

Regulation of Class I Major Histocompatibility Complex (MHC) by Nucleotide-binding Domain, Leucine-rich Repeat-containing (NLR) Proteins*

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Background: Multiple functions have been ascribed to NLRC5 including MHC-I transcription and cytokine responses.

Results: We generated *Nlrc5*^{-/-} mice and showed that Nlrc5 increases classical and nonclassical MHC-I and causes removal of the gene-silencing H3K27me3 histone modification on MHC-I promoter.

Conclusion: Nlrc5 regulates MHC-I expression.

Significance: NLRC5, with CIITA, constitutes an NLR subfamily that regulates MHC.

Most of the nucleotide-binding domain, leucine-rich repeat (NLR) proteins regulate responses to microbial and damage-associated products. Class II transactivator (CIITA) has a distinct function as the master regulator of class II major histocompatibility complex (MHC-II) transcription. Recently, human NLRC5 was found to regulate MHC-I in cell lines; however, a host of conflicting positive and negative functions has been attributed to this protein. To address the function of NLRC5 in a physiologic setting, we generated an *Nlrc5*^{-/-} strain that contains a deletion in the exon that encodes the nucleotide-binding domain. We have not detected a role for this protein in cytokine induction by pathogen-associated molecular patterns and viruses. However, *Nlrc5*^{-/-} cells showed a dramatic decrease of classical (H-2K) and nonclassical (Tla) MHC-I expression by T/B lymphocytes, natural killer (NK) cells, and myeloid-monocytic lineages. As a comparison, CIITA did not affect mouse MHC-I expression. *Nlrc5*^{-/-} splenocytes and bone marrow-derived macrophages were able to up-regulate MHC-I in response to IFN- γ ; however, the absolute levels of MHC-I expression were significantly lower than WT controls. Chromatin immunoprecipitation of IFN- γ -treated cells indicates that Nlrc5 reduced the silencing H3K27me3 histone modification, but did not affect the activating AcH3 modification on a MHC-I promoter. In summary, we conclude that Nlrc5 is important in the regulation of MHC-I expression by reducing H3K27me3 on MHC-I promoter and joins CIITA as an NLR subfamily that controls MHC gene transcription.

NLR³ proteins conduct a variety of functions, including inflammasome-associated function, NF- κ B/MAPK activation,

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³ The abbreviations used are: NLR, nucleotide-binding domain, leucine-rich repeat; NBD, nucleotide-binding domain; CIITA, class II transactivator;

interferon (IFN) regulation, cell death, and autophagy (1). These functions are primarily elicited in response to pathogen-associated molecular patterns (PAMPs) or damaged-associated molecular patterns (DAMPs). In contrast, the class II transactivator (CIITA) has remained the only NLR that has been verified in both human and mice as a master transactivator of all major histocompatibility II (MHC-II) genes as well as genes encoding their accessory proteins, such as the invariant chain and H-2DM proteins (2). CIITA is shown to conduct its function by association with the DNA-binding proteins, NF- κ B and Regulatory Factor X (RFX), which recognize their cognate binding motifs, the X and Y elements that are conserved in all MHC-II promoters (3, 4). The assembly of this enhanceosome is necessary for the modification of MHC-II promoters via histone acetylation and methylation. Additionally, CIITA interacts with insulator factor, CCCTC binding factor (CTCF), to assure against gene silencing (5).

In addition to the regulation of MHC-II, CIITA was simultaneously found by our group and another laboratory to regulate human MHC-I (6, 7). Both groups showed that mutant cell lines with a defective CIITA protein have defective MHC-I expression and that wild type CIITA complemented this defect. Additionally, CIITA mediates MHC-I gene transactivation through a regulatory DNA motif known as the α site in its promoter. Subsequently, others have shown that CIITA activates MHC-I uniquely in a TATA box binding protein (TBP)-associated factor-1-independent fashion (8).

Recently, human NLRC5 was found to positively regulate MHC-I in cell lines (9, 10), although another group obtained the opposite finding and concluded that NLRC5 reduced MHC-I and CD40 expression (11). The former group overexpressed NLRC5 in Jurkat T cells and found that the expression of class I MHC as well as B2M, Transporter associated with antigen processing (TAP), and Large multifunctional protease (LMP) was enhanced, whereas siRNA targeting NLRC5 caused

PAMP, pathogen-associated molecular pattern; DAMP, damaged-associated molecular pattern; BMDM, bone marrow-derived macrophages; NK, natural killer; Q-PCR, quantitative PCR; Q-RT-PCR, quantitative RT-PCR; FAM, 6-carboxyfluorescein; TAM, *N,N,N,N*-tetramethyl-6-carboxyrhodamine.

the reduction of MHC-I. Similar to CIITA, they found that the Walker A and B motifs common among ATP-binding proteins are critical for both the MHC-I-inducing function of NLRC5 as well as its nuclear translocation. Furthermore, overexpressed NLRC5 was detected on MHC-I promoter by chromatin immunoprecipitation (ChIP), suggesting a direct binding of NLRC5 to the MHC-I promoter.

NLRC5 is highly expressed by cells in the immune system, in particular by cells of the lymphocytic and myeloid-monocytic cell lineages (11–13). It can be found in the nucleus (9–11), although cytoplasmic localization (10, 14) is also reported, and an overexpressed NLRC5 has been shown to shuttle between the nucleus and cytoplasm. Its expression is highly inducible by IFN- γ and LPS (9, 11, 14), and IFN- γ and CMV induced NLRC5 promoter activation via the Jak/Stat pathway (14).

However, a host of additional functional roles has been attributed to this protein, although a caveat is that many of the functional studies on NLRC5 have been limited to cell lines. Two groups showed a negative regulatory function for NLRC5. Cui *et al.* (15) demonstrated the interaction of endogenous NLRC5 with endogenous I κ B kinases (IKK) α/β to reduce the phosphorylation state of IKKs. They further showed that siRNA directed at NLRC5 resulted in elevated TNF and IL-6 in response to LPS in both human and mouse macrophage cell lines. This group also found that gene knockdown resulted in elevated antivesicular stomatitis virus response including enhanced expression of type-I interferons. In agreement with this, Benko *et al.* (11) showed an inhibitory effect of NLRC5 overexpression on NF- κ B-, AP-1-, and IFN-sensitive response element reporter assays in the mouse macrophage line RAW264, whereas knocking down the gene resulted in an enhancement of IL-1 β , IL-6, TNF, CD40, and class I MHC in response to LPS or IFN- γ . By contrast, two other groups showed that NLRC5 positively regulates cytokine responses to nucleic acids and viruses. Kuenzel *et al.* (14) used a forced dimerization system involving the caspase recruitment domain (CARD) domain of NLRC5 and found elevated gamma interferon activation site (GAS)- and IFN-sensitive response element-driven reporter activation. These authors further found that NLRC5 overexpression moderately enhanced IFN- α and its downstream targets, OAS1 and P58 repressor protein kinase (PRKRIR), whereas siRNA targeting NLRC5 reduced the IFN- α pathway in response to CMV. In agreement with these authors, Neerinx *et al.* (13) found that siRNA targeting NLRC5 in primary human dermal fibroblasts caused a drop in IFN- γ and Rantes (regulated on activation normal T cell expressed and secreted) in response to poly(I:C) or Sendai virus.

