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The N-Terminal Domain of NLRC5 Confers Transcriptional Activity for MHC Class I and II Gene Expression

Andreas Neerincx,* Kristin Jakobshagen,* Olaf Utermöhlen,*† Hildegard Büning,†‡§ and Thomas A. Kufer*

Ag presentation to CD4+ and CD8+ T cells depends on MHC class II and MHC class I molecules, respectively. One important regulatory factor of this process is the transcriptional regulation of MHC gene expression. It is well established that MHC class II transcription relies on the NLR protein CIITA. Recently, another NLR protein, NLRC5, was shown to drive MHC class I expression. The molecular mechanisms of the function of NLRC5 however remain largely elusive. In this study, we present a detailed functional study of the domains of NLRC5 revealing that the N-terminal domain of human NLRC5 has intrinsic transcriptional activity. Domain swapping experiments between NLRC5 and CIITA showed that this domain contributes to MHC class I and MHC class II gene expression with a bias for activation of MHC class I promoters. Delivery of this construct by adeno-associated viral vectors upregulated MHC class I and MHC class II expression in human cells and enhanced lysis of melanoma cells by CD8+ cytotoxic T cells in vitro. Taken together, this work provides novel insight into the function of NLRC5 and CIITA in MHC gene regulation. The Journal of Immunology, 2014, 193: 000-000.

Cytotoxic T lymphocyte responses rely on the presentation of cytosolic peptides by MHC class I molecules to CD8+ T cells. Most cells do express MHC class I molecules, although expression varies among different cell types and can be upregulated upon infection. In contrast, expression of MHC class II molecules, which present Ags to CD4+ T cells, depends on the NLR protein CIITA and is restricted mainly to professional Ag presenting cells. MHC class I expression was proposed to be controlled mainly by NF-κB and IFN-sensitive response element motifs in the MHC class I promoter region (1). However, the exact mechanisms how expression of MHC genes is regulated is still poorly defined. Recently, several groups identified NLRC5 as a novel transcriptional regulator of MHC class I genes (2–8). Both NLRC5 and CIITA belong to the NLR protein family that makes poorly defined. Recently, several groups identified NLRC5 as a novel transcriptional regulator of MHC class I genes (2–8). Both NLRC5 and CIITA belong to the NLR protein family that makes poorly defined. Recently, several groups identified NLRC5 as a novel transcriptional regulator of MHC class II expression with a bias for activation of MHC class I promoters. Delivery of this construct by adeno-associated viral vectors upregulated MHC class I and MHC class II gene expression in human cells and enhanced lysis of melanoma cells by CD8+ cytotoxic T cells in vitro. Taken together, this work provides novel insight into the function of NLRC5 and CIITA in MHC gene regulation. The Journal of Immunology, 2014, 193: 000-000.

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Abbreviations used in this article: AA V, adeno-associated virus; CARD, caspase recruitment domain; DD, death-domain–like fold; HPRT, hypoxanthine phosphoribosyltransferase; lLRR, leucine-rich repeat domain; NACHT, ATPase domain present in NAIP, CIITA, HET-E, and TP1; NLS, nuclear localization signal; ppc, particle per cell; qPCR, quantitative PCR.

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Materials and Methods

Cells and cell culture

HEK293T, HeLa, and B16F1O1 cells were grown at 37°C with 5% CO2 in DMEM containing 10% heat-inactivated FCS and penicillin–streptomycin.
(100 IU/ml and 100 ng/ml, respectively). The cells were continuously tested for mycoplasma contamination by PCR.

**Plasmids**

Plasmids encoding NLRC5 and CIITA isoforms were described previously (22, 23). A mammalian GAL4-DDB expression plasmid was generated by cloning of GAL4-DDB from pGBK7(T) (Stratagene) into pCMV3.1-3xmyc-B to obtain pCMV3.1-3xmyc-GAL4-DDB. GAL4-DDB fusion protein was generated by PCR amplification of GAL4-DDB from pGBK7 (Stratagene) and cloned into pCDNA3.1-3xmyc-DD. GAL4-CARD-NOD1, GAL4-AD-p65, GAL4-AD163, GAL4-AD335, and GAL4-DD162 were generated by PCR amplification using FLAG-NLRC5, CIITA-FIII, or YFP-p65 and subsequently cloned into pCDNA3.1-3xmyc-GAL4-DDB. Chimeric constructs of NLRC5 and CIITA were generated by nested PCR using FLAG-NLRC5 (22) and CIITA-FIII (23) as templates.

