NLRC5 Controls Basal MHC Class I Gene Expression in an MHC Enhanceosome-Dependent Manner

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Nucleotide-binding domain and leucine-rich repeat (NLR) proteins play important roles in innate immune responses as pattern-recognition receptors. Although most NLR proteins act in cell autonomous immune pathways, some do not function as classical pattern-recognition receptors. One such NLR protein is the MHC class II transactivator, the master regulator of MHC class II gene transcription. In this article, we report that human NLRC5, which we recently showed to be involved in viral-mediated type I IFN responses, shuttles to the nucleus and activates MHC class I gene expression. Knockdown of NLRC5 in different human cell lines and primary dermal fibroblasts leads to reduced MHC class I expression, whereas introduction of NLRC5 into cell types with very low expression of MHC class I augments MHC class I expression to levels comparable to those found in lymphocytes. Expression of NLRC5 positively correlates with MHC class I expression in human tissues. Functionally, we show that both the N-terminal effector domain of NLRC5 and its C-terminal leucine-rich repeat domain are needed for activation of MHC class I expression. Moreover, nuclear shuttling and function depend on a functional Walker A motif. Finally, we identified a promoter sequence in the MHC class I promoter, the X1 box, to be involved in NLRC5-mediated MHC class I gene activation. Taken together, this suggested that NLRC5 acts in a manner similar to class II transactivator to drive MHC expression and revealed NLRC5 as an important regulator of basal MHC class I expression. The Journal of Immunology, 2012, 188: 4940–4950.
very well-characterized example of another NLR protein with a nuclear function (2). Class II transactivator (CIITA), the first described mammalian NLR protein, is the main transcriptional activator of MHC class II gene expression (15).Mutations in CIITA cause bare lymphocyte syndrome (15), a hereditary, generally fatal primary immunodeficiency caused by the complete absence of MHC (in humans: HLA) class II gene expression (16). CIITA is an atypical transcriptional activator that does not bind directly to DNA but is rather recruited to the highly conserved MHC class II promoters via protein–protein interactions with a multisubunit DNA-binding protein complex termed the MHC enhansome (16, 17). The MHC enhansome contains the X1 box-binding RFX complex, the X2 box-binding CREB, and the Y box-binding NF-Y complex (16, 17). Binding of these factors to the S/X/Y element in proximal promoters of MHC class II genes is highly cooperative, with an absolute dependence on RFX and NF-Y (18, 19). MHC class II genes are expressed in a complex pattern with professional APCs showing constitutive expression, whereas most somatic cell types are negative for MHC class II expression. However, expression in the latter can be induced by various stimuli, most notably IFN-γ. Both constitutive and inducible MHC class II expression is strictly dependent, qualitatively and quantitatively, on CIITA expression (15, 17, 18, 20–22).

In contrast to MHC class II genes, MHC class I genes are constitutively expressed on the majority of nucleated cells (23, 24). However, MHC class I gene expression regulation is not simple, because expression levels vary widely between different tissues, and expression is modulated by different stimuli, such as type I and II IFNs and other signals (23–25). MHC class I promoters are composed of a core promoter and upstream regulatory elements containing binding sites for NF-κB and IRF1 (24). Site-α, another upstream regulatory element, contains a cis-regulatory element similar to the S/X/Y region, and both constitutive and IFN-γ inducible MHC class I expression was reported to depend, at least in part, on the MHC enhansome and CIITA (26–28). In vivo binding of endogenous CIITA to the HLA class I gene promoter in B cells and dendritic cells was also shown (29). In contrast, many cell types, including murine and human resting T cells, express high levels of MHC class I in the absence of MHC class II and CIITA, and CIITA-deficient mice showed normal MHC class I expression levels in all tissues analyzed (30). Thus, both constitutive and inducible MHC class I expression mechanisms remain to be fully understood.

In this study, we identified a function for NLRC5 in activating MHC class I expression in a site-α/S/X/Y and MHC enhancsomederpendent manner. Endogenous NLRC5 expression levels correlate with constitutive MHC class I expression levels in various human tissues. Exogenous expression of NLRC5 in the murine melanoma cell line B16-F10, in which MHC class I expression is low to undetectable, induces endogenous MHC class I expression to levels found in primary murine lymphocytes. Overexpression of NLRC5 increases MHC class I reporter gene expression in human cell lines, whereas knockdown of NLRC5 reduces expression. NLRC5-activated MHC class I expression is dependent on an intact XI element in the HLA class I B gene promoter, and dominant-negative mutants of CIITA competitively inhibit NLRC5-dependent MHC class I gene activation, strongly suggesting that NLRC5 and CIITA work, at least in part, through the same interaction partners in the MHC enhansome. Functionally, we show in this article that the N-terminal effector domain, the central NACHT domain with an intact ATPase motif, and the C-terminal LRR domains are all required for activation of the MHC class I promoter. Furthermore, our data suggest that constant cytoplasmic/nuclear exchange is needed for full activity of NLRC5.