Our group used a gene knockdown approach to show that NLRC5 reduction resulted in the lack of inflammasome activation in THP-1 cells and in primary human peripheral blood monocytic cells and further that NLRC5 can reconstitute an inflammasome system (16). This result is supported by another group that also found that overexpressed NLRC5 can reconstitute an inflammasome (17). However, this same group produced an *Nlrc5*^{-/-} mouse strain and found that inflammasome activation, cytokine response to LPS and IFN- γ , type-I IFN and cytokine responses to poly(I:C), RNA virus, stimulatory DNA,

and DNA viruses were all normal in this strain. Furthermore, *in vivo* response to poly(I:C) was also normal in *Nlrc5*^{-/-} mice.

The composite results revealed a rather confusing picture regarding the function of NLRC5, and the study of *Nlrc5*^{-/-} mice did not identify a positive function (17). To readdress the function of NLRC5, we produced an *Nlrc5* gene deletion mouse targeting the NBD-encoding exon. We observed a profound effect of *Nlrc5* deletion on MHC-I expression in lymphocytes and NK cells and explored the effect of *Nlrc5* on the histone modification of an MHC-I promoter. The deletion of *Nlrc5* resulted in a less dramatic reduction of MHC-I on B cells and myeloid-monocytic cells, leading us to ask whether residual CIITA might account for the differential effect of *Nlrc5* on T/NK, which do not express CIITA *versus* B cells that do express CIITA. In addition, we also explored possible explanations for the discrepant finding where others describe a role for *Nlrc5* in regulating cytokine production.

EXPERIMENTAL PROCEDURES

Animal Care and Use—C57BL/6J, *Ciita*^{-/-} (18), and *Nlrc5*^{-/-} mice were bred in-house in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Generation of *Nlrc5*^{-/-} Mice—The *Nlrc5*^{-/-} NBD was ablated as described in the legend for Fig. 1. Exons 3 and 4 were replaced with the neomycin cassette, and the targeting vector was confirmed by restriction digest analysis and sequencing. The linearized vector was electroporated in IC1 (C57BL/6/N) embryonic stem cells (InGenious Targeting Laboratories). Homologous recombinants were verified by Southern blot analysis before injection into BALB/c blastocysts. The resulting chimeras were mated to female C57BL/6J mice, and the resulting heterozygotes were crossed to yield homozygous *Nlrc5*^{-/-} mice, which were screened using duplex PCR with each reaction containing the following primers: P1 WT NBD primer, 5'-TAGAGTTGTATCACTGTGTGGCTGAGACCC-3'; P2 Neo primer, 5'-GGAAGTTCGCTAGACTAGTACGCGTG-3'; P3 common primer, 5'-TTTAGCTGATAGATACAGAGCCTTGACC-3', yielding bands of 650 bp for WT and 330 bp for *Nlrc5* knock-out.

Primary Cell Isolation—Single cell splenocyte and thymocyte suspensions were made by gentle dissociation of tissues with a 1-ml syringe plunger over a 70- μ m strainer followed by red blood cell lysis using ACK buffer (Invitrogen), and viable cells were counted using Trypan blue staining and stimulated as indicated. Peritoneal macrophages were isolated by seeding peritoneal lavage cells in DMEM (Invitrogen)+10% FCS (HyClone®) at a density of 5×10^5 cells/well in 96-well plates. After 16 h, the nonadherent cells were removed by washing three times with PBS (Invitrogen), and the remaining adherent cells were used for stimulation. Bone marrow-derived macrophages (BMDM) were generated as described previously (19). All cultures were supplemented with $1 \times$ penicillin/streptomycin (Sigma).

Viral Infection—BMDM were seeded at 2×10^5 cells/well in flat bottom 96-well plates and infected with herpes simplex virus (HSV) (multiplicity of infection of 0.2, 1, and 10) or SeV

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(80 HA unit/ml). Supernatant was collected from triplicate wells of virus or mock-infected cells and tested for IFN- β secretion by ELISA.

PAMP, DAMP, and Cytokine Stimulation—BMDM were seeded at 2×10^5 cells/well in flat bottom 96-well plates and were left untreated or stimulated for 8 h with 200 ng/ml *Escherichia coli* K12 LPS (InvivoGen), 4 μ M CpG (InvivoGen), or 50 μ g/ml poly(I:C) (InvivoGen). Supernatant was collected for ELISA, and cells were harvested for mRNA transcript analysis. Inflammasome induction was mediated using an LPS prime of 200 ng/ml for 3 h followed by the addition of 400 μ g/ml Imject[®] alum (Pierce) for 6 h or 2 mM ATP (InvivoGen) for 40 min. IFN- γ stimulation of BMDM and splenocytes used 500 units/ml of recombinant murine IFN- γ (PeproTech) at the indicated time points.

Flow Cytometry—The following reagents were used for flow cytometric staining: CD3-FitC, CD19-eFluor[®]-450, biotin-H2-K^b, biotin-H2-D^b, Avidin-APC, CD11c-PE-Cy5, and CD4-PE (eBioscience); NK1.1-PE-Cy7 and CD8-Pacific Blue (BioLegend); I-A^b-PE (PharMingen); Live/Dead[®] Aqua and Violet (Molecular Probes); CD45-Pacific Orange (Invitrogen); CD11b-PE (Beckman Coulter). All data were collected on CyAn[™] (Beckman Coulter) or LSRII (BD Biosciences), and sorting was performed using MoFlo[™] or MoFlo[™] XDP (Beckman Coulter). All data were analyzed using FlowJo (TreeStar).

ELISA—Detection of murine TNF- α , IL-6, and IL-1 β was performed using BD OptEIA[™] kits (BD Biosciences) according to the manufacturer's instructions. IFN- β ELISA utilizes the following reagents: 4 μ g/ml rat anti-mouse IFN- β capture antibody (Yamasa Corp.); polyclonal rabbit anti-mouse IFN- β (1:2000) (R&D Systems); goat anti-rabbit IgG HRP (Cell Signaling Technology), which was used at 1:2000. Standard curve for IFN- β was generated using purified mouse IFN- β (PBL, Inc.).

Quantitative Real-time PCR—Total RNA was harvested using TRIzol[®] (Invitrogen) followed by reverse transcription using M-MLV (Invitrogen) and oligo(dT) according to manufacturer's instructions. Q-RT-PCR for murine *Tnfa*, *Il6*, and *Il1b* was normalized to *Gapdh* using SYBR[®]-Green (Applied Biosystems) and the following primer sets: osTnfa, 5'-CCCTCACACTCAGATCATCTTCT-3', oaTnf, 5'-GCTACGACGTGGGCTACAG-3'; osIl6, 5'-CAACCACGGCCTTCCCTACTT-3', oall6, 5'-CACGATTTCCAGAGAACATGTG-3'; osIl1b, 5'-CAACCAACAAGTGATATTCTCCATG-3', oall1b, 5'-GATCCACACTCTCCAGCTGCA-3'; osGapdh, 5'-CCTCGTCCCCTAGACAAAATG-3', oaGapdh, 5'-TCTCCAATTTGCCACTGCAA-3'. Q-RT-PCR for murine *H2-k1*, *B2m*, *Tla*, *Cd1d*, *I-Ab*, and *Nlrc5* was normalized to 18 S RNA using TaqMan[®] primer/probe sets (Applied Biosystems).