**HEK293T-based luciferase reporter assays**

To analyze transcriptional activity of NLRC5 N-terminal domains, the PathDetect In Vivo Signal Transduction Pathway trans-Reporting Systems (Agilent Technologies) was adapted for analyses in human HEK293T cells as illustrated in Fig. 1A. HEK293T-based reporter assays for MYC-GAL4-fusion constructs and for chimeric NLRC5-CIITA proteins were performed as described in Refs. 5 and 24. Expression of the indicated constructs was confirmed by lysing the six times 96 wells transfected with 10 ng plasmid each (for NLRC5-CIITA chimeric constructs) or 100 ng plasmid each (for GAL4 constructs) in 60 μl 2× Laemmli. Fifteen microliters of each sample was analyzed on a 10% SDS-PAGE.

**Indirect Immunofluorescence**

HeLa cells were grown on coverslips and transfected with 1 μg of the indicated NLRC5/CIITA plasmids or 500 ng GAL4 constructs for 24 h. After 24 h, cells were stimulated with 50 nM leptomycin B for 4 h or left untreated prior paraformaldehyde fixation (5% PFA in PBS) for 10 min. Cells were permeabilized with 0.1% Triton in PBS and blocked for 20 min using 3% BSA in PBS. Ab staining was performed using anti-MYC (Sigma-Aldrich; 1:1000 in 3% BSA), anti-NLRC5 3H8 (1:5; described in Ref. 22), or anti-CIITA K5 (clone TAC-102 from Biozol; 1:1000 in 3% BSA), anti-FLAG M2 (Stratagene; 1:10000 in 3% BSA), or anti-FLAG Ab (number 200471; Stratagene) and anti-mouse or -rabbit Alexa 488-labeled anti-mouse were diluted 1:500 in 3% BSA in PBS. Ab staining was performed using anti-MYC (Sigma-Aldrich; 1:10000 in 3% BSA), anti-NLRC5 3H8 (1:5; described in Ref. 22), or anti-CIITA K5 (clone TAC-102 from Biozol; 1:10000 in 3% BSA), anti-FLAG M2 (Stratagene; 1:10000 in 3% BSA), or anti-FLAG Ab (number 200471; Stratagene) and anti-mouse or -rabbit Alexa 488-labeled anti-mouse were diluted 1:500 in 3% BSA in PBS. Images were recorded on an Olympus FV-1000 laser scanning microscope and processed using ImageJ software.

**Transfection of B16F10 cells**

For transient transfection 1.5 × 104 B16F10 cells were nucleofected with 2 μg of indicated plasmid using the Nucleofector Kit V and protocol P-020 as described in the manufacturer’s protocol (Lonza). Immediately after nucleofection, cells were transferred into a 6-cm dish in 5 ml complete DMEM. Twenty-four hours after nucleofection, medium was changed, and 48 h after nucleofection, cells were analyzed.

**Production of recombinant AAV vectors**

HEK293 cells were seeded at 80% confluency and cotransfected with a total of 37.5 μg pRCVP2k.o (26), pSUB-CMV-FLAG-DD-335-CIITA or pEFGP (27), and pX6-80 (26) for production of AAV-DD-335-CIITA and the control vector AAV-GFP. Forty-eight hours posttransfection, cells were harvested, lysed, and purified by iodixanol density step gradient centrifugation (27). Genomic particle titers were determined by quantitative PCR (qPCR) (LightCycler System; Roche Diagnostics, Mannheim, Germany) using transgene specific primers (28).

**AAV vector-based transduction of adherent cells**

For AAV vector-based transduction of HEK293T cells, 12 × 103 or 5 × 104 cells were seeded in 96-well or 12-well plates. After 24 h, cells were transduced with 500–40,000 particles per cells. After 24 h, medium was replaced once. After an additional 24 h, the luciferase reporter system was transfected to the 96-well plates as indicated in Refs. 5 and 24, whereas the 12-well plates were left untreated. Seventy-two hours after transduction, the luciferase readout was performed as described previously (5, 24). The 12-well plates were used either for protein analyses and lysed in 150 μl 2× Laemmli buffer or for RNA extraction using RNeasy Extraction Kit (Qiagen). Fifteen microliters of Laemmli lysis was separated on a 10% SDS-PAGE, blotted onto a nitrocellulose membrane and protein expression was analyzed using anti-FLAG Ab (number 200471; Stratagene) and anti-myc (sc-789; Santa Cruz Biotechnology) both 1:1000 in 5% milk powder in PBS overnight at 4°C or anti-NLRC5 3H8 Ab (described in Ref. 22) 1:5 in 5% milk powder in PBS overnight at 4°C. MHC expression was detected using anti-HLA-B/C (a gift from A. Halenius, University of Freiburg, Germany) 1:3000 in 5% milk powder in PBS overnight at 4°C or anti-HLA-DR/DP (sc-51617; Santa Cruz Biotechnology); 1:200 in 5% milk powder in PBS overnight at 4°C. As loading control, either anti-β-actin-HRP (sc-47778-HRP; Santa Cruz Biotechnology); 1:1000 in 5% milk powder in PBS for 1 h at room temperature or anti-GAPDH (sc-25778; Santa Cruz Biotechnology) 1:1000 in 5% milk powder for 1 h at room temperature Abs were used. Signal was recorded using SuperSignal Femto Reagent (ThermoScientific) on a Fuji Luminescent Image Analyzer LAS-4000. For B16F10 cell transduction, 2.5 × 105 cells was transduced in 12-well plates. After 24, 48, and 72 h cells were seeded into a 24-well format, and 1 × 105 cells were seeded into a 12-well-format. After an additional 48 h, cells in 24-well plates were processed for flow cytometry analysis, and cells in 12-well format were used for RNA extraction using RNeasy Extraction Kit (Qiagen) and processed as described below.

