The Nucleotide-Binding Oligomerization Domain-Like Receptor NLRC5 Is Involved in IFN-Dependent Antiviral Immune Responses

Sven Kuenzel, Andreas Till, Michael Winkler, Robert Häsl, Simone Lipinski, Sascha Jung, Joachim Grötzinger, Helmut Fickenscher, Stefan Schreiber and Philip Rosenstiel

*J Immunol* 2010; 184:1990-2000; Prepublished online 8 January 2010; doi: 10.4049/jimmunol.0900557

http://www.jimmunol.org/content/184/4/1990

Supplementary Material  
http://www.jimmunol.org/content/suppl/2010/01/08/jimmunol.0900557.DC1

References  
This article cites 43 articles, 11 of which you can access for free at:  
http://www.jimmunol.org/content/184/4/1990.full#ref-list-1

Subscription  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

Permissions  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
The Nucleotide-Binding Oligomerization Domain-Like Receptor NLRC5 Is Involved in IFN-Dependent Antiviral Immune Responses

Sven Kuenzel,*1 Andreas Till,*1 Michael Winkler,† Robert Häsler,* Simone Lipinski,* Sascha Jung,‡ Joachim Grötzinger,‡ Helmut Fickenscher,† Stefan Schreiber,*8 and Philip Rosenstiel*

Nucleotide-binding oligomerization domain-like receptors (NLRs) are a group of intracellular proteins that mediate recognition of pathogen-associated molecular patterns or other cytosolic danger signals. Mutations in NLR genes have been linked to a variety of inflammatory diseases, underscoring their pivotal role in host defense and immunity. This report describes the genomic organization and regulation of the human NLR family member NLRC5 and aspects of cellular function of the encoded protein. We have analyzed the tissue-specific expression of NLRC5 and have characterized regulatory elements in the NLRC5 promoter region that are responsive to IFN-γ. We show that NLRC5 is upregulated in human fibroblasts postinfection with CMV and demonstrate the role of a JAK/STAT-mediated autocrine signaling loop involving IFN-γ. We demonstrate that overexpression and enforced oligomerization of NLRC5 protein results in activation of the IFN-responsive regulatory promoter elements IFN-γ activation sequence and IFN-specific response element and upregulation of antiviral target genes (e.g., IFN-α, OAS1, and PRKRIR). Finally, we demonstrate the effect of small interfering RNA-mediated knockdown of NLRC5 on a target gene level in the context of viral infection. We conclude that NLRC5 may represent a molecular switch of IFN-γ activation sequence/IFN-specific response element signaling pathways contributing to antiviral defense mechanisms.

Received for publication February 17, 2009. Accepted for publication December 5, 2009.

This work was supported by Deutsche Forschungsgemeinschaft Grant SF6 617-A20 (to P.R.) and Grant SF6 617-A24 (to H.F.) and the Clusters of Excellence “Inflammation at Interfaces” and “The Future Ocean” (to A.T., S.S., H.F., and P.R.). Work on the innate immune system. At least 22 members have been identified within the human genome (1, 2). The family members are characterized by three distinct functional domains: an N-terminal effector-binding domain, a centrally located NAIP, CIITA, HET-E, and TP1 domain (NACHT) and a carboxy-terminal leucine-rich repeat (LRR) domain. The effector-binding domain is usually composed of caspase-recruitment domain(s) (CARD), a Pyrin domain, or a baculovirus inhibitor of apoptosis protein domain. Current knowledge suggests that pathogen- associated molecular patterns (PAMPs) and other danger signals are directly or indirectly recognized by the LRRs. This recognition process induces NLR oligomerization and activation of NLR-dependent signal transduction pathways via induced-proximity signaling (3). In particular, activation of NF-κB, MAPKs, and proinflammatory caspases have been implicated as downstream events of NLR activation (4–6). Interestingly, several NLR family members (e.g., NOD1, NOD2, NLRP3/NALP3, NLRP1/NALP1, NLRA/MHC class II transcription activator) have been shown to contribute to susceptibility for chronic inflammatory diseases (7–15). Thus, it is assumed that NLR proteins represent pivotal components of the innate immune system and that impaired NLR function is closely linked to pathological conditions.

In this report, we describe the genomic organization, regulation, and cellular function of NLRC5 (also referred to as NOD27).

