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NLRC5 Deficiency Selectively Impairs MHC Class I-Dependent Lymphocyte Killing by Cytotoxic T Cells

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Nucleotide-binding oligomerization domain-like receptors (NLRs) are intracellular proteins involved in innate-driven inflammatory responses. The function of the family member NLR caspase recruitment domain containing protein 5 (NLRC5) remains a matter of debate, particularly with respect to NF-κB activation, type I IFN, and MHC I expression. To address the role of NLRC5, we generated Nlrc5-deficient mice (Nlrc5∆/∆). In this article we show that these animals exhibit slightly decreased CD8+ T cell percentages, a phenotype compatible with deregulated MHC T expression. Of interest, NLRC5 ablation only mildly affected MHC I expression on APCs and, accordingly, Nlrc5∆/∆ macrophages efficiently primed CD8+ T cells. In contrast, NLRC5 deficiency dramatically impaired basal expression of MHC I in T, NKT, and NK lymphocytes. NLRC5 was sufficient to induce MHC I expression in a human lymphoid cell line, requiring both caspase recruitment and LRR domains. Moreover, endogenous NLRC5 localized to the nucleus and occupied the proximal promoter region of MHC I expression in a human lymphoid cell line, requiring both caspase recruitment and LRR domains. Furthermore, NLRC5-deficient mice (%20 displayed nearly normal CD8+ T-cell percentages, and, in addition, we observed low NLRC5 expression in several murine and human lymphoid-derived tumor cell lines. Hence, loss of NLRC5 expression represents an advantage for evading CD8+ T cell-mediated elimination by downmodulation of MHC I levels—a mechanism that may be exploited by transformed cells. Our data show that NLRC5 acts as a key transcriptional regulator of MHC I in lymphocytes and support an essential role for NLRs in directing not only innate but also adaptive immune responses. The Journal of Immunology, 2012, 188: 000–000.

The online version of this article contains supplemental material.

Abbreviations used in this article: B2m, β2 microglobulin; BMDM, bone marrow-derived macrophage; CARD, caspase recruitment domain; cDC, conventional dendritic cell; ChIP, chromatin immunoprecipitation; NLR, nucleotide-binding oligomerization domain-like receptor; NLRC5, NLR CARD containing protein 5; NOD, nucleotide-binding oligomerization domain; poly(I:C), polyinosinic-polycytidylic acid; RNAi, RNA interference; SP, single positive; TEC, thymic epithelial cell; TEM, thymic epithelial cell; TIF, thymic epithelial cell; TIE, thymic endothelial cell.

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These data highlight the important contribution of NLRs to innate-driven inflammation and their broad involvement in the immune response.

Despite intense investigation, functions for only a few NLRs have been firmly established (12). NLR caspase recruitment domain (CARD) containing protein 5 (NLRC5) (also called NOD4) is a poorly characterized NLR family member with high structural homology to NOD1, NOD2, and CIITA (13, 14), and its function remains a matter of debate. Given the important similarity to NOD1 and NOD2, the capacity of NLRC5 to modulate the NF-κB and the type I IFN pathways has been assessed. Overexpression and RNA interference (RNAi)-mediated knockdown studies suggested that NLRC5 negatively regulates both the NF-κB and the type I IFN responses (13, 15). However, subsequent studies reported contradictory observations showing that NLRC5 promotes...
type I IFN production (16, 17). NLRC5 was also proposed to form an inflammasome, as its knockdown by RNAi led to decreased IL-1β secretion in response to a number of pathogens and pathogen-associated molecular patterns (18). Importantly, it was recently shown that myeloid cells derived from Nlrc5 knockout mice presented alterations in neither inflammasome activation nor in the NF-κB and type I IFN pathways (19). Finally, by analogy to CIITA, it was suggested that overexpression of NLRC5 in a human T cell line drives the expression of MHC class I and functionally related genes, such as β2 microglobulin (B2m) (14). However, another study showed that RNAi-mediated NLRC5 knockdown in a murine myeloid cell line led to increased MHC I expression (13). Therefore, it is currently unclear whether NLRC5 modulates the MHC I Ag presentation pathway, and with which outcome.

Whereas data on Nlrc5 mRNA tissue distribution consistently indicate expression by immune cells (13, 15, 17), the regulation of NLRC5 expression is also controversial. In fact, NLRC5 expression was shown to be triggered by the TLR3 ligand polyinosinic-polycytidylic acid [poly(I-C)], by viral infection, or by IFN-γ (13, 16, 17), suggesting a direct role for both type I and II IFNs. This hypothesis was, however, not directly addressed and seems difficult to reconcile with the proposed MyD88-dependent induction of NLRC5 upon LPS triggering of macrophages (15).

