Cutting Edge: Impaired MHC Class I Expression in Mice Deficient for Nlrc5/Class I Transactivator

Amlan Biswas, Torsten B. Meissner, Taro Kawai and Koichi S. Kobayashi

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MHC class I and class II are crucial for the adaptive immune system. Although regulation of MHC class II expression by CIITA has long been recognized, the mechanism of MHC class I transactivation has been largely unknown until the recent discovery of NLRC5/class I transactivator. In this study, we show using Nlrc5-deficient mice that NLRC5 is required for both constitutive and inducible MHC class I expression. Loss of Nlrc5 resulted in severe reduction in the expression of MHC class I and related genes such as β2-microglobulin, Tap1, or Lmp2, but did not affect MHC class II levels. IFN-γ stimulation could not overcome the impaired MHC class I expression in Nlrc5-deficient cells. Upon infection with Listeria monocytogenes, Nlrc5-deficient mice displayed impaired CD8+ T cell activation, accompanied with increased bacterial loads. These findings illustrate critical roles of NLRC5/class I transactivator in MHC class I gene regulation and host defense by CD8+ T cell responses. The Journal of Immunology, 2012, 189: 516–520.

Major histocompatibility complex class I and class II molecules play key roles in the activation of the adaptive immune system. MHC class I molecules present peptide Ags of intracellular origin such as tumor or viral Ags to CD8+ T cells, whereas MHC class II molecules present peptide Ags of extracellular sources to CD4+ T cells (1). The expression of both constitutive and inducible MHC class II requires the master transcriptional coactivator CIITA (2). Although CIITA itself lacks a DNA binding domain, CIITA can activate the promoters of MHC class II genes by engaging in a nucleoprotein complex called the MHC enhanceosome (3, 4), together with promoter-resident transcription factors, including the trimeric regulatory factor X protein complex, CREB/activating transcription factor 1 family members, and the NF-Y protein (5, 6). CIITA can also transactivate MHC class I genes at least in vitro (7, 8), although both bare lymphocyte syndrome patients with mutations in the CIITA gene and CIITA-deficient mice retain intact MHC class I expression, indicating that there is another mechanism for the activation of MHC class I in vivo (2, 9–12).

The recent discovery of a transactivator specific for MHC class I genes, NLRC5, identified a new addition to the regulators of MHC genes (13, 14). Both NLRC5 and CIITA belong to the NLR or nucleotide binding domain (NBD), leucine-rich repeat family of proteins, and phylogenetically they are most closely related to each other (13, 15). NLRC5, or class I transactivator (CITA), is also an IFN-γ–inducible nuclear protein, but NLRC5 specifically associates with and transactivates MHC class I promoters, resulting in the expression of MHC class I and related genes such as β2-microglobulin (β2m), Tap1, and large multifunctional protease 2 (13, 14). The NBD is a critical domain for the function of NLRC5, as the NBD is required for both nuclear import and transactivation of MHC class I genes (16). In the nucleus, NLRC5 participates in a MHC class I-specific enhanceosome, together with the regulatory factor X components and CREB/activating transcription factor 1 family transcription factors to activate MHC class I gene promoters (17). In addition to its function as a MHC class I transactivator, NLRC5 has been reported to be involved in the regulation of TLR and RIG-I–like receptor signaling, antiviral responses, and inflammasome activation (15, 18–21). However, those reports provide conflicting results, and their conclusions are not supported by the data obtained from the initial characterization of Nlrc5-deficient mice (14, 22).

Although the identification of NLRC5 as a MHC class I gene transactivator is significant, the role of NLRC5 in MHC class I expression in vivo had not been elucidated. To address this question, we investigated the function of NLRC5 using Nlrc5-deficient mice. In this study, we show that Nlrc5 is required for both constitutive and inducible expression of MHC class I. Nlrc5-deficient mice were more susceptible to...
infection with *Listeria monocytogenes*, as highlighted by impaired CD8+ T cell activation and increased bacterial burden in the infected organs, indicating a critical role of Nlrc5/CITA in MHC class I-dependent CD8+ T cell responses.

**Materials and Methods**

**Mice**

Nlrc5-deficient mice were provided by T. Kawai and S. Akira (Osaka University, Osaka, Japan) (22). Wild-type mice (F1 mice from 129SvEv and C57BL/6 mice) were obtained from Taconic. OT-1 TCR transgenic mice were a gift of H. Cantor (Dana-Farber Cancer Institute, Boston, MA). Mice were maintained under specific pathogen-free conditions and used in accordance with institutional and National Institutes of Health guidelines.