ChIP Assay—Total splenocytes were plated at a cell density 10×10^6 , stimulated with IFN- γ as indicated (500 units/ml), but only nonadherent lymphocytes were used for ChIP analyses as described previously (20, 21). Following cytokine stimulation, cells were cross-linked with 1% formaldehyde for 10 min. Cross-linking was stopped by the addition of 0.125 M glycine for 5 min. Cells were lysed in SDS lysis buffer and were sonicated to generate an average of 500 bp of sheared DNA. Sonicated lysates were precleared with salmon sperm-coated agarose

beads (Millipore), and then lysates were immunoprecipitated overnight with 5 μ g of indicated antibody for H3k27me3 (Abcam), acetylated H3 (Millipore), H3 (Millipore), and isotype control antibody (Millipore). Following a 2-h immunoprecipitation with 50 μ l of salmon sperm-coated agarose beads, samples were washed with each of the following buffers: low salt buffer, high salt buffer, LiCl, and Tris-EDTA, and samples were eluted with SDS elution buffer. Following elution, cross-links were reversed overnight with 5 M NaCl at 65 °C, and immunoprecipitated DNA was isolated using phenol:chloroform:isopropyl alcohol mix (Invitrogen) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using primers spanning the proximal promoter region of H2K1, forward, 5'-CCGCGACGCTGGATA-3', reverse, 5'-GGCGATTCGCGACTTCTG-3', and probe, 5'-FAM-AGTCCACGCAGCCCGCAGAACT-TAM-3'; I-A^b forward, 5'-CTGGATGCTTCCTGAGTTTTGG-3', reverse, 5'-TGGT-TTTACTACAGCTATGTTTTGCA-3', and probe, 5'-FAM-CAATTGGCAAGCTTTGACCCCAA-TAM-3'; and GAPDH forward, 5'-GAGCGGCCCGGAGTCT-3', reverse, 5'-GGATTACGGGATGGGTCTGA-3', and probe, 5'-FAM-AAGTATTAGGAACAACCCACGCGCC-TAM-3'. Values generated from the Q-PCR reaction were normalized to the total DNA levels (input), and the control IgG levels were subtracted from each immunoprecipitation sample analyzed.

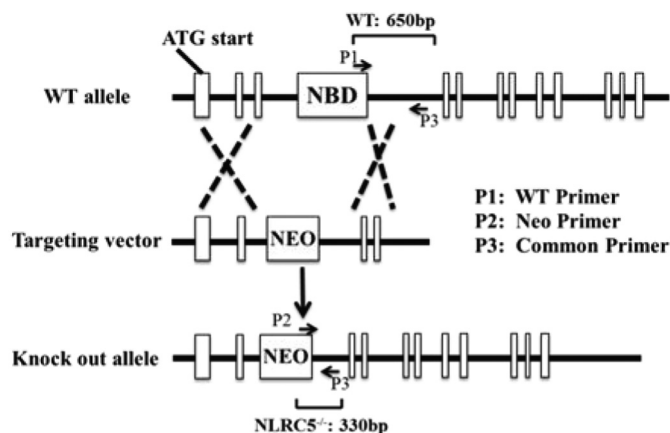
Statistical Analysis and Graphing—Statistical analysis was done using an on-line program through Vassar College, VassarStats: Website for Statistical Computation. Graphing was done using GraphPad Prism[®].

RESULTS

Production of an Nlrc5^{-/-} Mouse Strain by Targeted Deletion—We produced an *Nlrc5^{-/-}* strain by homologous targeted replacement of these residues (AKRPFQSYGSSPRRKNSSKKQQL) from exons 3 and the entire exon 4 with the *Neo* cassette (Fig. 1A). Exons 3 and 4 respectively encode a portion of the CARD-like domain and the entire NBD domain. The targeting construct was generated with an ~5-kb region of the 5' end flanking the targeted deletion and an ~2.3-kb region of the 3' end flanking sequence. The targeting vector was confirmed by restriction digestion and direct sequencing. The linearized vector was electroporated into IC1 C57BL/6N embryonic stem cells, and homologous recombinants were verified by Southern blot analysis before injection into BALB/c blastocysts. The resulting chimeras were mated to female C57BL/6J mice, and the resulting heterozygotes were crossed to yield homozygous *Nlrc5^{-/-}* mice. As indicated in Fig. 1B, a common reverse primer, a NEO primer, and an NBD primer generated the expected bands of 650 bp for the WT samples and 330 bp for the gene-targeted samples.

Deletion of Nlrc5 Does Not Affect Cytokine Production in Response to PAMPs and Viruses—As described earlier, previous work has indicated both positive and negative roles for Nlrc5 in cytokine production to PAMPs and viruses (11, 13–15). Additionally, studies in human cells have indicated a role for this protein in inflammasome activation (16, 17). To determine the contribution of mouse *Nlrc5* to these processes, we first assessed the production of *Tnfa*, *IL6*, and *IL-1b* mRNA in

A Targeted ablation of *Nlrc5* allele



B PCR analysis of WT and *Nlrc5*^{-/-} mice

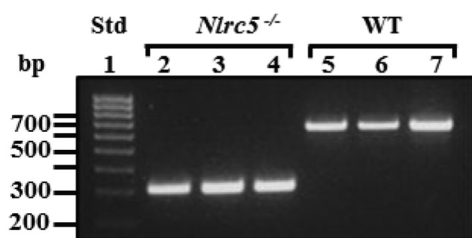


FIGURE 1. Targeted ablation of the *Nlrc5* NBD coding region. A, targeting construct with ~5-kb region of homology in the 5' end (targeting the NBD coding region and upstream exon) and ~2.3-kb homology 3' to the NBD coding region. Primers for duplex PCR genotype analysis are indicated: P1, WT *Nlrc5* NBD-specific primer; P2, neomycin-specific primer; P3, common primer. B, PCR products from WT and *Nlrc5*^{-/-} genomic DNA using primers described in A. Lane 1, DNA standard (Std); lanes 2–4, tail genomic DNA from three *Nlrc5*^{-/-} mice; lanes 5–7, tail genomic DNA from three WT mice.

response to LPS and CpG by BMDM. Cytokine mRNA levels were generally similar between WT and *Nlrc5*^{-/-} cells with the only statistically significant differences being less than 1.5-fold (Fig. 2A). This is reflected at the protein level for TNF and IL6. For IL-1 β production, we additionally tested response to LPS + ATP, or LPS + alum, because IL-1 β secretion requires two signals including the activation of pro-IL1 β production by LPS and inflammasome activation by ATP or alum to cause the post-translational processing of pro-IL1 β to mature IL-1 β via caspase-1. Again, deletion of *Nlrc5* did not cause a difference. Parallel experiments were performed with LPS-activated resting peritoneal macrophages because BMDM have been expanded *in vitro* and might generate artificial findings (Fig. 2B). However, no difference was found between WT and *Nlrc5*^{-/-} peritoneal macrophages. As an additional evaluation, we also tested for the production of interferon β (IFN- β) protein in response to a DNA virus, HSV, and an RNA virus, Sendai virus. No significant difference in cytokine production was observed between WT and *Nlrc5*^{-/-} BMDM with the exception of the high multiplicity of infection of HSV, which showed a statistically significant, but less than 25% decrease in IFN- β production by *Nlrc5*^{-/-} cells at 24 h (Fig. 2C). *Ifnb* mRNA induction by PAMPs including LPS, CpG, and poly(I:C) was similar between WT and *Nlrc5*^{-/-} BMDM (Fig. 2D). These results confirm those from a previous study, which did not find

a detectable role for *Nlrc5* in cytokine induction by various PAMPs and microbes (17).

