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

Francesco Staehli,* Kristina Ludigs,* Leonhard X. Heinz,† Queralt Seguín-Estévez,‡ Isabel Ferrero,§ Marion Braun,§ Kate Schroder,¶ Manuele Rebsamen,¶ Aubry Tardivel,* Chantal Mattmann,* H. Robson MacDonald,§ Pedro Romero,§ Walter Reith,§ Greta Guarda,*1 and Jürg Tsopp2,1,2

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 I 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, supporting an essential role for NLRs in directing not only innate but also adaptive immune responses. The Journal of Immunology, 2012, 188: 3820–3828.

The innate immune system is an essential component of our body’s defense strategy against invading pathogens and host-derived danger. Prompt detection of hazardous agents and early onset of the inflammatory response are guaranteed by dedicated receptors, such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These are abundantly expressed by innate immune cells and recognize pathogen- and danger-associated molecular patterns (1). Upon activation, a growing number of NLRs are known to assemble in inflammasomes, multiprotein platforms serving caspase-1 activation, resulting in the maturation of IL-1β (1–3). Other NLRs—namely, NOD1 and NOD2—form NF-κB activating complexes upon bacterial invasion (4–7) and were also recently shown to promote the induction of type I IFN (8, 9). Unique among NLRs, NLRC5Def deficiency selectively impairs MHC class I 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 H-2 genes. Consistent with downregulated MHC I expression, the elimination of Nlrc5−/− lymphocytes by cytotoxic T cells was markedly reduced 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.

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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 Nlrp3 knockout mice presented alterations in neither inflammasome activation nor in the NF-κB and type I IFN pathways (19). Finally, by analogy to C172, it was suggested that overexpression of NLR5 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 NLR5 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, NLR5 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−/−) revealed a modest decrease in CD8+ 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, NKT, 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 NLR5-dependent MHC I expression on lymphocytes for their cognate elimination by CD8+ T cells.

Materials and Methods

Mice

Generation of Nlrc5−/− mice (Ogene) is described in Supplemental Fig. 2. Nlrc5−/− mice were generated by crossing Nlrc5−/− to the CMV-Cre deleter strain (The Jackson Laboratory), leading to deletion of the loxP-flanked region also in germ cells, thus allowing selection of Nlrc5−/− deleted offspring and elimination of the CMV-Cre transgene in the following generation. C57BL/6 mice were purchased from Harlan; Nlrp3−/−, Nlrp3−/−, Stat1−/− (20), Ifnγ−/− (21), Ifnar1−/− (22), MyD88−/− (23), Trif−/− (23, 24), and OT-1 (25) mice were bred 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), 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+ T cells were enriched using anti-CD4 magnetic beads (Miltenyi Biotec), CD11b+, CD8+ T, and B cells were enriched using a two-step procedure. Anti-CD11b (clone M1/70), anti-CD8a (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). Thioglycollate-elicited macrophages (TEMs) were isolated 3 d after i.p. injection of a 10% thioglycollate solution.

Enrichment of primary human PBMC populations

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

FACS Abs, staining, and thymocyte gating strategy

Cells were precultured with anti-CD16/32 (2.4G2) to block FcRs, then surface staining was performed using the following Abs raised against CD8α (Ly-2), CD3e (145-2C11), CD4 (L3T4), CD11b (M1/70), CD11c (N418), CD19 (ID3), CD44 (IM7), CD62L (MEL-14), F4/80 (BM8), H-2Dk (28-14-8), H-2Kk (145-85.5.3), MHC II (MS11/14.15.2), 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 was used containing anti-CD220-, 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+ T cell proliferation assays, 1.5 × 105 CFSE (Sigma-Aldrich)-labeled, MACS-purified OT-I T cells were incubated together with 5 × 104 Nlrp3−/− and Nlrc5−/− 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, Nlrp3−/− and Nlrc5−/− MACS-purified target CD4+ and CD8+ T cells or T cell-depleted negative fractions were labeled with two levels of CFSE [briefly, CFSE labeling was done in PBS 8 min at room temperature, at the final concentrations of 1.25 (high) or 0.125 (low) μM] and pulsed with 75 nm with 10 μM SIINFEKL 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 μg/ml)–supplemented medium and used on day 3 or 4. For killing, 5 × 104 differentially labeled Nlrp3−/− and Nlrc5−/− T cells were incubated overnight with the indicated numbers of in vitro activated CD8+ T cells.

