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NLRC5 Limits the Activation of Inflammatory Pathways

Szilvia Benko,* Joao G. Magalhaes,† Dana J. Philpott,† and Stephen E. Girardin*

Nod-like receptors (NLRs) are intracellular sentinel proteins that are implicated in the detection of microbes and danger signals, thereby controlling several key innate immune pathways. The human genome encodes 22 NLR proteins, the function of many of which remains unknown. In this study, we present the identification and characterization of NLRC5, a NLR protein whose expression is found predominantly in cells of the myeloid and lymphoid lineages. NLRC5 expression was strongly induced by IFN-γ and more modestly by LPS and polyinosinic:polycytidylic acid. Overexpression of NLRC5 in HEK293T cells resulted in a global dampening of NF-κB–, AP-1–, and type I IFN-dependent signaling, most likely through transcriptional repression. Accordingly, NLRC5 was found to shuttle between the cytosol and the nucleus in a CrmA-dependent manner. Knocking down NLRC5 expression in RAW264.7 murine macrophages resulted in a potent upregulation of the proinflammatory responses to IFN-γ and LPS, including increased secretion of TNF, IL-6, and IL-1β, as well as cell surface expression of CD40. Strikingly, NLRC5 expression was also found to be critical for LPS-induced IL-10 production in RAW264.7 macrophages. Collectively, our results identify NLRC5 as a negative modulator of inflammatory pathways. ***The Journal of Immunology*, 2010, 185: 1681–1691.**

In vertebrates, the innate immune system relies on several families of pattern recognition molecules (PRMs) that mediate the activation of defense pathways following detection of microbial- and danger-associated molecular patterns (MAMPs and DAMPs, respectively). Whereas TLRs sense MAMPs and DAMPs exposed to the extracellular milieu, Nod-like receptors (NLRs) and Rig-I–like receptors have recently been identified as intracellular PRMs (1, 2). In humans, the NLR family is composed of 22 members having in common the juxtaposition of a NACHT (for domain present in NAIP, CIITA, HET-E, and TP1) domain flanked on the carboxyl-terminal side by a leucine-rich repeat (LRR) domain (3). Subfamilies of NLRs can be defined on the basis of the N-terminal region, which displays more variability. For instance, the well-studied NLR proteins Nod1 and Nod2 contain an N-terminal caspase activation and recruitment domain (CARD), which triggers the recruitment of the adaptor protein Rip2 and the activation of downstream signaling including NF-κB and MAPKs pathways (4). More recently, Nod1 and Nod2 have also been shown to induce the recruitment of the autophagy machinery at the site of bacterial invasion through a Rip2-independent interaction with ATG16L1 (5). Another large and relatively homogeneous subgroup of the NLR family is represented by the pyrin domain-containing NLR proteins (NLRPs), which display an N-terminal PYRIN domain. Studies on NLRP3 and NLRP1 have demonstrated the key role for NLRPs in the activation of caspase-1 inflammasomes in response to numerous MAMPs and DAMPs, resulting in the maturation and secretion of IL-1β and IL-18 (6). Other NLR proteins displaying well-defined N-terminal domains include NAIP (baculoviral inhibitory repeat domain) (7), NLRC4 (CARD) (8), and CIITA (CARD and acid-transactivation domain) (9). Finally, NLRC3 and NLRC5 have CARD-like N-terminal domains.

In addition to the well-established role of NLRs in triggering inflammatory pathways in response to microbes or danger signals, several lines of evidence have demonstrated that mutations and polymorphisms in genes encoding members of the NLR family are associated with susceptibility to inflammatory disorders. NLR genes associated with inflammatory disorders include *Nod2* (Crohn's disease and Blau syndrome), *Nod1* (asthma and atopic disorders, inflammatory bowel disease), *Nlrp3* (Muckle-Wells syndrome, familial cold autoinflammatory syndrome, chronic infantile neurologic cutaneous and articular syndrome, inflammatory bowel disease), and *Nlrp1* (vitiligo) (10). Finally, mutations in *CIITA* cause bare lymphocyte syndrome, a rare recessive condition in which the MHC class II expression is defective, resulting in severe immunodeficiency (11).

CIITA was the first identified member of the NLR family, and this protein displays unique features as compared with other NLRs. Contrary to Nod1, Nod2, NLRPs, and NLRC4, CIITA does not seem to trigger proinflammatory signaling pathways (such as NF-κB, MAPK, or caspase-1 inflammasome), but rather it acts as a transcriptional coactivator implicated in the regulation of MHC class II expression (12). Accordingly, CIITA has been shown to shuttle between the cytosol and the nucleus via a CrmA-dependent mechanism (13), and CIITA remains, up until now, the only known NLR protein that targets the nucleus. Note also that there are no known MAMPs or DAMPs that modulate the function of CIITA. This suggests that CIITA, unlike most other NLRs, likely would not act as a bona fide PRM, although this point remains difficult to establish with certainty.

In this study, we report the first characterization and analysis of NLRC5. Our results demonstrate that NLRC5 is highly expressed in cells of the myeloid and lymphoid lineages, and we establish that

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Abbreviations used in this paper: aMϕ, alveolar macrophages; Bl, blood; BMDC, bone marrow-derived dendritic cell; BMDM, bone marrow-derived macrophage; CARD, caspase activation and recruitment domain; Con A, concanavalin A; c.pl, cytoplasmic fraction; DAMP, danger-associated molecular pattern; DC, dendritic cell; IoM, ionomycin; IPS-1, IFN-β promoter stimulator-1; ISRE, IFN-stimulated regulatory element; LMB, leptomycin B; LRR, leucine-rich repeat; MAMP, microbial-associated molecular pattern; n, nuclear fraction; NLR, Nod-like receptor; NLRP, pyrin domain-containing NLR protein; poly(I:C), polyinosinic:polycytidylic acid; PRM, pattern recognition molecule; qPCR, quantitative PCR; RT, reverse transcriptase; shRNA, short hairpin RNA.

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NLRC5 expression is strongly regulated by IFN-γ in all immune cell populations analyzed. Microbial-derived molecules (in particular, LPS and polyinosinic:polycytidylic acid [poly(I:C)]) also triggered NLRC5 expression in several cell populations, albeit less potently than did IFN-γ. Overexpression studies in HEK293T cells showed that NLRC5 repressed the activation NF-κB, AP-1, and type I IFN-dependent signaling pathways, most likely by acting as a transcriptional repressor in the nucleus. In agreement with this, in silico analyses as well as subcellular localization studies identified that NLRC5 was found to shuttle from the cytosol to the nucleus via a CrmA-dependent mechanism. Silencing of NLRC5 expression in RAW264.7 murine macrophages resulted in a dramatic alteration of the response of these cells to IFN-γ and LPS. NLRC5-silenced RAW264.7 cells displayed exacerbated induction of a proinflammatory program, characterized by enhanced secretion of proinflammatory cytokines (TNF, IL-6, and IL-1β), reduced secretion of the anti-inflammatory cytokine IL-10 following LPS stimulation, and increased cell surface expression of the costimulatory molecule CD40, in response to activation by IFN-γ or LPS. Taken together, these results identify NLRC5 as key regulator of proinflammatory pathways in immune cells.

Materials and Methods

Reagents

PMA, ionomycin, and concanavalin A (Con A) were purchased from Sigma-Aldrich (St. Louis, MO); poly(I:C) was from InvivoGen (Cedarlane Laboratories, Hornby, Ontario, Canada); IFN-γ, CD3, and CD28 were from R&D Systems (Minneapolis, MN); mouse monoclonal anti-Flag M2 was from Sigma-Aldrich; mouse monoclonal anti-p84 was from Abcam (Cambridge, MA); and mouse monoclonal anti-tubulin Ab was from Sigma-Aldrich.

Cell culture of cell lines

HEK293T, HEK293, HeLa, MCF-7, and Caco-2 cells were cultured in DMEM; THP-1, Raji, Daudi, Jurkat, and RAW264.7 cells were cultured in RPMI 1640. Each medium was supplemented with 2 mM glutamine, 10% FCS (Invitrogen, Toronto, Ontario, Canada), and penicillin and streptomycin (Sigma-Aldrich). The medium for Caco-2 was supplemented with 1× nonessential amino acid (Invitrogen).

Macrophage, dendritic cell, and lymphocyte isolation

Bone marrow-derived dendritic cells (BMDCs) were obtained from mice as previously described (14). Mouse CD19 microbeads were used for the positive selection of mouse B lymphocytes, and CD11c microbeads were used for the isolation of mouse dendritic cells (DCs) from spleen. A $CD4^+$ T cell isolation kit and a $CD8a^+$ T cell isolation kit were used for the depletion of $CD4^+$ and $CD8^+$ lymphocytes from thymus and spleen. All microbeads and kits were purchased from Miltenyi Biotec (Auburn, CA).