Deletion of *Nlrc5* Affects Classical and Nonclassical MHC-I Expression but Not CD1 and MHC-II Expression—The work by Meissner *et al.* (9) showed that in human cell lines, the gene knockdown of *NLRC5* caused a reduction of MHC-I expression. Previously, we and others have noted the high levels of *NLRC5* gene expression in immune cells, particularly on T and B lymphocytes and NK cells (13, 16). To test this in a physiologic setting, we used CD45 as a marker for all hematopoietic cells and assessed MHC-I (H2-K^b and H2-D^b) expression by splenocytes. C57BL/6 lacks the *H2-L* allele, and thus expression of this gene cannot be tested in this strain. CD45⁺ hematopoietic cells from *Nlrc5*^{-/-} mice showed a significant reduction of both H-2K and H-2D expression. Among CD45⁺ cells, one population has a more dramatic loss of MHC-I than another, as revealed by the two peaks of MHC-I staining in the *Nlrc5*^{-/-} sample (Fig. 3A). A further separation of the cells shows that splenic T cells showed the most dramatic loss of MHC-I followed by NK cells, B lymphocytes, dendritic cells (CD11b^{int}/CD11c⁺), and macrophages (CD11b⁺, CD11c^{lo}). To further assess whether this is reflected at the transcriptional level, we sorted different populations, isolated RNA, and tested for the expression of a classical MHC-I gene (*H-2k1*), the common partner of MHC-I (*B2m*), and a nonclassical MHC-I gene (*Tla*). All three were largely reduced in the lymphoid lineage (Fig. 3B). In contrast, *Cd1d2*, which encodes the CD1 protein that presents lipids and is not generally co-regulated with MHC-I, is not altered in *Nlrc5*^{-/-} cells. Finally, *Nlrc5* deletion did not affect MHC-II gene expression represented by *I-A* in B cells, but it did cause a slight, but significant, decrease in MHC-II expression in CD11b⁺ cells. Decreased MHC-I expression in the absence of *Nlrc5* is not restricted to the spleen as CD4⁻CD8⁻ (double negative), as well as CD4 and CD8 single positive thymocytes, in *Nlrc5*^{-/-} mice all have reduced MHC-I (Fig. 3C). We observed similar MHC-I expression between WT and *Nlrc5*^{-/-} DP thymocytes, which may reflect low *Nlrc5* mRNA expression in the WT population (Fig. 3, C and D).

NLRC5 Does Not Alter the Extent of IFN-induced MHC-I Expression—Prior work with CIITA indicates that this protein regulates both basal and inducible expression of MHC-II (2, 22). To determine whether *Nlrc5* has a parallel function, we assessed the induced expression of MHC-I by IFN- γ , a known inducer of MHC-I. An analysis of T and B cells indicates that although the basal level of MHC-I is reduced in *Nlrc5*^{-/-} cells, the induction of MHC-I by IFN- γ in both populations remained intact (Fig. 4A). These data are consistent with MHC-I mRNA analysis showing similar relative increases in *H2-k1* message with IFN- γ treatment; however, the MHC-I levels in *Nlrc5*^{-/-} cells are consistently reduced when compared with WT controls (Fig. 4B). Additionally, IFN- γ -induced MHC-I expression occurs on *Nlrc5*^{-/-} and *Ciita*^{-/-} BMDM (Fig. 4C). IFN- γ -induced MHC-II surface expression was normal on *Nlrc5*^{-/-} BMDM but reduced on *Ciita*^{-/-} cells as expected (Fig. 4D). The decreased expression of MHC-I but not MHC-II in response to IFN- γ treatment is also reflected at the mRNA level in *Nlrc5*^{-/-} cells (Fig. 4E). These results indicate that unlike CIITA, which is crucial for both basal and IFN- γ

Nlrc5 Regulates Classical and Nonclassical Class I MHC

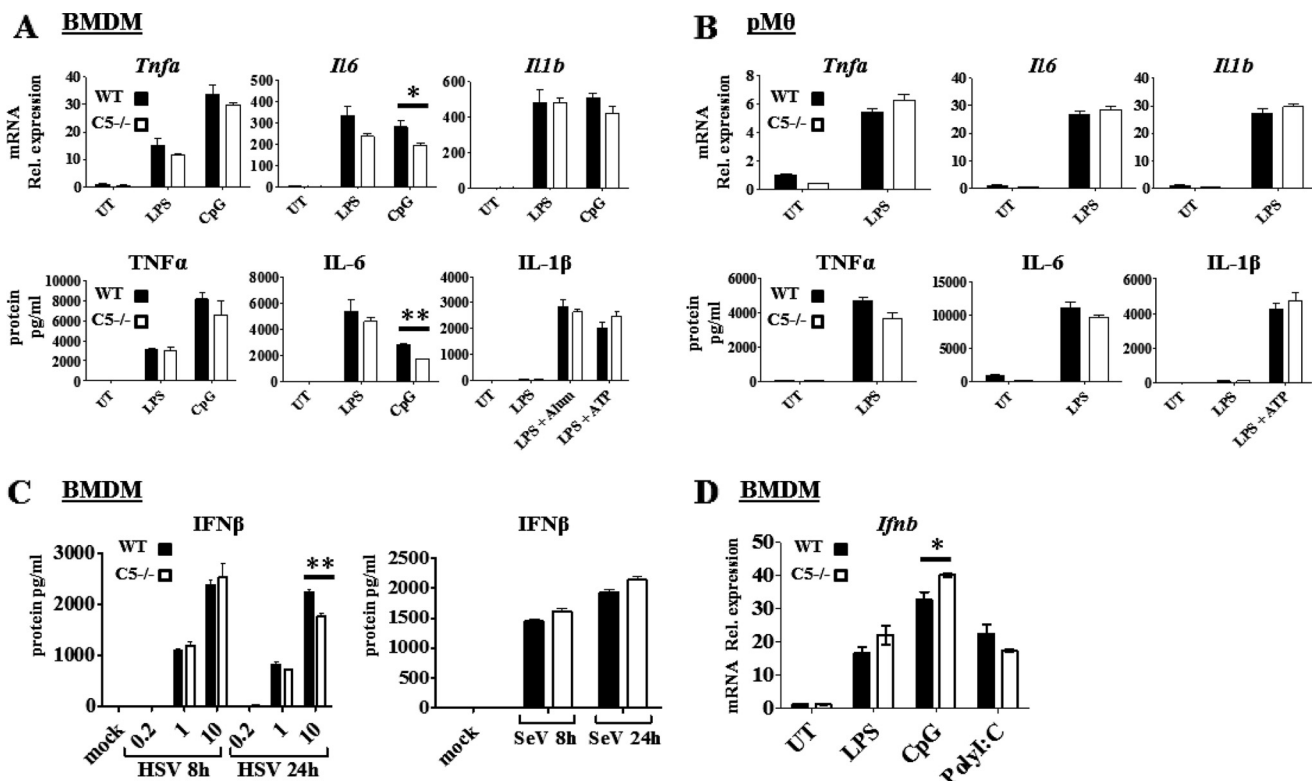


FIGURE 2. Cytokine expression by WT and *Nlrc5*^{-/-} macrophages in response to PAMP and DAMP stimulation and viral infection. *A* and *B*, Q-RT-PCR for mRNA (top) and ELISA for protein secretion (bottom) of indicated cytokines by BMDM (*A*) and peritoneal macrophages (*B*) 8 h after stimulation; data are graphed as -fold change over WT untreated control (UT). Rel. expression, relative expression. *C*, ELISA for IFN- β secretion by BMDM infected with HSV (left) or Sendai Virus (SeV) (right) at 8 and 24 h after infection. *D*, Q-RT-PCR for *Ifnb* mRNA expression in BMDM 8 h after PAMP stimulation. Data in *A*, *B*, and *D* are representative of three independent experiments. Data in *C* are representative of two independent experiments. Q-RT-PCR data are normalized to 18 S RNA expression. Error bars = S.E.; statistical analysis by two-tailed Student's *t* test, *, *p* < 0.05, **, *p* < 0.01.