Immunoblot analysis

Rabbit polyclonal anti-mouse NLR5 (aa 1–139) and anti-human NLR5 (aa 11–166) were generated by Adipogen. Other Abs used were monoclonal anti-tubulin (Sigma-Aldrich), anti-phosphorylated IsxBo, polyclonal anti-IsxBo, 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-γ (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.
NLRC5 constructs, transfection, and nuclear fractionation

The full-length human NLRC5 cDNA was assembled from three partial cDNA clones (GenBank: BX640842, BC063566, and a Jurkat cell-derived cDNA covering the 5' region of human NLRC5 cDNA). Full-length NLRC5, NLRC5 4ACD (aa 191–1866), and NLRC5 ΔLRR (aa 1–664) were cloned into a derivative of pCR3 (Invitrogen) in frame with an N-terminal VSVG tag. HEK 293T cells were transfected by the calcium-phosphate method. Jurkat cells were transfected with expression constructs or mock together with plasmid encoding SV40 Large T Ag and plasmid encoding enhanced GFP as previously described for BJAB (26), using 260 V. Cytoplasmic and nuclear fractions were prepared as previously described (29).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (30). Primer sequences are available upon request.

Statistical analysis

Statistical differences were calculated with an unpaired Student t test, two-tailed (GraphPad Prism version 5.0). Differences were considered significant when p ≤ 0.05 (*), very significant when p ≤ 0.01 (**), and extremely significant when p ≤ 0.001 (***)

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, Nlr55−/− 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 agonists of TLR3 and TLR4, known to strongly induce type I IFN production, and was abrogated when the autocrine effects of type I IFNs were prevented using Iftar1−/− or Stat1−/− BMDMs (Fig. 1D, Supplemental Fig. 1B–D). Consistent with previous reports, IFN-γ also induced NLRC5, an effect preserved in Iftar1−/− but lost in Stat1−/− BMDMs (Fig. 1D) (13, 16). However, upon LPS treatment, only a negligible role for autocrine IFN-γ was observed, as shown by the use of Iftar1−/− BMDMs and TEMs (Fig. 1D, Supplemental Fig. 1B–D).

Similar to myeloid cells, B lymphocytes express a range of TLRs and respond to their agonists. Indeed, LPS-dependent type I IFN was largely responsible for sustained NLRC5 induction, as demonstrated by decreased NLRC5 expression in Iftar1−/− B cells (Supplemental Fig. 1E). Of interest, exposure to type I IFN in particular induced the formation of a slower migrating band specifically detected by the NLRC5 Ab, suggestive of multilevel regulation of NLRC5 function (Fig. 1D, Supplemental Fig. 1C–E).

We also investigated the role of IFNs in NLRC5 expression by T cells. Although NLRC5 was detectable in ex vivo splenic CD4+ and CD8+ T cells, protein levels increased following TCR triggering, with more rapid kinetics in CD8+ T cells (Fig. 1E, Supplemental Fig. 1F). As demonstrated by the use of Stat1−/−, Iftar1−/−, and Iftar1−/− T lymphocytes, NLRC5 increase was fully dependent on autocrine IFN-γ signaling in CD4+, and partially in CD8+ T cells, whereas no role for autocrine type I IFN signaling was observed. However, we found that Nlr55−/− mRNA abundance transiently decreased upon TCR triggering, as shown in Supplemental Fig. 1F, highlighting the existence of important post-transcriptional mechanisms regulating NLRC5 protein levels. Interestingly, recovery to normal Nlr55 transcript amounts following TCR stimulation was dependent on autocrine IFN-γ signaling (Supplemental Fig. 1F). Accordingly, treatment of CD4+ and CD8+ T cells with IFN-γ was sufficient to increase Nlr55 mRNA (Fig. 1F). Altogether, these data show that both type I and type II IFNs positively regulate NLRC5 transcription through STAT1, but that additional posttranscriptional mechanisms exist.

Nlr55−/− mice exhibit minor differences in CD8+ T cell percentages

To study NLRC5 function, we generated Nlr55-deficient mice (Supplemental Fig. 2A–E). First, we investigated the role of NLRC5 in NF-κB signaling and type I IFN induction. However, NLRC5 ablation did not affect NF-κB activation, TNF, and IL-6 induction by LPS treatment, or production of IFN-β upon LPS and intracellular poly(I:C) stimulation of BMDMs (Supplemental Fig. 2F–I). Furthermore, Nlr55−/− T cells did not show altered TNF secretion upon TCR triggering (Supplemental Fig. 2J).