NLRC5 expression constructs

Full-length NLRC5 (1866 aa) cloned into pCMV6-XL6 expression vector was obtained from OriGene Technologies (Rockville, MD) and subcloned into pcDNA3.1 vector with a C-terminal Flag tag by using the restriction enzymes KpnI and XhoI (Fermentas, Burlington, Ontario, Canada). The generated product was fully sequenced.

Expression profile of human NLRC5 in organs

NLRC5 expression was determined in a panel of 48 human tissues by using Human Rapid-Scan expression panels (OriGene Technologies). The levels of NLRC5 among the 48 tissues are expressed as relative units normalized for human GAPDH expression.

Sequence phylogenetic analyses

Sequence alignment was made by using the ClustalW2 multiple alignment computer program. The phylogenetic tree of NLR NACHT and LRR domains was created using ClustalW and neighbor-joining/unweighted pair group method with arithmetic mean version 3.6a3 algorithms. Protein localization prediction was made by using the PSORT II algorithm.

Transient transfection and luciferase reporter assays

Transfection procedure and expression plasmids used in reporter assays were described previously (15). Transfection was performed with 75 ng of the β-galactosidase expression plasmid and 75 ng of the reporter plasmid NF-κB–, p53, AP-1–, or IFN-stimulated regulatory element (ISRE) luciferase plus 50, 100, 200, 400, or 600 ng of NLRC5-Flag expression vector where indicated. For positive controls, we transfected hemagglutinin-p53, MyD88, or IFN-β promoter stimulator-1 (IPS-1) expression vectors, or we treated the cells with 10 ng/ml IFN-γ or 0.1 μM PMA for 24 h. The pcDNA3.1 vector was used to balance the DNA concentration. Luciferase expression was normalized as a ratio to β-galactosidase activity. Assays were performed in triplicate, and data represent means ± SD of a representative experiment.

Immunofluorescence microscopy

Transfection and subsequent immunofluorescence were described previously (15). For the p65 translocation studies, HeLa cells grown on glass coverslips were transfected overnight with NLRC5 expression vector and stimulated with 10 ng/ml TNF-α for the indicated times. Nuclear translocation of the NF-κB p65 subunit was evaluated by immunofluorescence in cells overexpressing NLRC5 or not. Data shown are the means ± SEM of three independent experiments, and for each condition and time point a minimum of 500 cells were counted. Immunofluorescence images were obtained using a Zeiss Axiovert 200 microscope imaging system with a ×63 oil fluorescence objective.

Subcellular fractionation

HEK293 cells were transfected overnight with NLRC5-Flag expression vector. Subcellular fractionation was performed according to the standard protocol. Briefly, cells were resuspended in buffer A (1 M HEPES, 0.5 M KCl, 0.5 M EDTA, 0.3 M EGTA, 1 M DTT, supplemented with protease inhibitors) and incubated on ice. After addition of 10% Nonidet P-40, nuclei were spun out. Keeping the supernatant as the cytoplasmic fraction, the pellet containing the nuclei was resuspended in buffer B (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, supplemented with protease inhibitors). After 15 min incubation on ice, lysate was centrifuged and supernatant was kept as the nuclei fraction. Lysates were boiled for 10 min in SDS sample buffer, separated on acrylamide SDS-PAGE gel, and Western blot was performed.

Western blot analysis

Western blotting was performed as described previously (15). Proteins were visualized by a chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA).

Isolation of RNA from cell lines and mouse primary cells

Total RNA was extracted with TRIzol reagent (Invitrogen, San Diego, CA) and was isolated according to the manufacturer's instructions. The concentration and homogeneity of the RNA preparations were determined by measuring the absorbance at 260 and 280 nm by a spectrophotometer (NanoDrop ND1000; Promega Biosciences, San Luis Obispo, CA). To avoid contamination by genomic DNA, samples were treated by DNase I or in the case of the *Luc* quantitative PCR (qPCR) by Turbo DNase (Ambion, Austin, TX), and the final RNA preparations were stored at −80˚C.

TaqMan real-time qPCR

Reverse transcriptase (RT)-qPCR reactions were carried out as previously described (16). The real-time qPCR was performed with TaqMan assays (Applied Biosystems, Foster City, CA or Integrated DNA Technologies, San Diego, CA). The sequence of the primers used is displayed in Supplemental Fig. 7. The comparative C_T method was used to quantify transcripts, and the expression level was normalized to that of the human cyclophilin or mouse RPL19 (17). All PCR reactions were performed in triplicate in 10-μl volumes, with one control reaction that contained cDNA but no RT enzyme.

Lentiviral vector cloning and lentivirus packaging

Oligonucleotides for NLRC5 short hairpin RNA (shRNA) were ordered from Integrated DNA Technologies. The sense and antisense oligonucleotides were annealed in water and cloned into a pLKO.1 vector (Addgene, Cambridge, MA) using AgeI/EcoRI restriction sites (Fermentas). The constructed vector was confirmed by DNA sequencing with pLKO.1 sequencing primer. Packaging and purification of the lentivirus were performed according to classic procedures. Briefly, HEK293T cells were seeded 3×10^6 in a 10-cm

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culture dish. The following day, cells were cotransfected overnight with the lentiviral vector (1 μg) and the lentiviral packaging/envelope vectors psPAX2 (750 ng) and pMD2.G (250 ng). The next day the medium was replaced, and 40 and 64 h posttransfection the supernatants containing the lentiviral particles were collected, spun, and passed through a 0.45-μm filter.

Lentiviral transduction of RAW264.7 macrophages

RAW264.7 macrophages were seeded at 1.2×10^6 cell/2 ml on 6-well plates and left to attach overnight. The next day, lentiviruses were added to the cells in the presence of 10 μg/ml polybrene (Sigma-Aldrich), and the plates were spun at $1500 \times g$ for 1 h. After an overnight incubation, medium was replaced and supplemented with puromycin (Sigma-Aldrich).

Determination of secreted cytokine concentrations

Supernatants from RAW264.7 cells were harvested, centrifuged, and stored at −80˚C until used for cytokine measurements. The concentrations of IL-1β, IL-6, TNF, IL-10, IL-12p40, KC, and RANTES in cell culture supernatants were determined by ELISA according to the manufacturer's recommendations (DuoSet from R&D Systems). The detection limit of the assays was 15 pg/ml for IL-1β and IL-6, and 30 pg/ml for IL-10, TNF, and RANTES.

Flow cytometry

Flow cytometry was performed as described previously (18). FlowJo software was used for the analysis of the results. Data were collected on ~30,000 cells.

Statistical analysis

Significant differences between mean values were evaluated using a Student *t* test. Data are presented as means ± SD.

Results

Characterization of NLRC5

The human *NLRC5* gene (accession number NM_032206) is located in the 16q13 locus and encodes a protein that consists of 1866 aa, for a predicted molecular mass of 204 kDa. NLRC5 is therefore the largest protein of the NLR family. Sequence analysis of the protein showed that NLRC5 contains a NACHT domain encoded by one large exon, as well as an LRR domain possessing 27 LRRs that are encoded by 43 exons (Fig. 1*A*). However, whereas most NLRs display a well-characterized N-terminal domain (CARD, PYRIN, or BIR), the N-terminal region of NLRC5 displays no similarity to other defined domains, and sequence alignment from various animal species showed that this region is highly conserved among mammalian orthologs (Supplemental Fig. 1). Interestingly, no similarity was found between the mammalian and nonmammalian NLRC5 N-terminal regions (data not shown). Finally, phylogenetic analysis of the NACHT and LRR domains of NLR proteins demonstrated that NLRC5 displays a striking similarity to CIITA (Fig. 1*B*).

