Cutting Edge: NLRC5-Dependent Activation of the Inflammasome

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The nucleotide-binding domain leucine-rich repeat-containing proteins, NLRs, are intracellular sensors of pathogen-associated molecular patterns and damage-associated molecular patterns. 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 predominately expressed in hematopoietic cells and has not been studied for inflammasome function. RNA interference-mediated knockdown of NLRC5 nearly eliminated caspase 1, IL-1β, and IL-18 processing in response to bacterial infection, pathogen-associated molecular patterns, and damage-associated molecular patterns. This was confirmed in primary human monocytes. NLRC5, together with procaspase 1, pro–IL-1β, and the inflammasome adaptor ASC, reconstituted inflammasome activity that showed cooperativity with NLRP3. The range of pathogens that activate NLRC5 inflammasome overlaps with those that activate NLRP3. Furthermore, NLRC5 biochemically associates with NLRP3 in a nucleotide-binding domain-dependent but leucine-rich repeat-inhibitory fashion. These results invoke a model in which NLRC5 interacts with NLRP3 to cooperatively activate the inflammasome. The Journal of Immunology, 2011, 186: 000–000.

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 IFN pathway in HeLa and THP-1 cells and is required for robust levels of IFN secretion (3, 5). However, Benko et al. (1) demonstrated that NLRC5 is a negative regulator of the IFN, NF-κB, and AP-1 pathways in 293 cells. Furthermore, in the mouse monocyte cell line RAW 264.7, Nlr5 functioned in an inhibitory manner. Cui et al. (2) provided mechanistic detail by demonstrating that Nlr5 interacts with inhibitor of κB kinase α 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 and Jurkat T cells (4). However, an opposite effect has been described in RAW 264.7 cells, as RNA interference-mediated knockdown of Nlr5 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, pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs). In the absence of NLRC5, monocyte cell types process pro–IL-1β and pro–IL-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 nucleotide-binding domain leucine-

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rich repeat (LRR)-containing protein (NLR) 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 NLRC5 primers (Applied Biosystems). Transcripts were calculated by \( \Delta \Delta C_\text{T} \) method or relative expression by GePdi, Actin, or 18s rRNA. Microarray data were 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 open reading frames.

Cell culture

The human monocytic cell line THP-1 was transfected with short hairpin RNA (shRNA) (Supplemental Table I) containing lentivirus. Knockdown efficiencies were determined by immunoblot analysis. A total of 2 \( \times \) 10\(^5\) cells was transfected with Amaxa with short interfering RNA (siRNA) oligonucleotides (at 2–200 pM) using protocols T008 (THP-1) or V001 (monocytes).

Bacteria

Shigella flexneri (strain 12022), Klebsiella pneumoniae (strain 43816 serotype 2), Porphyromonas gingivalis (strain A7436), and Listeria monocytogenes (strain 432521) were obtained from American Type Culture Collection. Echerichia coli (strain LF82) was provided by Dr. R. Sartor (University of North Carolina at Chapel Hill) and Staphylococcus aureus (strain RN6390) from J.A. Duncan.

Infections and stimulations

THP-1 cells were grown to log phase growth. Bacteria were grown to stationary phase. Cultures were diluted 1:250 and grown for an additional 2 h then quantitated by OD. Cocultures were pulsed with gentamicin at 50 \( \mu \)g/ml (Life Technologies) after 1 h. Infections were harvested after an additional 2 h. 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-Aldrich) stimulations, cells were primed with 5 ng/ml ultrapure LPS from E. coli for 2 h then pulsed with agonist for an additional 4–6 h. For nigericin (Invivogen), \( \alpha \)-hemolysin (from J.A. Duncan) stimulation cells were primed as before but harvested after 2 h.

Cytokine, immunoblot, and cell death analyses

IL-1\( \beta \), IL-18, and TNF-\( \alpha \) were measured by ELISA (BD Biosciences and Bender Life Sciences). Immunoblot was performed as described and probed with anti-IL-1\( \beta \)-Abs from Cell Signaling Technology (2022 and 2021) and anti-hemagglutinin (Roche), RIPA buffer then resolved on 4–12% Bis-Tris gels. Immunoblots were probed with M2 (Sigma-Aldrich), anti-hemagglutinin (Roche), and anti-V5 (Invitrogen) mAbs.

Immunoprecipitations

HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) or Fugene\(^6\) (Roche). Cells were lysed in RIPA buffer in the presence of protease inhibitors and Boncea (Novagen). Immunoprecipitations were washed with RIPA buffer then resolved on 4–12% Bis-Tris gels. Immunoblots were probed with HRP-conjugated M2 (Sigma-Aldrich), anti-hemagglutinin (Roche), and anti-V5 (Invitrogen) mAbs.