**RNA sample analysis**

For RNA sample analysis, 1 μg RNA was used for reverse transcription using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and oligo-dT primer according to manufacturer’s instructions. 50 ng cDNA was analyzed in qRT-PCR using the SYBR Green 2× Mix (Bio-Rad) and analyzed on an iQ5 Cycler (Bio-Rad). All samples were normalized using murine hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene expression, and each sample was analyzed in triplicates. The following primer pairs were used for qPCR: CIITA_qPCR_fwd, 5′-CTG- AAGGATTTGGAAGCCTGGAGAACG-3′; CIITA_qPCR_rev, 5′-GTC- GCGGATCTTTGTTTCACAT-3′; mH2-Kb-F668, 5′-GCGGC- TCACACATATTC-3′; mH2-Kb-R596, 5′-CTTCCAGACCTGCT- CCCACCCTG-3′; mH2-LaAa-F668, 5′-CTGGTGCCCTGGGTGCTG-3′; mH2-LaAa-R773, 5′-TAAAGGCCTCTGTTCTGCAG-3′; mHPRT-F1, 5′-GCTTACGGGCGGAGGAG-3′; and mHPRT-R1, 5′-GCGGC- AAAAGGCGTGCTAGAG-3′.

**Cytotoxicity assays**

C57BL/6 mice were bred and kept strictly under barrier conditions at the Animal Facility of the Center for Molecular Medicine Cologne and were used when 8–12 wk old. The experiments with mice were conducted in agreement with local and national regulations.

Cytotoxicity was analyzed as described previously (29). In brief, CD8+ T lymphocytes were induced by i.v. inoculation of C57BL/6 mice with 105 infectious units (IU) of the LCMV strain WE. On day 8 postinfection, CD8+ T cells were immunomagnetically enriched from splenic single-cell suspensions (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). LCMV-specific cytotoxicity of primary CD8+ T cells was assessed in a standard chromium release assay. B16F10 cells were transduced with the indicated viral vectors as described above and after 48 h loaded for 1 h with the synthetic peptide representing the immunodominant H2-Dd-restricted epitope (gpg33–41) of the LCMV gpl at the indicated concentrations. After 4 h, the release of 51Cr was measured and lysis calculated as percent lysis ([cpm experimental − cpm spontaneous] × 100/[cpm maximal − cpm spontaneous]).

**Flow cytometry analysis**

For flow cytometry analysis, cells were harvested, washed twice using 0.1% FCS in PBS, and blocked for 1 h on ice using 1% BSA in PBS. After additional two washing steps, cells were incubated with allophycocyanin-conjugated anti-mouse H-MHC class I (H-2Kb) Ab (catalog number 175958; eBioscience) or APC-conjugated anti-mouse H-MHC class II (I-A/II-E) Ab (catalog number 175321; eBioscience) for 1 h on ice. Finally, cells were washed twice in 0.1% FCS in PBS and directly used for flow cytometry analyses using FACSCan instrument (BD Biosciences). Data were analyzed using the FACS DIVA Software (BD Biosciences).

**Statistical analysis**

Data were analyzed by two-sided paired Student’s t test using Microsoft Excel 2007.