NLRC5 is expressed predominantly in immune cells

We determined by qPCR the expression profile of NLRC5 using an array containing 48 various human organs and tissues. Interestingly, we observed that NLRC5 was highly expressed in immune-related tissues, including bone marrow, lymph node, spleen, and in PBLs, as well as in certain organs having mucosal surfaces, such as lung, small intestine, colon, and uterus (Fig. 2*A*). Therefore, these observations suggest that NLRC5 likely plays a role in immune regulation and that its expression might be critical for mucosal immune defense. In human cell lines, NLRC5 was expressed at high levels in THP-1 macrophages and at low but detectable levels in cells of epithelial (MCF-7, Caco-2, and HeLa) and lymphoid (Jurkat, Daudi, and Raji) origin (Fig. 2*B*). Similarly, in murine tissues, NLRC5 was highly expressed in lymph nodes, spleen, and thymus as well as in liver and lung (Fig. 2*C*). Surprisingly, NLRC5 expression in the murine colon and small intestine was low (Fig. 2*C*), which is at odds with the results found in human samples. Although we do not have a definitive explanation for these results, the data might reflect the fact that the mice from which the organs were collected were maintained in strict specific pathogen-free conditions and do not carry known murine intestinal pathogens.

To obtain a more detailed view of the cell types that express NLRC5 in immune tissues, we isolated mouse immune cells of myeloid and lymphoid origin from several organs and tissues and measured the basal mRNA expression of NLRC5 by qPCR (Fig. 2*D*). With regard to myeloid cells, we detected high expression of NLRC5 in bone marrow-derived macrophages (BMDMs), but not in peritoneal or alveolar macrophages. Splenic DCs also expressed NLRC5 in large quantities. Interestingly, the expression of NLRC5 in BMDCs was dependent on the differentiating conditions. We measured high mRNA expression in cells cultivated without IL-4, while cells differentiated in the presence of IL-4 showed low expression of NLRC5. As for cells of lymphoid origin, NLRC5 was highly expressed in thymic and splenic $CD4^+$ cells and moderately expressed in $CD8^+$ cells. Interestingly, NLRC5 was differentially expressed during splenic T cell development, as double-positive ($CD4^+CD8^+$) and double-negative ($CD4^-CD8^-$) populations displayed low expression of NLRC5, suggesting a potential role for this NLR during T cell development in the thymus. Finally, NLRC5 was also found expressed in splenic B cell populations (Fig. 2*D*).

NLRC5 expression is highly inducible by IFN-γ

We next examined how activation of immune cells alters NLRC5 expression. We first sorted and isolated splenic murine $CD4^+$ and $CD8^+$ T cells and stimulated cells overnight with various agonists. Whereas IFN-γ stimulation resulted in moderate induction of NLRC5 in these cells (Fig. 3*A*, 3*B*), activation with anti-CD3/anti-CD28 treatment did not alter the expression of NLRC5. Treating the cells with the polyclonal activator Con A did not change NLRC5 expression in CD4 cells, but it resulted in a moderate increase in $CD8^+$ cells. In the case of splenic B lymphocytes, IFN-γ treatment resulted in a very potent induction of NLRC5 expression, while activation with PMA plus ionomycin, but not Con A, also effectively triggered NLRC5 expression (Fig. 3*C*). The TLR4 agonist LPS also induced a moderate increase in NLRC5 expression in splenic B lymphocytes (Fig. 3*C*). In murine BMDMs, treatment with IFN-γ induced NLRC5 mRNA expression. However, LPS stimulation did not alter NLRC5 mRNA expression in these cells, whereas poly(I:C) potently induced expression of the gene (Fig. 3*D*). Next, we differentiated bone marrow cells to DCs (BMDCs) either in the presence or absence of IL-4, which results in a different polarization profile of BMDCs. IFN-γ treatment in both cell populations resulted in a strongly elevated NLRC5 mRNA expression (Fig. 3*E*). Interestingly, LPS and poly(I:C) stimulations resulted in different results in the two cell types. LPS, but not poly(I:C), induced NLRC5 expression in BMDCs cultured in the absence of IL-4 (Fig. 3*E*). In contrast, poly(I:C), but not LPS, induced NLRC5 expression in BMDCs cultured in the presence of IL-4 (Fig. 3*E*).

We also performed similar studies in cells of human origin. First, we isolated PBMCs from a healthy donor and stimulated these cells with IFN-γ. Whereas stimulation for 24 or 48 h only moderately induced NLRC5 expression, culture of PBMCs in the presence of IFN-γ for 72 h resulted in a strong potentiation of NLRC5 expression (Fig. 3*F*). Similarly, NLRC5 expression was considerably induced by IFN-γ stimulation in human Jurkat (Fig. 3*G*) and THP-1 cell lines (Fig. 3*H*). Taken together, these results demonstrate that IFN-γ is a general activator of NLRC5 expression in all of the cell populations that we have tested. Our data also show that, besides IFN-γ, the effect of other stimuli (general activator of lymphocytes or microbial-derived molecules) on NLRC5 expression appears to be cell specific.

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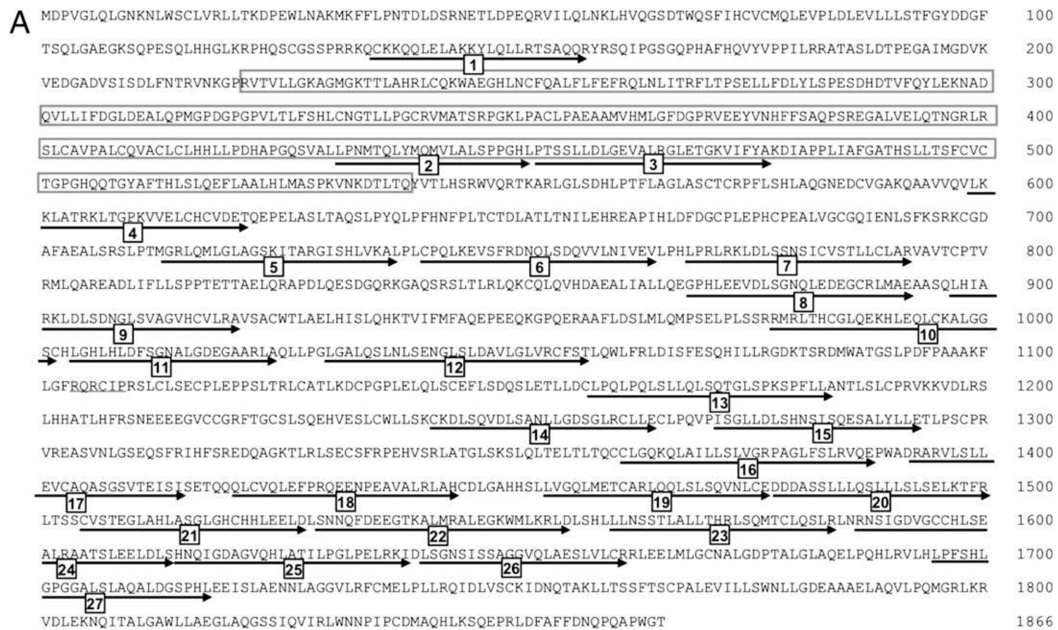

FIGURE 1. Characterization of NLRC5. *A*, Amino acid sequence of human NLRC5. The NACHT domain is shown in boxes and the LRR repeats are indicated by arrows and numbers. *B*, The phylogenetic trees of the NACHT (*left*) and the LRR (*right*) domains were created using ClustalW and the neighbor-joining/unweighted pair group method with arithmetic mean version 3.6a3 algorithms.

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NLRC5 localizes to the cytoplasm and the nucleus

Using the publicly available algorithm PSORT II (www.psort.org) that predicts protein subcellular localization on the basis of several parameters, we next observed that NLRC5 and CIITA were the two only NLR proteins displaying a predicted nuclear localization (Supplemental Fig. 2). Indeed, in the case of CIITA, it is well characterized that the protein shuttles from the cytosol to the nucleus, where it functions as a transcriptional coactivator and, by binding to transcription factors on the HLA class II promoter, regulates the gene expression of the Ag-presenting molecule MHC class II. We cloned the NLRC5 coding sequence in frame with a C-terminal Flag tag and verified the expression of Flag-tagged NLRC5 (NLRC5-Flag) by Western blotting in human HEK293T cells (Fig. 4*A*). Next, we overexpressed NLRC5-Flag in human epithelial HeLa cells and observed by immunofluorescence that in resting conditions, NLRC5 was found predominantly in the cytosol (Fig. 4*B*); however, a pool of the protein was found in the nucleus in resting conditions, as the nuclear staining was above background in fluorescence quantification (data not shown), and some cells also displayed clear nuclear staining (Supplemental Fig. 3*A*). HeLa cells were then treated with leptomycin B (LMB), a molecule that inhibits CrmA-mediated nuclear export, therefore provoking the nuclear accumulation of proteins that shuttle between the cytosol and the nucleus. LMB treatment resulted in a strong accumulation of NLRC5 into the nucleus (Fig. 4*C*), thereby demonstrating that the protein shuttles from the cytosol to the nucleus in a CrmA-dependent manner. As a control, we observed that LMB treatment did not alter the cytosolic distribution of the NLR proteins NLRX1 and Nod1 (Supplemental Fig. 3*A*). Finally, nuclear and cytosol fractionation of NLRC5-

