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NLRC5 Deficiency Does Not Influence Cytokine Induction by Virus and Bacteria Infections

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Nucleotide-binding domain and leucine rich repeat containing gene family receptors (NLRs) are cytosolic proteins that respond to a variety of pathogen and host components to induce inflammatory cytokines. NLRC5 is a recently identified member of the NLR family that has been implicated in positive and negative regulation of antiviral innate immune responses. To clarify whether NLRC5 controls antiviral innate immunity in vivo, we generated NLRC5-deficient mice. Macrophages and dendritic cells derived from NLRC5-deficient mice induced relatively normal levels of IFN-β, IL-6, and TNF-α after treatment with RNA viruses, DNA viruses, and bacteria. The serum cytokine levels after polyinosinic-polycytidylic acid infection were also comparable between control and NLRC5-deficient mice. NLRC5 overexpression promoted IL-1β production via caspase-1, suggesting that NLRC5 constitutes an inflammasome. However, there was no reduction of IL-1β in NLRC5-deficient cells in response to known inflammasome activators, suggesting that NLRC5 controls IL-1β production through an unidentified pathway. These findings indicate that NLRC5 is dispensable for cytokine induction in virus and bacterial infections under physiologic conditions. ***The Journal of Immunology*****, 2011, 186: 994–1000.**

The innate immune system senses various molecular motifs within pathogens known as pathogen-associated molecular patterns (PAMPs) through germline-encoded pattern-recognition receptors (PRRs). TLRs are membrane-bound PRRs that recognize various PAMPs on the cell surface or within endolysosomes and induce the expressions of inflammatory cytokines and type I IFN (1–4). RIG-I–like receptors (RLRs), RIG-I and MDA5, which are composed of an RNA helicase domain and caspase recruitment domains (CARDs), are cytoplasmic PRRs that sense RNA viruses to induce antiviral innate immune responses (1, 4–6). Cells express additional cytoplasmic PRRs that recognize dsDNA derived from viruses, bacteria, and damaged host cells. Absent in melanoma 2 (AIM2) was identified as a dsDNA sensor that triggers caspase-1–dependent IL-1β production (7–13). This pathway is required for host defense against *Francisella tularensis* infection (12, 13). However, a cytosolic DNA sensor responsible for the induction of type I IFN production has not yet been discovered. The signaling pathways through RLRs, DNA sensors, and several TLRs culminate in the activation of the transcription factors NF-κB and IFN regulatory factor (IRF) 3. NF-κB is sequestered in the cytoplasm by interactions with IκB proteins in the unstimulated condition. PAMP stimulation triggers IκB kinase (IKK)α- and IKKβ-mediated phosphorylation of IκBs and subsequently induces their degradation, thereby allowing NF-κB to translocate into the nucleus and regulate inflammatory cytokine expressions (1–4). IRF3 also resides in the cytoplasm in the unstimulated condition. IRF3 is phosphorylated by an IKK-related kinase, TBK1, and translocates into the nucleus to regulate the expression of IFN-β (1–4).

The nucleotide-binding domain and leucine-rich repeat containing gene receptor (NLR) family contains more than 20 members (14, 15). The NLR members mainly consist of three distinct domains: an N-terminal domain that varies among members and consists of signaling modules such as pyrin, CARD, baculovirus inhibitor repeat, or acidic domains; a central nucleotide-binding and oligomerization domain; and C-terminal leucine-rich repeats (LRRs). NLRC1 and NLRC2 recognize bacterial components and activate NF-κB and MAPKs to induce inflammatory cytokines (14). In contrast, NLRP3, NLRC4, and NAIP5 constitute inflammasomes, together with caspase-1, that induce the maturation of IL-1 family cytokines and cell death after stimulation, with a wide array of inflammasome activators (16–21).

NLRC5 is a member of the NLR family and was recently reported to regulate antiviral innate immune responses. It was shown that NLRC5 binds to IKKα and IKKβ and inhibits their activation, thereby serving as a negative regulator of NF-κB activation. Moreover, the CARD of NLRC5 directly interacts with those of RLRs and suppresses NF-κB and IRF3 activation (22). On the contrary, another study showed that NLRC5 overexpression induces IFN-β promoter activation and enhances RLR-mediated antiviral

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Abbreviations used in this article: AIM2, absent in melanoma 2; BMDM, bone marrow-derived macrophage; CARD, caspase recruitment domain; DC, dendritic cell; GMDC, GM-CSF-induced bone marrow dendritic cell; IKK, IκB kinase; IRF, IFN regulatory factor; LRR, leucine-rich repeat; moi, multiplicity of infection; MSU, monosodium urate; NDV, Newcastle disease virus; NLR, nucleotide-binding domain and leucine-rich repeat containing gene family receptor; PAMP, pathogen-associated molecular pattern; PEC, peritoneal exudate cell; poly (dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; poly-IC, polyinosinic-polycytidylic acid; PRR, pattern-recognition receptor; RLR, RIG-I–like receptor; WT, wild type.