In this study we show that NLRC5 expression mostly relies on the transcription factor STAT1 and is dependent on type I and II IFNs. Splenocyte analysis of Nlrc5 knockout mice (Nlrc5<sup>−/−</sup>) revealed a modest decrease in CD8<sup>+</sup> T cell percentages and a tendency to increased NK cell proportion, supporting the hypothesis of an altered MHC I display. Whereas the expression of H-2 genes was only mildly affected in myeloid and intermediate so in B cells, it was strikingly decreased in T, NK, and NK lymphocytes. Accordingly, cytotoxic T cell-mediated killing of Nlrc5-deficient T lymphocytes was severely impaired. Moreover, NLRC5 expression was found to be low in several B cell- and T cell-derived tumor lines. Altogether, our results highlight the importance of NLRC5-dependent MHC I expression on lymphocytes for their cognate elimination by CD8<sup>+</sup> T cells.

Materials and Methods

Mice

Generation of Nlrc5<sup>−/−</sup> mice (Oxgene) is described in Supplemental Fig. 2. Nlrc5<sup>−/−</sup> mice were generated by crossing Nlrc5<sup>+/−</sup> to the CMV-Cre deleter strain (The Jackson Laboratory), leading to deletion of the ltv-p-flanked region also in germ cells, thus allowing selection of Nlrc5-deleted off-spring and elimination of the CMV-Cre transgene in the following generation. C57BL/6 mice were purchased from Harlan; Nlrc5<sup>+/−</sup>, Nlrc5<sup>−/−</sup>, Stat1<sup>−/−</sup> (20), Ifnγr1<sup>−/−</sup> (21), Mtb88<sup>−/−</sup> (23), Trif<sup>−/−</sup> (23, 24), and OT-I (25) mice were used in our animal facility and treated in accordance with the Swiss Federal Veterinary Office guidelines. Unless otherwise specified, 6- to 12-wk-old mice were used.

Tumor cell lines

Human Jurkat and lymphoma cell lines were maintained as previously described (26). Murine tumor cell lines were grown in DMEM supplemented with 10% FCS, 50 μM β-mercaptoethanol, and antibiotics. EL-4 cells are a spontaneous variant derived from EL-4 cells in this article called EL-4<sup>−</sup>, characterized by reduced surface MHC I levels (H. Robson MacDonald, unpublished observations).

Media and reagents

The medium used for all T cell in vitro experiments was previously described (27). Ultrapure LPS and poly(I:C) were from Invivogen, IFN-α11 and IFN-β from PBL IFN Source. CpG 1826 from Microsynth, and IFN-γ from Peprotech. Unless otherwise specified, LPS was used at 100 ng/ml, IFN-β at 1000 U/ml, IFN-γ at 10 ng/ml, poly(I:C) at 1 μg/ml, CpG at 1 μg/ml, and IFN-α at 1000 U/ml.

Enrichment of splenic cell populations, bone marrow-derived macrophage and bone marrow-derived dendritic cell differentiation

CD4<sup>+</sup> T cells were enriched using anti-CD4 magnetic beads (Miltenyi Biotec). CD11b<sup>+</sup>, CD8<sup>+</sup> T, and B cells were enriched using a two-step procedure. Anti-CD11b (clone M1/70), anti-CD8α (Ly-2), and anti-B220 (RA3-6B2) were followed by the appropriate anti-fluorochrome magnetic beads (Miltenyi Biotec). Bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells were differentiated as previously described (27). Thiglycolate-elicited macrophages (TEMs) were isolated 3 d after i.p. injection of a 10% thioglycollate solution.

Enrichment of primary human PBMC populations

We obtaineduffy coats from the Lausanne Blood Transfusion Center. Mononuclear cells were isolated from heparinized blood by density centrifugation over a Ficoll–Paque gradient (GE Healthcare). CD4<sup>+</sup> cells, CD8<sup>+</sup> T cells, monocytes, B cells, and NK cells were enriched using anti-CD4, -CD8, -CD14, -CD19, and -CD56 magnetic beads, (Miltenyi Biotec). Cellular enrichment was confirmed by FACS and was at least 90%.