### Materials and Methods

#### Cells and cell culture

Primary human dermal fibroblasts were obtained and maintained as described (7).

HEK293T, HeLa, and B16-F10 cells were cultured in DMEM, and THP1 cells were cultured in VLE RPMI 1640 (Biolog) containing 10% heat-inactivated FCS, 2 mM glutamine, and 100 U/ml penicillin and streptomycin in 5% CO2 at 37°C.

#### Plasmids

Plasmids encoding myc-tagged NLRC5 full-length and deletion constructs were cloned into pCDNA3.1-myc-6B using restriction digestion cloning (EcoRV; XhoI) from the previous described FLAG-tagged NLRC5 (7). Plasmids encoding truncated versions of NLRC5 were generated by PCR cloning in pCDNA3.1-myc-B. These truncated versions contain the following amino acids (referred to NP_115582): myc-NLRC5 isoform 3 aa 1–720, myc-NLRC5 ΔDD-nuclear localization signal (NLS) aa 110–1866, myc-NLRC5 ΔDD aa 1–133, and myc-NLRC5 LRR aa 589–1866. Naturally 2xSV40-NLS-tagged NLRC5 was cloned by PCR into pCDNA3.1-3xmyc-B using the following primers: fwd: 5′-GGGGATATCAAGGATC

#### Luciferase assays

A total of $3 \times 10^4$ HeLa or HEK293T cells/well was seeded in 96-well plates. Cells were transfected with 10 ng of pCDNA3.1. The transfection was performed using X-tremeGene 9 (Roche), as indicated in the manufacturer’s protocol. Luciferase activity was measured 16–24 h after transfection and normalized to β-galactosidase activity (36).

#### Indirect immunofluorescence microscopy

HeLa cells were seeded on coverslips in 24-well plates. Cells were transfected with 1 μg myc-tagged NLRC5 using Lipofectamine 2000 (Invitrogen). After overnight incubation, cells were treated with 50 nM Leptomycin B (LepB; Sigma-Aldrich) or left untreated. After 4 h of LepB treatment, cells were fixed using 4% paraformaldehyde (Roth) in PBS for 10 min at room temperature. Cells were permeabilized using 0.5% Triton X-100 (Roth) in ice-cold PBS for 5 min. After permeabilization, cells were blocked with 3% BSA for 20 min at room temperature. Myc-tagged protein was detected using mouse anti-myc Ab 9E10 (Sigma) diluted 1:1000 in 3% BSA, followed by incubation with rabbit anti-mouse Alexa Fluor 488 (1:500) in 3% PBS. DNA was stained using DAPI. Cells were mounted in ProLong Gold Antifade reagent (Invitrogen). Images were recorded with a Cell™ microscope (Olympus) and processed using ImageJ software.

#### Transfection of B16-F10 cells

B16-F10 cells were transfected using PEI-max (Polysciences). Transient transfections were analyzed 2–4 d after transfection. For the selection of stable transfecants, cells were selected with 200 μg/ml hygromycin B (EBS-NPL–based expression vectors) or 7.5 μg/ml blasticidin (EBSB-PL–based vectors) for at least 2 wk. CIITA-transfected cells were sorted for MHC class II expression with anti-IA/IE mAb 2G9 (BD Biosciences) and anti-β-galactosidase or empty vector plasmid were described (35). All plasmids were verified by sequencing.

#### Luciferase reporter constructs for MHC class I promoter activation

Luciferase reporter constructs for MHC class I promoter activation were described (34). The HLA class II DRA promoter reporter plasmid contains a fragment from position −150 to +31 bp with respect to the transcription start site amplified from Raji genomic DNA and cloned into the polynucleotide PGL3 (Promega). Generation of an HLA-B250′TCGCA′ luciferase reporter plasmid was conducted, as described in Gobin et al. (34). Plasmids encoding β-galactosidase or empty vector plasmid were described (35). All plasmids were verified by sequencing.

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Small interfering RNA knockdown

Small interfering RNA (siRNA) duplexes specific for NLRC5 and control siRNA were described (7). For siRNA-based knockdown in HeLa cells, 50,000 cells were seeded into 24-well plates, 6 h prior to siRNA transfection. After incubation, cells were transfected with 10 nM siRNA using HiPerfect (Qiagen), as described in the manufacturer’s protocol. Seventy-two hours posttransfection, triplicates were pooled, and RNA was extracted using the RNeasy extraction kit (Qiagen).

Knockdown in THP1 cells and primary human dermal fibroblasts was performed as described (7). RNA was extracted using the RNeasy extraction kit (Qiagen). For quantitative RT-PCR analysis, 1 μg RNA was reverse transcribed using the First-Strand cDNA Synthesis Kit (Fermentas).