Results

Expression, cloning, and characterization of NLRC5

NLRC5 is expressed predominantly in the lymphocytic and macrophage/monocytic cell lineages with low expression in nonhematopoietic cells based on public gene profile database (Supplemental Fig. 1A, 1C). By real-time PCR analysis of human and mouse tissues (Supplemental Fig. 1B, 1D), NLRC5 is expressed preferentially in immune tissues relative to nonimmune tissues. The gene is encoded by 49 exons, and its open reading frame is 5601 nt (46 exons). NLRC5 has an N-terminal caspase recruitment domain-like domain and an extended C terminus consisting of an expanded LRR, making it the largest member of the NLRs (Supplemental Fig. 2A) with an approximate molecular mass of 200 kDa compared with the molecular masses of other NLRs ~100 kDa each. NLRC5 is well conserved in vertebrate evolution; orthologs are found in human, chimpanzee, cow, rat, and mouse (Supplemental Fig. 2B) (8). Thus, NLRC5 is a conserved NLR member with preferential expression in hematopoietic 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 cell line. shRNA for NLRC5 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 of NLRC5 transcript (Supplemental Fig. 3A) and protein (Supplemental Fig. 3B). The latter was determined by immunoblot using an mAb specific for the N terminus of NLRC5 (Supplemental Fig. 3C, 3D). E. coli-infected cells with shNLRC5 produced significantly reduced levels of secreted IL-1\( \beta \) compared with cells with control shRNAs in two different lentivirus vectors (Fig. 1A, 1B, Supplemental 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-1\( \beta \) than cells with either siNLRC5 or siNLRP3 upon E. coli infection.

![Figure 1](http://www.jimmunol.org/DownloadedFrom/20170428/)
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 (Supplemental Fig. 4A–C). Additionally pro–IL-1β levels are comparable (Supplemental Fig. 4D). These data suggest that NLRC5 has a role in IL-1β inflammasome formation in transformed and nontransformed human monocytic cells.

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 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 (Supplemental Fig. 5A–D) except at high concentrations of S. aureus, IL-1β/IL-18 secretion is independent of NLRC5 (Supplemental Fig. 5B, 5D). 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 PAMPs, DAMPs, and pore-forming toxins, all of which can activate the NLRP3 inflammasome, possibly via the release of endogenous secondary signals such as ATP (9) or reactive oxygen species (10). The PAMPs induce significantly less IL-1β in the absence of NLRC5 (Fig. 2D). Treatment with MSU or Alum 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, 11, 12). 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 multiplicities of infection (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, 12). 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 infected cells for TNF-α. TNF-α levels from shNLRC5-containing cells are not significantly different from control cell lines postinfection 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 because they result in the further release of inflammatory molecules (13, 14). Interestingly, pathogen-induced cell death that is 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.

**FIGURE 2.** NLRC5 is necessary for IL-1β production in response to bacterial pathogens and DAMPs. E. coli (MOI of 5) (A), S. aureus (MOI 5) (B), and S. flexneri (MOI 5) (C) were used to infect THP-1 cells. Supernatants were analyzed for IL-1β. D and E, THP-1 cells were stimulated with PAMPs, DAMPs, and toxins. MSU (100 μg/ml), Alum (100 μg/ml), nigericin (40 μM), and α-hemolysin 1 μg/ml (α-HL). Supernatants were harvested and assayed for IL-1β (D, E). 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. D–G, Values represent mean ± SD of duplicate samples of shNLRC5 (open bars) or shControl (closed bars). All experiments were done at least three times. *p < 0.05, FSL, FSL-1; Lman, lipomannan; LTA, lipoteichoic acid; P3Cys, Pam3Cys; UnTx, untreated.
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 coimmunoprecipitation experiments with NLRC5 and itself, NLRP3, AIM2, ASC, procaspase 1, and pro–IL-1β. Epitope-tagged NLRC5 interacts with itself, NLRP3, and ASC (Fig. 3A, Supplemental Fig. 6A). In contrast, it did not interact with the other inflammasome components AIM2, procaspase 1, pro–IL-1β, or additional cytosolic proteins such as TNFR-associated factor 2 and JNK (Supplemental Fig. 6B, 6C). To determine if the interactions of NLRC5 are specific to inflammasome-forming NLRs, we show that NLRC5 does not interact with another NLR, CIITA (Supplemental Fig. 6D).

To determine the domain of NLRC5 that is necessary and sufficient for NLRP3 binding, truncation mutants were used in coimmunoprecipitation experiments (Fig. 3B). The nucleotide-binding domain (NBD) appears to be necessary and sufficient to bind NLRP3, as it coimmunoprecipitates with NLRP3. The N-terminal caspase recruitment domain (N-term) or C-terminal LRR of NLRC5 failed to coimmunoprecipitate 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 the 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 (15). 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, pro–IL-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 nucleotide-binding oligomerization domain 1 (NOD1) is significantly less than the amount seen with NLRC5. When NLRC5 was cotransfected 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 (10), although the function of a majority of NLR proteins remains unclear. NLRC5 is primarily expressed in adaptive and innate immune systems. The conserved expression pattern of NLRC5 orthologs suggests a critical biological role in immunity. As its expression in monocyctic 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 (8).