Materials and Methods

Cell lines and transfection

Human cervical carcinoma HeLaS3 cells (ACC161), human acute monocytic cell line THP-1 (ACC16), human colonic carcinoma cell lines CaCo2 (ACC169), and HT-29 (ACC299) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Human foreskin fibroblasts (HFFs) were isolated from human foreskin and cultivated up to passage number 25 in DMEM. HeLaS3 and THP-1 were cultured in RPMI 1640 and CaCo2 and HT-29 were cultured in DMEM. All media were from PAA Laboratories (Paschberg, Austria). Media were supplemented with 10% FCS and penicillin/streptomycin (each at 50 µg/ml); all cell lines were grown in 5% CO2 at 37°C. One day before transfection, cells were typically seeded at a density of 5 × 103 cells/ml on six-well plates or at 2 × 104 cells on a 96-well plate. Transfection of plasmids was performed using FuGene6 (Roche, Basel, Switzerland), small interfering RNA (siRNA) transfection was performed using Lipofectamine2000 (Invitrogen, Carlsbad, CA). For all procedures, standard protocols were used according to the manufacturers’ manuals.
FIGURE 1. Characterization of NLRC5 gene and the resulting protein NLRC5. A, The NLRC5 gene consists of 39 exons spanning a region of 96 kbp. The resulting protein is characterized by an N-terminal CARD, a central NACHT, and a region containing LRRs that are scattered along the C-terminal region. B, Amino acid sequence of NLRC5. The dashed line indicates the predicted CARD, the underlined region represents the NACHT domain, and the numbered boxes illustrate the position of LRR units. C, Tissue-specific mRNA expression profile of NLRC5. NLRC5 displays highest expression in brain, lung, prostate, and tonsil.
Bacterial and viral infection

Infection of different epithelial cell lines with *Listeria monocytogenes* serotype 1/2a strain EGD was performed as described before (16). HFF cells were infected with human CMV AD169-BAC as described (17, 18). To inactivate and remove extracellular viral particles, HFF cells were infected with human CMV AD169-BAC as described (17, 18). To inactivate and remove extracellular viral particles, HFF cells were additionally washed with citrate buffer (pH 3.0).

Plasmid construction

Expression plasmid for NLRC5 was generated by amplifying full-length cDNA coding for NLRC5 from pancreas cDNA and inserting cDNA coding for NLRC5 into destination vectors by Gateway cloning (Invitrogen). The promoter region of NLRC5 was cloned into pGL3 vector (Promega, Madison, WI) by using restriction sites EcoRI and XbaI. Primers were as follows: forward 5'-ATGGACCCCCTGGCTCCA-3' and reverse 5'-TACATGAAGTCCCACT-3'. The resulting deletion constructs (pGL3-DEL) were transfected with 40 ng wild-type promoter construct (pGL3-NLRC5) or the deletion constructs (pGL3-DelB/STAT, pGL3-DelSTAT) in combination with 10 ng reference plasmid pRL-TK (Promega). After 24 h, cells were harvested and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and a MicroLumatPlus Luminometer (Berthold Technologies, Bad Wildbad, Germany).

Luciferase assay

For quantification of promoter transactivation and pathway-specific reporter gene activity, transient transfection was used. HeLaS3 cells were seeded in a 96-well plate at a density of 1.5 x 10^4 per well. The next day, cells were transfected with 40 ng wild-type promoter construct (pGL3-NLRC5) or the deletion constructs (pGL3-DelB/STAT, pGL3-DelSTAT) in combination with 10 ng reference plasmid pRL-TK (Promega). After 24 h, cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and a MicroLumatPlus Luminometer (Berthold Technologies, Bad Wildbad, Germany).

Native gel electrophoresis

Native glycine polyacrylamide gels were used for analysis of NLRC5-FKBP oligomerization upon addition of exogenous dimerizer AP20187. Intracellular delivery of polycationic IFN-γ-antibody complex (Sigma-Aldrich) followed by boiling for 5 min and sonication. Lysed cells were cleared from debris by centrifugation, and supernatants were collected. A total of 15 μg protein extract was separated by denaturing SDS-PAGE and

**FIGURE 2.** A. Subcellular localization of NLRC5. GFP-NLRC5 was overexpressed in HeLaS3 cells. Nuclear DNA was counterstained using DAPI. Fluorescence microscopy reveals a spot-like cytosolic localization. B. Characterization of an Ab raised against NLRC5. Overexpression of GFP-NLRC5 fusion protein followed by immunoblotting revealed a specific signal of the expected size (230 kDa). The presence of two individual bands is estimated to be caused by posttranslational modification of unknown nature. As control, GFP alone and NLR family member NOD2 fused to GFP were overexpressed in parallel. No signals could be detected using anti-NLRC5 Ab, whereas anti-GFP Ab exhibited strong signals as expected. C. Using the evaluated Ab, cytosolic localization of endogenous NLRC5 protein could be confirmed. Irrelevant primary Ab was used as negative control.
transferred onto polyvinylidene difluoride membranes (Millipore). After blocking, membranes were probed with specific primary Abs, washed, and incubated with HRP-conjugated IgG as secondary Ab. Proteins were visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ). To determine even transfer and equal loading, membranes were stripped and reprobed with Ab specific for β-actin (Sigma-Aldrich).