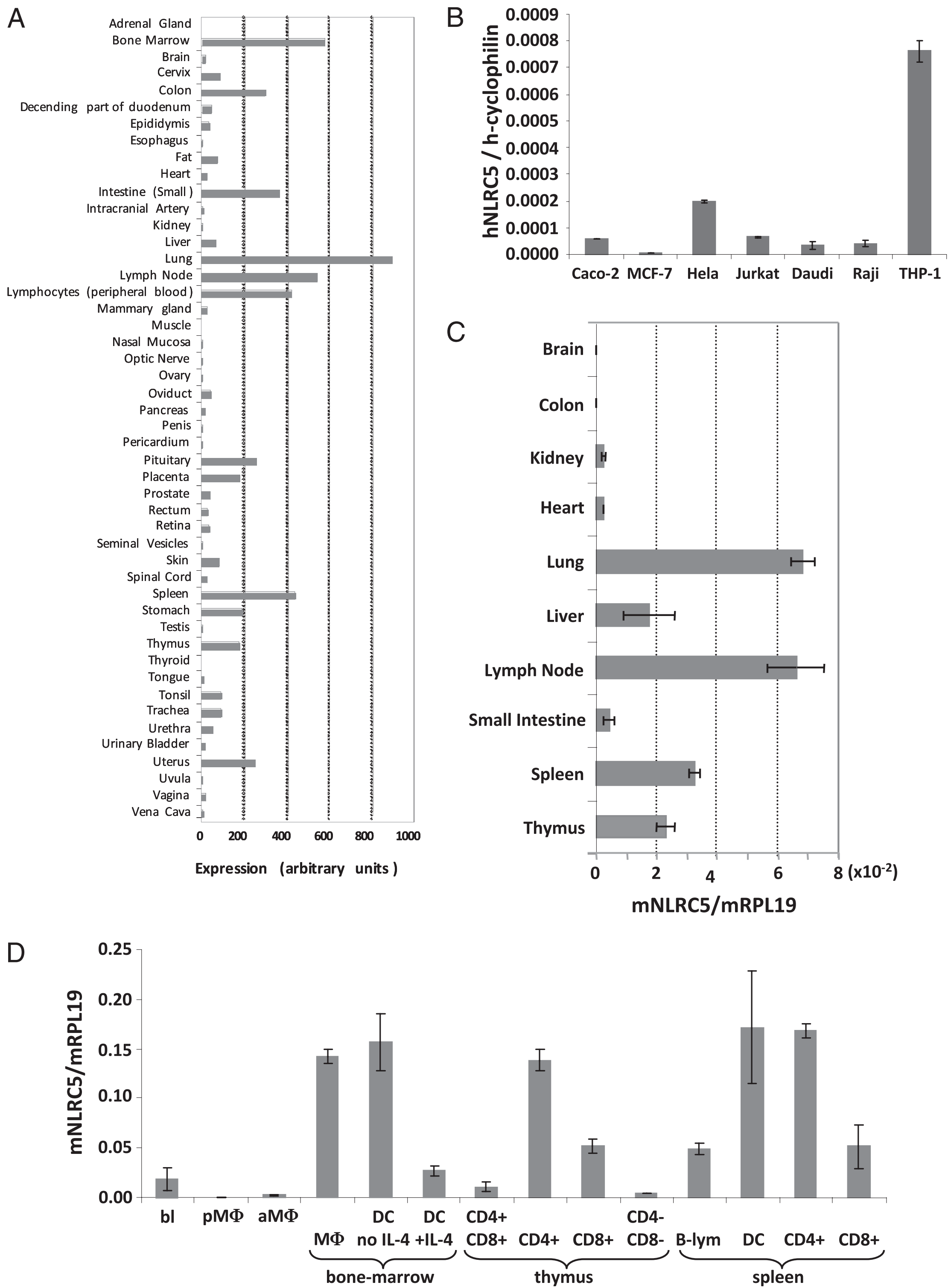

FIGURE 2. Expression profile of NLRC5 mRNA in human and mouse tissues and cell populations. *A–D*, Expression of NLRC5 mRNA in human (*A*, *B*) and murine (*C*, *D*) tissues and cell populations was determined by qPCR. *A*, Expression profile of NLRC5 mRNA in various human organs using the Human Rapid-Scan expression panels. *B*, Expression of NLRC5 mRNA in human cell lines. *C*, Expression profile of NLRC5 in various mouse organs. *D*, Expression of mouse NLRC5 in immune cells. aMϕ, alveolar macrophages; Bl, blood; pMϕ, peritoneal macrophages.

overexpressing cells was performed, followed by Western blotting using anti-Flag, anti-tubulin (cytosol marker), and anti-p84 (nuclear marker) Abs in both HEK293T cells (Fig. 4*D*) and HeLa cells (Supplemental Fig. 3*B*), thus demonstrating cytosolic and nuclear distribution of NLRC5. Collectively, these results show that, in addition to CIITA, NLRC5 is the second identified NLR protein that shuttles between the cytosol and the nucleus.

NLRC5 represses proinflammatory signal transduction pathways

We transfected the NLRC5-Flag expression vector along with AP-1–, p53-, NF-κB–, or ISRE-responsive luciferase reporter constructs in HEK293T cells. As positive controls, we cotransfected plasmids that code either for the response element binding protein (p53 for p53-luc) or an upstream activator in the pathway (IPS-1 for

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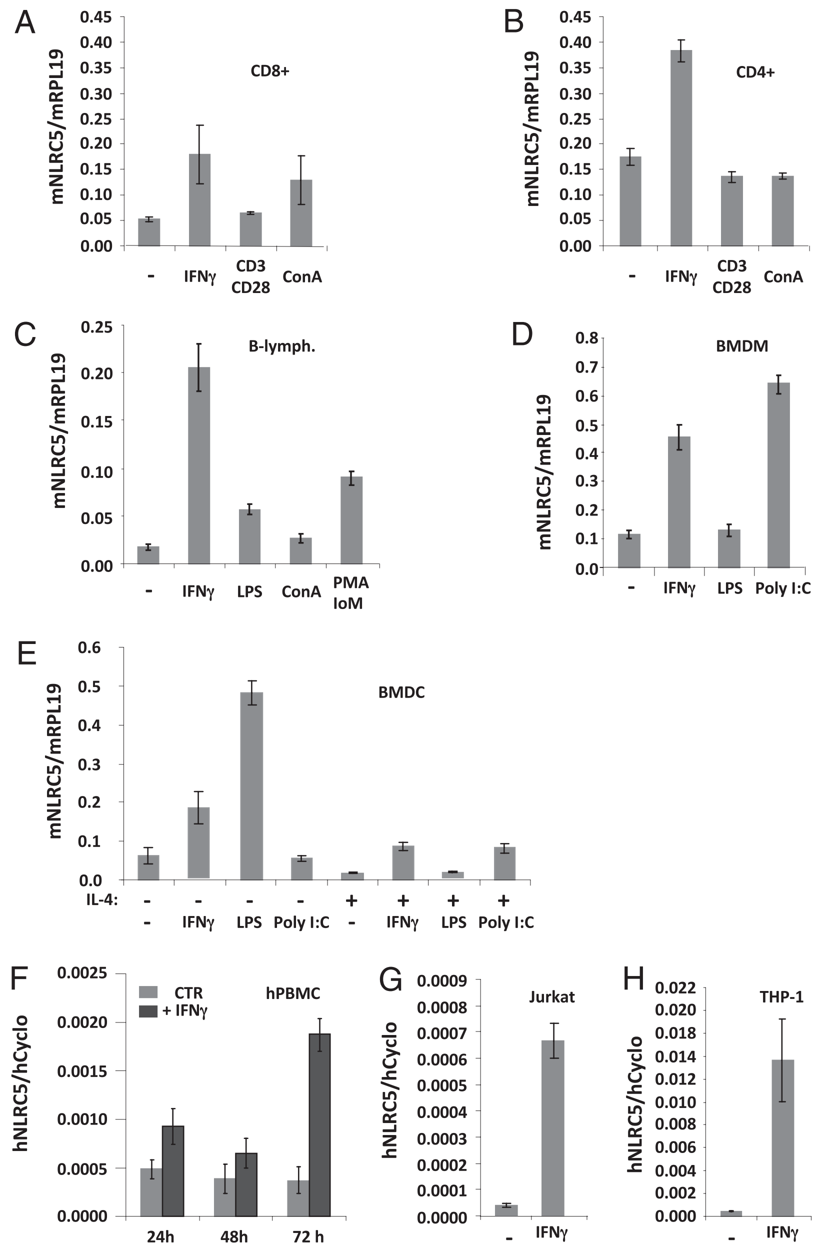