Quantitative PCR analysis

For quantitative PCR analyses, 50 ng cDNA was analyzed in a total volume of 25 μl using the iQ SYBR Green Supermix (Bio-Rad), as described in the manufacturer’s protocol. For amplification of the following primers were used: NLRC5_fwd: 5′-CTGCAGCCAAGTTGCTTGGG, NLRC5_rev: 5′-TCAGCTAGGAAAGTCTGATG-3′; HLA-B_fwd: 5′-CTACCTTGGAGGATCAAC-3′; HLA-B_rev: 5′-CACACCGAGGCCAGC-3′; GAPDH_fwd: 5′-CAAGAGGACCACCTTGTGCAAAGC-3′; GAPDH_rev: 5′-TCTTCTTTTGTTGCTTGGC-3′. For gene-expression profiling, multiple tissue cDNA panels (Clontech) were used. All quantitative PCR reactions were run on a Bio-Rad iQ5 cycler, and data were evaluated by the iQ5 system software (version 2.0) using the ΔΔCT method.

Flow cytometry

For staining of surface HLA class I molecules, THP-1 cells were incubated with anti-HLA-ABC Ab (BD Pharmingen) or control Ab-allophycocyanin mouse IgG1 (BD Pharmingen). FACS analysis of THP1 cells was performed using a BD FACSCan and FACSData analysis software. B16-F10 cells were stained for MHC class II expression with Alexa Fluor 647-coupled anti-IA/IE mAb 2G9 (BD Biosciences), for HMC class I expression with biotinylated anti H-2K(b) Ab AF6-88.5 (BD Biosciences), followed by secondary staining with Streptavidin-allophycocyanin (BD Biosciences), or appropriate controls. Cells were analyzed on a FACScCalibur instrument using CellQuest software (BD Biosciences).

Protein-stability assay

For inhibition of protein neo-synthesis, HEK293T cells were treated with 30 μg/ml cycloheximide for 1, 3, or 6 h and subsequently lysed in Laemmli buffer, followed by Western blot analysis.

Immunoprecipitation of endogenous NLRC5

HeLa cells were seeded to a confluence of ~80% and stimulated overnight using 100 μg/ml poly(I:C) to induce NLRC5 expression (7). After stimulation with poly(I:C), cells were treated with 50 nM LepB for 4 h. Cells were fractionated using the QIAGEN QProteome Kit, as described in the manufacturer’s protocol. The immunoprecipitation was performed using an anti-NLRC5 3H8 Ab (7) bound to Protein G Sepharose Beads (GE Healthcare) for 4 h at room temperature. Cytoplasmic and nuclear immunoprecipitation samples were analyzed by SDS-PAGE and subsequent Western blot analysis. Detection Abs used were anti-NLRC5 3H8 (7), anti-tubulin (T7186; Sigma-Aldrich), and anti-lamin A/C (2032; Cell Signaling).

Statistical analysis

Data are presented as mean ± SD. Significance was assessed with a two-sided Student t test.

Results

NLRC5 contributes to MHC class I gene expression

Several recent reports analyzed NLRC5 with conflicting results for many of the reported functions, including its ability to activate MHC class I transcription. To address this unresolved issue, we first cotransfected various amounts of an NLRC5 expression vector in HEK293T cells together with HLA class I A and B gene promoter reporter constructs (27, 36). Expression of NLRC5 significantly activated HLA-A and HLA-B luciferase reporter expression in this system (Fig. 1A).

NLRC5 and CIITA are most closely related to each other within the NLR gene family in their NACHT and LRR domains (12, 37, 38), and CIITA is the only other NLR protein with a clearly defined function in transcriptional activation. This suggested a functional analogy between these two NLR proteins; therefore, we compared the ability of NLRC5 and CIITA to activate HLA class I and II reporter gene constructs in HEK293T cells. NLRC5 expression resulted in a significant and dose-dependent HLA class I induction, whereas its effect on an HLA class II DRA promoter reporter construct was negligible (Fig. 1B). In contrast, CIITA activated the HLA class II DRA promoter strongly and showed a low, but reproducible, activation of the HLA class I B promoter (Fig. 1B).

Knockdown of NLRC5 in human myeloid macrophage-like differentiated THP1 cells via siRNA robustly reduced mRNA levels of endogenous NLRC5 and led to a decreased expression of HLA-B mRNA (Fig. 1C) and HLA class I cell surface expression (Supplemental Fig. 1A). Of note, two independent NLRC5-specific siRNA duplexes yielded similar results (Fig. 1C). We observed similar results using HeLa cells (Fig. 1D). In all cases, transfection of control siRNA had no effect on NLRC5 or MHC class I gene expression compared with untreated cells (Fig. 1C, 1D). We also analyzed nontransformed primary human dermal fibroblasts in which we induced high levels of NLRC5 expression by treatment with poly(I:C) (7). For both NLRC5-specific siRNA duplexes, knockdown of NLRC5 correlated with a reduction in endogenous HLA class I B mRNA expression levels (Fig. 1E). Moreover, a similar result was obtained with cells derived from a second donor and when NLRC5 expression was induced by Sendai virus (Supplemental Fig. 1B).

Taken together, this strongly suggests that NLRC5 is involved in the control of constitutive MHC class I gene expression.