**Fluorescence microscopy**

HeLa S3 cells were seeded on sterile coverslips at 2 × 10⁵ cells/well in six-well plates and grown overnight. The next day, cells were transiently transfected with overexpression plasmid encoding GFP-NLRC5. After 24 h, the cells were washed twice in ice-cold PBS, fixed for 20 min at room temperature with 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany), and stained with DAPI (Roche) for 15 min. Alternatively, untransfected cells were fixed and stained with anti-NLRC5 Ab (1:250 in PBS/BSA) (or irrelevant primary Ab as negative control) followed by staining with FITC-conjugated secondary anti-rabbit Ab. Slips were transferred onto glass slides and examined using an apotome-equipped Axio Imager Z1 microscope (Zeiss, Jena, Germany).

**RNA isolation and RT-PCR**

Total RNA was isolated from human cell lines using the RNeasy kit (Qiagen, Valencia, CA), and 250 ng total RNA was reverse-transcribed to cDNA using the Advantage RT-for-PCR kit (Clontech). Resulting cDNA was analyzed using standard semiquantitative RT-PCR procedures and the following primers: 5'-TGACTTCCTTCTCGCTGACAG-3' (NLRC5-for), 5'-CATGTGCCGCAAGTCCGCTGAG-3' (NLRC5-rev), 5'-ATTCAATCTGGCGTCTGAG-3' (IFN-α-for), 5'-CATCACAGGAGTCCGCTGAT-3' (IFN-α-rev), 5'-ATGCCAGAAGTGCGTTCGACAGG-3' (PKRIR-for), 5'-AGCCTCATGTCATCCAGAGATGACT-3' (PKRIR-rev), 5'-TGGTCTCCTGAGGCAAGGGC-3' (OAS1-for), 5'-TGGTACCAGTGCGTACTGACCGG-3' (OAS1-rev).

---

**FIGURE 3.** Regulation of NLRC5. A and B, HeLaS3 were transfected with NLRC5 promoter construct and stimulated for 24 h as indicated. Luciferase assay revealed a specific increase in NLRC5 promoter transactivation after stimulation with IFN-γ, whereas no other stimulus induced NLRC5 promoter activity. All measurements were performed in triplicate. ***p < 0.01. C, Using RT-PCR and quantitative real-time PCR, a significant upregulation of NLRC5 mRNA was shown within 8 h after stimulation with IFN-γ. ***p < 0.01. Note that similar results were obtained using THP-1, CaCo2, and HT-29 cells (see Supplemental Fig. 4). D, Dose dependency of IFN-γ-mediated upregulation of NLRC5 mRNA levels as demonstrated by RT-PCR and real-time PCR (measurements in duplicate). E, NLRC5 protein level is upregulated by stimulation with IFN-γ within 6–12 h after stimulation.
A biosystems/Ambion) was used. GUCUGCtg-3


Expression was determined by normalization to housekeeping gene

F: 5′-AGAAGCAUAGCCU-3′, R: 5′-GAAAGCAUAGCCU-3′ (GAPDH-rev). To analyze the tissue-specific expression pattern of NLRC5, we used commercially available human cDNA tissue panels (Clontech) and RT-PCR. Relative expression was determined by normalization to housekeeping gene GAPDH using densitometry (ImageJ software, http://rsb.info.nih.gov/ij/).

**Real-time PCR**

cDNA derived from stimulation and infection experiments was analyzed for NLRC5 and IL-8 mRNA levels using TaqMan Gene Expression Assays (NLRC5: Hs00260008_m1, IL-8: Hs00174103_m1; housekeeping gene ACTB: Hs99999903_m1; Applied Biosystems, Foster City, CA) on the ABI Prism 7900HT Fast Sequence Detection System (Applied Biosystems). All other target genes were quantified using the primer sets given above and the 

Prism 7900HT Fast Sequence Detection System (Applied Biosystems). All values were generated using the Mann-Whitney test. Transcripts were calculated based on the ratio between treated sample and untreated control; p values were generated using the Mann-Whitney U test. Transcripts were considered differentially expressed when the p value was < 0.05.

