucleofected with siRNAs specific for *NLRP3* and *NLRC5* and with a non-silencing (siControl) control siRNA, 48 hours cells were stimulated as in (A). D) Human primary monocytes were transfected with siRNA to NLRC5 and a non-silencing siRNA (siControl) and stimulated as in (C). In D) values were normalized to non-silencing control. In A-C) experiments were repeated at least three independent times. Untreated samples were below the level of detection. Asterisks denote $p < 0.05$. (E) THP-1 cell lysates were probed for active IL-1β in response to *E. coli* infection and active capase-1 (F).

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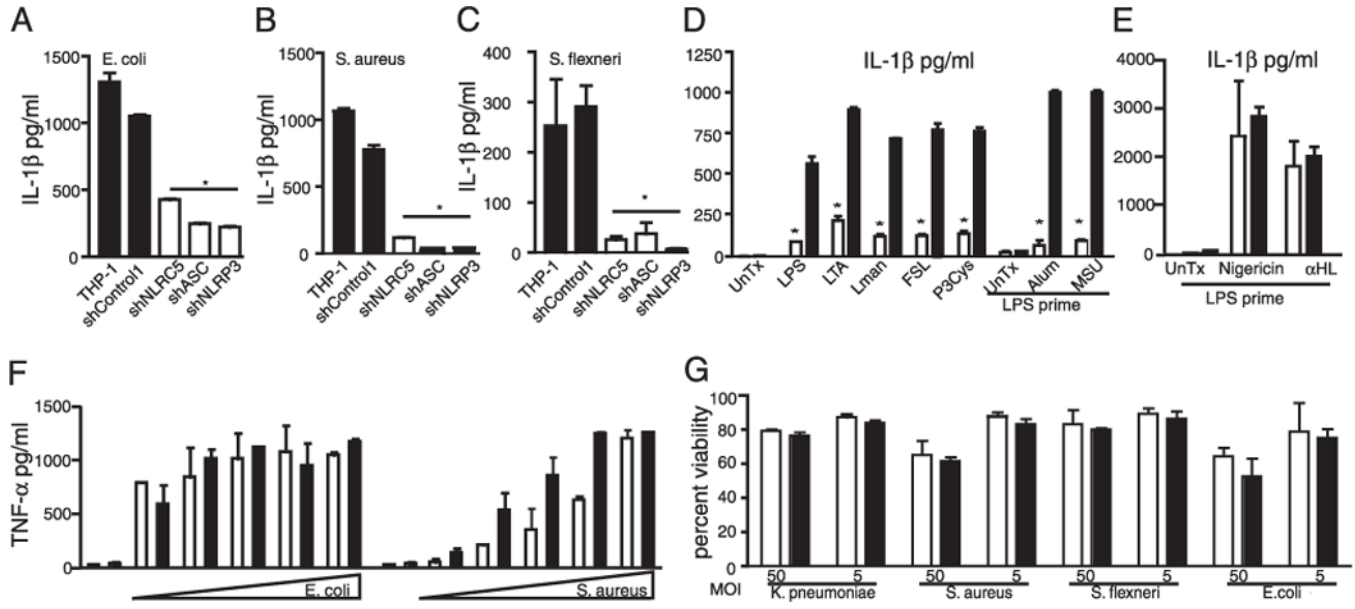

Figure 2. NLRC5 is necessary for IL-1β production in response to bacterial pathogens and DAMPs

A) E. coli (MOI of 5) B) S. aureus (MOI 5) C) S. flexneri (MOI 5) D) were used to infect THP-1 cell lines. Cell-free supernatants were analyzed for IL-1β. Asterisks denote $p < 0.05$. D-E) THP-1 cells were stimulated with PAMPs and DAMPs. Untx = untreated; LPS = lipopolysaccharide; LTA = lipoteichoic acid; Lman = lipomannan; FSL = FSL-1; P3Cys = Pam3Cys; MSU = monosodium urate and Alum. Cell-free supernatants were harvested and assayed for IL-1β. E) and treated with nigericin (40μM) and α-hemolysin 1μg/ml(αHL). F) TNF-α secretion in response to pathogenic bacteria infection. G) Cell death measured by release of ATP by Toxilight assay during infection with high (50) and low (5) MOIs. Values represent mean ±standard deviation of duplicate samples. All experiments were done at least three individual times.

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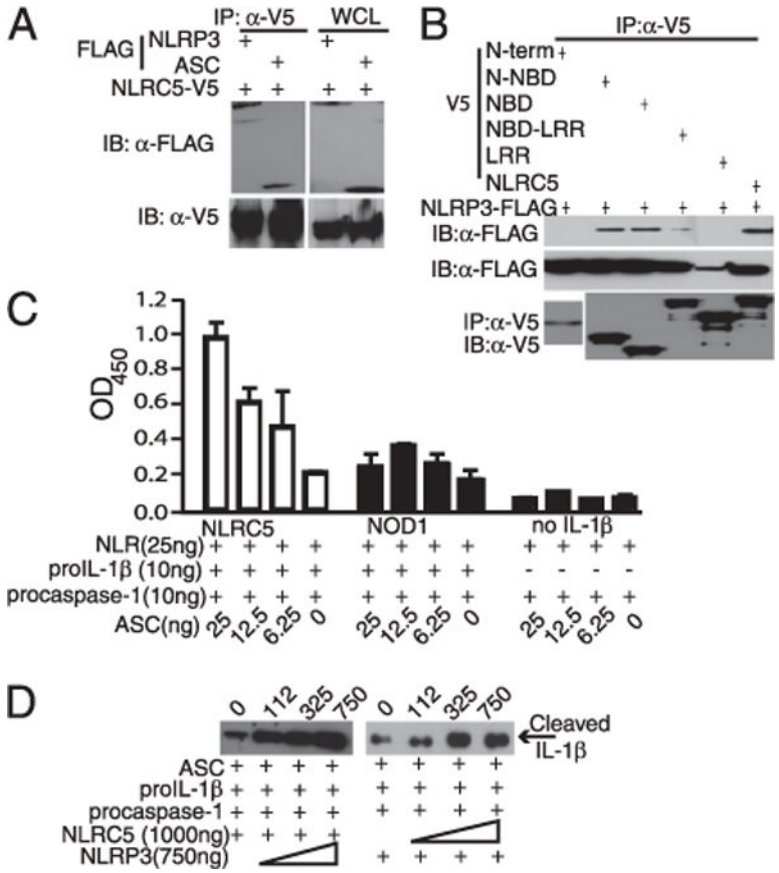

Figure 3. NLRC5 interacts with inflammasome components

A) FLAG™ tagged NLRP3 and ASC were co-transfected with NLRC5-V5. Lysates were immunoprecipitated with α-V5 and probed with α-FLAG™ mAbs. NLRC5 specifically interacts with NLRP3 and ASC. B). Truncation mutants of NLRC5 were co-transfected with NLRP3-FLAG. Lyastes were immunoprecipated with α-V5 mAb and probed with α-FLAG™. C). HEK293T cells were transfected with caspase-1 and IL-1β in the presence of increasing amounts of ASC in the presence or absence of NLRs. Supernatants were analyzed for IL-1β by ELISA. D) HEK293T cells were transfected with increasing concentrations of NLRP3 or NLRC5 in the presence of ASC, pro-caspase-1 and –IL-1β. Cell lysates were probed for cleaved IL-1β.