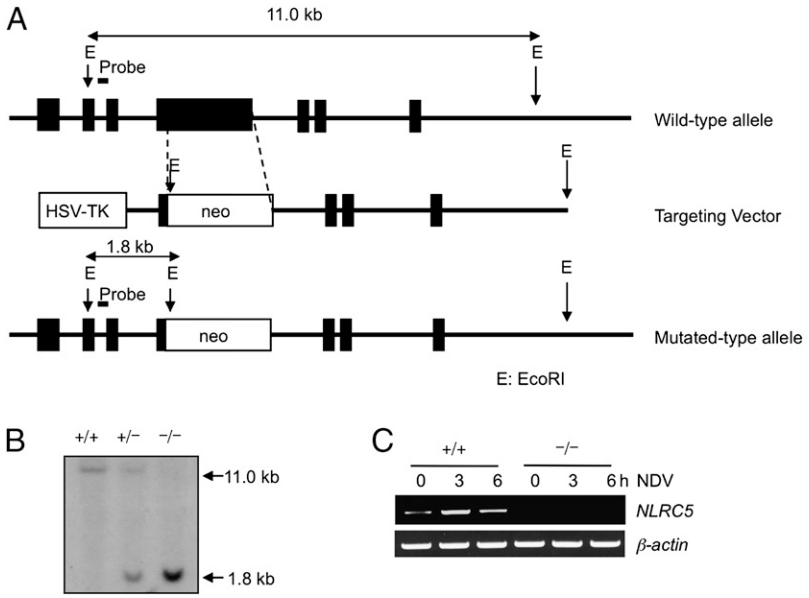

FIGURE 1. Generation of NLRC5-deficient mice. *A*, Schematic diagrams of the genomic structure of the murine *NLRC5* gene. The targeting vector and the predicted mutated allele are shown. Black boxes denote exons. E, EcoRI sites in the genome. *B*, Southern blot analysis of progenies from heterozygote intercrosses. Genomic DNA was extracted, digested with EcoRI, electrophoresed, and hybridized with the probe indicated in *A*. The analysis produced a single 11.0-kb band for WT mice (+/+), a 1.8-kb band for homozygous mutant mice (−/−), and both bands for heterozygous mice (+/−). *C*, GMDCs were prepared from WT (+/+) and NLRC5-deficient (−/−) mice and infected with NDV (moi = 1.0) for the indicated times. Isolated total RNA samples were subjected to RT-PCR analyses for the expressions of NLRC5 and β-actin mRNAs.

responses (23, 24). However, the in vivo function of NLRC5 in antiviral immune responses remains unclear. In this study, we have generated NLRC5-deficient mice and analyzed their phenotypes in terms of antiviral innate immune responses.

Materials and Methods

Generation of NLRC5-deficient mice

The *NLRC5* gene was isolated from genomic DNA extracted from embryonic stem cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 1.5-kb fragment encoding the NLRC5 open reading frame (exon 4) with a neomycin-resistance gene cassette, and an HSV-thymidine kinase gene driven by the PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was electroporated into embryonic stem cells, G418 and ganciclovir doubly-resistant colonies were selected and screened by PCR and further confirmed by Southern blot analysis. Homologous recombinant embryonic stem cells were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain NLRC5$^{-/-}$ and NLRC5$^{+/+}$ mice. Mice were bred and maintained in a specific-pathogen-free facility at the Research Institute for Microbial Diseases in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. All mouse protocols were approved by the Osaka University Animal Care and Use Committee. All mice were killed at 5–9 wk of age.

Plasmids

The cDNA fragments of human NLRC5 and NLRC5 ΔLRR (aa 1–655) obtained by RT-PCR (Takara) were cloned into the pFlag-CMV-2 vector (Sigma-Aldrich). The sequences of all the constructs were confirmed using an ABI PRISM Genetic Analyzer (Applied Biosystems). Expression