FACS Abs, staining, and thymocyte gating strategy

Cells were preincubated with anti-CD16/32 (2.4G2) to block FcRs, then surface stained using the following Abs raised against CD8a (Ly-2), CD3ε (145-2C11), CD4 (L3T4), CD11b (M1/70), CD11c (N418), CD19 (IID3), CD44 (IM7), CD62L (MEL-14), F4/80 (BM8), H-2<sup>K<sub>b</sub></sup> (AF6-85.5.3), MHC II (M5/1/4,15.2), CD1d (1B1), NK1.1 (PK136), HLA-ABC (W6/32), TCRγδ (GL3) (all from eBioscience), B220 (RA3-6B2), and CD25 (7D4) (both from BD Biosciences). Allophycocyanin-Cy7–labeled streptavidin was from eBioscience. Stainings were performed with an appropriate combination of fluorophores. To gate on thymocytes, the lineage mixture used contained anti-B220, -NK1.1, -F4/80, -CD11c, and -TCRγδ. Propidium iodide (Sigma-Aldrich) was used to exclude dead cells from in vitro cultures. Samples were acquired on a FACSCanto flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

In vitro T cell proliferation and cytotoxic T cell-mediated killing

For CD8<sup>+</sup> T cell proliferation assays, 1.5 × 10<sup>6</sup> CFSE (Sigma-Aldrich)-labeled, MACS-purified OT-I T cells were incubated together with 5 × 10<sup>4</sup> Nlrc5<sup>−/−</sup> and Nlrc5<sup>−/−</sup> BMDMs and the indicated doses of OVA (Calbiochem). The third day, cells were harvested and proliferation assessed by FACS. For OT-I–mediated killing of OVA-pulsed target cells, Nlrc5<sup>−/−</sup> and Nlrc5<sup>−/−</sup> MACS-purified target CD4<sup>+</sup> and CD8<sup>+</sup> T cells or T cell-depleted negative fractions were labeled with two levels of CFSE [briefly, CFSE labeling was performed in PBS 8 min at room temperature, at the final concentrations of 1.25 (high) or 0.125 (low) μM] and pulsed for 75 min with 10 μM SfNFEK peptide (Anaspec) at 37°C. For effector T cell generation, MACS-purified T cells were stimulated in vitro with 10 μg/ml plastic-bound functional grade purified anti-CD3 (145-2C11), 2 μg/ml soluble anti-CD28 (37.51) (eBioscience), and human rIL-2 (Glaxo) for 2 d. Effector cells were then maintained in IL-2 (5 ng/ml)–supplemented medium and used on day 3 or 4. For killing, 5 × 10<sup>6</sup> differentially labeled Nlrc5<sup>−/−</sup> and Nlrc5<sup>−/−</sup> targets were incubated overnight with the indicated numbers of in vitro activated CD8<sup>+</sup> T cells.

Immunoblot analysis

Rabbit polyclonal anti-mouse NLRC5 (aa 1–139) and anti-human NLRC5 (aa 1–116) were generated by Adipogen. Other Abs used were monoclonal anti-tubulin (Sigma-Aldrich), anti-phosphorylatedIkKα, polyclonal anti-IkBα, anti-caspase-3, anti-phosphorylated IKKα and -β (all from Cell Signaling), anti-lamin B1, and anti-β-actin (both from Abcam).

ELISA

Cell culture supernatants were assayed for mouse TNF, IL-6 (eBioscience), and IFN-β (PBL IFN Source) according to the manufacturer’s instructions.

Quantitative PCR

RNA extraction, retrotranscription to cDNA, and expression analysis were done as previously described (28). Primer sequences are available upon request.
**Results**

**NLRC5 is highly expressed by lymphocytes**

To start, we investigated NLRC5 expression at the protein level and found it particularly abundant in lymph nodes and spleen (Supplemental Fig. 1A). NLRC5 was also clearly detectable in primary lymphoid organs and tissues highly populated by immune cells, such as liver and intestine (Supplemental Fig. 1A). A more detailed examination of purified splenic cell subsets revealed high NLRC5 expression in the lymphoid lineage, particularly in CD4^+ and CD8^+ T cells, whereas it was present to a lesser degree in myeloid cells (Fig. 1A). A similar pattern was confirmed in human cells, where it was also detected in CD56^+ NK/NKT cells (Fig. 1B). NLRC5 is therefore constitutively expressed in immune cells, particularly by lymphocytes.