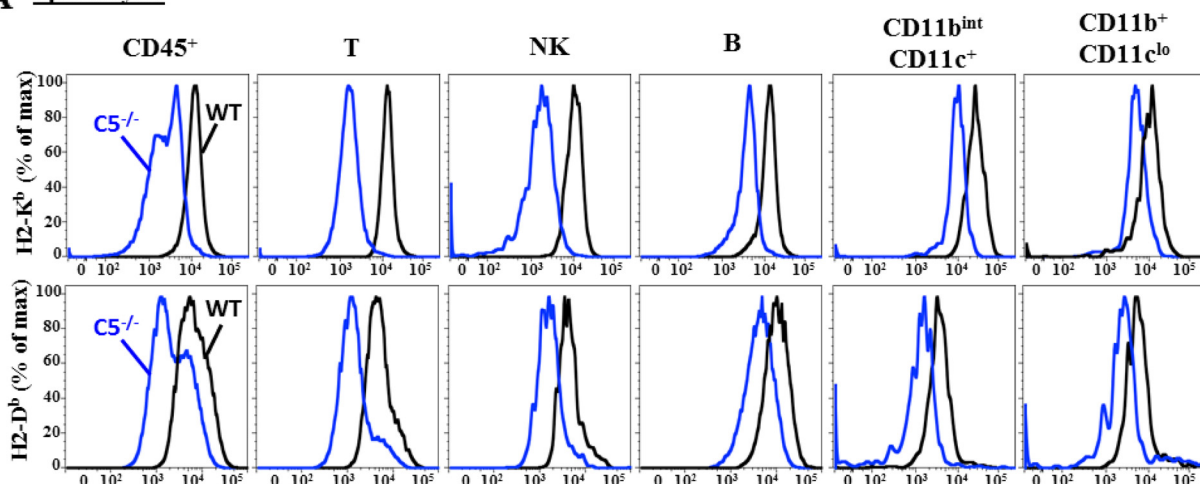
induced MHC-II expression, *Nlrc5* does not affect the extent of IFN- γ -induced up-regulation of MHC-I, but rather is necessary for basal constitutive MHC-I expression, which then affects the final amount of MHC-I expressed by cells after IFN- γ stimulation.

CIITA Does Not Regulate MHC-I Expression in Mouse B Lymphocytes—The earlier experiments indicate that the effect of *Nlrc5* deletion on classical *H-2K* and *B2m* expression is most dramatic in T cells and less so in B cells (Fig. 3, *A* and *B*). Prior work has shown that CIITA can regulate MHC-I in human cells, and furthermore, CIITA is highly expressed by B cells (6, 7). Thus one possibility is that CIITA and *Nlrc5* both regulate MHC-I expression in B cells, whereas CIITA expression does not affect MHC-I expression by T cells because the level of CIITA is negligible in mouse T cells. An assessment of *Nlrc5*^{-/-}, *Ciita*^{-/-}, and WT cells indicates that although *Nlrc5*^{-/-} T and B cells express less H-2K antigen, H-2K expression by *Ciita*^{-/-} T and B cells is similar to wild type (Fig. 5*A*). This indicates that *Ciita* deletion does not result in a detectable loss of MHC-I expression by B cells in the murine system. We also do not detect a decrease in MHC-II expression *Nlrc5*^{-/-} B cells (Fig. 5*B*), suggesting that *Nlrc5* control of MHC expression is restricted to MHC-I.

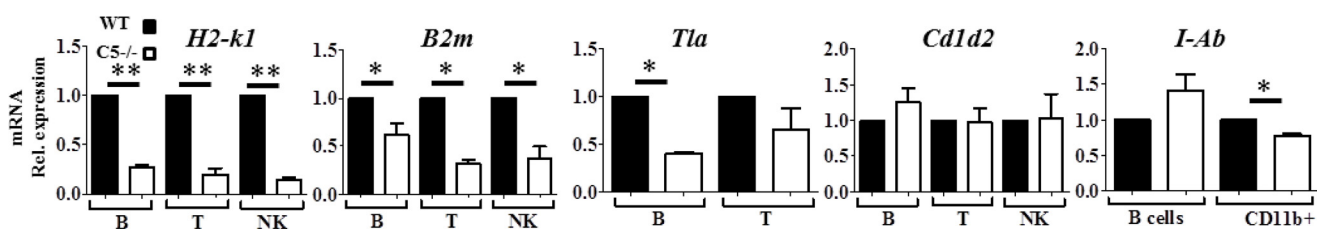
***Nlrc5* Is Required for the Removal of the Silencing Mark H3K27me3 from the MHC-I Proximal Promoter**—Although others have noted the regulation of MHC-I by *Nlrc5*, the mechanism has not been explored. Gene expression occurs within

the highly ordered context of chromatin, and open chromatin structure is required for transcription initiation. To determine the histone modification of the MHC-I promoter, levels of acetylation and trimethylation of lysine 27 on histone H3 were assessed in lymphocyte-enriched splenocytic populations and BMDM. After IFN- γ treatment, WT cells progressively lost the gene silencing H3K27me3 modification at the MHC-I promoter, *H-2K1*, which agrees with IFN- γ induction of this gene. By contrast, *Nlrc5*^{-/-} cells did not show reduced H3K27me3 modification and even exhibited increased methylated state at the 4-h time point (Fig. 6*A*). Both WT and *Nlrc5*^{-/-} cells showed similar acetylation of H3 at the MHC-I promoter upon IFN- γ treatment, which is associated with increased chromatin accessibility and gene transcription (Fig. 6*B*). The unabated and increased H3K27me3 in *Nlrc5*^{-/-} cells was specific to the MHC-I promoter as normal demethylation and acetylation were generally observed for the MHC-II (Fig. 6, *C* and *D*) and GAPDH (Fig. 6, *E* and *F*) proximal promoters upon IFN- γ treatment. An exception is the increased H2K27me3 at I-A^b promoter in the BMDM + IFN- γ group at the 4-h time point, which needs to be explored further in the future. Increased H3K27me3 at the MHC-I promoter region is not due to increased histone levels as control assays demonstrated no significant changes in total histone H3 associated with MHC-I or MHC-II (Fig. 6, *G* and *H*). These data are consistent with a mechanism in which the MHC-I promoter in *Nlrc5*^{-/-} cells becomes partially accessible in response to IFN- γ through