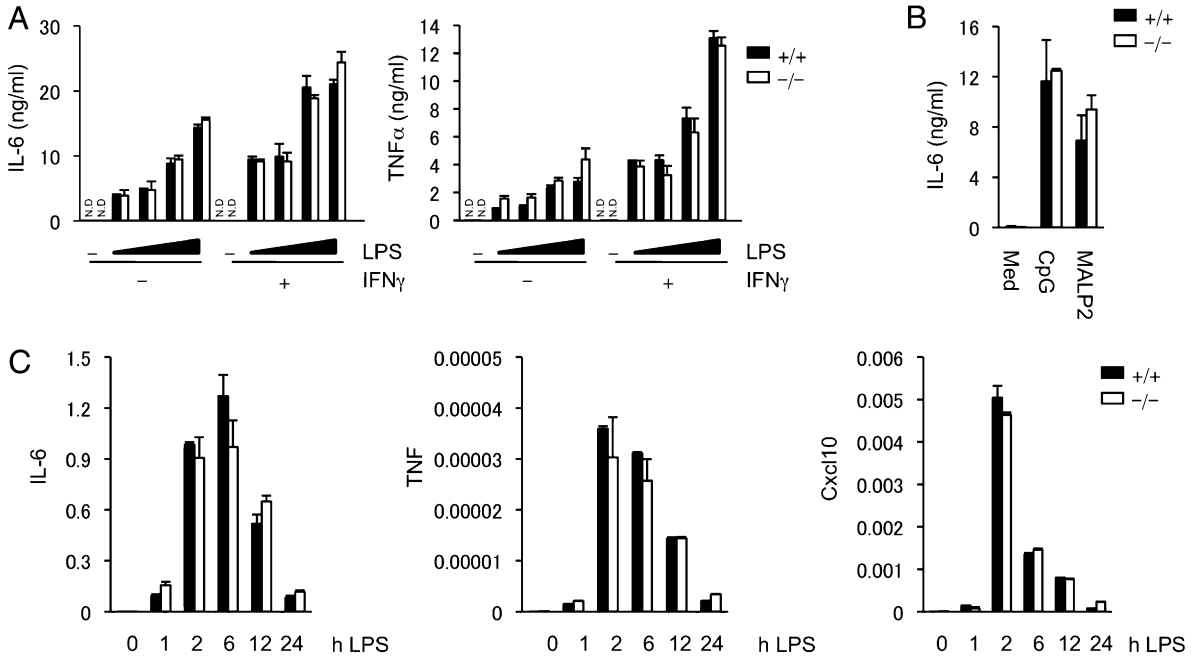

FIGURE 2. Responses to TLR ligands in NLRC5-deficient mice. *A*, GMDCs prepared from WT (+/+) and NLRC5-deficient (−/−) mice were pretreated with or without IFN-γ (10 ng/ml) and stimulated with medium (*lanes 1*, *6*), 1 ng/ml (*lanes 2*, *7*), 10 ng/ml (*lanes 3*, *8*), 100 ng/ml (*lanes 4*, *9*) or 500 ng/ml (*lanes 5*, *10*) LPS for 24 h. The culture supernatants were analyzed for their levels of IL-6 and TNF-α by ELISA. *B*, GMDCs were stimulated with CpG DNA 1 μM and MALP2 100 ng/ml for 24 h. The culture supernatants were analyzed for their levels of IL-6 by ELISA. *C*, GMDCs were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA were subjected to quantitative PCR analyses for expression of IL-6, TNF, and Cxcl10. ND, not detected.

plasmids for pro–IL-1β and procaspase-1 were provided by Dr. Jürg Tschopp (University of Lausanne, Switzerland).

Cells, viruses, bacteria, and reagents

HEK293 cells were cultured in DMEM supplemented with 10% FCS. Peritoneal exudate cells (PECs) were collected 3 d after i.p. injection of 4% thioglycollate medium. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μM 2-ME, and 10 ng/ml murine GM-CSF (PeproTech) or 40 ng/ml murine macrophage CSF (PeproTech) for 6–8 d or 4 d to allow differentiation to GM-CSF-induced bone marrow dendritic cells (GMDCs) or bone marrow-derived macrophages (BMDMs), respectively. Newcastle disease virus (NDV) and HSV-1 (F-strain) were described previously. *Salmonella typhimurium* (SL1334) (25) or *Listeria monocytogenes* (EGD) were grown in Bacto Brain Heart Infusion medium (BD Biosciences) while *Francisella turalensis* spp novicida U112 was grown in Bacto Tryptic Soy Broth medium (BD Biosciences) with shaking at 37˚C. After determination of their number by the absorbance at 600 nm, the bacteria were washed twice and infected at each multiplicity of infection (moi) indicated in the figure legends. LPS from *Salmonella minnesota* Re-595, ATP, and calf thymus DNA were purchased from Sigma-Aldrich. CpG ODN 1668, polyinosinic-polycytidylic acid (poly-IC), and poly(deoxyadenylic-eoxythymidylic) acid [poly (dA:dT)] were purchased from Invivogen. MALP2 was purchased from Alexis Biochemicals. For stimulation, poly-IC or poly (dA:dT) was mixed with the FuGENE 6 transfection reagent (Roche) at a ratio of 1:1 (volume/weight) in OptiMEM and incubated for 15 min prior to use for stimulation.

Transfection, immunoprecipitation, and immunoblotting

HEK293 cells (1×10^6) seeded on 100-mm dishes were transiently transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For immunoprecipitation, cells were harvested at 36 h after transfection and lysed in lysis buffer comprising 1.0% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), and 150 mM NaCl. Precleared cell lysates were incubated with an anti-FLAG M2 mAb (Sigma-Aldrich) for 12 h by rotation. The beads were washed four times with lysis buffer. The immunoprecipitants were eluted by boiling with Laemmli sample buffer, separated in a 4–20% polyacrylamide gradient gel, and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with an HRP-conjugated anti-FLAG mAb (Sigma-Aldrich) for 1 h. After washing with TBS-T (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20), the peroxidase activity was detected with an ECL system (PerkinElmer Life Sciences). Immunoblotting for caspase-1 was described previously (26).