MHC class I gene expression correlates with NLRC5 expression levels

MHC class I expression is near ubiquitous on nucleated cells, but it shows strong differences in expression levels between different cell lines and tissues. Based on the previous findings, we asked whether NLRC5 levels correlate with basal HLA class I gene expression in human tissues. In agreement with our previous report (7), we found NLRC5 expression levels to vary widely between different tissues (Fig. 2A). Comparison of HLA class I B mRNA expression levels with those of NLRC5 revealed a close correlation between the expression levels of both genes in tissues with very low (skeletal muscle and heart), intermediate (liver, kidney, prostate, thymus, and colon), or high expression (CD8+ T cells). However, in some tissues, such as brain and lung, the expression levels were more disparate (Fig. 2A). These results are compatible with a positive role for NLRC5 in MHC class I gene expression but also suggest that, at least in certain tissues, other factors contribute to the overall MHC class I expression levels.

Several tissues and cell lines show very low to undetectable MHC class I expression (24, 28). One example is the murine melanoma cell line B16 and its various sublines (28, 39). B16-F10 cells were transfected with an empty vector or with vectors expressing EGFP-NLRC5 or EGFP-CIITA. Analysis of transiently transfected cells showed that NLRC5 robustly induced MHC class I expression, whereas CIITA induced MHC class II expression. Expression of a Walker A mutant of NLRC5 (GFP-NLRC5 K234A) had no effect on MHC class I expression (data not shown).

After 2 wk of selection with appropriate antibiotics, NLRC5-transfected B16-F10 cells were sorted once with Ab and magnetic beads for MHC class I+ cells, whereas CIITA-transfected cells were enriched for MHC class II+ cells (Fig. 2B, 2C). In parallel experiments, we were unable to enrich for MHC class I+ or class II+ cells in vector- or NLRC5-K234A–transfected cells,
showing that B16-F10 cells are negative or nearly negative for MHC class I and II (Fig. 2B1, 2C1) and that NLRC5-K234A does not activate MHC class I expression (data not shown).

Stable expression of EGFP-NLRC5 induced endogenous MHC class I gene and protein expression levels very substantially in two independently transfected and selected cell populations (Fig. 2B3, 2B4). Remarkably, MHC class I levels in the EGFP+ cells were comparable to those found on freshly isolated murine splenic lymphocytes (Fig. 2B2). The distinctive kink in the dot plots showing EGFP-NLRC5 expression on the x-axis and MHC class I [H2K(b)] on the y-axis (Fig. 2B3, 2B4) suggests a threshold mechanism, where only a limited amount of NLRC5 expression is needed to fully activate endogenous MHC class I gene expression.

EGFP-CIITA also induced MHC class I expression in B16-F10 cells, albeit less efficiently than did NLRC5 (Fig. 2B5 versus Fig. 2B1; see Supplemental Fig. 1C for a histogram overlay). As expected, MHC class II expression was strongly induced by EGFP-CIITA in the same cells (Fig. 2C5). The level of EGFP-CIITA expression is very low in these stable transfectants, because EGFP expression could not be detected (Fig. 2B5, 2C5). However, we did detect EGFP expression readily in the same cells 2 d after transfection, demonstrating the integrity of the construct (data not shown). EGFP-NLRC5 expression had no effect on MHC class II expression in B16-F10 cells (Fig. 2C3, 2C4; compare EGFP+ and EGFP- cells).

These results show that endogenous NLRC5 and MHC class I mRNA expression levels are closely correlated in various cell lines and tissues and that NLRC5, on its own, is able to induce high levels of endogenous MHC class I gene expression in B16-F10 cells. NLRC5-mediated MHC class I expression is dependent on nuclear shuttling and the integral protein fold of NLRC5.

NLRC5 shares the canonical tripartite NLR structure consisting of an N-terminal effector domain, a central nucleotide-binding domain, and a C-terminal LRR region (7). However, our knowledge of the contribution of these domains to the subcellular localiza-
tion, nuclear trafficking, and MHC class I activation of NLRC5 remains fragmentary. Therefore, we generated various NLRC5 deletions and point mutants and analyzed their ability to shuttle through the nucleus by immunofluorescence analysis, as well as their capacity to activate MHC class I transcription by HLA class I B promoter reporter assays in HeLa cells (Fig. 3). Wild-type (wt) NLRC5 was localized very predominantly in the cytoplasm, but inhibition of Crm1-dependent nuclear export with LepB led to a strong nuclear accumulation of NLRC5 (Fig. 3A). These experiments show that NLRC5 transits through the cell nucleus, which is in agreement with recently reported findings (12, 13).

The requirement of a functional ATPase domain in NLRC5 for subcellular localization and transport and the ability to activate MHC class I transcription were analyzed by mutating the conserved lysine residue (K234) in the predicted Walker A motif of NLRC5 to alanine (K234A) (7, 38). In contrast to wt NLRC5, NLRC5 K234A was localized very predominantly in the cytoplasm, but inhibition of Crm1-dependent nuclear export with LepB led to a strong nuclear accumulation of NLRC5 (Fig. 3A). These experiments show that NLRC5 transits through the cell nucleus, which is in agreement with recently reported findings (12, 13).