**Results**

The N-terminal effector domain of NLRC5 has transcriptional activity

NLR proteins have a typical tripartite structure, including an N-terminal effector domain, a central NACHT domain and a C-
terminal leucine-rich-repeat domain (LRR). In CIITA, the N-terminal domain contains classical acidic transactivator motifs and drives the transcriptional activity for MHC class II expression (30). The N-terminal domain of NLRC5 shows a death-domain-like fold (DD), although it lacks structural homology to classical members of this family, including caspase recruitment domains (CARDs) and pyrin domains, in that helix 3 is replaced by an ordered loop in NLRC5 (31). Acidic transactivator motifs are not identifiable in the N-terminal domain of NLRC5. In order to gain insight into the function of this domain, we tested whether the N-terminal effector domain of NLRC5 might confer transcriptional activity by other means. To this end, we adopted the mammalian one hybrid system (32), which was successfully applied to study the function of CIITA (33, 34). On the basis of the modeling and secondary structural database information (psipred v3.0), we defined the N-terminal domain of NLRC5 as aa 1–139 (according to NP_115582). We fused these first 139 aa of human NLRC5 to the yeast Gal4 DNA binding domain (Gal4-DBD) and tested whether this construct (Gal4-DD) could drive transcription of a Gal4 binding site containing luciferase reporter in HEK293T cells (Fig. 1A, right panel). As a positive control, the well-defined transactivator domain from the NF-kB subunit p65 protein (Gal4-AD-p65) was used (32). As expected, Gal4-AD-p65 showed strong transcriptional activity (Fig. 1B). Interestingly, the construct containing the N-terminal effector domain of NLRC5 (Gal4-DD) also induced luciferase expression, albeit to a lesser extent. As negative controls, we used the Gal4-DBD alone (Gal4-Cut), and in addition, the NOD1 N-terminal CARD domain fused to the Gal4-DBD (Gal4-CARD-NOD1). Both of these controls did not induce reporter gene transcription (Fig. 1B). Expression of all constructs was verified by Western blotting (Fig. 1E) and subcellular localization was determined by indirect immunofluorescence analysis (Supplemental Fig. 1). Although the C-terminal LRR region (aa 589–1866) of NLRC5 is needed for MHC class I promoter activation of the full-length NLRC5 (3–5, 35), a Gal4-LRR construct showed no detectable transcriptional activity. However, this Gal4-LRR construct failed to shuttle to the nucleus (Supplemental Fig. 1), impairing interpretation of the results. Nuclear shuttling of this construct could not be rescued by fusion of two SV40-derived nuclear localization signals (data not shown).

The N-terminal domain of CIITA contains a transactivator domain, followed by a region rich in proline/serine/threonine, called P/S/T region. Although it contributes to MHC class II activation, the function of this P/S/T domain still remains largely elusive (20, 36). To compare the transcriptional activity of the N-terminal domains of NLRC5 and CIITA, we generated fusion proteins containing Gal4-DBD fused to either the acidic domain of CIITA (Gal4-AD163) or to the combined acidic and P/S/T domain of CIITA (Gal4-AD335) and tested these constructs together with Gal4-DD in the transactivation assay. Both Gal4-AD163 and Gal4-AD335 showed a strong transcriptional activity. In agreement with previous studies (33, 34), the acidic domain of CIITA alone (Gal4-AD163) was sufficient to induce transcription (Fig. 1C). Of note, the shorter form of the CIITA transactivator domain (AD163) was more potent in activating reporter gene expression than the CIITA transactivator domain containing the P/S/T motif (AD335), although the latter was reported to contribute to CIITA specificity (20, 36). As shown above, NLRC5 DD alone was transcriptionally active, although to a lesser extent than the short (AD163) or long (AD335) N-terminal domain from CIITA (Fig. 1C). A database-based structural prediction (psipred version 3.0) of the NLRC5 DD revealed an extended helix from aa 135 to 161. To analyze whether this helix contributes to transcriptional activation, we generated an extended N-terminal effector domain fused to Gal4-DBD (Gal4-DD162). However, Gal4-DD162 was less active than Gal4-DD (Fig. 1D).

Taken together, this revealed that the N-terminal 139 aa of NLRC5 but not the LRR domain confer transcriptional activity.

**NLRC5/CIITA chimeric proteins are able to activate both MHC class I and II transcription**

Besides the fact that the N-terminal domains of both NLRC5 and CIITA enhance basal transcription, we recently showed that NLRC5 specifically confers MHC class I expression, whereas CIITA most prominently induces MHC class II expression (5). The underlying mechanism for discrimination between MHC promoters by NLRC5 and CIITA remains elusive. To address this question, we conducted domain-swapping experiments between NLRC5 and CIITA via the generation of a series of chimeric constructs containing combinations of the different domains of NLRC5 and CIITA.