ELISA

HEK293 cells (1×10^5) were seeded on 24-well plates. After 12 h, the cells were transiently transfected with a total of 600 ng of various plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 36 h after transfection, the cell culture supernatants were analyzed for IL-1β by ELISA. For mouse cell experiments, the culture supernatants of cells ($1–5 \times 10^5$ cells per well) seeded on 96-well plates were collected and analyzed for their cytokine levels by ELISA. The ELISA kit for IFN-β was purchased from PBL Biomedical Laboratories. Mice were injected i.p. with poly-IC (200 μg). Sera were prepared before (0 h) and after (4, 8, and 24 h) poly-IC injection and analyzed for their IFN-β, IL-6, and RANTES levels by ELISA. Mouse IL-1β, IL-6, RANTES, and TNF-α and human IL-1β ELISA kits were purchased from R&D Systems. All ELISAs were performed according to the manufacturers' instructions.

RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed with RTace (Toyobo) according to the manufacturer's instructions. PCR was then performed with the following primers: *NLRC5*, 5′-ATGGACGCTGAGAGCATC-3′ and 5′-GGAGGTTCCCATGGCAGA-3′; *Ifnβ*, 5′-CCA-

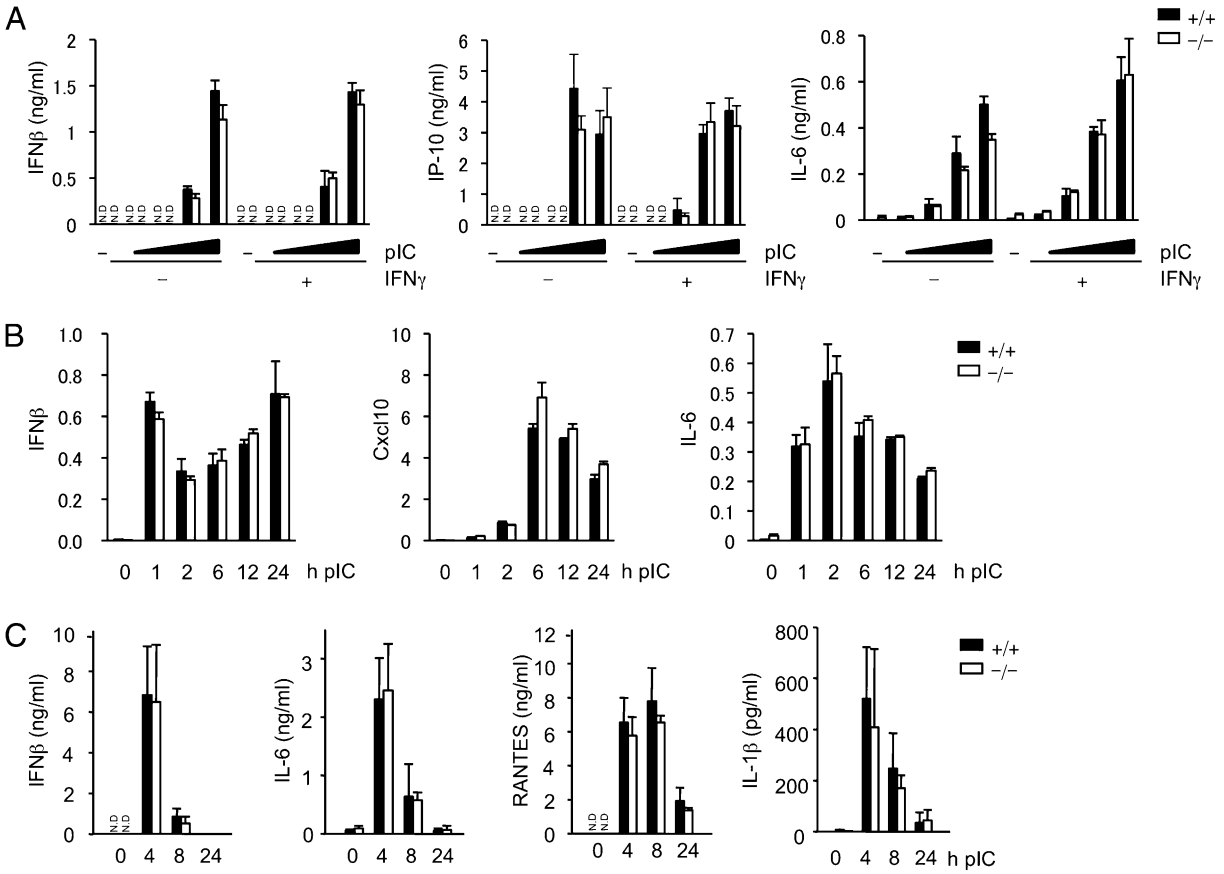

FIGURE 3. Responses to poly-IC in NLRC5-deficient mice. *A*, GMDCs prepared from WT (+/+) and NLRC5-deficient (−/−) mice were pretreated with or without IFN-γ and stimulated with medium (*lanes 1*, *6*), 1 μg/ml (*lanes 2*, *7*), 10 μg/ml (*lanes 3*, *8*), 50 μg/ml (*lanes 4*, *9*) or 100 μg/ml (*lanes 5*, *10*) poly-IC (pIC) for 24 h. The culture supernatants were analyzed for their levels of IFN-β, IP-10, and IL-6 by ELISA. *B*, GMDCs were stimulated with 100 μg/ml poly-IC for the indicated periods. Total RNA were subjected to quantitative PCR analyses for expression of IFN-β, Cxcl10, and IL-6. *C*, WT ($n = 5$) and NLRC5-deficient ($n = 4$) mice were injected i.p. with poly-IC. The IFN-β, IL-6, RANTES, and IL-1β levels were measured in serum samples prepared at the indicated times. ND, not detected.