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We showed previously that NLRC5 is expressed in various isoforms. In this study, we focused our attention on isoform 3, which lacks the entire LRR domain and is expressed in several human tissues (7). NLRC5 isoform 3 localized predominantly in the nucleus, even in the absence of LepB (Fig. 3A), yet it completely failed to activate MHC class I transcription (Fig. 3B). This indicates that the C-terminal LRR domain is involved in nuclear export of NLRC5, as well as in transcriptional activation.

CIITA contains, at its N-terminal end, a “classical” acidic-activation domain that activates transcription in yeast when tethered to a promoter (40, 41). CIITA is expressed from three independent promoters, generating three isoforms (FI, FIII, and FIV) (42). Although CIITA FIII and FIV start at their N-terminal end with the acidic activation domain, CIITA FI contains an additional domain N-terminal of the acidic domain, which has homology to a CARD domain and was reported to contribute to MHC class II gene activation (43). The N-terminal domain of NLRC5 adopts a death domain fold, but it lacks obvious homology to the CARD and PYD found in other NLRs, including the CARD of CIITA FI, or known transcriptional activation domains (7). In contrast, the results shown above suggested that NLRC5 functions as a transcriptional activator. Accordingly, we investigated whether the N-terminal NLRC5 effector domain is necessary for MHC class I transcriptional activation. A truncated form of NLRC5, lacking the N-terminal part of the effector domain but still containing a recently identified nuclear localization signal at amino acid positions 122–134 (NLRC5DD NLS) (12), was found in the nucleus in ∼50% of HeLa cells in the absence of
LepB. Nuclear localization of this construct was increased by inhibiting nuclear export (Fig. 3A); however, it was unable to activate the MHC class I promoter (Fig. 3B, Supplemental Fig. 2A).

These experiments show that the N-terminal death-fold domain, a functional ATPase domain, and the C-terminal LRR domain of NLRC5 are all required to induce MHC class I gene expression, whereas the requirements for nuclear shuttling are different. As expected from the previous experiments, the N-terminal death domain alone showed an exclusively nuclear localization in the absence of LepB, whereas the LRR region alone was completely cytoplasmic, irrespective of the presence or absence of LepB (Fig. 3A). Neither construct was able to activate MHC class I transcription in HeLa or HEK293T cells (Fig. 3B, Supplemental Fig. 2A). All constructs were expressed at comparable levels in HEK293T and HeLa cells, showing that the differences in reporter gene activation reflect protein activity and not protein levels (data not shown, Supplemental Fig. 2B).

FIGURE 3. Requirements for NLRC5 nuclear shuttling and MHC class I activation. (A) Photomicrographs of indirect immunofluorescence analysis of HeLa cells transfected with the indicated plasmid for 24 h. Cells were fixed untreated (left panels) or after treatment with 50 nM LepB for 4 h to block nuclear export (right panels). Signals obtained with the myc tag-specific 9E10 Ab are shown in green. DNA staining with DAPI is shown in blue. Scale bar, 10 μm. (B) HLA-B250 reporter luciferase assays in HeLa cells in which increasing amounts of the corresponding constructs were transiently transfected. Luciferase activity, normalized to β-galactosidase activity, is shown as fold activation to cells transfected with empty vector ("0" set to 1). Data represent mean ± SD (n = 3). All constructs were tested in a single transfection series. (C) Schematic representation of the constructs. The N-terminal NLS is highlighted in red. Numbers indicate amino acid residues. ED, Effector domain; LRR, LRR domain.
The previous experiments were carried out by ectopic expression of epitope-tagged protein constructs. Overexpression can easily lead to saturation of transport pathways and, thus, may yield artificial results. Therefore, we also analyzed the subcellular localization of endogenous NLRC5 in HeLa cells by cytoplasmic and nuclear protein fractionation and Western blotting (Fig. 4, upper panel). To obtain detectable amounts of NLRC5, cells were pretreated with poly(I:C) to induce NLRC5 expression. This revealed a strong nuclear accumulation of endogenous NLRC5 after blocking of nuclear export by LepB, whereas NLRC5 was mainly cytoplasmic in untreated cells (Fig. 4). Blotting for α-tubulin and α-Lamin A/C demonstrated the absence of cross-contamination of cytoplasmic and nuclear fractions (Fig. 4, middle and lower panels). Of note, induction of NLRC5 expression by poly(I:C) treatment did not correlate with accumulation of NLRC5 in the nucleus. This is reminiscent of our observation that overexpressed NLRC5 strongly activated MHC class I gene expression, beside being mainly localized in the cytoplasm also in HeLa cells (Fig. 3).

Taken together, these results show that NLRC5 shuttles through the nucleus and that MHC class I promoter activation is dependent on a functional ATPase domain and on the presence of both the death domain and LRR region of NLRC5, suggesting that the integral NLRC5 fold is needed for subcellular transport and transactivation function.