**NLRC5 is induced by a STAT1-dependent pathway**

We next investigated the regulation of NLRC5 expression. In BMDMs, Nlrc5 transcript levels were found to be already increased 4 h after exposure to LPS (Fig. 1C, Supplemental Fig. 1B). Accordingly, NLRC5 protein augmented following incubation with LPS in a time-dependent manner in both BMDMs and TEMs (Fig. 1D, Supplemental Fig. 1C, 1D). Of interest, this up-regulation was dependent on the TLR4 adaptor Trif and not on MyD88, as shown by the use of Trif^-/- and MyD88^-/- BMDMs (Supplemental Fig. 1B–D). This finding suggested a role for autocrine IFN signaling in the induction of NLRC5, a hypothesis also supported by the ability of IFNs to induce NLRC5 at both the mRNA and protein level (Fig. 1C, 1D). Accordingly, NLRC5 induction was particularly high when using agonists of TLR3 and TLR4, known to strongly induce type I IFN, with more rapid kinetics in CD8^+ T cells (Fig. 1D, Supplemental Fig. 1E). Furthermore, Nlrc5^-/- mice did not show altered TLR-triggered IFN-beta production upon TLR triggering (Supplemental Fig. 2D). We next sought to analyze the lymphoid compartment of Nlrc5^-/- mice, given the prominent expression of NLRC5 in these cells. Irrespective of their age, Nlrc5^-/- mice exhibited only mild differences in the proportions of lymphocyte subsets (Fig. 2, Supplemental Fig. 3A). We consistently observed a slight decrease in the percentage of CD8^+ T cells in spleen, lymph nodes, and blood. We therefore analyzed the ability of Nlrc5^-/- or Nlrc5^+/+ T cells to proliferate and survive upon TCR triggering in vitro, and the activation state ex vivo, and did not observe major differences (Supplemental Fig. 3B, 3C). Of interest, NK cells showed a tendency to increased percentages in the spleen and the blood of young animals (8–12 wk old) (Fig. 2, Supplemental Fig 3A). Importantly, whereas spleen cellularity in young Nlrc5^-/- mice was similar, compared with that in Nlrc5^+/+ controls, older (34- to 36-wk-old) Nlrc5^-/- mice often exhibited enlarged spleens (Supplemental Fig. 3D). Taken together, these results reveal a mild alteration in the homeostasis of the CD8^+ lymphoid compartment in Nlrc5^-/- animals.

**Selected Nlrc5-deficient lymphocyte subsets display markedly reduced MHC I levels**

The differences in CD8^+ T cell percentages prompted us to assess the levels of MHC I, as these cells require MHC I for their thymic selection and peripheral maintenance. Indeed, splenocyte analysis revealed a dramatic reduction in the display of both H-2D and H-2K MHC I molecules by Nlrc5^-/- CD4^+ and CD8^+ T, NK, NKT, and γδ T cells (Fig. 3A); an intermediate reduction among B cells; and a mild decrease in conventional dendritic cells (cDCs) (Fig. 3B). As STAT1 is crucial for NLRC5 expression (Fig. 1E), we assessed MHC I levels in T cells derived from Stat1-deficient mice. We observed defective MHC I expression by these cells, and the
defect was more pronounced for H-2K, as in the case of Nlrc5 deficiency (Fig. 3C). Moreover, the partial dependency of NLRC5 expression on STAT1 observed in CD8+ T cells (Fig. 1E) was reflected in their milder defect in MHC I expression (Fig. 3C). To delineate the specificity of NLRC5 in controlling MHC I expression, we also investigated the levels of MHC II and the non-classical MHC I CD1d on APCs (Fig. 3B). Both these MHC molecules were only moderately altered, emphasizing the specificity of NLRC5 in controlling the display of MHC I proteins.

We then assessed whether the perturbed levels of MHC I observed on splenocytes could also be found in the thymus, the organ where T cell selection proceeds. Indeed, MHC I expression was dramatically decreased on single positive (SP) thymocytes (Fig. 3D). Moderately decreased levels of H-2 molecules were also apparent on cDCs and on cortical and medullary thymic epithelial cells (TECs) (Supplemental Fig. 3E, 3F). Nonetheless, thymocyte development did not present overt abnormalities in Nlrc5D/D mice, except for a slight decrease in CD8+ SP (Supplemental Fig. 3G, 3H).

Altogether, these findings demonstrate that NLRC5 is essential for MHC I display on T, NKT, and NK cells. Of interest, although the display of MHC I in TECs and thymic cDCs is partially reduced in Nlrc5D/D mice, CD8+ T cell numbers and activation state are fairly normal.