CAGCCCTCTCCATCAACTATAAGC-3′ and 5′-AGCTCTTCAACTGGAG-AGCAGTTGAGG-3′; *Rantes*, 5′-ATGAAGATCTCTCTGCAGCTGCCCTC-ACC-3′ and 5′-CTAGCTCATCTCCAAATAGTTGATG-3′; *Il6*, 5′-GACAA-AGCCAGAGTCCTTCAGAGAG-3′ and 5′-CTAGGTTTGCCGAGTAGAT-CTC-3′; *TNFα*, 5′-ATGAGCACAGAAAGCATGATC-3′ and 5′-TACAGGC-TTGTCACTCGAATT-3′; and *β-actin*, 5′-GACATGGAGAAGATCTGGCA-CCACA-3′ and 5′-ATCTCCTGCTCGAAGTCTAGAGCAA-3′.

Quantitative PCR

Total RNA was isolated and cDNA was synthesized as described in the RT-PCR section. Quantitative PCR analysis was performed using the 7500 real-time PCR system (Applied Biosystems) using real-time PCR master mix (Toyobo) and TaqMan probe for IFN-β, IL-6, TNF-α, and Cxcl10 probes purchased from Applied Biosystem. Primers for 18s rRNA were used as an internal control.

Statistical analysis

Statistical significance between groups was determined by two-tailed Student *t* test and ANOVA test. Differences were considered as significant when $p \leq 0.05$, and highly significant when $p \leq 0.01$.

Results

Generation of NLRC5-deficient mice

To understand the physiologic roles of NLRC5, we generated NLRC5-deficient mice using a standard gene targeting method. We designed a targeting vector to disrupt exon 4 harboring the intermediate region between the CARD and nucleotide-binding and oligomerization domain of NLRC5 (Fig. 1*A*). The heterozygosity and homozygosity of the obtained mice were verified by Southern blot analysis (Fig. 1*B*), and nullizygosity was confirmed by RT-PCR analysis using total RNA isolated from expected NLRC5-deficient and wild type (WT) GMDCs after NDV infection (Fig. 1*C*). Mutant homozygous mice for the disrupted NLRC5 allele were born at the expected Mendelian ratio, grew healthy until 48 wk of age under specific pathogen-free conditions, and were fertile. No obvious changes in hematopoietic cell development were observed in NLRC5-deficient mice as determined by FACS analysis (Supplemental Fig. 1).

Normal inflammatory cytokine and type I IFN productions after stimulation with agonists for TLRs, RLRs, and a DNA sensor

Initially, we examined the responses to bacterial LPS, a TLR4 ligand. We pretreated GMDCs derived from WT and NLRC5-deficient mice with or without IFN-γ and stimulated them with different concentrations of LPS (1–500 ng/ml). The production of IL-6 and TNF-α was comparable between WT and NLRC5-deficient cells at all concentrations examined, as measured by ELISA (Fig. 2*A*). Moreover, IL-6 production after treatment with CpG DNA (TLR9 ligand) or MALP2 (TLR2 ligand) was also comparable between WT and NLRC5-deficient GMDCs (Fig. 2*B*). We then stimulated GMDCs with LPS for different periods of time and subjected their total RNA to quantitative PCR analyses for mRNAs encoding IL-6, TNF-α, and IP-10 (Cxcl10). The expressions of these genes were comparable between WT and NLRC5-deficient cells at all time points examined (Fig. 2*C*). Moreover, results of the regular RT-PCR indicated that WT and NLRC5-deficient GMDCs displayed similar induction of TNF-α, IL-6, and RANTES after LPS treatment (Supplemental Fig. 2). Thioglycollate-elicited PECs derived from WT and NLRC5-deficient mice also displayed comparable production of IL-6 and RANTES after LPS treatment (Supplemental Fig. 3). These results indicate that NLRC5 is dispensable for TLR2-, TLR4-, and TLR9-induced cytokine production.

Next, we addressed responses to poly-IC, which is a dsRNA analog that acts as a ligand for MDA5 and TLR3. We pretreated GMDCs with or without IFN-γ and then stimulated them with various concentrations of poly-IC to measure IFN-β, IP-10, and IL-6 by ELISA. The production of these cytokines was normal in NLRC5-deficient GMDCs (Fig. 3*A*). Furthermore, the induction of IFN-β, Cxcl10, and IL-6 mRNA was also normal in the absence of NLRC5 at the tested time points (Fig. 3*B*). We next injected poly-IC into mice and measured the serum cytokine levels by ELISA. The levels of IFN-β, IL-6, RANTES, and IL-1β were comparable between WT and NLRC5-deficient mice (Fig. 3*C*).