Nuclear–cytoplasmic shuttling of NLRC5 enhances MHC class I promoter activation

To analyze the role of nuclear shuttling of NLRC5 in MHC class I signaling, we asked whether forcing nuclear localization of NLRC5 would result in increased MHC class I promoter activation. Therefore, we generated NLRC5 constructs containing either a myc epitope tag or a myc epitope followed by a double SV40 NLS sequence at the N terminus (myc-2xNLS-NLRC5). As shown in Fig. 5A, this construct showed a predominantly nuclear localization, even in cells that had not been treated with LepB. Unexpectedly, myc-2xNLS-NLRC5 showed a reduced capacity to activate the HLA class I A and B promoters and did not show a dose-dependent induction of the MHC class I promoter, as observed for wt myc-NLRC5 (Fig. 5B, 5C). This was not due to a change in protein stability or turnover, as assessed by Western blotting after blocking of protein synthesis using cycloheximide (CHX) for the indicated time.
This indicates that nuclear localization, as well as constant cytoplasmic/nuclear shuttling, is essential for full NLRC5 activity.

**NLRC5-mediated MHC class I promoter activation is mediated by the MHC enhanceosome**

Activation of MHC class I genes is dependent on a core promoter and upstream regulatory elements containing NF-κB- and ISRE-binding elements and a regulatory region originally called site-α (26). Site-α was shown to contain a region homologous to the S/X/Y composite cis-acting crucial for MHC class II gene activation (26, 28, 34). The MHC enhanceosome complex, and in particular the RFX complex, was shown to be required both for basal MHC class I expression (in absence of CIITA) and for CIITA-dependent activated MHC class I expression (26). Given the close homology of NLRC5 and CIITA, we anticipated that the site-α/S/X/Y element is also involved in NLRC5-mediated MHC class I promoter activation. Gobin and colleagues (26) showed that a mutated X1 box element (“TCGCA”) exhibits a strongly reduced activity for both basal (CIITA-independent) and CIITA-dependent MHC class I expression. Therefore, we tested whether NLRC5 could activate an HLA class I B promoter reporter construct containing a mutated X1 box element (“TCGCA”) (Fig. 6A). Luciferase reporter assays in HEK293T cells revealed an inability for overexpressed NLRC5 to activate this defective X1-Box HLA class I B250 construct (“TCGCA”), whereas NLRC5 robustly turned on the wt B250-luciferase construct in the same experiment (Fig. 6B, left panel). The parallel experiment with CIITA confirmed the results of Gobin and colleagues (26) (Fig. 6B, right panel). Interestingly, we found in this and similar experiments that CIITA isoform I activated MHC class I expression more efficiently compared with isoform III (compare Fig. 6B, right panel with Fig. 1B, left panel, and Fig. 6D). Taken together, these findings suggest that MHC class I activation by both NLRC5 and CIITA is dependent on a functional X1 box.

**FIGURE 6.** MHC class I promoter activation by NLRC5 relies on a functional X-box motif. (A) Schematic representation of the B250 promoter, highlighting the wt (B250) and mutated X1 box (B250”TCGCA”) mutation used. (B) HEK293T cells were transfected with increasing amounts of wt NLRC5 or CIITA (isoform 1) in the presence of either the HLA-B250-luciferase construct or the X1 box mutant (HLA-B250”TCGCA”) luciferase construct. Luciferase activity, normalized to β-galactosidase activity, is shown as fold activation to mock-transfected cells (set to 1). Data represent mean + SD (n = 3). (C) HEK293T cells were transfected with 1 ng NLRC5 or 1 ng CIITA alone or together with 1, 5, or 10 ng of CIITA L335. B250 (left panel) and DRA (right panel) luciferase activity, normalized to β-galactosidase activity, is shown as fold activation to mock-transfected cells (set to 1). Data represent mean + SD (n = 3). (D) HEK293T cells were transfected with 1 ng NLRC5 and increasing amounts of CIITA. HLA-B250 reporter-mediated luciferase activity, normalized to β-galactosidase activity, is shown as fold activation to mock-transfected cells (set to 1). Data represent mean + SD (n = 3). *p < 0.05, **p < 0.005.
CIITA is recruited to the MHC enhanceosome through multiple protein–protein interactions with RFX5, RFXAP, CREB, NF-YB, and NF-YC (32, 44–46). Most of the interaction surfaces in CIITA were mapped to the NACH and LRR regions (reviewed in Ref. 18), which are the regions that show the highest sequence similarity between NLRC5 and CIITA. We reasoned that NLRC5 and CIITA might share, at least in part, similar interaction surfaces and, therefore, be able to compete with each other for binding to the MHC enhanceosome. Therefore, we coexpressed NLRC5 with a strongly dominant-negative N-terminally truncated CIITA mutant. CIITA-NLS-L335 is expressed at 30–50-fold higher levels compared with wt CIITA because of its greatly increased stability, and it displaces CIITA efficiently from the MHC class II promoter (33, 47, 48). As we showed before, CIITA-NLS-L335 very efficiently suppressed CIITA-dependent MHC class II gene expression in a dose-dependent manner (Fig. 6C, right panel). Very interestingly, we also observed a strong reduction in NLRC5-dependent MHC class I promoter activation when CIITA-NLS-L335 was coexpressed with NLRC5, suggesting a competition between the two factors for the same binding sites (Fig. 6C, left panel). Because the previous experiments indicated that both NLRC5 and CIITA act via site-α/S/X/Y and the MHC enhanceosome in the MHC class I promoters, we wanted to test for a possible cross-talk between these two factors. Therefore, we cotransfected a constant amount of NLRC5 with increasing amounts of CIITA and measured the activation of the HLA class I B reporter construct. As shown in Fig. 6D, we observed an additive effect of CIITA on NLRC5-dependent MHC class I expression. This further supports the hypothesis that NLRC5 and CIITA occupy the same binding interface.