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, whereas 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 (11); however, recent studies have challenged the notion that IL-1β response to *Salmonella* relies exclusively upon Nlr4 (16). Similarly, Miao et al. (17) has recently demonstrated Nlrc4-dependent inflammasome activation in response components of the type 3 se-
cretion system in a broad panel of Gram-negative bacteria. Likewise, both Nlrp3- and Nlrc4-dependent inflammasome activation in response to \textit{S. flexneri} infection are reported (7, 18). These differences might reflect differences in bacterial strains, host species, infected cell types, or duration and multiplicities of infection. Thus, the emerging 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 (19). A prime example is NOD2, which was initially described as an epithelial cell and/or macrophage sensor of muramyl dipeptide that induces NF-\(\kappa\)B activation (20, 21). Others have shown that NOD2 functions as an inflammasome component that facilitates NLRP1-dependent activation in monocytic cells (22). 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 human epithelial cell lines and primary fibroblasts (3, 5), as a negative regulator of mice deficient in or the biological systems used remains to be seen. The genera-

References

Supplemental Figure legends

Supplemental Figure 1. NLRC5 expression profiles in human and mice. A) Human data was mined from the Genome Institute of the Norvartis Research Foundation GeneAtlas U133A microarray. One of three representative probes is shown. B) Real-time PCR analysis of pooled human RNA samples (commercially available) was analyzed from NLRC5 expression. C) Mouse data was mined from the GeneAtlas MOE430 microarray. One probe was available for this array. D) Real-time PCR analysis from pooled C57/B16 mice.

Supplemental Figure 2. NLRC5 protein structure and alignment A) Graphic representation of NLRC5 compared to NLRP3 and NLRC4. B) Human (h), chimpanzee (p), cow (b), mouse (m), and rat (r) predicted amino acid sequences were aligned using Clustal X and manipulated using Boxshade. Identities between species are indicated by black shading and white lettering. Conserved residues are indicated by gray shading with black lettering. Non-conserved residues are not shaded.

Supplemental Figure 3. NLRC5 specific shRNA and monoclonal antibody A) Real time PCR analysis of shNLRC5 and control cell lines. B) Immunoblot (mAb raised against recombinant NLRC5) analysis of immunoprecipitated (rabbit polyclonal sera raised against recombinant NLRC5) NLRC5 in knocked-down cells (shNLRC5) and mutant shNLRC5 (shControl). C) mAb 2B1.20 is specific for NLRC5. 293T cells were transfected with either epitope tagged NLRC5 or NOD2 and probed with 2B1.20. D) 2B1.20 recognized the N-terminus of NLRC5. E) Recombinant lentivirus were generated using the pGipZ vectors from Open Biosystems. shControl is a non-silencing lentivirus generated from an irrelevant oligonucleotide sequence (Open Biosystems). NLRC5#1 and #2 represent independent sequences that are separate from FG12-based lentiviruses. Cells were infected with E. coli at an MOI of 25. Values are representative of at least three individual infections.

Supplemental Figure 4 shNLRC5 reduces NLRC5 but not NLRP3 and PYCARD (ASC) expression. A-C) real-time PCR analysis for inflammasome transcripts during bacterial infection. Values are normalized to Actb levels and normalized to untreated samples. A) NLRC5, B) NLRP3, C) PYCARD(ASC). D) Cell lysates were probed after 1 hour of infection with E. coli with an anti-IL-1β antibody to detect the pro form.

Supplemental Figure 5. NLRC5 regulates IL-1β and IL-18 production upon bacteria infection. A and B) Bacteria were serially diluted (1:2) at a starting MOI of 50 and used to infect cells containing lentivirus expressing shRNA. IL-1β levels were analyzed by ELISA. Bacteria are separated into panels based upon scalar value of IL-1β secretion. Cell-free supernatants were analyzed for IL-1β by ELISA. Asterisks denote p<0.05. C and D) Cells were infected at an MOI of 5 (C) or MOI of 50 (D) with bacteria and analyzed for IL-18 secretion. Values represent mean ±
standard deviation of duplicate samples. Experiments were repeated at least three times. Values were normalized to mshNLRC5 as 100% values. Asterisks denote *p*<0.05

**Supplemental Figure 6. Interaction of NLRC5 with itself and NLRP3 but no other proteins**

A) NLRC5 interacts with NLRP3 and NLRC5. HA-tagged NLRC5 was co-transfected into HEK293T cells with either V5-tagged NLRP3 or NLRC5. B) NLRC5 does not interact with AIM2, procaspase-1 or proIL-1β nor C) TRAF2 and JNK nor D) the NLR protein CIITA.
Supplemental Figure 3

A

percent relative expression

293T 293T NLRC5-V5 293 T NOD2-V5

primers#1 primers#2 primers#3

B

C5-V5 tag THP-1 shNLRC5 shControl

260 kDa

NLRC5

160 kDa

Actin

C

IB: α-NLRC5 2B1.20

IB: α-V5

D

N-term N-NBD NBD NBD-LRR LRR NLRC5

1 2 3 4 5 6

shNS shNLRC5#1 shNLRC5#2

E

IL-1β pg/ml average±SD

E. coli

pGipZ vector
Supplemental figure 4

A

B

C

D

ShNLRC5, shControl

E.coli (MOI)

Pro-IL-1β

GAPDH
Supplemental Figure 6

A

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shRNA and siRNA sequences used. Underlined positions denote substitutions relative to the original.