**Cell culture and reagents**

Generation and stimulation of bone marrow-derived macrophages and dendritic cells have been described previously (23). Splenocytes and thymocytes were cultured in RPMI 1640 supplemented with 10% FBS, 55 μM 2-ME (Life Technologies), and penicillin/streptomycin (Life Technologies). Murine IFN-γ was from BioLegend. Peritoneal cells were isolated without pretreatment with thioglycolate.

**RNA isolation and quantitative PCR**

RNA isolation and quantitative PCR were performed, as previously described (13). Primer sequences are listed in Supplemental Table I.

**Western blot analysis**

Western blot analysis was performed, as described previously (13), using the following Abs: anti–H2-Kb, anti-β2m (gifts of T. Hansen, Washington University), and anti-heat shock protein (Hsp) 90 Ab (F-8; Santa Cruz Biotechnology).

**Flow cytometric analysis**

FACS analysis was performed, as previously described (13), using the following Abs: FITC anti-mouse B220, FITC anti-mouse CD11c, allophycocyanin anti-mouse CD4, PE/Cy5 anti-mouse CD8, allophycocyanin anti-mouse F4/80, PE anti-mouse I-Ab/I-Ad (eBioscience), PE anti-mouse H2, and allophycocyanin anti-mouse IFN-γ (BioLegend).

**OT-1 cell coculture assay**

MACS-sorted CD8+ T cells from OT-1 mice with CD45.1 background were CFSE labeled and cocultured with SIINFEKL (1 μM) pulsed irradiated splenic B220+ cells from either Nlrc5+/- or Nlrc5-/- mice at a ratio of 1:25 (B:T) for 72 h. Proliferation of OT-1 T cells was determined by FACS analysis of the CFSE dilution in CD45.1 gated cells.

**Statistical analysis**

Data were subjected to one-way ANOVA for analysis of statistical significance using Prism (GraphPad). Results are given as the mean ± SEM. A p value <0.05 was considered to be significant.

**Results and Discussion**

To clarify the role of NLRC5 in MHC class I gene expression in vivo, we analyzed Nlrc5-deficient mice. Heterozygous littermates (+/-) and F1 mice from a C57BL/6 × 129SvEv breeding (+/+), with a similar mixed genetic background to the knockout mice (-/-/-) were used as controls. As can be seen in Fig. 1A, MHC class I (H2-Kb) transcript levels in the thymus, spleen, kidney, and ileum were significantly reduced in Nlrc5-deficient mice compared to that of wild-type or heterozygous mice, confirming that MHC class I expression is indeed largely dependent on Nlrc5. The expression of MHC class I-related genes, β2m, Tap1, Lmp2, as well as the nonclassical MHC class I gene, H2-M3, was impaired in the thymus and spleen of Nlrc5-deficient mice (Fig. 1B). In contrast, Nlrc5 deficiency did not affect the expression of MHC class II (H2-Db) and CIITA gene expression in splenocytes, as determined by qPCR, n = 3. Error bars represent ±SEM. ns, not significant. (C) Analysis of Nlrc5, MHC class II (H2-Db), and CIITA gene expression in organs by flow cytometry. The surface expression of MHC class I (H2-Kb) and β2m protein expression in the spleen and thymus from mice with the indicated genotype. The expression of Hsp90 is shown as a loading control. *p < 0.05, **p < 0.01; ns, not significant.
origin or bone marrow derived. Interestingly, the surface expression of MHC class I on these bone marrow-derived cells was largely unaffected (Supplemental Fig. 1A), suggesting that there appears to be a compensatory posttranscriptional mechanism to rescue the MHC class I deficiency in cultured cells. Together, these findings suggest that Nlrc5 plays a major role in the regulation of MHC class I gene expression, albeit the degree of the requirement for Nlrc5 in MHC class I expression varies between different cell types.