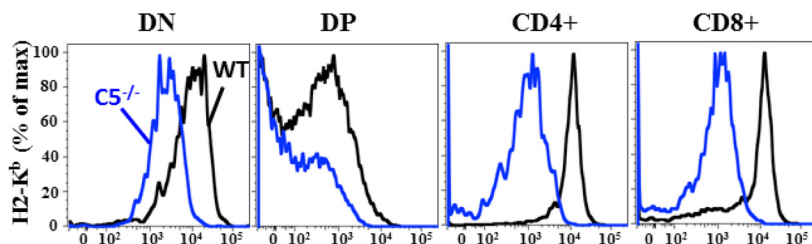
A Splenocytes



B Sorted Splenic Populations



C Thymus



D WT Thymus

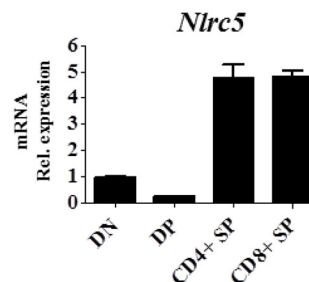


FIGURE 3. Flow cytometric and Q-RT-PCR analysis of MHC expression in WT and *Nlrc5*^{-/-} mice. *A*, surface H2-K^b and H2-K^d expression in WT (black) and *Nlrc5*^{-/-} (blue) splenocyte populations. *B*, Q-RT-PCR for *H2-k1*, *B2m*, *Tla*, *Cd1d2*, and *I-Ab* mRNA expression in sorted splenic B, T, and NK populations. *Rel. expression*, relative expression. *C*, flow cytometric analysis of H2-K^b expression in WT (black) and *Nlrc5*^{-/-} (blue) thymocyte populations. % of max, percentage of maximum; DN, double negative; DP, double positive. *D*, Q-RT-PCR for *Nlrc5* mRNA expression in sorted WT thymocyte populations. Flow cytometric data in *A* and *C* are representative of five experiments; Q-RT-PCR data are combined from at least two independent experiments and normalized to 18 S RNA expression. Error bars = S.E. Statistical analysis by two-tailed Student's *t* test, *, *p* < 0.05, **, *p* < 0.01.

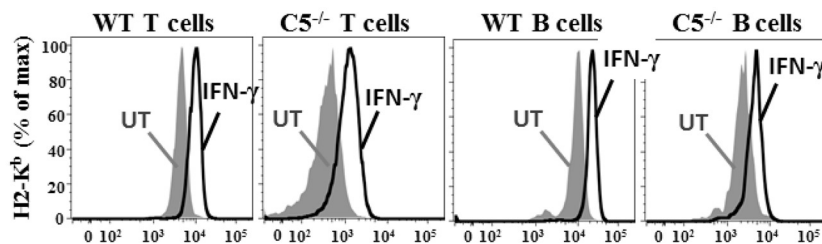
increased H3 acetylation; however, the inability to reach WT levels of MHC-I expression after IFN- γ stimulation is likely due to an inability of *Nlrc5*^{-/-} cells to remove H3K27me3 at the MHC-I promoter.

Detection of a 3'-End Transcript That Originates from *Nlrc5* in WT and *Nlrc5*^{-/-} Mice—While this manuscript was under review, Tong *et al.* (23) reported that deletion of *Nlrc5* by targeting the C-terminal LRR1 also decreased MHC-I expression, but additionally increased NF- κ B-dependent cytokine and type-I interferon production in response to various stimuli. Our studies did not indicate an NF- κ B or a type-I interferon phenotype, and we considered the possibility that our *Nlrc5* knock-out strategy (which targeted the nucleotide-binding domain) may allow for expression of *Nlrc5* variant proteins that fail to induce normal MHC-I expression, but are sufficient to dampen

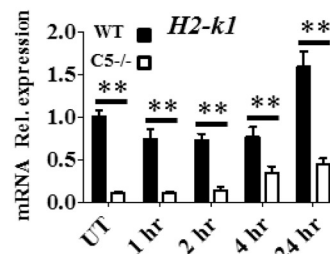
NF- κ B and type-I interferon responses. We examined *Nlrc5* mRNA expression in several resting and stimulated cell subsets using a primer/probe set that anneals to the 3' end of the mRNA (Fig. 7A, *Primer/Probe*). We found equivalent, if not increased, levels of *Nlrc5* mRNA transcripts containing this end of the mRNA in the *Nlrc5*^{-/-} splenic populations when compared with WT (Fig. 7B). Stimulation of total splenocytes or BMDM with IFN- γ (Fig. 7, C and D) or stimulation of BMDM with Toll-like receptor (TLR) agonists (Fig. 7E) resulted in up-regulation of this 3' end transcript equal to, or greater than, WT control samples. These data suggest that *Nlrc5* may be autoinhibitory at the mRNA level and leaves open the possibility that alternative splice forms of *Nlrc5* that contain the 3' end, although unable to maintain normal MHC-I expression, could be sufficient for normal regulation of NF- κ B and type-I interferon responses.

Nlrc5 Regulates Classical and Nonclassical Class I MHC

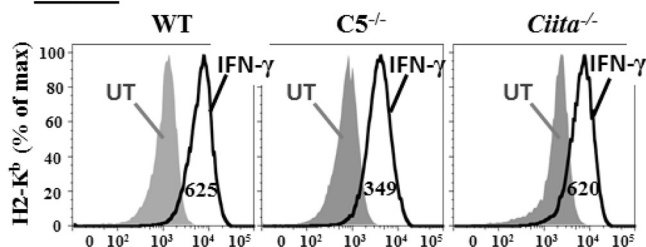
A Splenocytes



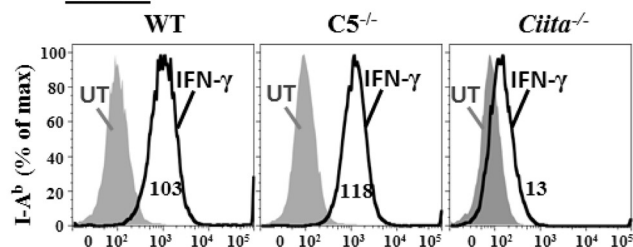
B Splenocytes



C BMDM



D BMDM



E BMDM

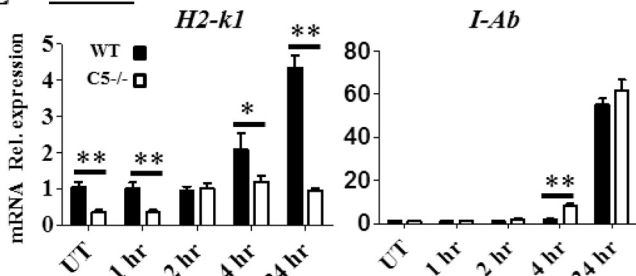
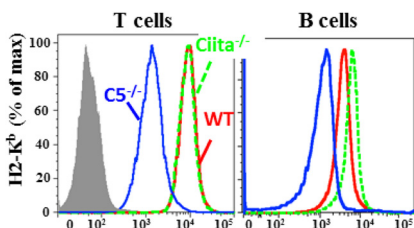