For CIITA, it was reported that deletion of the acidic domain (aa 1–163) or the acidic and the proline/serine/threonine domain (aa 1–335) both result in dominant-negative CIITA proteins (19, 25, 36–38). Accordingly, we used two deletions of the N-terminal domain of CIITA; including the acidic domain alone (aa 1–163) and the acidic and P/S/T domain (aa 1–335) (according to NP_000237; refer to Ref. 39). These N-terminal regions (aa 1–163 and aa 1–335) were exchanged in CIITA by the corresponding fragment of NLRC5 (aa 1–142) as shown in Fig. 2A. We tested the ability of these chimeric fusion constructs to activate MHC class I or II promoters in gene-reporter assays in HEK293T cells that do not express endogenous NLRC5 (22) or CIITA (40). As reported, expression of NLRC5 and CIITA led to significant activation of the HLA-B250 MHC class I or HLA-DRA MHC class II promoter, respectively (Fig. 2B, 2C). Replacement of the N-terminal part of CIITA by the N-terminal effector domain of NLRC5 (DD-163-CIITA) led to a potent activation of both the HLA-B250 and the HLA-DRA reporters (Fig. 2B, 2C). Induction of MHC class I and II promoters by this chimeric construct was at least as strong as with NLRC5 or CIITA wild-type constructs also with lower concentrations of transfected plasmids (Fig. 2C). We noted that the construct lacking the P/S/T domain of CIITA (DD-335-CIITA) showed a lower, albeit not in all cases statistical significant activity compared with DD-163-CIITA (Fig. 2B, 2C). Western blot analysis showed that DD-335-CIITA was expressed at higher levels compared with DD-163-CIITA (Fig. 3). Notably, the CIITA “backbone” itself had no activity toward MHC class I or class II promoters as shown for a nuclear localization signal (NLS) containing deletion constructs of the first 335 aa of CIITA (CIITA NLS-L335) (Fig. 2B). Moreover, the exchange of the N-terminal domain of NLRC5 by the p65 transactivator domain resulted in a construct that lacked HLA-B or HLA-DRA activity, suggesting that intrinsic features of the NLRC5 DD are important for MHC gene expression (data not shown).

To conclude, although the P/S/T region of CIITA is not necessary for transcriptional activity (Fig. 1C) (33), this domain contributes to MHC class I and II induction as observed for the DD-163-CIITA construct (Fig. 2B). This is in line with its reported function in CIITA-dependent MHC class II induction (36) and lack of MHC class I or II activation of CIITA constructs with a deletion of this domain (5). Our results also suggest that MHC class I and II activation by the NLRC5/CIITA chimeric protein is determined by the effector domain (DD) of NLRC5.

The acidic domain of CIITA (Gal4-AD163) showed strong transcriptional activator function (Fig. 1C); however, fusion of this fragment to the NACHT and LRR domain of NLRC5 led to constructs that were not detectable inside the nucleus even after blocking nuclear export by leptomycin B (data not shown). This
suggests that the NLS located in the effector domain of NLRC5 (aa 121–122; aa 132–134) (3) is needed for functionality and that the CIITA C-terminal domain(s) cannot compensate for this requirement. To substantiate these results, we quantified MHC class I and II protein expression in HEK293T cells. As shown in Fig. 2D, overexpression of wild-type NLRC5 and DD-335-CIITA strongly induced MHC class I protein expression, whereas the well-described inactive K234A Walker A mutant of NLRC5 (NLRC5 K234A) (4, 5) failed to induce MHC class I expression, respectively. In agreement with our data above, only DD-335-CIITA and CIITA induced MHC class II protein expression.

To formally exclude that activity of the NLRC5 effector domain depends on structural clues provided by a NACHT-LRR backbone we generated a fusion protein of the NLRC5 N-terminal domain and NOD1 lacking the CARD domain, termed DD-NOD1 (Supplemental Fig. 2A). NOD1 was chosen as it is one of the founding members of the NLR family (9, 41). The DD-NOD1 did not induce the HLA-B250 promoter even upon stimulation with the NOD1 elicitor TriDAP (Supplemental Fig. 2B), although it was well-expressed and showed nuclear localization (Supplemental Fig. 2C).

To investigate the contribution of the LRR and NACHT domains to MHC activation, we constructed a series of NLRC5 and CIITA chimeric proteins with swapped LRR and NACHT domains. In CIITA, the LRR domain contributes to nuclear transport and MHC class II activation (37, 40, 42), whereas in NLRC5, the LRR domain plays a pivotal role in MHC class I activation and is involved in nuclear export (5). To test whether the LRR domains of NLRC5 and CIITA confer specificity for MHC class I versus MHC class II activation, we generated two LRR chimeric proteins, containing either the NLRC5 N terminus and the CIITA LRRs (NLRC5-562-LRR-762) or vice versa (CIITA-762-LRR-562) (Fig. 4A). These LRR chimeric constructs were tested in HEK293T-based gene reporter assays. As shown in Fig. 4B, neither the NLRC5-562-LRR-762 nor the CIITA-762-LRR-562 construct did activate MHC class I or II promoters. Expression of these constructs was confirmed in Western blot analysis, and indirect immunofluorescence experiments revealed that the NLRC5-562-LRR-762 was able to localize to the nucleus even without leptomycin B treatment (Fig. 4C). This is in agreement with the presence of an NLS located in the N-terminal effector domain of NLRC5 (3) and a role of the NLRC5 LRR region in nuclear export (5). To address
whether the NACHT domains contribute to the specificity, we exchanged these between NLRC5 and CIITA. The resulting constructs, AD-NLRC5-LRR and DD-CIITA-LRR were well expressed and showed nuclear location; however, they failed to activate both MHC class I or class II promoters (Fig. 4B, 4C). Although it should be noted that the DD-CIITA-LRR was not nuclear localized in all cells after leptomycin B treatment (Fig. 4C).