We next sought to analyze the lymphoid compartment of Nlr55−/− animals, given the prominent expression of NLRC5 in these cells. Irrespective of their age, Nlr55−/− 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 Nlr55−/− or Nlr55−/− 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 Nlr55−/− mice was similar, compared with that in Nlr55−/− controls, older (34- to 36-wk-old) Nlr55−/− 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 Nlr55−/− animals.

Selected Nlr55-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, spleenocyte analysis revealed a dramatic reduction in the display of both H-2D and H-2K MHC I molecules by Nlr55−/− CD4+, 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 Nlrc5/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 Nlrc5/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 Nlrc5/D and Nlrc5/D T cells (Fig. 4A). Interestingly, we detected decreased amounts of both mRNAs in Nlrc5/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
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 II, MHC II, MHC II, MHC II, MHC II, MHC II, MHC II.
Safeguard mechanism allowing CD8+ T cells to identify and eliminate infected or transformed cells. We thus investigated the cytolytic T lymphocyte-dependent killing of Nlrc5Δ/Δ T cell targets, which present a strong defect in MHC I display. For this purpose, Nlrc5Δ/Δ and Nlrc5Δ/Δ-/- 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. Nlrc5Δ/Δ target T lymphocytes were much less efficiently killed by OT-I CD8+ T cells, compared with their Nlrc5Δ/Δ counterparts (Fig. 6A, Supplemental Fig. 4C). Furthermore, when unpulsed Nlrc5Δ/Δ and Nlrc5Δ/Δ-/- T cells were used as targets, or polyclonal effector cells employed as killers, no difference in target cell killing was observed. These results are consistent with the notion that Nlrc5Δ/Δ T cells are less effective killers than their WT counterparts, even when pulsed with a high-affinity target peptide.

**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 Nlrc5Δ/Δ and Nlrc5Δ/Δ-/- BMDCs to prime transgenic OT-I CD8+ T cells with type I or II IFN. Of interest, IFNs were able to induce H-2D and H-2K expression in both Nlrc5Δ/Δ and Nlrc5Δ/Δ-/- BMDCs in a type I and II IFN-dependent manner. However, IFN-γ stimulation resulted in a greater overall difference in H-2K expression between Nlrc5Δ/Δ and Nlrc5Δ/Δ-/- BMDCs, compared with their Nlrc5Δ/Δ counterparts (Fig. 6A, Supplemental Fig. 4C). Furthermore, when unpulsed Nlrc5Δ/Δ and Nlrc5Δ/Δ-/- T cells were used as targets, or polyclonal effector cells employed as killers, no difference in target cell killing was observed. These results are consistent with the notion that Nlrc5Δ/Δ T cells are less effective killers than their WT counterparts, even when pulsed with a high-affinity target peptide.
ferences in the survival of two target cell populations was ob-
served (Supplemental Fig. 4C). This finding indicates that the
preferential killing of \textit{Nlrc5} \textsuperscript{fl/fl} target T cells is the result of
coag enate elimination. We also used T cell-depleted splenocytes, as
the vast majority consisting of B cells, as target cells (Fig. 6B).
Although much less pronounced, a survival advantage of T cell-
depleted \textit{Nlrc5} \textsuperscript{fl/fl} target cells was also observed, in agreement
with the milder defect in MHC I display detected in B cells (Fig.
3B).

Given that low NLRC5 expression confers an advantage to
escape CD8 \textsuperscript{+} T cell-mediated elimination, and that downregu-
lation of surface MHC I molecules is known to mediate immune
surveillance evasion (36), we investigated NLRC5 levels in sev-
eral T cell- and B cell-derived tumor lines (Fig. 6C, 6D). Notably,
the tested murine thymoma (EL-4, EL-4 6.1.10, and ST-4), T cell (LSTRA, RMA, RMA-S, and YAC), and B lymphoma
cells (L1210 and M12.4.1) had lower NLRC5 expression than
did primary splenocytes or B cells (Fig. 6C). Importantly, the
tested human cell lines behaved similarly, with Jurkat T cells and
half of the B cell lymphomas (U2932, SUDHL-4, SUDHL-6, and
BJAB) expressing negligible NLRC5 levels (Fig. 6D). For the
C57BL/6 murine and the human cell lines, we also measured
\textit{H-2D} \textsuperscript{C} expression in the cognate elimination
mechanisms negatively regulating transcript levels.