**siRNA-mediated knockdown of NLRC5**

Synthetic siRNAs targeting NLRC5 were purchased from Applied Biosystems/Ambion. Target sequences were as follows: siRNA1 (ID 141058), F: 5′-CCGGUGGGAAGAAUAGUGAtm-3′, R: 5′-UCACAUUUCUCACCCGCGt-3′; siRNA2 (ID 141060), F: 5′-CCACACUUCAAGAGCACAGt-3′; R: 5′-CUGUUCGAAUAGUUGGtic-3′; siRNA3 (ID 141059), F: 5′-CCAGAGCGGCUUAGUUCU-3′, R: 5′-GAAGCAGUAGGCU-GUCUCGt-3′. As control, unspecific siRNA Negative Control #2 (Applied Biosystems/Ambion) was used.

**Results**

**Genomic organization and expression of NLRC5**

In the human genome, NLRC5 is located at the locus 16q13, spanning a region of ∼96 kbp. The gene contains 39 exons, and the translation start site is located in the third exon. The gene structure is conserved among many vertebrates and seems to have occurred by a single gene duplication event early in mammalian evolution (Supplemental Fig. 1). The predicted protein consists of 1866 aas and shows the typical tripartite domain structure characterizing NLR family members (21). The N terminus comprises a predicted CARD domain spanning amino acids 1–91. The predicted NACHT domain is located between position 220 and 383. The predicted C-terminal LRR region consists of 713 aa residues and shows an unusual architecture compared with other NLRs, where the 20 LRR units are scattered irregularly along the domain (Fig. 1A, 1B).

To describe the tissue-specific expression pattern of NLRC5, we designed exon-spanning primer pairs and used human cDNA tissue panels for RT-PCR analysis. NLRC5 mRNA is expressed ubiquitously, with highest expression levels in the brain, lung, and prostate (Fig. 1C) and medium to high expression in a variety of tissues including the heart, digestive tract, and thymus. The lowest expression was detected in spleen, lymph nodes, and leukocytes. To assess the subcellular localization of the NLRC5 protein, we generated a GFP-NLRC5 fusion construct for transient transfection of HeLaS3 cells. Fluorescence microscopy revealed a spot-like expression of the GFP-NLRC5 fusion protein in the cytosol (Fig. 2A). Using Lyso-Tracker and MitoTracker staining (Invitrogen), we found no evidence for lysosomal or mitochondrial localization of NLRC5 (Supplemental Fig. 3 and data not shown). Next, we used peptide immunization to raise an Ab against the NLRC5 protein. Validation of the Ab using control plasmids (Fig. 2B) showed that neither GFP nor GFP-NOD2 were recognized by the NLRC5 Ab, but a specific signal was detectable that corresponds to the predicted 230-kDa size of GFP-NLRC5. Interestingly, all experiments revealed the existence of a double-band pattern that might be caused by translational modifications of yet unknown nature. Using this validated Ab, we could demonstrate that NLRC5 protein expression is restricted to the cytosolic compartment (Fig. 2C).

**NLRC5 expression is specifically regulated by IFN-γ**

For characterization of the transcriptional regulation of NLRC5, the promoter region was cloned into the pGL3B luciferase reporter gene vector. The resulting reporter gene construct (pGL3-NLRC5) was used. A 3′UTR sequence (Fig. 2) was fused to a firefly luciferase reporter gene and co-transfected into HeLaS3 cells with a CMV promoter-driven luciferase gene. Transfections were performed in triplicate.