We have previously demonstrated that NLRC5 is required for MHC class I gene expression upon IFN-γ stimulation (13). Therefore, we examined the impact of Nlrc5 deficiency on IFN-γ-inducible MHC class I expression by stimulating splenocytes from Nlrc5-deficient mice with IFN-γ. As previously shown (13, 15, 20, 21), IFN-γ treatment resulted in the upregulation of Nlrc5 transcript, which correlated with the induction of MHC class I gene (H2-Kb) expression in both wild-type and heterozygous splenocytes (Fig. 3A) (13). In Nlrc5-deficient splenocytes, however, IFN-γ stimulation could not rescue the impairment of MHC class I expression, when compared with the levels of MHC class I observed in the controls (Fig. 3A). In contrast, upregulation of Stat1 transcripts, which is induced by IFN-γ stimulation in a Nlrc5-independent manner (13), was comparable among the three genotypes, indicating that the JAK/STAT signaling cascade downstream of the IFN-γ receptor is intact in Nlrc5-deficient cells. Interestingly, there was a small, but distinct, induction of MHC class I expression in Nlrc5-deficient splenocytes upon IFN-γ stimulation, supporting the existence of a Nlrc5-independent mechanism(s) of MHC class I expression (Fig. 3A). In agreement with the transcript data, IFN-γ stimulation did not restore the expression of MHC class I (H2-Kb) and β2m at the protein level in Nlrc5-deficient splenocytes and thymocytes (Fig. 3B). Moreover, flow cytometric analysis showed that IFN-γ stimulation did not rescue the reduced MHC class I surface expression in CD4+, CD8+ T cells, B cells, F4/80+ macrophages, and CD11c+ dendritic cells obtained from the spleen of Nlrc5-deficient mice (Fig. 3C).

To address whether the impaired MHC class I expression caused by Nlrc5 deficiency has an impact on immune responses, OT-1 CD8+ T cells were cocultured with peptide-loaded B cells. OT-1 T cells cultured with Nlrc5-deficient B cells displayed impaired proliferation, indicating that NLRC5 is indeed required for Ag-specific stimulation of CD8+ T cells (Fig. 4A). The role of NLRC5 in MHC class I-
mediated immune responses was further investigated by infection studies using an intracellular bacterium, L. monocytogenes, because a CD8+ T cell response is critical for the host defense against this bacterium. Although Listeria infection clearly induced Ag-specific CD8+ T cell activation in the spleen and liver of wild-type mice, as demonstrated by increased numbers of IFN-γ-positive cells after ex vivo stimulation with heat-killed bacteria (Fig. 4B, Supplemental Fig. 1C), in Nlrc5-deficient mice, Ag-specific CD8+ T cell activation was impaired and the mice harbored increased numbers of the bacterium in both spleen and liver (Fig. 4B, 4C). This Listeria-susceptible phenotype contradicts the previously proposed function of NLRC5 as a TLR inhibitor, as in that case the Nlrc5-deficient mice should be more protected rather than being more susceptible to infection. Indeed, Nlrc5-deficient macrophages expressed normal levels of IL-6, TNF-α, IL-12 p40, and IL-1β at both transcript and protein levels upon LPS, CpG oligo, and poly(I:C) stimulation, confirming the observations made in the previous study using Nlrc5-deficient mice (Supplemental Fig. 1D, 1E) (22). These data collectively demonstrate a critical role of NlrC5/CITA in MHC class I-mediated immune responses in vivo.

In addition to our previous study in which we used human lymphoid and epithelial cell lines (13), the current study compellingly demonstrates the critical role of NLRC5 in both constitutive and inducible MHC class I expression in vivo using Nlrc5-deficient mice. Moreover, we show that NLRC5 is required for CD8+ T cell responses in an Ag-specific manner. Ag peptide-loaded Nlrc5-deficient B cells failed to activate OT-1 T cells efficiently. Strikingly, our Listeria infection study demonstrated that CD8+ T cells obtained from Nlrc5-deficient mice displayed impaired Ag-specific activation, and the mice had increased bacterial loads in the spleen and liver. Therefore, we conclude that NLRC5 plays a critical role in MHC class I-mediated immune responses in vivo. This study also reveals that the requirement for NLRC5 in MHC class I gene expression varies between different cell types. The reduced MHC class I phenotype was most prominent in CD4+ and CD8+ T cells and less prominent in B cells (Fig. 2A–C). Also, macrophages and dendritic cells retained residual MHC class I expression (Fig. 2A). This may suggest that an alternative, NLRC5-independent mechanism of MHC class I transactivation exists. Interestingly, similar residual expression of MHC class II genes has been reported in CIITA-deficient mice; CIITA-deficient dendritic cells retained MHC class II expression, although expression levels were significantly reduced (12). These observations indicate that although NLRC5/CIITA and CIITA are critical for the expression of MHC class I and class II, respectively, APCs may possess alternative mechanisms to ensure the efficient presentation of
Ags to T cells. In summary, we demonstrated the critical role of NLRC5/CITA in MHC class I expression and CD8+ T cell responses in vivo. Further analysis of Nlrc5-deficient mice will certainly extend our understanding of MHC class I biology, and, hence, may improve therapeutic interventions in the field of infectious diseases, transplantation, and cancer immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

References