In conclusion, these data demonstrate the importance of
NLRC5-dependent MHC I expression in the cognate elimination
of lymphocytes by cytotoxic T cells and consistently show that
NLRC5 expression is low in a large number of lymphoid tumor

cells.

Discussion

In this article, we report the predominant distribution and function
of an NLR in cells of the lymphoid lineage, as NLRC5 is abund-
antly 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 \textit{Nlrc5} promoter region, where, in addition, an
NF-\kappaB binding site has been identified (16). The latter may ac-
count 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-\gamma.

In agreement with Kumar et al. (19), we did not observe defects
in NF-\kappaB activation or in type I IFN induction in \textit{Nlrc5}-deficient
cells. We found NLRC5 to be crucial for the transcriptional regu-
lation of MHC I expression in vivo. First, the levels of \textit{H-2D}
and \textit{H-2K} proteins and transcripts were markedly reduced in \textit{Nlrc5} \textsuperscript{fl/fl}
T cells, compared with \textit{Nlrc5} \textsuperscript{+/+} cells. Second, NLRC5 was also
found to localize in the nucleus and occupy the conserved SXY
promoter sequence of these genes, in agreement with overex-
pression 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 tran-
scription together with a specific enhanceosome.

We identified a key role for the CARD and LRR regions
of NLRC5 in driving MHC I 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 \textit{Nlrc5} \textsuperscript{fl/fl}
lymphoid T, NK, 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 \textit{Nlrc5} \textsuperscript{+/+} cells, although the expression of
MHC I in \textit{Nlrc5} \textsuperscript{fl/fl} 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 \textit{Nlrc5} \textsuperscript{fl/fl}
origin, which became even less apparent following inflamma-
tory challenge. Accordingly, overt differences in the priming of
transgenic OT-I T cells by \textit{Nlrc5} \textsuperscript{fl/fl} BMDMs were not observed.
However, as OT-I T cells bear a TCR with high affinity for the

\begin{figure}
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\caption{Impaired cytotoxic T cell-mediated killing of \textit{Nlrc5} \textsuperscript{fl/fl} target lymphocytes. (A and B) Target T cells isolated from \textit{Nlrc5} \textsuperscript{fl/fl} and \textit{Nlrc5} \textsuperscript{+/+} (A), and the T cell-depleted fractions (B), labeled with dim and bright CFSE concentrations, respectively; pulsed with SIINFEKL peptide; and mixed in a 1:1 ratio. The indicated proportions of effector OT-I cells were then supplied for an overnight period (E:T ratio). Histograms show the relative abundance
and the T cell-depleted fractions (B) representative of at least two experiments. HD, Healthy donor controls.}
\end{figure}
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-s/-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-s/- 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-s/- animals that may perturb NK cell education. Intriguingly, Nlrc5-s/- 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-s/- 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 surface protein expression, in accordance with the existence of additional posttranscriptional regulatory mechanisms controlling MHC expression (39). Taken together, our results suggest that transformed cells may take advantage of Nlrc5 down-regulation, and future studies aimed at evaluating Nlrc5 levels in primary transformed lymphocytes will be crucial to verify this hypothesis.

Several viruses devote part of their restricted genome to encode proteins interfering with MHC I expression (40, 41). By decreasing MHC I display, infected cells evade CD8+ T cell-mediated elimination. According to our results, the modulation of Nlrc5 by viruses having lymphocyte tropism, such as HIV, would represent an attractive way to escape cytotoxic T cells. It is also predictable that NLRC5 plays an important role in transplant rejection. In this context, therapeutic approaches targeting Nlrc5 could reduce immune rejection without hampering CD8+ T cell priming.

NLRs are well known for their potent activity in the onset of inflammatory responses, particularly in innate immune cells. Our findings clearly identify Nlrc5 as a second NLR family member regulating MHC expression. These observations raise the fascinating hypothesis that NLRs may control MHC expression in a much broader manner than has been recognized so far with the well-studied CIITA system. If so, the possibility exists that additional transcriptional regulators belonging to the NLR family 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 presiding over the adaptive branch of the immune system.