Taken together, these results show that NLRC5 activates MHC class I gene expression via MHC enhanceosome binding to the site-α/S/X/Y element and also suggest that NLRC5 interacts, at least in part, with the same protein partners as does CIITA via overlapping interaction sites.

Discussion

We show in this study that human NLRC5 plays a role in basal or constitutive MHC class I expression in a manner quite similar to the mode of action of CIITA. Of note, in an independent study, Meissner et al. (49) obtained very similar results that strongly support this conclusion. Despite the very different expression patterns of MHC class I and II genes, there is a certain overlap in the molecular mechanisms controlling the expression of these genes. The MHC enhanceosome binds to the site-α/S/X/Y element in the MHC class I promoter, and Gobin et al. (26) showed a strongly reduced constitutive MHC class I expression in RFX-deficient fibroblast cell lines, indicating that the MHC enhanceosome plays an important role in the constitutive expression of MHC class I genes in the absence of CIITA. Although CIITA is indispensable for MHC class II gene expression, it has been well established that this factor is not required for constitutive MHC class I expression, even though it is clearly capable of increasing it. This begged the question whether a CIITA-equivalent factor binding to the MHC enhanceosome might be involved in MHC class I gene activation, at least in tissues and cells that are negative for CIITA expression. The results presented in this article provide compelling evidence that NLRC5 is such a factor. NLRC5 mRNA expression levels are correlated to constitutive MHC class I expression levels in several cell lines and tissues; ectopic NLRC5 expression activates HLA class I promoter reporter constructs in a dose-dependent manner, as well as increasing HLA class I cell surface expression in THP1 cells; and knockdown of NLRC5 reduces MHC class I transcription both in immortalized cell lines and primary cells.

Furthermore, and quite remarkably, stable expression of NLRC5 in the murine melanoma cell line B16-F10, which shows low to undetectable basal MHC class I expression, is sufficient to induce endogenous MHC class I expression to the same level as found in freshly isolated lymphocytes. It appears that low levels of NLRC5 are sufficient for full activation of MHC class I expression in a threshold-dependent manner. This is in contrast to the mode of action of CIITA, which activates endogenous MHC class II transcription in a dose-dependent manner over a wide range of concentrations (22, 31).

The integrity of the RFX-binding X1 box within the site-α/S/X/Y element of the HLA class I B promoter is necessary for NLRC5-dependent transcription. So far, only the requirement for RFX binding to the site-α/S/X/Y has been rigorously demonstrated (26). However, because the sequence, spacing, and orientation of the S/W/Z, X1, X2, and Y elements are highly conserved between MHC class I and class II promoters, we can extrapolate that the complete RFX/CREB/NF-Y enhanceosome binds to the site-α/S/X/Y element in a manner similar to the MHC class II promoters. This notion is reinforced by the observation that a strongly dominant-negative CIITA mutant (CIITA-NLS-L335) inhibits NLRC5-dependent MHC class I activation in a dose-dependent manner. This also suggests that NLRC5 and CIITA, which are the two most closely related NLR proteins, interact with the MHC enhanceosome via overlapping protein interfaces. This is further supported by the observation of an additive effect on MHC class I gene expression when both CIITA and NLRC5 are coexpressed. Thus, the results presented in this article strongly suggest that NLRC5 is involved in the constitutive activation of MHC class I genes.

NLRC5 and CIITA show important similarities with respect to MHC enhanceosome-dependent MHC gene activation, as well as with regard to their intracellular transport and localization. Both show a highly complex nucleo-cytoplasmic shuttling behavior that is necessary for transactivation function and that involves all domains of both proteins. We confirmed the initial observation that ectopically expressed NLRC5 is localized predominantly in the cytoplasm, but transits through the nucleus in a Crm-1–dependent manner (12, 13). Very importantly, the same is true for endogenous NLRC5, as shown in this study by subcellular protein fractionation after LepB treatment. Both the integrity of the Walker A motif within the ATPase domain (NACH) and an NLS in the N-terminal effector domain are required for nuclear localization, whereas the C-terminal LRR region appears to be involved in nuclear export, as shown by the predominantly nuclear localization of NLRC5 isoform 3 lacking the LRR region and the exclusively cytoplasmic localization of the NLRC5 LRR domain when expressed on its own.