FIGURE 3. NLRC5 is an inducible gene. NLRC5 expression by qPCR in primary mouse cells (*A–E*), human primary cells (*F*), and human cell lines (*G*, *H*). Gene expression is shown as the ratio of NLRC5 transcripts relative to mouse RPL19 or human cyclophilin expression (±SD) measured in triplicates. *A* and *B*, Mouse T lymphocytes were isolated from the spleen and treated with 10 ng/ml IFN-γ, 3 ng/ml anti-CD3, and anti-CD28 Abs, or with 10 μg/ml Con A for 24 h. *C*, Mouse B lymphocytes were isolated from spleen and treated with 10 ng/ml IFN-γ, 3 ng/ml anti-CD3, and anti-CD28 Abs, 10 μg/ml Con A or 0.1 μM PMA plus 0.5 μM ionomycin for 24 h. *D*, Bone marrow cells were differentiated to macrophages for 5 d (BMDM) and then treated for 24 h with 10 ng/ml IFN-γ, 100 ng/ml LPS, or 100 μg/ml poly(I:C). *E*, Bone marrow cells were differentiated to DCs (BMDC) for 7 d in the absence or in the presence of IL-4. Cells were then stimulated with 10 ng/ml IFN-γ, 100 ng/ml LPS, or poly(I:C) for 24 h. *F*, Human PBMCs were separated from peripheral blood by centrifugation using Ficoll-Paque and were stimulated with 10 ng/ml IFN-γ for various times. *G* and *H*, Jurkat T lymphocyte cell line (*G*) or THP-1 human macrophage-like cell line (*H*) were stimulated for 24 h with 10 ng/ml IFN-γ. IoM, ionomycin.

ISRE-luc, MyD88 for Igκ-luc), or we treated the cells with reagents that are known to activate specific pathways (PMA for AP-1-luc or TNF for Igκ-luc). We first observed that the overexpression of NLRC5 in HEK293T cells was not sufficient to stimulate any of the classical signal transduction pathways studied here (Supplemental Fig. 4). Strikingly, NLRC5 overexpression resulted in the potent downregulation of NF-κB– (Fig. 5*A*) and type I IFN-dependent (Fig. 5*B*) signal transduction. NLRC5 was also found to repress AP-1–dependent signaling, albeit more moderately than NF-κB– and type I IFN-dependent pathways (Fig. 5*C*). In contrast, overexpression of NLRC5 did not alter p53-dependent signaling (Fig. 5*D*). To gain insights into the mechanism through which NLRC5 mediates the repression of signal transduction pathways, we further analyzed NF-κB signaling and quantified by immunofluorescence the effect of NLRC5 overexpression on the nuclear translocation of NF-κB p65 following TNF stimulation in HeLa cells. Interestingly, while NLRC5-expressing cells displayed an initial delay in NF-κB p65 translocation, no difference was observed by 30 min post-TNF stimulation (Fig. 5*E*). This observation suggests that NLRC5 overexpression mainly affects the NF-κB pathway at a level downstream of p65 NF-κB nuclear translocation; however, the delayed kinetics of p65 NF-κB nuclear translocation also shows that NLRC5 could dampen NF-κB signaling in its cytosolic steps. The fact that NLRC5 could dampen multiple signal transduction pathways, and that this repression could be, at least in part, attributed to the nuclear pool of NLRC5, suggests that the protein could either act as a general transcriptional repressor or repress a posttranscriptional step. To test these two possibilities, we repeated the experiment presented in Fig. 5*A* and measured both luciferase activity and the expression of the luciferase gene by qPCR. We observed a strong correlation between luciferase activity and mRNA expression (Fig. 5*F*), therefore strongly suggesting that NLRC5 overexpression blocks the NF-κB pathway at a transcriptional level in human epithelial cells.

Silencing of NLRC5 in RAW264.7 macrophages

To gain further insight into the function of NLRC5, we used a lentiviral-mediated shRNA delivery and expression system to silence NLRC5 expression in the murine macrophage cell line RAW264.7. Whereas NLRC5 was expressed at low levels in

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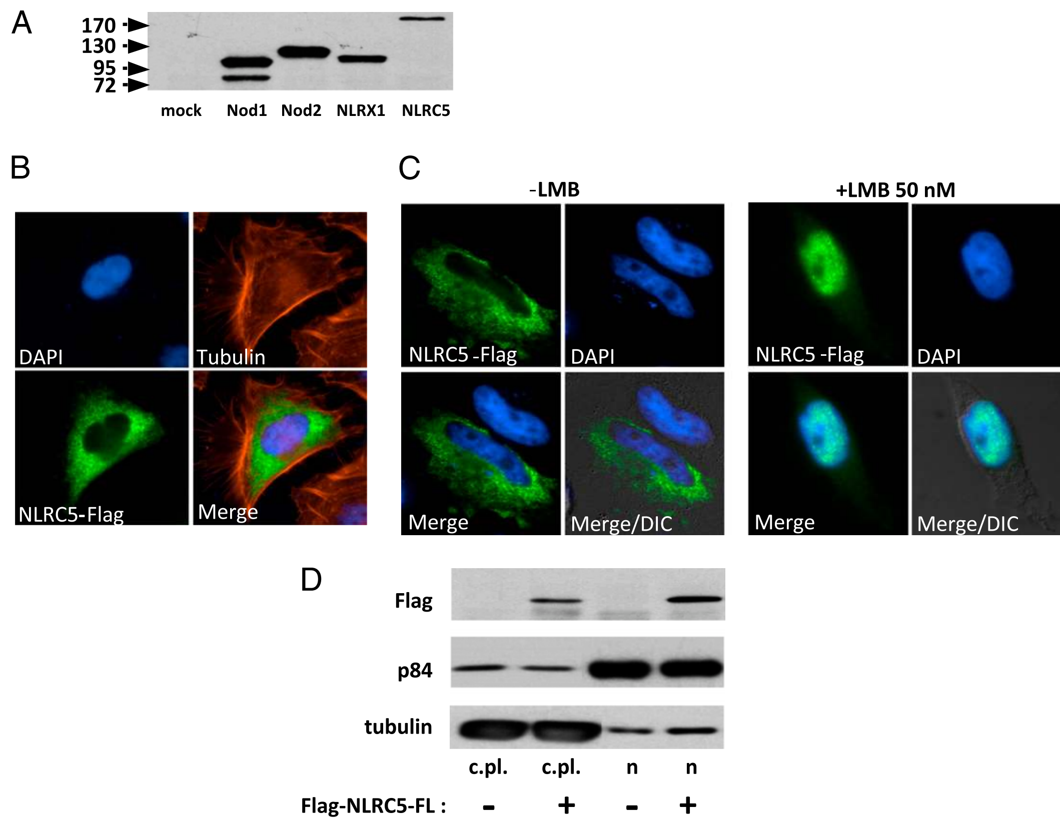

FIGURE 4. NLRC5 localizes to the cytosol and the nucleus. *A*, HEK293T cells were transfected with NLRC5-Flag, Nod1-Flag, Nod2-Flag, or NLRX1-Flag expressions vectors, and protein expression was determined by Western blotting using anti-Flag Ab. *B*, HeLa cells were grown on coverslips, transfected with NLRC5-Flag, fixed, and analyzed by immunofluorescence using anti-Flag and anti-tubulin Abs as indicated. Nuclei were stained using DAPI. *C*, HeLa cells were grown on coverslips, transfected with NLRC5-Flag, stimulated with vehicle (*left panels*) or 50 nM LMB (*right panels*) for 4 h before fixation, and analyzed by immunofluorescence using an anti-Flag Ab. Nuclei were stained using DAPI (original magnification ×63). *D*, Cytoplasmic and nuclear fractions of NLRC5-Flag–transfected HEK293T cells were analyzed by Western blotting using Abs against Flag, tubulin (cytoplasmic fraction), and p84 (nuclear fraction). c.pl, cytoplasmic fraction; n, nuclear fraction.