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Disclosures
The authors have no financial interests of conflicts of interest.

References


Supplementary Figure 1. Further characterization of NLRC5 expression

(A) NLRC5 expression in murine tissues was determined by immunoblot analysis. (B) The abundance of Nlrc5 mRNA relative to Hprt mRNA upon LPS was determined by quantitative PCR in BMDMs from wt, MyD88−/−, Trif−/−, Ifnar1−/−, Ifn−/−, and Stat1−/− origin at the indicated times. (C and D) NLRC5 expression was determined by immunoblot analysis in BMDMs (C) or in TEM (D) derived from wt, Stat1−/−, MyD88−/−, Trif−/−, Ifnar1−/−, and Ifn−/− mice stimulated for the indicated times with LPS. (E) Wt or Ifnar1−/− B cells were stimulated for the indicated times with LPS. NLRC5 expression was determined by immunoblot analysis. (F) Wt and Ifnr−/− CD4+ lymphocytes were stimulated on plastic-coated α-CD3 antibody in the presence of soluble α-CD28 and IL-2 for 2 days. Nlre5 protein (left panel) and transcript (right panel, relative to Hprt mRNA) abundance were assessed by immunoblot and quantitative PCR, respectively, at the indicated days (d). Data are representative of at least two experiments.
Supplementary Figure 2. Generation of conditional Nlrc5 knockout mice and involvement of NLRC5 in NF-κB activation and type I IFN production

(A) Schematic representation of the region spanning exons 1-9 of the murine Nlrc5 gene. The 5'UTR of murine Nlrc5 mRNA, which is currently not included in the NCBI reference sequence (NM_001033207.3, http://www.ncbi.nlm.nih.gov/nuccore/254692965), was determined experimentally by analysis of the cDNA spanning the region from the predicted FANTOM3 transcriptional start cluster (TC ID: T08F059700E3) and the NACHT domain. The sequence was deposited in NCBI dbEST (http://www.ncbi.nlm.nih.gov/nucest/H0079270.1, http://www.ncbi.nlm.nih.gov/nucest/H0079271.1) and reveals four non-coding exons before the annotated ATG start site in exon 5. (B) Nlrc55/4 mice were generated on a C57BL/6 background by targeted gene disruption. The two loxP sites shown in the targeting vector allow CRE-mediated deletion of the ATG start-containing exon 5. (C) Nlrc5 locus after CRE-mediated deletion of exon 5. (D) PCR amplification of wild-type, floxed and disrupted alleles from wild-type (wt/wt), homozygous floxed (fl/fl), heterozygous floxed (wt/fl), heterozygous disrupted (wt/Δ) and homozygous disrupted (Δ/Δ) mice. (E) The deletion of Nlrc5 was revealed by immunoblot analysis of BMDC lysates after stimulation with poly(I:C). (F) Nlrc55/β and Nlrc55/Δ BMDCMs were stimulated for the indicated times with LPS. The phosphorylation of the IKK complex and the presence of IkBa and its phosphorylated form (pIkBa) were determined by immunoblot analysis of cell lysates. (G) Secretion of TNF was assessed in the culture media of Nlrc55/β and Nlrc55/Δ BMDCMs after overnight stimulation with LPS. (H) Expression of IL-6 by Nlrc55/β and Nlrc55/Δ BMDCMs (left panel, mRNA relative to Hprt; right panel, protein) was measured at the indicated times after LPS stimulation. (I) IFN-β production was assessed in the culture media of Nlrc55/β and Nlrc55/Δ BMDCMs stimulated for the indicated times with LPS or intracellular poly(I:C). Intracellular poly(I:C) was delivered with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. (J) Nlrc55/β and Nlrc55/Δ effector T lymphocytes were restimulated for the indicated times on plastic-coated α-CD3 antibody (1 μg/ml) and secretion of TNF was assessed in the culture media. (E-J) Data are representative of at least two experiments.
Supplementary Figure 3. Detailed characterization of different cell populations in Nlrc5^+/− mice