Endogenous CIITA shows a relatively even distribution between the cytoplasm and nucleus, and it is also exported from the nucleus in a Crm-1–dependent manner (31). At least three potential NLS motifs have been described in CIITA (reviewed in Ref. 50); however, none of these motifs behaves like a conventional NLS motif, and many other mutations, especially within the NACH domain and LRR regions of CIITA, lead to an inhibition of nuclear import and loss of transactivation potential (31). Our previous studies suggested that the efficiency of nuclear import of CIITA, rather than its steady-state concentration in the nucleus, is rate limiting for MHC class II gene activation (31, 32). This is largely reminiscent of the results obtained in this study for NLRC5, which show that increasing NLRC5 protein levels do correlate with enhanced MHC class I expression but not with nuclear accumulation of NLRC5. Moreover, we found in this study that an N-terminal fusion of an exogenous SV40-derived NLS to myc-tagged NLRC5 was very efficient in increasing nu-
clear localization but paradoxically reduced the MHC class I gene-transactivation potential of myc-2xNLS-NLRC5. Although we cannot formally exclude that the added 2xNLS element disturbed the integrity of the N-terminal domain of NLRC5, we do not think that this is very likely, because N-terminal addition of the myc epitope tag or even EGFP did not appear to have an effect on the activity and transport behavior of NLRC5 (Figs. 2, 3, data not shown). Furthermore, blocking of nuclear export of NLRC5 with LepB also reduced MHC class I transactivation (data not shown). There are several possible explanations for this complex transport behavior, which are not mutually exclusive. Trans-activation potential might be dependent on cytoplasmic modifications of the proteins that are not carried out correctly if nuclear import is mediated via a "classical" importin-α-dependent pathway. It is possible that nuclear import is indirect and dependent on the interaction with protein partners in the cytoplasm. The involvement of the LRR domain in this process makes this plausible, because LRR domains are best known as protein-interaction domains. Lastly, it is possible that nuclear export is somehow linked to the transactivation function of NLRC5 and CIITA.

Interestingly, it was recently shown for several plant NLRs that nuclear/cytoplasmic transition is needed for full activity, analogous to our findings for NLRC5 (51). In plants, nucleotide-binding and LRR domain-containing proteins (also called R proteins) also play pivotal roles in the detection of invading pathogens. For example, the Arabidopsis thaliana R protein RPS4 has to translocate to the nucleus to develop complete functionality (52, 53). Moreover, Cheng and colleagues (53, 54) reported a cytoplasmic and nuclear localization of the Arabidopsis thaliana R protein SNC1 and components of the nuclear pore control nuclear shuttling of these proteins (53). This raises the exciting possibility that nuclear shuttling of the human NLRs CIITA and NLRC5 is also controlled by unidentified components of the nuclear pore.

Based on our previous study, which showed an involvement of NLRC5 in type I IFN responses and the data discussed in this article, we propose that NLRC5 has a dual function. We assume that NLRC5 contributes to cytosolic control of type I IFN responses, as well as to the basal induction of MHC class I gene expression. Together, both responses likely enhance innate and adaptive immune responses to viral and bacterial infection. Many pathogens interfere with MHC class I-dependent Ag presentation to escape CD8 T cell responses. Moreover, MHC class I downregulation is often observed in malignant tumors, which rely on similar immune-evasive strategies as do infectious pathogens.

The findings presented in this article indicate that NLRC5 is an important player in the control of MHC class I-dependent immune responses and, thus, represents a valuable new target for the development of novel strategies to regulate MHC class I expression in viral infection and cancer immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

References


Figure S1 (relates to Figures 1 and 2):

A. PMA differentiated THP1 cells were transfected with 50 nM of siRNA for 72 h. Flow cytometry analysis of MHC class I surface expression in these cells is shown. Data is shown as median fluorescence intensity + S.D. (see also Figure 1C).

B. Primary human dermal fibroblasts from donor 2 were transfected with 10 nM of NLRC5 specific siRNA or scrambled control siRNA (siCTL) for 72 h and subsequently treated with 10 μg/ml poly(I:C) or 80 HAU/ml Sendai virus for 16 h. Quantitative RT-PCR of NLRC5 and HLA-B expression is shown as the mean of the relative expression to GAPDH + S.D. (n=3). (relates to Figure 1E).

C. Histogram visualizing the effect of CIITA and MHC class I expression, representing the data shown in Figure 2B (panels 1 and 5) as a histogram overlay. (see also Figure 2B).
Figure S2 (relates to Figure 3):

A. HLA-B250 reporter luciferase assays in HEK293T cells where increasing amounts of the corresponding constructs were transiently transfected. Luciferase activity, normalized to β-galactosidase activity is shown as fold activation to cells transfected with empty vector (“0” set to 1). Data represents mean ± S.D. (n=3). All constructs were tested in a single transfection series. (relates to Figure 3B).

B. Western blot analysis showing comparable expression of the NLRC5-constructs in HEK293T. Probing for α-tubulin served as control for equal loading. * indicates unspecific background.