FIGURE 4. Regulatory elements controlling NLRC5 promoter activity. A, The NLRC5 promoter region contains two predicted STAT consensus binding sites (one of which is overlapping with a predicted NF-κB consensus site). Individual and combined deletion of these two regulatory elements significantly impairs the IFN-γ response of the promoter construct. *p < 0.05. Note that the empty vector backbone activity (vector control) accounts for <10% of the NLRC5 promoter construct. B, Preincubation with pharmacological JAK inhibitor reduces the regulatory influence of IFN-γ on NLRC5 promoter activity. *p < 0.05. All experiments were performed in triplicate.
FIGURE 5. Role of viral infection for regulation of NLRC5. A, Infection of HFFs with human CMV, but not with heat-inactivated virus particles, upregulates NLRC5 mRNA levels within 24 h postinfection. This effect is abolished if cells are preincubated with JAK inhibitor (30 nM). B, Indicates a regulatory role of the JAK/STAT pathway. C, Supernatant (SN) of CMV-infected HFF is capable of inducing upregulation of NLRC5. **p < 0.01 (A–C). D, Preincubation of supernatant derived from CMV-infected HFF with antagonizing IFN-γ Ab impairs the upregulation of NLRC5. NLRC5 mRNA levels were quantified by TaqMan real-time PCR (Applied Biosystems; measurements in duplicate). *p < 0.05; **p < 0.01.
was transfected into HeLaS3. Baseline reporter gene expression by the empty vector backbone accounted for 10% of the NLRC5 promoter construct (data not shown). Cells were stimulated with 10 different stimuli, including cytokines and a panel of PAMPs. Alternatively, cells were infected with cytoinvasive Listeria monocytogenes to check for direct response to bacterial infection. As shown in Fig. 3A and 3B, only stimulation with IFN-γ resulted in a significant increase in luciferase activity, whereas none of the other stimuli, including IFN-α and IFN-β, affected NLRC5 promoter transactivation. These findings were supported by quantitative real-time PCR data showing that NLRC5 mRNA levels were significantly increased at 8 h after stimulation with IFN-γ (Fig. 3C). Similar findings were obtained in a variety of other cell lines (THP-1, CaCo2, and HT-29; Supplemental Fig. 4). Moreover, this effect was dose-dependent, as shown by RT-PCR and quantitative real-time PCR (Fig. 3D). As control, quantification of IL-8 mRNA levels was used to demonstrate responsiveness of the cells to the various stimuli (Supplemental Fig. 5A–C). Using the validated anti-NLRC5 Ab, we could demonstrate that NLRC5 protein expression exhibits the same response to IFN-γ stimulation because NLRC5 protein levels were remarkably increased 6 to 12 h after stimulation with IFN-γ (Fig. 3E).

Next, we aimed to understand the regulatory mechanisms underlying the IFN-γ–dependent effect on NLRC5 expression. Using bioinformatical prediction tools, the NLRC5 promoter region was analyzed for the presence of potential cis-regulatory elements. The computational analysis revealed the presence of two STAT consensus binding sites. The STAT family of transcription factors represents a central component of multiple signal transduction cascades, including the IFN and IL-6 pathways (22, 23). The NLRC5 promoter region contains two individual potential STAT binding sites located at positions −21336 and −2452, respectively. Interestingly, the STAT site at −21336 partially overlaps with a predicted NF-κB consensus binding site. To further analyze a possible involvement of these binding sites in NLRC5 promoter activation, we created deletion constructs by site-directed mutagenesis. Individual or combined deletion of the predicted regulatory elements resulted in significant decrease of IFN-γ–dependent promoter transactivation (Fig. 4A).

**FIGURE 6.** Signal transduction events downstream of NLRC5. Pathway-specific cis-reporter gene constructs (A–D) were cotransfected in combination with either GFP-tagged NLRC5, CARD-FKBP fusion construct, or the appropriate control vectors. In addition, FKBP-expressing cells were stimulated with exogenous oligomerizer AP20187 to enforce oligomerization of the (FKBP)2 domain. Although NLRC5 does not influence AP-1 or NF-κB activity (A and B), our data demonstrate a significant enhancement of ISRE- and GAS-dependent reporter gene activity by overexpression and by enforced oligomerization of the CARD domain of NLRC5 (C and D). Values represent mean of relative luciferase activity. *p < 0.05; **p < 0.01. All experiments were performed in triplicate.
Moreover, pretreatment with a specific inhibitor of JAK activity prior to IFN-γ stimulation decreased promoter transactivation almost completely (Fig. 4B). Because JAK activity is essential for phosphorylation and nuclear translocation of STATs (24), these data further emphasize the role of IFN-γ–dependent JAK/STAT signaling for NLR5 transcriptional regulation. Viral infection contributes to transcriptional regulation of NLR5 via autocrine IFN-γ

The secretion of the cytokine IFN-γ is a pivotal cellular response to viral infection (25). In order to elucidate whether the expression of NLR5 is directly linked to viral infection, we used a well-established viral infection model that is based on the infection of HFFs with human CMV (17, 18). To differentiate between effects that originate from direct viral infection and effects depending on sole presence of inactive virus particles, heat-inactivated virus particles were used in a parallel experimental setup. Six, 12, or 24 h postinfection with CMV, expression levels of NLR5 mRNA were checked by quantitative real-time PCR. Efficiency of the viral infection was checked by concomitant detection of CMV-IE2. As shown in Fig. 5A, a strong increase in NLR5 expression was detectable that was peaking at 12 h postinfection. Remarkably, only intact virus particles were capable of inducing this effect, whereas heat-inactivated CMV did not alter NLR5 mRNA levels within 24 h (Fig. 5A). Thus, the ability to efficiently infect human cells appears to be a prerequisite for regulation of NLR5 expression.