FIGURE 4. Flow cytometric and Q-RT-PCR analysis of H2-K^b expression by WT and *Nlrc5*^{-/-} splenocytes or BMDM in response to INF- γ treatment. A, surface H2-K^b expression on *in vitro* cultured splenocytes, untreated (gray filled) or INF- γ -treated for 24 h (black). % of max, percentage of maximum; UT, untreated control. B, Q-RT-PCR for *H2-k1* mRNA expression by *in vitro* cultured splenocytes from WT or *Nlrc5*^{-/-} mice at the indicated time points after INF- γ treatment. C, surface H2-K^b expression on BMDM, untreated (gray filled) or INF- γ -treated at 24 h (black). D, surface I-A^b expression on BMDM, untreated (gray filled) or INF- γ -treated at 24 h (black). Numbers under the histogram in C and D are mean fluorescence intensities. E, Q-RT-PCR for *H2-k1* and *I-Ab* mRNA expression in WT or *Nlrc5*^{-/-} BMDM treated with INF- γ . Q-RT-PCR data are combined from at least two independent experiments and normalized to 18 S RNA expression. Error bars = S.E. Data are representative of two independent experiments. Statistical analysis by two-tailed Student's *t* test, *, *p* < 0.05, **, *p* < 0.01. Rel. expression, relative expression.

A MHC I Expression



B MHC II Expression

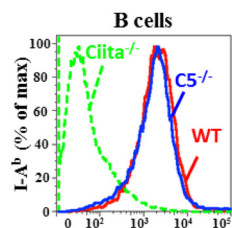


FIGURE 5. Flow cytometric analysis of MHC-I and MHC-II surface expression by WT, *Nlrc5*^{-/-}, and *Ciita*^{-/-} mice. A, surface H2-K^b expression in WT (red), *Nlrc5*^{-/-} (blue), and *Ciita*^{-/-} (green dashed) splenic B and T cells. Gray, isotype control. % of max, percentage of maximum. B, flow cytometric analysis of I-A^b expression in WT (red), *Nlrc5*^{-/-} (blue), and *Ciita*^{-/-} (green dashed) B cells. Data are representative of three independent experiments.

DISCUSSION

This study describes an assessment of the physiologic function of NLRs by studying a targeted deletion of the NBD domain of *Nlrc5*. The NBD domain for most NLRs contains Walker A and B motifs, which are conserved among ATP-binding proteins. A study of *Nlrc5* has shown that mutation of the Walker A

and B motifs destroyed the ability of this protein to induce MHC-I transcription and nuclear translocation (9). Interestingly, previous data similarly showed the critical role of the Walker A and B motifs within CIITA in mediating nuclear translocation as well as MHC-II transactivation function (24–26). Others have shown that bacterially expressed partially purified NBD domain of NLR4/ipaf binds ATP (27). Nucleotide binding by purified, full-length NLRP3 and NLRP12 was previously reported by our group with studies showing that both NLRs bind ATP and dATP but not CTP, GTP, or UTP and exhibit ATPase activity (28, 29). Others have shown that similar mutations of NOD2 also led to the loss of function (30). Thus the NBD domain plays important functions among many NLRs examined.

The gene expression pattern of *Nlrc5* indicates that it is highest in T lymphocytes, NK cells, and B cells. Indeed it has the most profound effect on MHC-I expression by T lymphocytes and NK cells, whereas its effect on MHC-I expression by B cells is less than the former two cell types. One can speculate that perhaps B cells express other MHC-I-regulating NLRs. A highly

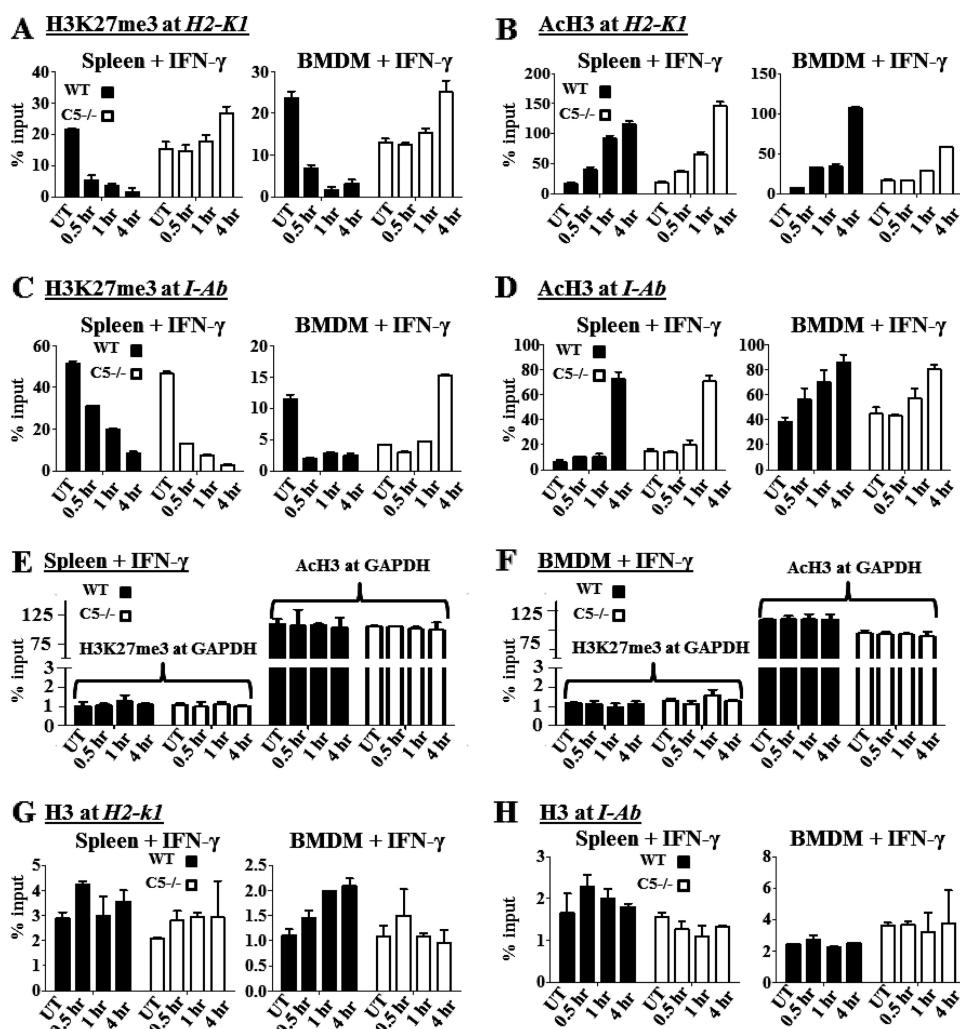


FIGURE 6. Levels of H3K27me3 and acetylated H3 at MHC-I (*H-2K1*) and MHC-II (*I-Ab*) proximal promoters in WT and *Nlr5*^{-/-} lymphocyte-enriched splenocytes and BMDM. ChIP assays were carried out in cells stimulated as indicated with IFN- γ . Lysates were immunoprecipitated with control antibody, antibody to H3K27me3 (A, C, E, and F), acetylated H3 (B, D, E, and F), or total H3 (G and H), and associated DNA was isolated and analyzed via Q-PCR using primers spanning the proximal promoter region of *H2-K1* (A, B, and G), *I-Ab* (C, D, and H), and *Gapdh* (E and F). Values for immunoprecipitations represent the mean \pm S.E. of three independent experiments. UT, untreated control.