RAW264.7 cells as compared with BMDMs in basal conditions, its mRNA levels were strongly upregulated by LPS and IFN-γ (Supplemental Fig. 5*A*). RAW264.7 cells were next transduced for 2–5 d with viral particles encoding shRNA constructs against either murine NLRC5 (shNLRC5) or a scramble control (shCTR), and NLRC5 expression was determined by qPCR. The optimal conditions for transient knockdown of NLRC5 expression was found to be between day 3 and day 4 posttransduction (Supplemental Fig. 5*B*), consistently providing silencing efficiencies >60% in IFN-γ–stimulated cells, and these conditions were used for further studies.

NLRC5 silencing selectively amplifies the induction of proinflammatory cytokines in RAW264.7 macrophages

RAW264.7 macrophages were transduced with lentiviral particles encoding shNLRC5 or shCTR for 3 d, stimulated overnight with either LPS or IFN-γ, and RNA and cell culture supernatants were collected to determine NLRC5 expression (Fig. 6*A*) and cytokine secretion by ELISA (Fig. 6*B*), respectively. Interestingly, silencing of NLRC5 resulted in significant upregulation of proinflammatory cytokines (IL-6, IL-1β, and TNF) secretion, as well as reduced levels of the anti-inflammatory cytokine IL-10 (Fig. 6*B*). In contrast, LPS-mediated secretion of RANTES was found to be unaffected by NLRC5 silencing. Collectively, these results suggest that NLRC5 plays a critical role in limiting the inflammatory cytokine program in RAW264.7 macrophages stimulated with LPS. The effect of NLRC5 silencing on cytokine secretion induced by IFN-γ was more complex; whereas IFN-γ was a poor activator of cytokine secretion in shCTR-transduced cells, we observed that NLRC5 silencing resulted in a significant upregulation of both the proinflammatory cytokine TNF and the anti-inflammatory cytokine IL-10 (Fig. 6*B*), without affecting the levels of the other mediators tested in this study. To determine whether NLRC5 modulated cytokine levels at either transcriptional or posttranscriptional levels, we performed kinetics experiments to determine by qPCR the levels of cytokine expression in shNLRC5 versus shCTR RAW264.7 cells stimulated with LPS. Interestingly, cytokine mRNA levels strongly correlated with protein secretion (Fig. 7), therefore implying that NLRC5 modulates cytokine expression mainly at a transcriptional level. Importantly, the most dramatic effect of NLRC5 knockdown on cytokine mRNA expression, following LPS stimulation, was the strong reduction observed for IL-10 mRNA (Fig. 7*D*).

Silencing of NLRC5 augments the cell surface expression of CD40 and MHC class I

CD40 is a costimulatory molecule that is expressed at the cell surface of activated APCs. Using flow cytometry, we observed that NLRC5-silenced cells displayed enhanced cell surface expression of CD40 following stimulation with LPS and IFN-γ, as compared with CTR-silenced cells (Fig. 8*A*). Quantifications showed that while LPS or IFN-γ stimulation resulted in an ∼3-fold upregulation of CD40 cell surface expression in shCTR-transduced cells, LPS or IFN-γ stimulation resulted in a 9-fold and a 12-fold higher CD40 expression in shNLRC5-transduced cells, respectively (Fig. 8*B*). However, the increased cell surface expression of CD40 in NLRC5-silenced cells did not correlate with altered levels of

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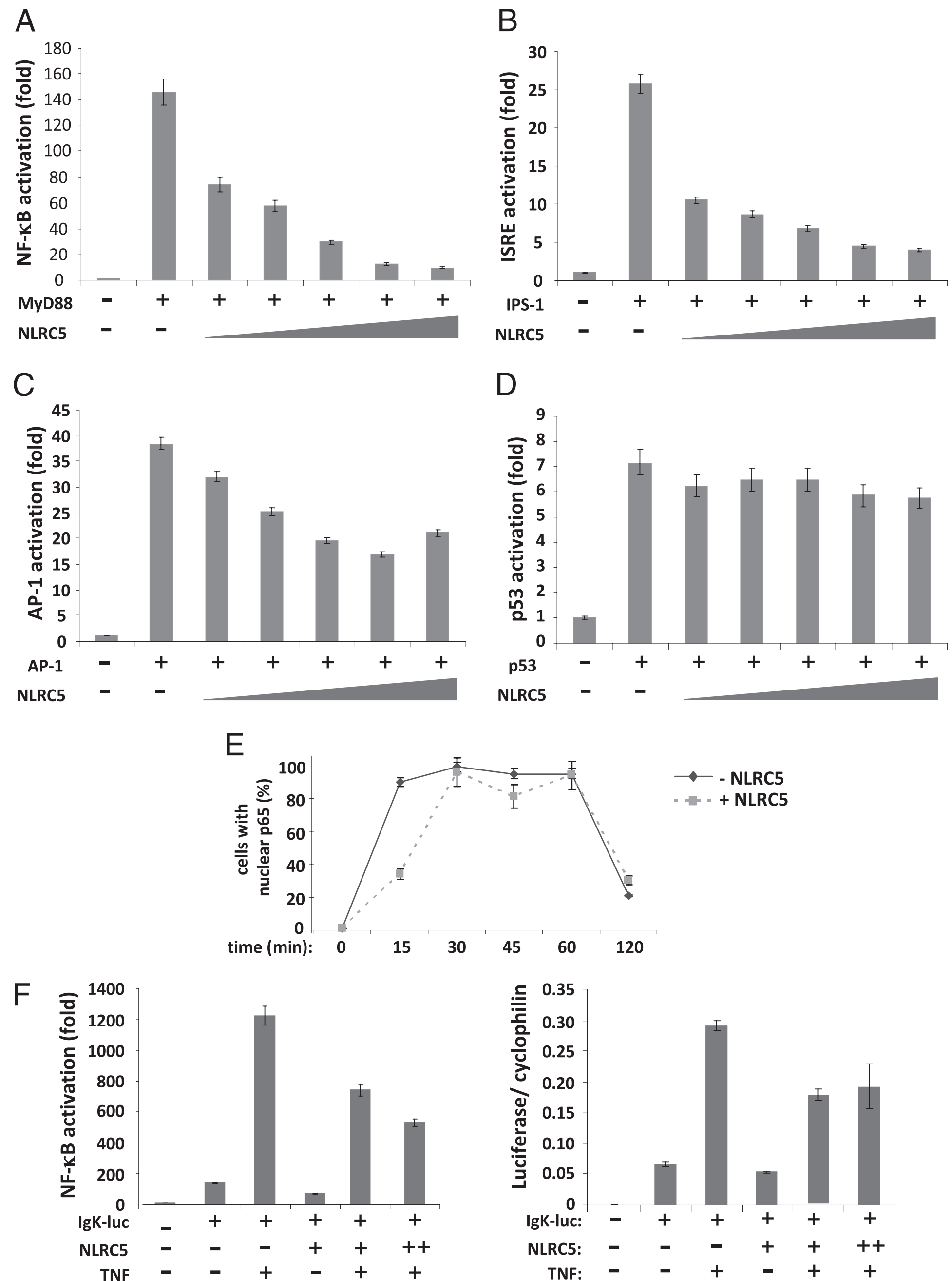

FIGURE 5. NLRC5 represses proinflammatory signal transduction pathways. *A–D*, HEK293T epithelial cells were transfected with increasing amounts (50, 100, and 200 ng) of NLRC5 expression vector together with luciferase-reporter constructs responsive to NF-κB (*A*), ISREs (*B*), AP-1 (*C*), or p53 (*D*). Overexpression of p53 or IPS-1 or treatment with PMA or TNF was used as a positive control. *E*, HeLa cells grown on glass coverslips were transfected with 200 ng of NLRC5-Flag expression vector and stimulated the following day with 100 ng/ml TNF for various times, as indicated. Cells were fixed, permabilized, and analyzed by immunofluorescence using anti-p65 and anti-Flag Abs. NF-κB p65 nuclear translocation in NLRC5$^+$ or NLRC5$^-$ cells was performed on randomly selected fields. *F*, HEK293T epithelial cells were transfected overnight with 200 ng of NLRC5 expression vector together with luciferase-reporter construct responsive to NF-κB. Cells were then stimulated with 100 ng/ml TNF for 4 h, and samples were collected for luciferase (*left panel*) or qPCR analysis of firefly *Luc* gene expression. Data shown are the means ± SEM of duplicates and are representative of two or three independent experiments.

CD40 mRNA (Fig. 8*C*); this surprising result could possibly be attributed to the decreased secretion of IL-10 in the cells (see *Discussion*). Finally, similar results were obtained when analyzing the cell surface expression of MHC class I Ag-presenting molecules (Supplemental Fig. 6). Taken together, analyses of cytokine secretion profiles by ELISA and of the cell surface expression of macrophage activation markers in NLRC5-silenced cells all indicated that NLRC5 plays a pivotal role in limiting the induction of inflammatory pathways upon stimulation with LPS or IFN-γ.