NLRC5 drives H-2 gene transcription and requires both CARD and LRR domains

To delineate the mechanism by which NLRC5 regulates MHC I expression, we assessed the abundance of H-2K and H-2D mRNA in Nlrc5D/D and Nlrc5D/D T cells (Fig. 4A). Interestingly, we detected decreased amounts of both mRNAs in Nlrc5D/D T cells. As MHC I is formed by the association of the H-2 chain with B2m, we tested the abundance of B2m mRNA and found that the latter was affected to a milder extent. We next overexpressed

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**FIGURE 1.** NLRC5 expression is high in lymphocytes, and its induction is STAT1 dependent. (A and B) NLRC5 expression in the indicated splenocyte populations isolated from C57BL/6 animals (A), or among the indicated human PBMC populations (B), was determined by immunoblot analysis. Tubulin was used as loading control. (C) The abundance of Nlrc5 mRNA (relative to Hprt) upon LPS or IFN-β stimulation was determined by quantitative PCR in BMDMs at the indicated times. (D and E) NLRC5 expression was determined by immunoblot analysis in the indicated cell types and stimulatory conditions. (D) BMDMs derived from wild-type (wt), Ifnar1−/−, Ifnγ−/−, and Stat1−/− mice were stimulated for 16 h with poly(I:C), LPS, CpG, IFN-α, IFN-β, or IFN-γ. (E) Splenic CD4+ and CD8+ T cells from either wt, Ifnar1−/−, Ifnγ−/−, or Stat1−/− mice were stimulated for 2 d on plastic-coated anti-CD3 Ab, in the presence of soluble anti-CD28 and IL-2. Cell lysates were collected before stimulation, at day (d) 2 or 5. (F) The abundance of Nlrc5 mRNA following 16 h of IFN-γ stimulation was determined by quantitative PCR in CD4+ and CD8+ T cells. One representative experiment of at least two is shown (A–F). **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 2.** Nlrc5 deficiency mildly affects lymphocyte homeostasis. Percentages of splenic CD4+ (CD3+, CD4+), CD8+ (CD3+, CD8+), γδ (CD3+, TCRγδ+) T cells, NKT cells (NK1.1+, CD3+), NK cells (NK1.1+, CD3−), and B cells (CD19+) from Nlrc5D/D and Nlrc5D/D mice are shown. Data illustrate mean ± SEM; results for individual mice are depicted (n = 7) and are representative of at least three independent experiments, although the increase in γδ T cell percentages is not always observed. *p ≤ 0.05, ***p ≤ 0.001.
NLRC5 in the T lymphocyte-derived Jurkat cell line and measured MHC I expression by FACS. We found that whereas constructs lacking the CARD or the LRR domain were inactive, full-length NLRC5 strongly increased HLA-AB and -C levels (Fig. 4B). These data support the hypothesis that NLRC5 acts as a transcriptional regulator; we therefore tested the ability of NLRC5 to shuttle into the nucleus. Indeed, we detected NLRC5 in both cytoplasmic and nuclear fractions of splenocytes (Fig. 4C), in both the presence and the absence of IFN-β treatment.

To exert its transcriptional regulatory activity, CIITA is recruited to the promoters of MHC II genes by a DNA-bound protein complex called “enhanceosome” (31–33). Enhanceosome factors bind cooperatively to the SXY sequence, a conserved regulatory module found in the proximal promoter regions of MHC II, MHC

**FIGURE 3.** MHC I expression in lymphocytes is regulated by NLRC5. (A and B) Expression of different MHC molecules on the indicated splenic Nlrc5fl/fl and Nlrc5D/D subpopulations was analyzed. Graphs illustrate H-2D and H-2K geometric mean fluorescence intensities (MFIs) (A). Graphs show MFIs of H-2D, H-2K, MHC II, and CD1d expression by B cells and cDCs (CD11c+CD11b−) (B). (C) Graphs illustrate H-2D and H-2K MFIs of wt and Start1−/− CD4+ and CD8+ T cells. (D) Graphs depict H-2K and H-2D MFIs on double positive (DP) [lineage−, CD4+, CD8−], CD4+ SP (lineage−, CD3+, CD4+, CD8−), and CD8+ SP (lineage−, CD3+, CD4−, CD8+) thymocyte subpopulations. Graphs depict mean ± SEM of MFIs (n = 3), and results are representative of at least two independent experiments (A–D). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
I, and related genes (34, 35). By analogy, we assessed the ability of NLRC5 to occupy the proximal SXY module of the H-2D, H-2K, and B2m genes by ChIP. As shown in Fig. 4D, endogenous NLRC5 was efficiently recruited to the promoter regions of these genes in T cells, whereas binding to the promoter of an unrelated gene (TBP) was not observed. The specificity of our Ab in the ChIP assay was ensured by the use of Nlrc5−/− deficient T cells. Altogether, these observations indicate that NLRC5 acts as a transcriptional regulator of MHC I genes and requires both the CARD and the LRRs for this activity. However, mRNA levels of B2m are less affected than those of the H-2D and H-2K genes, suggesting the existence of additional mechanisms promoting B2m transcription.