This revealed that the NACHT domains of CIITA and NLRC5 are not interchangeable, indicating that the NACHT-LRR module might act as a functional unit. Taken together, our data showed that the N-terminal domain of NLRC5 can compensate the function of the N-terminal domain of CIITA and moreover that this domain confers enhanced MHC class I promoter activation. Of note, MHC transcriptional activity in any case was linked to the NACHT-LRR backbone of CIITA or CIITA FIII.
NLRC5 as the DD of NLRC5 fused to an unrelated NLR backbone (NOD1) did not induce MHC promoters.

Restoring MHC expression on melanoma cells by delivery of chimeric NLRC5/CIITA constructs using AAV vector transduction

The chimeric protein containing the N terminus of NLRC5 and the NACHT domain and C terminus of CIITA (DD-335-CIITA) was a very strong inducer of MHC class I and MHC class II gene expression with higher activity than wild-type CIITA or NLRC5. Moreover, this construct had a smaller m.w., favoring its use in vector-mediated transduction experiments. Low MHC class I surface expression is seen on many malignantly transformed cells and was proposed as a mechanism avoiding immune recognition of these cells by CD8+ cytotoxic T lymphocytes. We tested the functionality of our synthetic construct to enhance MHC expression in melanoma cells. Using an electroporation-based transfection protocol, we expressed NLRC5, CIITA, and DD-335-CIITA in B16F10 cells. As shown before (5), expression of NLRC5 led to enhanced MHC class I (H-2Kb) mRNA and protein surface expression (Supplemental Fig. 3A, 3B). In contrast, CIITA induced mainly MHC class II (H2-IA) expression (Supplemental Fig. 3A, 3B). The DD-335-CIITA induced both MHC classes and showed a much stronger effect on MHC class I and class II compared with either NLRC5 or CIITA, respectively (Supplemental Fig. 3A, 3B). Notably, this widely used transfection protocol led to enhanced surface expression of MHC class I also in mock transfected cells (Supplemental Fig. 3B). To overcome general limitations by the use of liposome- and electroporation-based transfections and in particular the observed unspecific effects on MHC expression, we generated AAV vector particles able to deliver DD-335-CIITA cDNA to a broad range of human and murine cell types. First, we tested the functionality of this vector in HEK293T cells and measured MHC class I or MHC class II transcriptional activation using luciferase gene reporter assays. Transduction with doses of 5000 particles per cell (ppc) of AAV-DD-335-CIITA strongly activated both MHC class I and class II promoters (Fig. 5A), whereas the AAV-GFP control vector did not activate MHC class I or II promoters over background (Fig. 5A). Induction of MHC class I and II transcription induced by transfection of 10 ng NLRC5 or CIITA plasmid was comparable to that obtained by 500 ppc AAV-DD-335-CIITA (Fig. 5A). Protein levels of DD-335-CIITA, HLA-/B/C, and HLA-RD/DP were verified by Western blot analysis and positively correlated with the ppc used (Fig. 5B).

We next applied these AAV vector particles to murine melanoma cells (B16F10) and analyzed MHC class I (H-2Kb) and II (H2-IA) mRNA levels compared with cells transfected with AAV-GFP or nontreated controls (Fig. 6C). As expected, expression of DD-335-CIITA enhanced MHC class I and II mRNA expression (Fig. 6D).

To assess the functional relevance of enhanced MHC class I expression on B16F10 cells, we conducted CTL-mediated cytotoxicity assays. To this end, we used CD8+ splenocytes derived from LCMV infected C57BL/6 mice as effector cells against B16F10 target cells loaded with the LCMV-derived, H-2Db-restricted peptide gp33–41 in ex vivo cytotoxicity assays. B16F10 cells were transduced with AAV-DD-335-CIITA or AAV-GFP as control. AAV-DD-335-CIITA resulted in the expression of DD-335-CIITA and higher MHC class I (H-2Kb) and MHC class II (H2-IA) mRNA levels compared with cells transduced with AAV-GFP or nontreated controls (Fig. 7A). Accordingly, we measured higher H-2Kb and H2-IA surface expression in the DD-335-CIITA transduced cells (Fig. 7B). The B16F10 cells transduced with DD-335-CIITA were approximately two times more effectively lysed by CTLs than target cells transduced with the GFP expressing control vector (Fig. 7C, left panel). The specific lysis of the B16F10 cells at a given E:T cell ratio thereby correlated with the amount of peptide used for loading; however, at all peptide concentrations DD-335-CIITA expressing cells were more efficiently lysed (Fig. 7C, right panel). Thus, DD-335-CIITA–induced MHC class I surface expression was sufficient to lead to augmented Ag-specific CTL lysis of tumor cells. Similar effects were observed when DD-335-CIITA was delivered by electroporation. Although, this method led to enhanced Ag-specific CTL lysis already in mock treated cells compared with untreated controls, in any case DD-335-CIITA delivery enhanced lysis more efficiently than delivery of NLRC5 or CIITA (Supplemental Fig. 3C).