Discussion

We report in this study the first characterization of NLRC5, a member of the NLR family of intracellular proteins implicated in innate immunity. One of the most striking features of NLRC5 is that the expression of the gene appears to be tightly controlled and subject to key levels of regulation. Indeed, we commonly observed >100-fold NLRC5 mRNA induction when cells were stimulated with IFN-γ and, for some cell populations, with LPS or poly(I:C). The observed substantial upregulation of NLRC5 expression upon inflammatory stimulation was not restricted to immune cells; poly (I:C) stimulation, which recapitulates some aspects of viral infection, also resulted in significant induction of NLRC5 mRNA levels in stimulated epithelial cells (data not shown). These observations are strongly suggestive of a role for NLRC5 in controlling cellular responses in inflammatory conditions.

Our results have identified a key role for NLRC5 in dampening inflammatory pathways in macrophages. Because NLRC5 expression is also strongly induced by inflammatory cues [IFN-γ, LPS, poly(I:C)], our view is that this NLR protein may not be a constitutive repressor of inflammation, but rather it would act to limit the intensity or the duration of inflammatory signaling. Strikingly, our results in NLRC5-silenced RAW264.7 cells demonstrated that NLRC5 does not simply dampen cytokine secretion upon inflammatory stimulation, as RANTES and IL-10 secretion were

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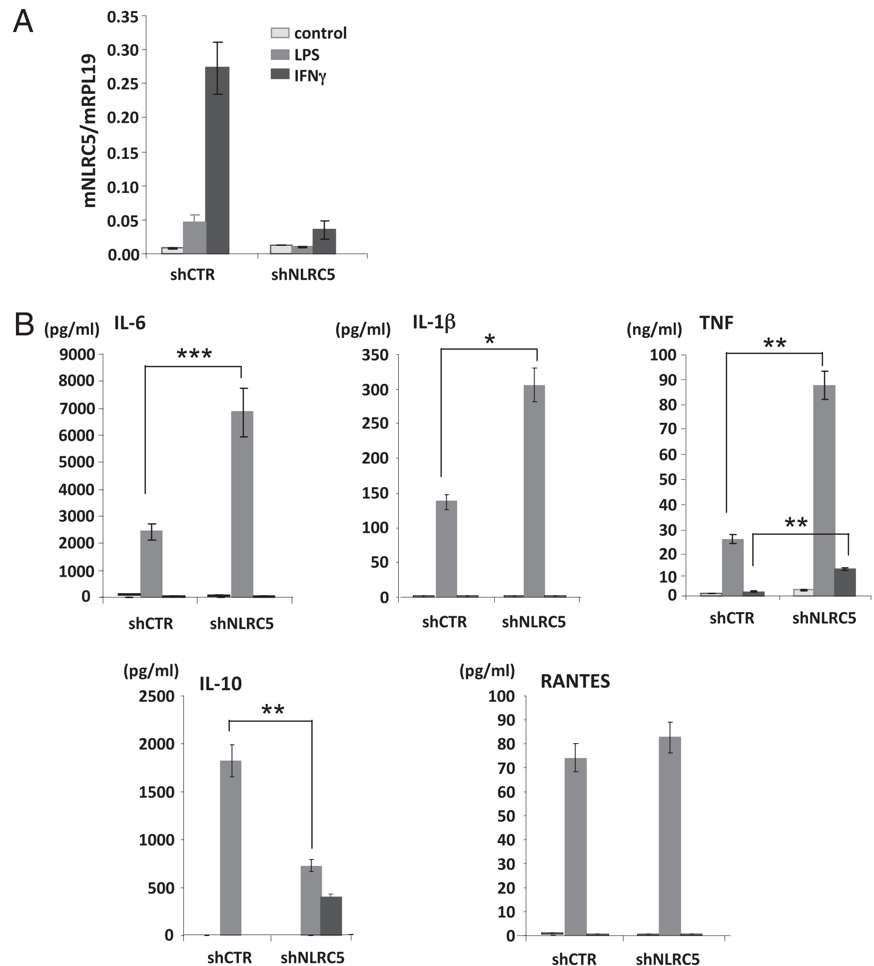

FIGURE 6. NLRC5 silencing amplifies the secretion of proinflammatory cytokines in RAW-264.7 macrophages. *A* and *B*, RAW264.7 macrophages were transduced with lentiviruses expressing either shCTR or shNLRC5 shRNAs, and on the third day they were stimulated with 100 ng/ml LPS or 10 ng/ml IFN-γ for 24 h. After harvesting the cells, RNA was purified and NLRC5 expression was measured by qPCR (*A*). Cell supernatants were used to measure cytokine secretion by ELISA (*B*). $*p < 0.1$; $**p < 0.04$; $***p < 0.0004$.

unaffected and reduced by NLRC5 knockdown, respectively, in LPS-stimulated cells. These results suggest that, at least in the case of LPS stimulation, NLRC5 orchestrates a coordinated cellular response that includes the upregulation of anti-inflammatory mediators (IL-10) and the limitation of the induction of proinflammatory cascades (proinflammatory cytokines IL-1β, TNF, and IL-6 as well as costimulatory molecules). In the case of IFN-γ stimulation, we obtained similar results, with the notable exception that NLRC5 appeared to limit both proinflammatory and anti-inflammatory pathways, since IL-10 secretion was upregulated in NLRC5-silenced cells stimulated with IFN-γ.

Besides NLRC5, several NLR family members possibly act as negative regulators of inflammatory pathways. In particular, NLRP12 (also known as Monarch-1) has been shown to repress IL-1R–associated kinase-1–mediated proinflammatory signaling, resulting in the dampening of the response of macrophages to TLR agonists and *Mycobacterium tuberculosis* infection (18). NLRP12 is found to be constitutively expressed in macrophages, and NLRP12 mRNA levels are downregulated by these proinflammatory stimuli. These observations suggest that, in contrast to NLRC5, NLRP12 may act as a constitutive brake on inflammation, which would need to be removed to allow full induction of inflammatory pathways. Similarly, NLRC3, which is preferentially expressed in T cells, may act as a constitutive suppressor of T cell activation pathways since its expression is profoundly inhibited by T cell-activating molecules, such as CD3, CD28, or PMA/ionomycin (19). However, the putative inhibitory role of NLRC3 on T lymphocytes was only suggested by overexpression studies and awaits confirmation using gene silencing or in NLRC3-deficient mice.

Using overexpression studies in HEK293T and HeLa cells, we identified a key role for NLRC5 in dampening proinflammatory signal transduction pathways (type I IFN, NF-κB, and AP-1) at a transcriptional level. Interestingly, our data also suggest that, at least for NF-κB signaling, NLRC5 likely blocks signal transduction in the nuclear steps (downstream of NF-κB p65 nuclear translocation). Because this NLR protein blocks several independent proinflammatory pathways, we speculate that it may act as a direct transcriptional modulator by selectively interfering with the action of transcription factors on the promoters of inflammatory (such as IL-6, TNF, or IL-1β) or anti-inflammatory (IL-10) genes. Further investigation is required to elucidate the mechanism through which NLRC5 could tip the transcriptional balance toward a less inflammatory environment.