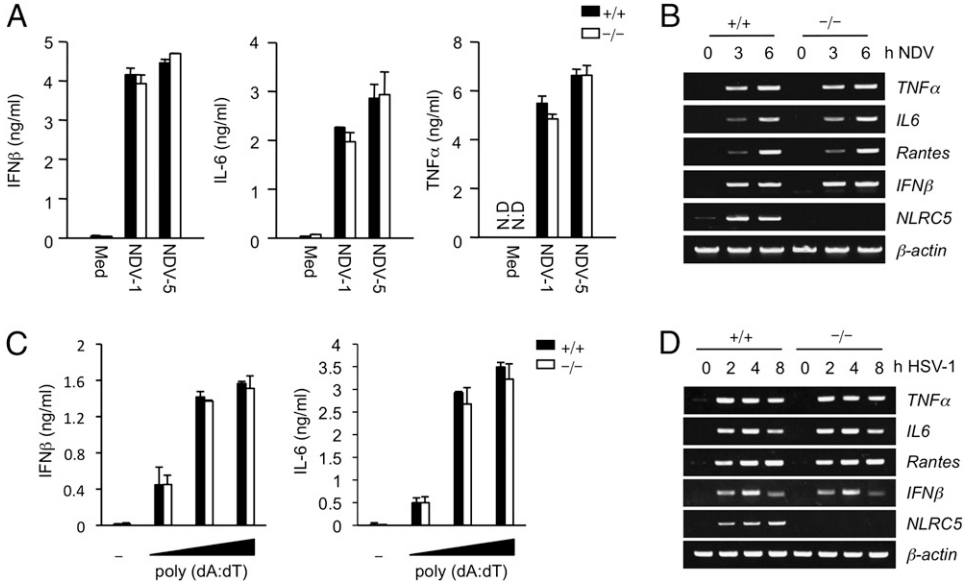

FIGURE 4. Responses to RNA virus, DNA virus, and immunostimulatory DNA in NLRC5-deficient mice. *A*, GMDCs were prepared from WT (+/+) and NLRC5-deficient (−/−) mice and infected with NDV (NDV-1, moi = 1.0; NDV-5, moi = 5) for 24 h. The culture supernatants were analyzed for their levels of IFN-β, IL-6, or TNF-α by ELISA. *B*, Total RNA prepared from GMDCs infected with NDV (moi = 1.0) for the indicated times were subjected to RT-PCR analysis for the expressions of the indicated genes. *C*, GMDCs were unstimulated (−) or stimulated with poly (dA:dT) (0.1, 1.0, or 10 μg/ml) for 24 h. The culture supernatants were analyzed for the production of IFN-β and IL-6 by ELISA. *D*, GMDCs were infected with HSV-1 (moi = 10) for the indicated times, and isolated total RNA samples were subjected to RT-PCR analyses for the mRNA expressions of the indicated genes. Similar results were obtained from two independent experiments. ND, not detected.

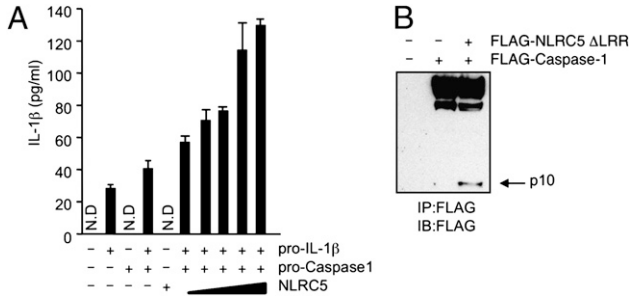

FIGURE 5. Induction of IL-1β production by NLRC5 overexpression. *A*, HEK293 cells were cotransfected with pro–IL-1β (150 ng) together with procaspase-1 (50 ng) plus NLRC5-FL (10, 50, 100, 200, or 400 ng) expression plasmids as indicated. The total amount of DNA in all transfection reactions was kept constant by the addition of an irrelevant plasmid. At 36 h after transfection, the culture supernatants were assayed for their IL-1β levels by ELISA. The data shown are the means ± SD of triplicate experiments. *B*, HEK293 cells were transfected with FLAG-NLRC5 ΔLRR alone or together with FLAG–procaspase-1. At 36 h after transfection, whole cell lysates were subjected to immunoprecipitation (IP) with an anti-FLAG Ab and then immunoblotted (IB) with anti-FLAG Ab. ND, not detected.

Next, we evaluated the contribution of NLRC5 to RIG-I–mediated pathways. The productions of IFN-β, IL-6, and TNF-α (Fig. 4*A*) and the inductions of TNF-α, IL-6, RANTES, and IFN-β mRNAs postinfection with NDV, which is recognized by RIG-I, were also comparable between WT and NLRC5-deficient GMDCs (Fig. 4*B*).