To investigate if autocrine JAK/STAT signaling in response to infection is involved in these regulatory events, we infected cells in presence of JAK inhibitor to block JAK/STAT signaling. Blockade of JAK-dependent pathways resulted in almost complete abrogation of NLR5 upregulation (Fig. 5B). These results were confirmed by the fact that the supernatant of infected cells was able to upregulate NLR5 mRNA in cells that were not infected (Fig. 5C). To analyze whether autocrine IFN-γ secretion initiated by viral infection is responsible for regulation of NLR5, we blocked endogenous IFN-γ by addition of an inhibitory Ab. As shown in Fig. 5D, supernatant of infected cells preincubated with anti–IFN-γ Ab resulted in impaired induction of NLR5.

**FIGURE 7.** NLR5-dependent regulation of antiviral target genes. A, Overexpression of GFP-NLR5 in HeLaS3 cells results in increased expression of IFN-α, PRKIR, and OAS1, whereas expression of chemokine IL-8 is unaffected. *p < 0.05. B, siRNA-mediated knockdown of NLR5 results in reduction of endogenous mRNA levels of target genes, as demonstrated by RT-PCR and quantitative RT-PCR. *p < 0.05; **p < 0.01.
NLRC5 oligomerization activates IFN-specific response element- and IFN-γ activation sequence-dependent signal transduction pathways

Next we asked which signaling pathways are activated downstream of NLRC5 activation. Because it is a common phenomenon of NLRs that ectopic overexpression leads to ligand-independent autoactivation of downstream signaling events (3), we used overexpression of GFP-NLRC5 to screen for potential signaling cascades initiated by NLRC5 activity. In addition, we overexpressed a fusion protein consisting of the N-terminal CARD of NLRC5 fused (FKBP), whose oligomerization could be exogenously triggered by addition of the synthetic oligomerizer AP20187 (for illustration, see Supplemental Fig. 6). As shown by native glycine gel analyses, addition of the exogenous oligomerizer resulted in enforced oligomerization of CARD-FKBP monomers (Supplemental Fig. 7). Next, both GFP-NLRC5 and CARD-FKBP constructs were expressed in parallel with a number of pathway-specific reporter gene constructs that could monitor the activation of specific regulatory promoter elements (Fig. 6). No activation of AP-1 or NF-κB signaling pathways was detectable (Fig. 6A, 6B). This was surprising because several members of the NLR family have been shown to directly activate NF-κB and/or MAPK signaling cascades (26–28). In contrast, our data point to a specific role of NLRC5 for the activation of signaling pathways that uses the consensus binding sites IFN-specific response element (ISRE) and IFN-γ activation sequence (GAS) (Fig. 6C, 6D). These regulatory elements represent the major binding sites for STAT family members. Supporting these data, we demonstrated increased and prolonged phosphorylation of STAT1 downstream of IFN-γ in the presence of overexpressed NLRC5 (Supplemental Figs. 8, 9).

In order to analyze whether this activation profile was also detectable on the target gene level, we overexpressed GFP-NLRC5 in epithelial cells and used RT-PCR and real-time PCR to characterize potential NLRC5-dependent downstream target genes. As shown in Fig. 7A, the overexpression resulted in remarkable increase in mRNA levels of IFN-α, PRKRIR, and OAS1 24 h after transfection, whereas expression of IL-8 was not affected. Next, we checked the influence of siRNA-mediated knockdown of NLRC5 on the target gene level. As demonstrated in Fig. 7B, efficient knockdown of NLRC5 by three individual siRNAs results in reduction of endogenous mRNA levels of the investigated target genes as compared with control. To analyze whether this effect is also relevant in the context of viral infection, we next transfected HFF cells with siRNA and quantified expression of IFN-α in response to CMV. siRNA-mediated knockdown of NLRC5 significantly reduces induction of IFN-α after CMV infection (Fig. 8A), further emphasizing the role of NLRC5 as an essential component of antiviral signaling pathways. Supporting this view, NLRC5 knockdown also significantly reduces induction of GAS-driven luciferase activity in response to poly I:C, as shown in Fig. 8B.

Taken together, our data argue for the NLR family member NLRC5 as being both target and activator of signaling pathways contributing to antiviral defense mechanisms.