To conclude, our work showed that the N-terminal domain of NLRC5 has transcriptional activity toward MHC class I and class II promoters. A chimeric construct of this domain on a CIITA backbone yielded a protein with higher activity toward MHC class I and class II promoters than each NLRC5 or CIITA itself. This DD-335-CIITA protein was able to significantly enhance MHC class I and class II expression in mammalian cells leading to increased lysis of B16F10 melanoma cells by Ag-specific CTLs.

Discussion

Regulation of MHC class I and MHC class II protein expression is pivotal for cell-mediated adaptive immune responses in mammals (43). In particular, the quite restricted and highly inducible expression of MHC class II on APCs upon activation was studied in detail leading to the identification of CIITA as the major transcriptional regulator of MHC class II genes (10, 39, 44). CIITA is a member of the NLR protein family. Only recently the CIITA related protein NLRC5 was identified as a key regulator of MHC class I gene transcription (3). We and others showed that NLRC5 and CIITA use the same docking site at the promoter sequences and both use a similar MHC enhanceosome complex (5, 35). This

![Figure 3](http://www.jimmunol.org/DownloadedFrom/)
suggests that features of the NLRC5 and CIITA proteins themselves contribute to promoter discrimination of these two NLRs.

In this study, we identified that the N-terminal domain of NLRC5 has intrinsic transcriptional activity and that this domain contributes to regulation of MHC promoters. However, the transcriptional activity of the NLRC5 N-terminal domain was low compared with typical transcriptional activator domains, such as that of p65 or CIITA. A plausible explanation for this low activity could be that this domain recruits transcriptional enhancers rather than containing intrinsic transcriptional activity itself. In agreement with this, replacement of the first 335 aa of CIITA with the NLRC5 N-terminal domain was sufficient to enhance the transcriptional activity of this construct toward MHC class I promoters (Fig. 2B). Interestingly, whereas the transcriptional activity of the N-terminal domain of NLRC5 is 5- to 10-fold weaker compared with the CIITA N-terminal counterpart in GAL4-DBD fusion experiments, the fusion of the NLRC5 N-terminal domain to the CIITA NACH/LRR region completely restored MHC class activity.

**FIGURE 4.** Exchange of the LRR and NACHT domains between CIITA and NLRC5 is not compatible. (A) Schematic overview of NLRC5-CIITA chimeric constructs used in this figure. (B) Five nanograms of NLRC5 or CIITA constructs and 1, 5, or 10 ng chimeric constructs were transfected together with 20 ng MHC-reporter and 10 ng β-galactosidase encoding plasmid. Luciferase activity was normalized to β-galactosidase expression. Depicted are mean ± SD (n = 3). (C) Indirect immunofluorescence was performed as described in Fig. 3. Indirect immunofluorescence micrographs of staining using the indicated Abs (green) are shown together with a merge of this signal with DNA staining (blue). Bar, 10 µm. Expression of the constructs is shown by Western blot, probing for β-actin as loading control (right panels). LepB, leptomycin B; RS, reporter system (cell transfected with the reporter constructs only as control).
I and class II transcriptional activity. To date, the exact role of the proline/serine/threonine (P/S/T) domain of CIITA is not known; however, it is important for CIITA-mediated MHC class II expression (36). Although the P/S/T domain positively contributes to MHC induction in the chimeric NLRC5/CIITA construct DD-163-CIITA, this domain is not absolutely required for its MHC expression (36). Although the P/S/T domain positively contributes to MHC induction in the chimeric NLRC5/CIITA construct DD-163-CIITA, this domain is not absolutely required for its MHC

**FIGURE 5.** AAV vector–mediated delivery of DD-335-CIITA to human cells. (A) HEK293T cells were transduced with the indicated AAV vector constructs and analyzed after 48 h. Twenty nanograms of MHC-reporter (MHC class I, HLA-B250; MHC class II, HLA-DRA) and 10 ng β-galactosidase encoding plasmid were transfected, and luciferase activity was analyzed. Luciferase activity was normalized to β-galactosidase expression and is shown as fold induction over background (RS). Depicted are mean ± SD (n = 3). (B) HEK293T cells were transduced with AAV vectors and analyzed after 72 h. Protein expression was analyzed by Western blot using the indicated Abs, probing for GAPDH served as loading control. RS, reporter system (cell transfected with the reporter constructs only as control).