**NLRC5-dependent MHC I expression is not crucial for CD8+ T cell priming**

Given the fact that IFNs regulate MHC I expression, we next asked whether IFN-induced MHC I was also affected by Nlrc5 deficiency. To this end, we stimulated Nlrc5−/− and Nlrc5−/− T cells with type I or II IFN. Of interest, IFNs were able to induce H-2D and H-2K expression in both Nlrc5−/− and Nlrc5−/− CD4+ and CD8+ T cells (Fig. 5A, Supplemental Fig. 4A). As a consequence, although still clearly present, the fold difference in MHC I levels between Nlrc5−/− and Nlrc5−/− T cells diminished, in particular upon IFN-β exposure. Similar results were obtained in BMDMs, although the overall difference in H-2K expression between Nlrc5−/− and Nlrc5−/− cells was minor, compared with what was observed in T cells (Supplemental Fig. 4B). The mild decrease in MHC I levels found on Nlrc5−/− APCs led us to attest to the competence of Nlrc5−/− and Nlrc5−/− BMDMs to prime transgenic OT-I CD8+ T cells, whose cognate peptide (SIINFEKL) is presented in the context of H-2Kb. Indeed, OT-I T cell proliferation induced by unpulsed Nlrc5−/− and Nlrc5−/− T cells were used to immunoprecipitate cross-linked chromatin fragments derived from Nlrc5−/− or Nlrc5−/− T cells. Immunoprecipitates were analyzed by quantitative PCR for the abundance of SXY sequences of the H-2D, H-2K, and B2m promoters, and for the promoter sequence of TATA binding protein (TBP). Relative promoter binding is shown. The average and SD of three experiments are depicted.

Thus, consistent with the milder defect in MHC I levels in professional APCs, which is further attenuated under inflammatory conditions, NLRC5 does not play a major role in CD8+ T cell priming.

**NLRC5 expression by target lymphocytes is essential for their cognate elimination**

MHC I expression on nearly all nucleated cells is an essential safeguard mechanism allowing CD8+ T cells to identify and eliminate infected or transformed cells. We thus investigated the cytolytic T lymphocyte-dependent killing of Nlrc5−/− T cell targets, which present a strong defect in MHC I display. For this purpose, Nlrc5−/− and Nlrc5−/− T cells were labeled with distinguishable dim and bright CFSE levels, respectively; pulsed with the SIINFEKL peptide; and incubated overnight with OT-I CD8+ effector T cells. Nlrc5−/− target T lymphocytes were much less efficiently killed by OT-I CD8+ T cells, compared with their Nlrc5−/− counterparts (Fig. 6A, Supplemental Fig. 4C). Furthermore, when unpulsed Nlrc5−/− and Nlrc5−/− T cells were used as targets, or polyclonal effector cells employed as killers, no differences were observed.

**FIGURE 4.** NLRC5 occupies the promoters and drives transcription of MHC I genes. (A) Abundance of H-2D, H-2K, and B2m mRNA (relative to Hprt) in Nlrc5−/− CD8+ T cells (mean and SD). **p = 0.01. (B) Jurkat cells were cotransfected with plasmids encoding EGFP and empty vector (−), human full-length NLRC5 (FL), NLRC5 lacking the CARD (∆CARD) or lacking the LRR region (∆LRR). The graph shows the average MFI of surface HLA-A, -B, and -C expression among EGFP cells of two independent experiments. (C) NLRC5 presence in cytoplasmic (Cy) and nuclear (Nu) fractions of splenocytes treated or not with IFN-β for a 16-h period was determined by immunoblot analysis. Lamin B1 and caspase-3 were used as nuclear and cytoplasmic markers, respectively. Results are representative of at least three independent experiments (A–C). (D) Abs specific to mouse NLRC5 were used to immunoprecipitate cross-linked chromatin fragments derived from Nlrc5−/− or Nlrc5−/− T cells. Immunoprecipitates were analyzed by quantitative PCR for the abundance of SXY sequences of the H-2D, H-2K, and B2m promoters, and for the promoter sequence of TATA binding protein (TBP). Relative promoter binding is shown. The average and SD of three experiments are depicted.