A Schematic of Full Length *Nlr5* Transcript

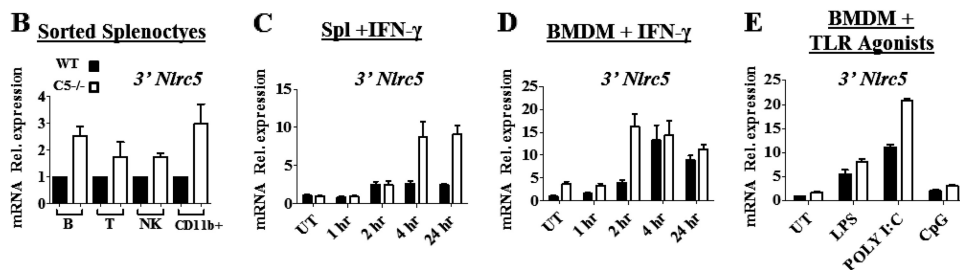
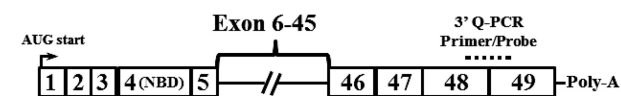


FIGURE 7. Q-RT-PCR for 3' end of *Nlr5* transcript in splenocytes and BMDM from WT or *Nlr5*^{-/-} mice. A, schematic depicting the full-length *Nlr5* transcript including the AUG start, the NBD encoded by exon 4, and the 3' Q-RT-PCR primer/probe set spanning exons 48–49 (dashed line). B–D, Q-RT-PCR with primer/probe set depicted in A. B, sorted resting splenocytes. C, total splenocytes cultured *in vitro* with IFN- γ for the indicated times. D, BMDM treated with IFN- γ for the indicated times. E, BMDM 8 h after PAMP stimulation. Data in B are combined from at least two independent experiments, and data in C–E are representative of at least two independent experiments. Q-RT-PCR data are normalized to 18 S RNA expression. Error bars = S.E. Rel. expression, relative expression; UT, untreated control.

Nlrc5 Regulates Classical and Nonclassical Class I MHC

probable candidate is CIITA, which has been found by many laboratories to update MHC-I in human cells (6, 7). The initial studies documenting the role of CIITA in MHC-I regulation were considered physiologically relevant as they relied on mutant cell lines with defective CIITA, as opposed to artificial overexpression systems. The physiologic relevance of CIITA in murine MHC-I is readdressed in this study, but we failed to find its role in the regulation of mouse MHC-I. In addition, our study also shows that although constitutive MHC-I expression is reduced in *Nlrc5*^{-/-} cells, the extent of IFN- γ -induced MHC-I expression remained unaltered despite the overall reduction in MHC-I expression when compared with WT cells. Finally, our study indicates that *Nlrc5* regulates the transcription of both classical MHC-I as well as the nonclassical *Tla* gene. Thus *Nlrc5* likely controls MHC-I through conserved motifs found in classical and nonclassical MHC-I promoter. Additionally, our data hint at the possibility that *Nlrc5* has a slight effect on MHC-II expression. For example, *I-Ab* mRNA by CD11b⁺ cells is slightly reduced in the absence of *Nlrc5*, and H3K27me3 modification of *I-Ab* promoter revealed a different kinetics in control *versus* *Nlrc5*^{-/-} cells. A recent study showed that NLRC5 can associate with RFX, a DNA-binding protein that regulates MHC-II (31). Hence additional work is necessary to assess whether *Nlrc5* cross-regulates MHC-II under some conditions.

The above data suggest that unlike CIITA, which is necessary for promoter loading of multiple transcription factors and histone modification factors including histone acetylases and methylases at MHC-II promoters (32), *Nlrc5* might play a more defined role in its regulation of MHC-I. Consistent with this working model, the data show that although *Nlrc5* is an important regulator of proper chromatin dynamics at MHC-I promoter, the effect is rather specific. Our study demonstrates that decreased expression of MHC-I in *Nlrc5*^{-/-} knock-out cells is associated with increased levels of H3K27me3 but normal histone 3 acetylation. These observations underscore the dominance of H3K27me3 in down-regulating the transcriptional status of MHC-I chromatin in the absence of *Nlrc5*.

Aside from the regulation of MHC-I, our study has not been able to identify other functions of *Nlrc5* that have been identified by previous studies, including our own, which showed that two interference RNAs targeting *Nlrc5* caused a drastic reduction of inflammasome activation by an array of stimuli in human cell lines and primary peripheral blood mononuclear cells (16). Although it is difficult to reconcile the opposing functions found for *Nlrc5* in cytokine induction by PAMPs reported in the literature, there are a number of explanations for the inability of *Nlrc5*^{-/-} cells to confirm previous findings. 1) For the previous studies performed in human cells, it is possible that there are species-specific differences, which are ample among the other NLRs. For example, NLRP1 from humans and mice serve similar functions in inflammasome activation, but the former is responsive to muramyl dipeptide (MDP), whereas the latter is activated by *Bacillus anthracis* lethal toxin (33). 2) Along this same line, it is possible that there are gene redundancies in mice that are not found in humans. Mice have 38 NLR genes, whereas humans only have 22 NLR genes. As an example, whereas humans have one copy of the NAIP gene,

mice have six copies of the gene (34, 35). 3) It is possible that the function of the *Nlrc5* is cell type- and stimulus-dependent, and we have not identified the correct stimulus in the appropriate cell types. However, the reduction of MHC-I in *Nlrc5*^{-/-} cells was observed in all cell types surveyed, so the ability of *Nlrc5* to regulate MHC-I is not cell-specific, although the degree in which *Nlrc5* controls MHC-I does vary with the cell type. 4) It is possible that deleting the NBD domain is not sufficient to eliminate all functions of *Nlrc5* and that the gene deletion might have resulted in a functional protein without NBD. At the time of this submission, another group used a different gene-targeting strategy to show that *Nlrc5* deletion resulted in a loss of MHC-I gene expression (36). During the review of this manuscript, two other groups have also shown a role for *Nlrc5* in MHC-I expression, but one found an additional role for *Nlrc5* in dampening cytokine response (23), whereas another found a role for this gene in inflammasome activation (37). As the various groups used different gene deletion strategies, it is possible that residual transcripts in the different gene deletion strains might be able to regulate functions other than MHC-I expression. The detection of a 3' end transcript that originates from *Nlrc5* in the strain that we produced suggests that this possibility deserves a further investigation.

The above caveats do not take away from the definitive results that support the findings of Meissner *et al.* (9) observed in cell lines. Together, these results indicate that *Nlrc5* is a *bona fide* MHC-I gene transcriptional regulator. It is interesting that CIITA and *Nlrc5* serve similar functions, the human CIITA and *Nlrc5* are on the same chromosome (38), and their NBD and leucine-rich repeat (LRR) are most closely related on a phylogenetic tree (11). Since the discovery of the NLR family, some have argued that CIITA does not belong to this family because it is the only NLR protein with a transcriptional function. The discovery of *Nlrc5* in MHC-I gene transcription clearly negates this concern and indicates that CIITA has at least one closely related relative with similar function. Thus CIITA and *Nlrc5* represent bridges between innate immune genes and regulators of MHC that play pivotal roles in adaptive immunity.

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