We then examined the role of NLRC5 in cytosolic dsDNA-driven innate immune responses. We stimulated GMDCs with B-DNA (poly [dA:dT]) and found that the productions of IFN-β and IL-6 were comparable between WT and NLRC5-deficient GMDCs (Fig. 4*C*). Moreover, the induction of IFN-β and IL-6 mRNA after stimulation with poly (dA:dT) or calf thymus DNA or infection with *L. monocytogenes*, which is known to introduce DNA into the cytosol, was also comparable between WT and NLRC5-deficient BMDMs (Supplemental Fig. 4). Infection with HSV-1, a DNA virus, resulted in comparable levels of TNF-α, IL-6, RANTES, and IFN-β mRNA induction in both WT and NLRC5-deficient GMDCs (Fig. 4*D*). These results suggest that NLRC5 is dispensable for cytosolic DNA-induced innate immune responses.

Activation of caspase-1 and induction of IL-1β production by NLRC5 overexpression

We investigated further whether NLRC5 has the ability to activate caspase-1. HEK293 cells were transiently cotransfected with expression plasmids encoding pro–IL-1β or procaspase-1 together with control or NLRC5 expression plasmids, followed by measurement of the IL-1β levels in the culture supernatants by ELISA. IL-1β production was increased when pro–IL-1β and caspase-1 were coexpressed with NLRC5 (Fig. 5*A*). To further confirm that caspase-1 is activated by NLRC5, we examined the cleavage of caspase-1. FLAG–caspase-1 was cleaved in cells expressing NLRC5 ΔLRR, whose overexpression showed higher production of IL-1β than full length NLRC5 (unpublished observations) as determined by immunoblot analysis using an anti-FLAG Ab (Fig. 5*B*).

Normal induction of IL-1β after stimulation with NLRP3, NLRC4, and AIM2 activators

The finding that NLRC5 overexpression promotes IL-1β secretion prompted us to investigate whether NLRC5 regulates IL-1β production. We first prepared various cell types such as PECs, BMDMs, and GMDCs and titrated the amount of NLRP3 inflammasome activators to determine optimal concentration for IL-1β production. Our results (some data not shown) and previously reported results showed a similar pattern of IL-1β production irrespective of cell types such as PECs, BMDMs, or GMDCs (26, 27). Using optimal concentration of inflammasome activator, PECs were stimulated with monosodium urate (MSU), LPS plus ATP or nigericin, zymosan, or curdlan, which are known to activate NLRP3 inflammasome and induce comparable levels of IL-1β production be-

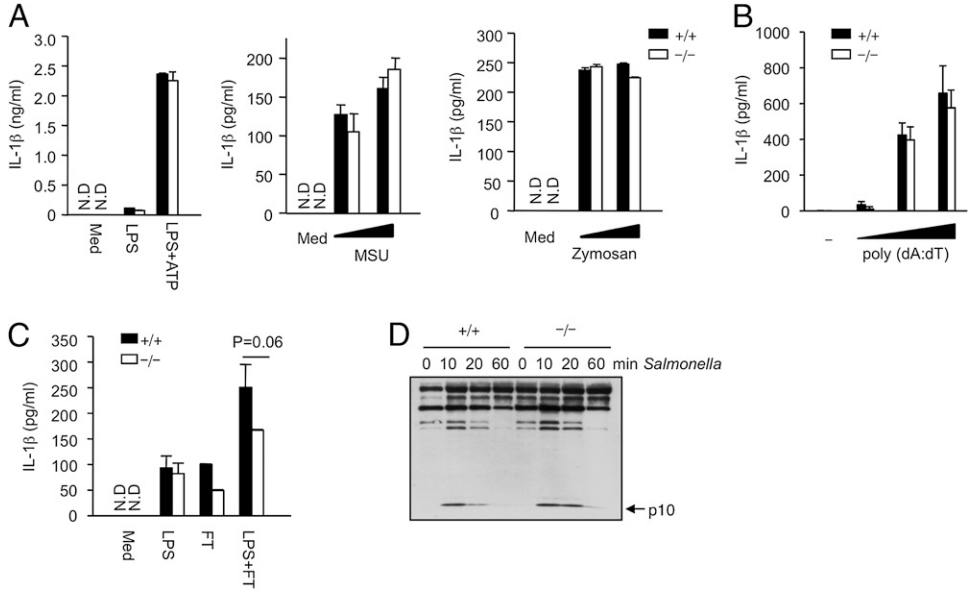

FIGURE 6. IL-1β production after stimulation or infection with NLRP3 and AIM2 activators or bacteria in NLRC5-deficient mice. *A*, PECs from WT (+/+) and NLRC5-deficient (−/−) mice were stimulated with LPS (1 μg/ml) or LPS plus ATP (10 mM); 0.5 or 1.0 mg/ml of MSU; or zymosan (1 or 2 mg/ml) for 24 h and the production of IL-1β was measured in supernatant by ELISA. *B*, GMDCs were unstimulated (−) or stimulated with poly (dA:dT) (0.1, 1.0, or 10 μg/ml) for 24 h. The culture supernatants were analyzed for the production of IL-1β by ELISA. *C*, BMDMs were stimulated with LPS (1 μg/ml) or infected with *F. tularensis* (FT; moi = 100) or LPS (1 μg/ml) plus FT. After 24 h, the production of IL-1β was measured in supernatant by ELISA. The data shown are the means ± SD of triplicate experiments. *D*, LPS-stimulated BMDMs were infected with *Salmonella* (moi = 50) for the indicated times. Cell lysates were immunoblotted with an Ab against the p10 subunit of caspase-1.

tween WT and NLRC5-deficient macrophages (Fig. 6*A*, Supplemental Fig. 5). Moreover, we found that IL-1β production by curdlan was comparable between WT and NLRC5-deficient dendritic cells (DCs; Supplemental Fig. 6), suggesting that NLRC5 was dispensable for the NLRP3 inflammasome-triggered IL-1β production in macrophages and GMDCs.