**FIGURE 5.** Nlrc5 deficiency does not prevent MHC I induction by IFN and CD8+ T cell priming by APCs. (A) Histograms show H-2D and H-2K fluorescence by Nlrc5−/− and Nlrc5−/− splenic T cells treated for 16 h with IFN-β or IFN-γ. (B) CFSE profile of OT-I cells cocultured for 3 d with Nlrc5−/− and Nlrc5−/− BMDMs, supplied with the indicated doses of OVA (μg/ml) and LPS (10 ng/ml). Data are representative of at least three experiments (A and B).
NLRC5 expression is low in a large number of lymphoid tumor of lymphocytes by cytotoxic T cells and consistently show that NLRC5-dependent MHC I expression in the cognate elimination mechanisms negatively regulating transcript levels. 

In this article, we report the predominant distribution and function of an NLR in cells of the lymphoid lineage, as NLRC5 is abundantly expressed and required for the regulation of MHC I expression in lymphocytes. We found that STAT1 plays a crucial role in NLRC5 induction, consistent with the presence of STAT1 binding sites in the Nlr5 promoter region, where, in addition, an NF-κB binding site has been identified (16). The latter may account for the remaining NLRC5 expression detectable in STAT1-deficient cells. In vitro stimulation of BMDMs highlighted the role of autocrine type I IFN signaling in NLRC5 induction, whereas NLRC5 expression by TCR-triggered T cells mostly relied on autocrine IFN-γ.

In conclusion, these data demonstrate the importance of NLRC5 in driving MHC I expression in vivo. First, the levels of H-2D and H-2K proteins and transcripts were markedly reduced in Nlr5-deficient cells, compared with Nlr5 wild-type cells. Second, NLRC5 was also found to localize in the nucleus and occupy the conserved SXY promotor sequence of these genes, in agreement with overexpression data by Meissner et al. (14). These results highlight the analogy between CIITA and NLRC5, and future studies will tell whether NLRC5 as well acts as a coactivator of MHC I transcription together with a specific enhanceosome.

We identified a key role for the CARD and LRR regions of NLRC5 in driving MHC I α-chain transcription. The LRR portion is particularly extended and could be involved in the interaction with other transcriptional regulators, similarly to the homologous region of CIITA (32).

Importantly, we show that the extent of NLRC5-dependent MHC I expression differs depending on the cell type and on the activation state of the cell. In fact, resting Nlr5-deficient lymphoid T, NKT, and NK cells were virtually devoid of MHC I expression, whereas the defect was generally milder in myeloid and B cells, reflecting the distribution of NLRC5 expression. The mild MHC I reduction observed in APCs could also be explained by the presence of CIITA in these cells, as this transcriptional regulator participates in MHC I expression as well (37). IFN treatment also induced MHC I in Nlr5-deficient cells, although the expression of MHC I in Nlr5-deficient cells remained markedly lower compared with that in control cells. These results confirm the importance of NLRC5 but indicate that under inflammatory circumstances other factors can concur in MHC I regulation (38). Similar to what we observed for ex vivo cDCs, we found a mild reduction in the levels of MHC I displayed on in vitro-generated BMDMs of Nlr5-deficient origin, which became even less apparent following inflammatory challenge. Accordingly, overt differences in the priming of transgenic OT-I T cells by Nlr5-deficient BMDMs were not observed. However, as OT-I T cells bear a TCR with high affinity for the

**Discussion**

In this article, we report the predominant distribution and function of an NLR in cells of the lymphoid lineage, as NLRC5 is abundantly expressed and required for the regulation of MHC I expression in lymphocytes. We found that STAT1 plays a crucial role in NLRC5 induction, consistent with the presence of STAT1 binding sites in the Nlr5 promoter region, where, in addition, an NF-κB binding site has been identified (16). The latter may account for the remaining NLRC5 expression detectable in STAT1-deficient cells. In vitro stimulation of BMDMs highlighted the role of autocrine type I IFN signaling in NLRC5 induction, whereas NLRC5 expression by TCR-triggered T cells mostly relied on autocrine IFN-γ.