**FIGURE 6.** AAV-DD-335-CIITA functionally restores MHC I and II expression on B16F10 melanoma cells. The murine melanoma cell line B16F10 was transduced with 5,000–40,000 ppc AAV-GFP or AAV-DD-335-CIITA. (A) Transduction efficiency of AAV-DD-335-CIITA in B16F10 was analyzed 4 h post-transduction (p.t.) using qRT-PCR. Depicted are the mean ± SD relative to HPRT expression. (B) Seventy-two hours postinduction, the percentage of GFP-positive cells was determined using flow cytometry for AAV vector–treated B16F10 cells. (C) DD-335-CIITA mRNA expression was analyzed using qRT-PCR. Each sample was analyzed in triplicates. Depicted are mean ± SD relative to HPRT expression. (D) Endogenous murine H-2Kb and H2-IA mRNA expression was analyzed 72 h p.t. using qRT-PCR compared with untreated cells set to 1.
transcriptional activity as shown by the activity of DD-335-CIITA lacking the P/S/T.

NLRC5 and CIITA are both shuttling between cytoplasm and nucleus but differ in their distribution, with NLRC5 mainly being present in the cytoplasm, whereas CIITA is present in both cytoplasm and nucleus. NLRC5 fused to a strong viral NLS that enhanced its nuclear localization however does result in diminished MHC class I transcriptional activity (5, 35). Although our NLRC5/CIITA chimeras exclusively localized to the nucleus, they displayed enhanced activity compared with NLRC5 and CIITA. Thus, a divergent regulation between NLRC5 and CIITA, which relies on their different NACHT and in particular LRR domains is plausible. It is noteworthy, that exchange of the LRRs or NACHT domains between NLRC5 and CIITA yielded nonfunctional constructs. Given that NLRs are regulated by intermolecular interactions involving the LRRs and the NACHT domain, this might indicate that the NACHT-LRR acts as one functional unit. Evidence for this is provided by the crystal structures of the NLR-related protein APAF-I (reviewed in Ref. 45). Also, the structure of NLRC4, a bona fide NLR protein, shows that interactions both between two subdomains of the NACHT domain and of the LRR with the nucleotide-binding domain are essential for controlling NLRC4 activity (46). Moreover, it was shown that the region between the NACHT and the LRR of NOD2 is important for the regulation of NOD2 activity (47).

The NLRC5/CIITA chimeric construct (DD-335-CIITA) we generated is considerably shorter than NLRC5 and shows improved activity to induce MHC I and MHC II promoters compared with NLRC5 and CIITA. We used this construct for the generation of AA V vectors for gene-delivery of this construct. AA V vectors were chosen as they represent well-established gene delivery tool also in humans with a so far excellent safety record and transduction efficiencies that conferred clinical benefit (reviewed in Refs. 48 and 49).

To test the efficiency of our construct in restoring MHC class I and class II gene expression, we used the melanoma cell line B16F10, which expresses low levels of MHC class I (50). In vitro

FIGURE 7. Transduction with AAV-DD-335-CIITA renders B16F10 more susceptible for CTL-mediated lysis. Murine melanoma B16F10 cells were transduced with 40,000 ppc AAV-DD-335-CIITA or AAV-GFP or were left untreated (Ctrl) and analyzed after 48 h. (A) Endogenous H-2Kb and H2-IA as well as DD-335-CIITA transgene mRNA expression was analyzed by qRT-PCR. Expression was normalized to HPRT and is shown as fold induction compared with untreated B16F10 cells. Mean ± SD from measurements in triplicate is shown. Data from one of three representative experiments is shown. (B) Flow cytometrical analysis of H-2Kb and H2-IA/IE surface expression on the cells from (A). Data from one of three representative experiments is shwon. (C) Lysis of the B16F10 cells from (A) by Ag-specific CD8+ T lymphocytes was measured by a cytotoxicity assay as described in Materials and Methods. Specific lysis at different E:T ratios (left graph) and specific lysis at E:T 50% with different concentrations of loading peptide (right graph) is shown as mean ± SEM of two independent experiments.
delivery of our novel construct through AAV vectors to these cells increased MHC class I and class II expression and subsequently led to a more efficient killing of transduced cells by Ag-specific CD8⁺ cytotoxic T lymphocytes. This showed that our construct is able to enhance MHC class I Ag presentation in a physiological context. Importantly, when directly compared with NLRC5 and CIITA this construct was a far better inducer of MHC gene expression and CTL-mediated lysis. We also realized that induction of murine H-2Kb in B16F10 seemed to be lower than the induction of HLA-B/C in human HEK293T cells, indicating that either cell type or species specific factors contribute to this process.

To conclude, we show that the N-terminal domain of NLRC5 confers transcriptional activity and contributes to the activation of MHC class I and class II promoters. Moreover, we describe the generation of a NLR5/CIITA chimera protein that is able to strongly increase MHC class I and class II promoters. Vector-based delivery of this construct increased recognition of murine type or species specific factors contribute to this process.

References

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Disclosures

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