Next, we simulated GMDCs with a different amount of an AIM2 inflammasome activator, poly (dA:dT). The production of IL-1β was comparable between WT and NLRC5-deficient GMDCs (Fig. 6*B*). Furthermore, the production of IL-1β was also comparable between WT and NLRC5-deficient PECs (data not shown). We then infected the cells with *Francisella tularensis*, which induces IL-1β production through AIM2 inflammasome. The production of IL-1β by PECs infected with *F. tularensis* was modestly reduced in culture supernatants from NLRC5-deficient cells compared with those from WT cells (Fig. 6*C*). However, when *F. tularensis* was infected into LPS-pretreated macrophages, there was no significant difference in IL-1β production between WT and NLRC5-deficient cells (Fig. 6*C*), suggesting that NLRC5 is not required for AIM2 activator-induced IL-1β production in macrophages and GMDCs. Finally, we examined caspase-1 activation triggered by *S. typhimurium*, which activates NLRC4 and NLRP3 inflammasomes (28), in NLRC5-deficient macrophages. In both WT and NLRC5-deficient BMDMs, caspase-1 activation was similarly induced as determined by immunoblot analysis using an anti–caspase-1 p10 Ab (Fig. 6*D*). Furthermore, in vivo poly-IC–induced IL-1β production was comparable in both WT and NLRC5-deficient mice (Fig. 3*C*). These observations suggest that NLRC5 is not required for IL-1β production in response to various tested stimulators of NLRP3, NLRC4, and AIM2 inflammasomes in macrophages.

Discussion

Recently, it was shown that NLRC5 has a role to control antiviral innate immune responses. Cui et al. (22) indicated that NLRC5 interacts with IKKα and IKKβ to suppress NF-κB activation; they also showed that NLRC5 interacts with RIG-I via its CARD to suppress RLR signaling. In contrast, reports by Kuenzel et al. (23) and Neerincx et al. (24) indicated that NLRC5 increases IFN-β and NF-κB promoter activation and enhances RLR-mediated antiviral innate immune responses. Therefore, it may be possible that NRLC5 positively and negatively controls RLR-mediated signaling pathways.

In our reporter analyses, overexpression of NLRC5 in HEK293 cells significantly suppressed NF-κB activation induced by MyD88, TRAF6, and TRAF2, which are downstream molecules for TLR or TNFRs, but did not abrogate IKKβ-mediated NF-κB activation (Supplemental Fig. 7). These findings suggest that NLRC5 negatively regulates NF-κB activation upstream of IKKβ in TLR and TNFR signaling. Notably, we found that NLRC5 formed a complex with TBK1 (Supplemental Fig. 8A, 8B). We performed yeast two-hybrid screening using TBK1 as a bait to identify NLRC5 (Supplemental Fig. 8A). NLRC5 interacted with TBK1 but not IKKi, another IKK-related kinase, in yeast and HEK293 cells (Supplemental Fig. 8A, 8B). NLRC5 overexpression suppressed TBK1-mediated IFN-β promoter activation in a dose-dependent manner (Supplemental Fig. 8C). Our in vitro data implicated that NLRC5 negatively regulates TBK1 and NF-κB activation. Although the reason for the discrepancies between our data on in vitro characterization of NLRC5 and the findings in the previous in vitro studies is unknown, it is possible that differences in the expression plasmids and cell lines used in each study influenced the results.

It is noteworthy that, under in vivo conditions, NLRC5 was not required for the induction of inflammatory cytokines and type I IFN by macrophages and DCs in response to LPS, poly-IC, dsDNA, NDV, HSV-1, and *L. monocytogenes*. These findings indicate that NLRC5 deficiency does not affect TLR, RLR, and DNA sensor signaling, at least in mice. However, it cannot be ruled out the possibility that NLRC5 plays a critical role in certain cell types or in unidentified signaling pathways.

Our findings that NLRC5 overexpression induced caspase-1–dependent IL-1β production implicated that NLRC5 forms an inflammasome. However, NLRC5 deficiency did not abrogate IL-1β production in response to various stimulations, including LPS plus ATP or nigericin, MSU, curdlan, poly-IC, poly (dA:dT), and *F. tularensis* infection, which activate NLRP3 or AIM2 inflammasomes in macrophages. Moreover, *Salmonella*-induced caspase-1 activation, which was NLRC4 and NLRP3 inflammasome dependent, was intact in NLRC5-deficient macrophages (28). Therefore, NLRC5 inflammasomes may be activated by unknown pathogenic or endogenous activators, or they may play roles in other cell types. Future identification of NLRC5 inflammasome activators is required to understand the physiologic role of NLRC5 in innate immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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