In agreement with Kumar et al. (19), we did not observe defects in NF-κB activation or in type I IFN induction in Nlr5-deficient cells. We found NLRC5 to be crucial for the transcriptional regulation of MHC I expression in vivo. First, the levels of H-2D and H-2K proteins and transcripts were markedly reduced in Nlr5-deficient T cells, compared with Nlr5 wild-type cells. Second, NLRC5 was also found to localize in the nucleus and occupy the conserved SXY promotor sequence of these genes, in agreement with overexpression data by Meissner et al. (14). These results highlight the analogy between CIITA and NLRC5, and future studies will tell whether NLRC5 as well acts as a coactivator of MHC I transcription together with a specific enhanceosome.

We identified a key role for the CARD and LRR regions of NLRC5 in driving MHC I α-chain transcription. The LRR portion is particularly extended and could be involved in the interaction with other transcriptional regulators, similarly to the homologous region of CIITA (32).

Importantly, we show that the extent of NLRC5-dependent MHC I expression differs depending on the cell type and on the activation state of the cell. In fact, resting Nlr5-deficient lymphoid T, NKT, and NK cells were virtually devoid of MHC I expression, whereas the defect was generally milder in myeloid and B cells, reflecting the distribution of NLRC5 expression. The mild MHC I reduction observed in APCs could also be explained by the presence of CIITA in these cells, as this transcriptional regulator participates in MHC I expression as well (37). IFN treatment also induced MHC I in Nlr5-deficient cells, although the expression of MHC I in Nlr5-deficient cells remained markedly lower compared with that in control cells. These results confirm the importance of NLRC5 but indicate that under inflammatory circumstances other factors can concur in MHC I regulation (38). Similar to what we observed for ex vivo cDCs, we found a mild reduction in the levels of MHC I displayed on in vitro-generated BMDMs of Nlr5-deficient origin, which became even less apparent following inflammatory challenge. Accordingly, overt differences in the priming of transgenic OT-I T cells by Nlr5-deficient BMDMs were not observed. However, as OT-I T cells bear a TCR with high affinity for the
cognate peptide, it is still possible that priming of low- and intermediate-affinity TCR-bearing T cells is affected by reduced MHC I expression in Nlrc5-deficient APCs.

As MHC molecules play a crucial role in thymocyte selection processes, we analyzed MHC expression on thymic subpopulations and in thymocyte development. MHC I was strongly reduced among SP thymocytes, in agreement with the appearance of Nlrc5 expression at this differentiation stage (13). Moreover, although TECs and thymic cDCs displayed diminished MHC I levels, only a minor decrease in Nlrc5-deficient CD8+ T cell proportion was observed. The fairly normal number of peripheral CD8+ T cells was not the consequence of lymphopenia-driven proliferation in the periphery, as splenic CD8+ T lymphocytes did not show a significantly altered activation state. On the one hand, the slight decrease in CD8+ T cell percentage in Nlrc5-deficient mice echoes the absence of major defects in priming by APCs. On the other hand, it is tempting to speculate that CD8+ T cells may undergo altered thymic selection, skewing the TCR repertoire toward receptors with higher cross-reactivity, compensating for the slightly reduced MHC I levels encountered in the thymus.

The presence of normal MHC I levels on some cell types, and decreased levels on others, leads to a MHC I chimerism in Nlrc5-deficient animals that may perturb NK cell education. Intriguingly, Nlrc5-deficient animals presented a trend toward increased NK cell numbers. Although lowered MHC I expression favors NK cell-mediated killing, it also represents an effective way for potentially hazardous cells to evade cytotoxic T cells. Indeed, lymphocytes derived from Nlrc5-deficient animals were less efficiently killed by CD8+ T cells, compared with their Nlrc5+ counterparts. In agreement with a physiologic relevance of the reduced recognition and lysis of Nlrc5-deficient cells, Nlrc5 expression was low in several murine transformed cell lines. In addition, several human tumor cell lines showed decreased Nlrc5 expression, particularly Jurkat T cells and the tested germinal center B cell-like diffuse large B cell lymphomas. Importantly, reconstitution of Nlrc5 expression was sufficient to restore MHC I display in Jurkat T cells. Moreover, only tumors expressing Nlrc5 showed a substantial amount of transcripts encoding MHC I α-chain genes. Nonetheless, the levels of MHC I transcripts were not always correlating with higher cross-reactivity, compensating for the slightly reduced MHC I expression at this differentiation stage (13). Moreover, although Nlrc5-deficient APCs may participate in this process, the evidence that Nlrc5 governs Ag presentation thus sheds new light on the function of NLRs in immunity, making them instrumental players not only in the innate response but also in preserving over the adaptive branch of the immune system.

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Disclosures

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References