(A) Percentages of CD4^+ (CD3^+CD4^+), CD8^+ (CD3^+CD8^+), γδ (CD3^−TCRγδ^+) T cells, NK cells (CD3^−NK1.1^+), NK cells (CD3 NK1.1^−) and B cells (CD19^+) in spleen, lymph nodes and blood of young (12-week-old, n=5) and old (34- to 36-week-old, n=5 for Nlrc5^+/− mice and n=4 for Nlrc5^−/− mice) Nlrc5^+/− and Nlrc5^−/− mice were analyzed by flow cytometry. (B) Nlrc5^+/− and Nlrc5^−/− CD4^+ and CD8^+ T cells were labeled with CFSE and stimulated on plastic-coated α-CD3 antibody. Survival (left panel) and proliferation (right panel) were assessed at day 4 by flow cytometry. (C) Expression of CD44 and CD62L by Nlrc5^+/− and Nlrc5^−/− CD4^+ and CD8^+ splenic T cells is shown. (D) Spleen cellularity of 8- to 9-week-old (n=7) (left) and 32- to 35-week-old (n=10) (right) Nlrc5^+/− and Nlrc5^−/− mice. (E) Thymic cDCs were analyzed for H-2D and H-2K expression. Graphs illustrate mean ± SEM of geometric mean fluorescence intensities (MFIs) (n=3). (F) mTECs and cTECs were analyzed for H-2D and H-2K expression. Graphs illustrate averages of MFIs (n=2). TECs were prepared using a modified protocol from Klein et al. (Klein et al., 2000). In brief, individual thymi were cut into small pieces and washed 3 times in DMEM under stirring for 10 minutes at room temperature to remove thymocytes. TECs were then extracted from the thymic fragments by two sequential steps of incubation with 3mg/ml Collagenase IV (Worthington) in DMEM at 37°C for 15’. Supernatants from both Collagenase incubations and the residual thymic fragments were filtered trough a 70 mm nylon cell strainer. The enriched TEC suspension was washed in PBS 2% FCS 5mM EDTA and stained for FACS analysis using the following mAb-conjugated mix: α-CD45 (30F11), α-BP1 (6C3) (both from BioLegend), α-MHCII (M5/114.15.2) (eBioscience), α-H-2Db (B22/24g) and α-H-2Kb (B8.24.3). 7AAD was used to exclude dead cells. TECs were identified as MHCII^+ cells among the CD45^+ 7AAD- gated cells, and cortical TEC were distinguished from medullar TEC by their expression of BP1. (G) Thymus cellularity of Nlrc5^+/− and Nlrc5^−/− mice (n=7). (H) Proportion of DN (lin CD4^−CD8^+), DP (lin CD4^+CD8^+), CD4^+ (lin CD3^−CD4^+CD8^−) and CD8^+ (lin CD3^−CD4^−CD8^+) SP thymocytes in Nlrc5^+/− and Nlrc5^−/− mice. Results are representative of at least two independent experiments (A-H).
Supplementary Figure 4. Inducibility of MHCI, decreased cognate killing of \( Nlrc5^{5/4} \) T lymphocytes, and expression of \( MHCI \) mRNA in lymphoid-derived tumor lines

(A and B) H-2D and H-2K expression on \( Nlrc5^{5/0} \) and \( Nlrc5^{5/4} \) CD4+ and CD8+ splenic T cells (A) or BMDMs (B) treated for 16 hours with IFN-β, IFN-γ or LPS. Graphs illustrate averages of geometric mean fluorescence intensities (MFIs) (n=2). (C) \( Nlrc5^{5/0} \) and \( Nlrc5^{5/4} \) T lymphocytes were labeled with dim and bright CFSE concentrations, respectively, pulsed or not with SIINFEKL peptide and mixed in a one to one ratio. The indicated numbers of effector OT-I or polyclonal CD8+ T cells were then supplied for an overnight period (effector to target ratio, E:T). The percentage of surviving \( Nlrc5^{5/0} \) and \( Nlrc5^{5/4} \) T cell targets is quantified (average). (D) H-2D and H-2K mRNA expression relative to \( Hprt \) mRNA were determined by quantitative PCR in a panel of different murine thymoma (EL-4, EL-4 6.1.10) and T cell-derived (RMA and RMA-S) tumor lines (mean and SD). (E) \( HLA-A \) mRNA expression relative to \( Hprt \) mRNA was determined by quantitative PCR in a panel of different human B lymphoma cell lines and in Jurkat T cells (healthy donor, HD).