Discussion

In this report, we describe the genomic organization, regulation, and aspects of cellular function of the NLR family member NLRC5. This gene family has attracted much attention because mutations in several NLR genes have been linked to a variety of inflammatory diseases (29). For example, different genetic variants...
of NOD2 are associated with susceptibility for Crohn’s disease or Blau syndrome (7–10, 30), whereas NLRP3/NALP3 has been linked to rare hereditary fever syndromes and Crohn’s disease (11–14, 31, 32). These genetic findings underscore the importance of NLRs as surveillance proteins for host defense and immunity and point to their potential as novel therapeutic targets (33). Among the NLR family members identified and characterized so far, NLRC5 displays an unusual architecture of its carboxy-terminal LRR because its repeat units are irregularly scattered along the domain. The LRR domain of NLR is generally considered to represent the sensor region of the proteins. The molecular structures that are recognized by NLR proteins represent either conserved molecular signatures of bacteria (PAMPs) or endogenous molecules that arise from conditions associated with cellular danger (danger-associated molecular patterns) (34). Interestingly, some NLRs are described to constitute highly selective sensor platforms that only respond to a specific molecular elicitor, whereas others exhibit a promiscuous selectivity by responding to a variety of stimuli. For example, NLRC4/IPAF responds to cytosolic presence of the bacterial protein flagellin, whereas NLRP3/NALP3 is activated by various endogenous danger-associated molecular patterns like monosodium urate crystals or low potassium ion concentration (35). Nevertheless, it is still unclear whether this response is caused by direct physical interactions of the respective NLR with its elicitor or indirectly by mediators of unknown nature. The identification of physiological elicitors of NLR signaling and the dissection of the exact interaction mode of putative ligand and cognate receptor still remain important tasks for future studies. Because both the identity of a potential physiological elicitor of NLRC5 and the downstream signaling events were unknown, we used enforced oligomerization of an FKBP fusion protein and overexpression to simulate cellular events that trigger oligomerization of NLRC5. Our data demonstrate that NLRC5 oligomerization can efficiently trigger GAS- and ISRE-dependent promoter activity. In subsequent experiments, a panel of known PAMPs was used to test stimulus specificity of NLRC5-dependent GAS/ISRE activation. However, from the tested compounds (e.g., LPS, flagellin, single-stranded RNA, muramyl dipeptide), the biochemical identity of a potential endogenous NLRC5 elicitor could not be elucidated (data not shown).

Our data demonstrate a direct link between signaling events triggered by viral infection and transcriptional regulation of NLRC5 and argue for autocrine IFN-γ activating JAK/STAT signaling as a key player of this autoregulatory loop. Moreover, we show that activation of NLRC5 implies specific activation of ISRE and GAS elements and upregulation of IFN-α, PRKRIR, and OAS1 without affecting transcription of chemokine IL-8. These findings are underscored by our data demonstrating that knockdown of NLRC5 significantly impairs induction of these target genes in the context of CMV infection. IFN-α transcriptional regulation has been shown to be dependent on JAK/STAT signaling via the IFN-stimulated gene factor 3 transcription factor complex (36). PRKRIR encodes for a protein that was first identified as a regulator of P58(IPK), a cellular inhibitor of the RNA-dependent protein kinase (37). In addition, OAS proteins represent a group of IFN-inducible enzymes contributing to activation of RNaseL that degrade viral RNAs and thus inhibit viral replication. Conversely, IL-8 transcriptional regulation, which was used as a control, is mainly dependent on NF-κB and AP-1 transcription activity, thus supporting our data on a specific induction of GAS/ISRE by NLRC5 activation. Given the regulatory role of IFN-γ on NLRC5 gene regulation, these data argue for a specific role of NLRC5 as an endogenous amplifier of antiviral signaling pathways.

By now, only few members of the NLR family have been associated with antiviral immune responses, whereas the majority of NLRs have been shown to contribute to innate defense mechanisms against pathogenic bacteria (38–40). The recently characterized NLR family member NLRX1 interacts with mitochondrial antiviral signaling adaptor in the outer membrane of mitochondria and represents a checkpoint modulating mitochondrial antiviral responses (41). Furthermore, NLRX1 is capable of amplifying NF-κB and JNK pathways activated by different proinflammatory stimuli via production of reactive oxygen species (42). A recent study reports on the specific detection of influenza virus by components of the ASC/NLRP3 inflammasome, but the direct requirement for NLRP3 in the sensing process is still under debate (43). Our data add NLRC5 to the list of NLR family members involved in mechanisms that respond to viral infection and emphasize the role of NLRs as general cytosolic surveillance proteins involved in both bacterial and viral infection and endogenous danger response.

In summary, we have characterized the genomic organization of NLRC5 and describe complex regulatory mechanisms identifying NLRC5 as target gene of IFN-γ and mediator of IFN-mediated antiviral signaling pathways. Further analyses on NLRC5 regulation and function will help us to fully understand the impact of NLR family members on molecular defense strategies against viral pathogens.