By comparing the effect of NLRC5 silencing on LPS-induced cytokine secretion (ELISA) and expression (qPCR), we clearly noticed a strong correlation, thus showing that the anti-inflammatory effects of NLRC5 are mediated at a transcriptional level. In contrast, while NLRC5-silenced RAW264.7 cells displayed increased cell surface expression of CD40 as compared with control cells, we did not observe a correlation at the mRNA level, arguing for the implication of an additional posttranscriptional level of regulation. Although we do not have a definitive explanation for this observation, we speculate that the blunted levels of IL-10 in NLRC5-silenced RAW264.7 might participate in this regulation. Indeed, several reports have identified a key role for this anti-inflammatory cytokine in dampening the expression and/or stability of inflammatory genes, such as cytokines or costimulatory molecules (20, 21). Importantly, and along these lines, the most dramatic effect of NLRC5 silencing in

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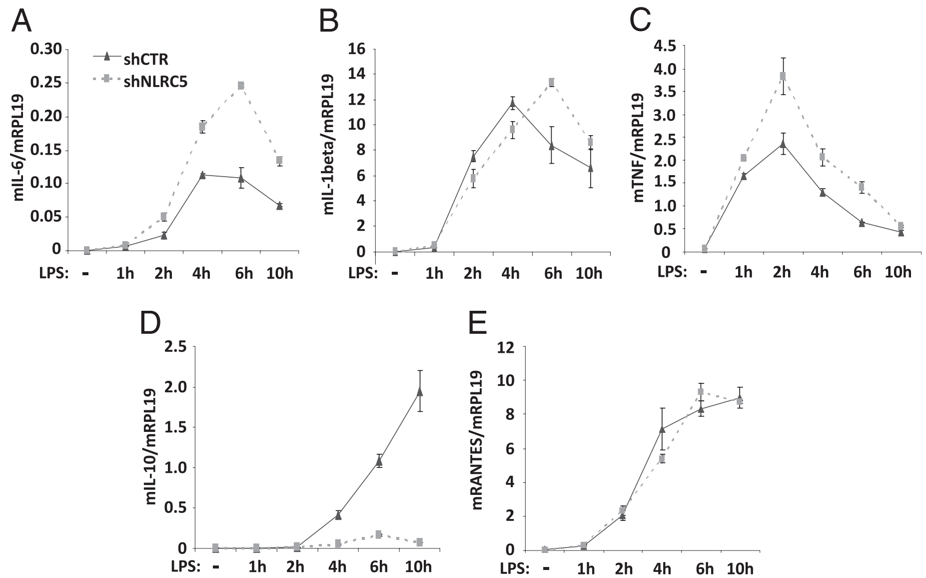
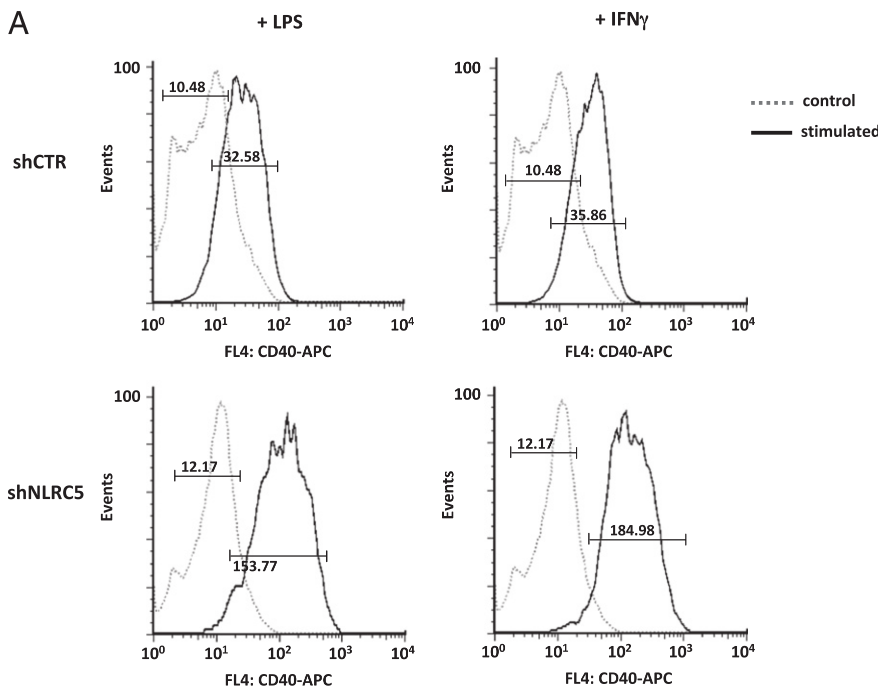
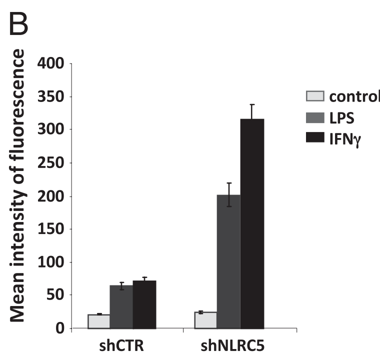


FIGURE 7. NLRC5 silencing modulates the expression of cytokines in RAW264.7 macrophages. *A–E*, RAW264.6 macrophages were transduced with lentiviruses expressing either shCTR or shNLRC5 shRNAs, and on the third day they were stimulated with 100 ng/ml LPS for 24 h. After harvesting the cells, RNA was purified and expression of IL-6 (*A*), IL-1β (*B*), TNF (*C*), IL-10 (*D*) and RANTES (*E*) measured by qPCR.

our assays was the reduction in IL-10 expression following LPS stimulation. Further investigation will be needed to identify if the major mechanism responsible for the downregulation of proinflammatory mediators by NLRC5 is through direct inhibition of proinflammatory signal transduction pathways or via the induction of IL-10.

IL-4 was recently shown to play a key role in DC polarization by repressing IL-10 and promoting IL-12 secretion following LPS stimulation (22). Interestingly, our results show that BMDCs grown in the presence of IL-4 displayed a strongly blunted expression of NLRC5 as compared with those grown in the absence of this cytokine (see Figs. 2*D*, 3*E*). Therefore, and in light of our results





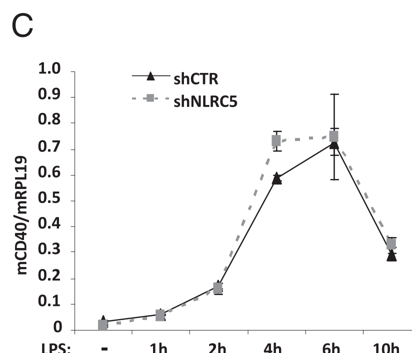


FIGURE 8. NLRC5 silencing augments LPS- and IFN-γ–induced expression of CD40 costimulatory molecule in RAW264.7 cells. *A*, RAW264.7 cells were transduced with lentiviruses expressing either shCTR or shNLRC5 shRNA, and on the third day they were stimulated with 100 ng/ml LPS or 10 ng/ml IFN-γ for 24 h. Cells were stained with anti-mCD40–allophycocyanin. The dotted and bold lines indicate the nontreated control and stimulated cells, respectively. The horizontal bars represent the positive area defined by the stained cells. *B*, Mean intensity of fluorescence is represented in column chart (calculated from the positive area defined by the stained cells). *C*, The expression of CD40 mRNA was determined as in Fig. 7.

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linking NLRC5 to IL-10 secretion in LPS-stimulated RAW264.7 macrophages, it will be interesting to determine whether the downregulation of NLRC5 expression contributes to the inhibitory effect of IL-4 on IL-10 production in response to LPS.

Two recent articles that were published while this manuscript was in revision also reported the initial characterization of NLRC5 (23, 24). Kuenzel et al. (24) proposed that NLRC5 was involved in the induction of IFN-dependent antiviral defense and, in agreement with our results, demonstrated that NLRC5 expression was potently induced by IFN-γ and poly(I:C) stimulation. However, in contrast to our results, the authors proposed that NLRC5 could activate, albeit modestly, ISRE-dependent reporter genes. Cui et al. (23) identified, similar to our results, that NLRC5 was expressed predominantly in immune cells and tissues and acted as a negative regulator of proinflammatory signaling. Using shRNA-mediated NLRC5 silencing in various macrophage cell populations, including RAW264.7 cells, the authors identified a key role for NLRC5 in dampening the expression of several inflammatory mediators (including IL-6, TNF, and type I IFNs) in response to LPS stimulation. Interestingly, Cui et al. (23) demonstrated that NLRC5 acted on NF-κB and type I IFN pathways directly by interacting and inhibiting the function of the IκB kinase complex and the sensors Rig-I and Mda-5, respectively. These results could possibly explain the delayed translocation of NF-κB p65 that we observed in TNF-stimulated NLRC5-expressing cells (see Fig. 5*E*). However, the study of Cui et al. did not investigate the potential role for NLRC5 as a modulator of proinflammatory signaling in the nucleus, nor did it evaluate the impact of NLRC5-dependent IL-10 secretion in LPS-stimulated macrophages.

In summary, we have characterized NLRC5, a new member of the NLR family of intracellular proteins implicated in immune regulation, and identified a crucial role for NLRC5 in limiting the activation of inflammatory pathways in murine macrophages. Because NLRC5 appears to modulate pathways downstream of IFN-γ and LPS, our results strongly suggest that NLRC5, rather than being a bona fide PRM like several other NLR proteins, might act as a regulator of cell signaling pathways. Finally, our observations suggest that NLRC5 might represent an interesting target for modulating immune responses in inflammatory disorders, autoimmune diseases or sepsis.

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Disclosures

The authors have no financial conflicts of interest.

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