Acknowledgments

We thank Tanja Kaacksteen, Melanie Schlaphkohl, Dorina Oelsner, Yasmin Brodtmann, and Alina Gräff for their technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


Supplementary information

'The NOD-like receptor NLRC5 is involved in Interferon-dependent antiviral immune responses'

(initially entitled 'The NOD-like receptor NLRC5 is regulated by viral infection through IFN-γ dependent signaling')

Supplemental Figure Legends.

S1. The gene tree was created using the TreeBeST method (http://treesoft.sourceforge.net/treebest.shtml) and annotates the evolutionary origin of NLRC5 within the NLR family. The internal nodes are colour-coded to discriminate duplication or speciation events. Red-squared nodes represent gene duplications events, blue-squared nodes represent speciation events. The data suggest that the gene emerged from a duplication ancestor node early in mammalian evolution.

S2. Schematic representation of the NLRC5-specific constructs used in this study.

S3. Subcellular localisation of NLRC5. GFP-NLRC5 fusion protein was overexpressed in HeLaS3 cells, and cells were fixed using 4% PFA followed by staining with LysoTracker. GFP-NLRC5 is localized in the cytosol exhibiting a spot-like expression pattern and does not co-localize with lysosomes or mitochondria (not shown). DAPI was used to stain nucleic DNA.

S4. Different human cell lines were assayed for NLRC5 mRNA expression levels using TaqMan real-time PCR after stimulation with different cytokines, pathogen-associated molecular patterns (PAMPs) or living bacteria (Listeria monocytogenes): EGF (100ng/ml), TNF-α (10ng/ml), LPS (10µg/ml), Flagellin (500ng/ml), IFN-γ (1000u/ml) or infected with Listeria monocytogenes (multiplicity of infection = 100 bacteria/cell).
Expression levels of NLRC5 were normalized to expression of housekeeping gene β-Actin. Only stimulation with IFN-γ resulted in significant upregulation of NLRC5 mRNA expression. Values represent means of three independent experiments.

S5 (A,B,C). The same cDNAs were checked for expression of IL-8 using TaqMan Gene Expression Assay Hs00174103_m1 to check for responsiveness of the cells towards various stimuli. As expected, stimulation with Flagellin, Listeria, LPS and TNF-α (but not IFN-γ or EGF) resulted in upregulation of IL8 in various cell lines.

S6. Schematic illustration describing the mode of action of (FKBP)2 fusion constructs. Addition of exogenous oligomerizer AP20187 leads to an oligomerization of the CARD of NLRC5 mediated by the two serially fused FKBP portions of the fusion protein. In analogy to other NLRs this event is estimated to result in activation of NLRC5-specific signal transduction pathways.

S7. Enforced oligomerization of NLRC5. Incubation of CARD-NLRC5 – expressing cells with oligomerizer AP20187 (24h) leads to oligomerization of the CARD-FKBP fusion protein as shown by native gel analysis.

S8, S9. Phosphorylation of STAT1. We transfected HeLaS3 cells as indicated, stimulated with IFN-γ (1000u/ml) for 10 min in the presence or absence of oligomerizer AP20187 (S8) or as indicated (S9) and investigated the phosphorylation status of STAT1 by phospho-specific immunoblotting. Presence of oligomerized NLRC5-CARD or FLAG-NLRC5 results in increased and prolonged STAT1 phosphorylation.
Kuenzel Suppl. Figure
NLRC5 induction by different stimuli (fold change)

- EGF 1h
- EGF 2h
- EGF 8h
- Flagellin 1h
- Flagellin 2h
- Flagellin 8h
- IFN-γ 1h
- IFN-γ 2h
- IFN-γ 8h
- L. monocytogenes 1h
- L. monocytogenes 2h
- L. monocytogenes 8h
- LPS 1h
- LPS 2h
- LPS 8h
- TNF-α 1h
- TNF-α 2h
- TNF-α 8h

- THP-1
- CaCo2
- HeLaS3
- HT-29

** Indicates significant induction.
kDa

~100

~50

WB: HA
**Relative Phosphorylation**

- **IB: pSTAT1**
- **IB: STAT1**
- **IB: β-Actin**

**Graph**
- **FKBP**
- **CARD-FKBP**

**Relative STAT1 phosphorylation (%)**
- **AP20187 (h): 0/2**
- **IFN-γ (10 min): -/- + +**

*Note: Data represents relative STAT1 phosphorylation levels under different conditions.*
Kuenzel Suppl. Figure