Cutting Edge: Requirement of MARCH-I-Mediated MHC II Ubiquitination for the Maintenance of Conventional Dendritic Cells


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MARCH-I (membrane-associated RING-CH I) has been suggested as a physiological E3 ubiquitin ligase for both MHC class II (MHC II) and B7-2. In this study, we show that MARCH-I-mediated MHC II ubiquitination is necessary for the maintenance of conventional dendritic cell (cDC) functions in the steady state. MARCH-I-deficient cDCs accumulated MHC II and B7-2 and exhibited low Ag-presenting ability for exogenous Ags and low cytokine-producing ability upon stimulation in vivo. Importantly, MHC II, but not B7-2, was required for impaired cDC function induced by loss of MARCH-I in vivo. Moreover, MHC II knockin mice whose MHC II was not ubiquitinated showed dysfunction of cDC similar to that of MARCH-I knockout mice. These results suggest that the accumulation of MHC II resulting from loss of ubiquitination caused cDC abnormality; therefore, MARCH-I may function as a housekeeper of cDC in the steady state. The Journal of Immunology, 2009, 183: 6893–6897.

Recently, several groups have reported important findings on the regulation of MHC II expression in the steady state in vivo. In immature dendritic cells (DCs), surface MHC II molecules are replaced with newly synthesized molecules via the degradation of preexisting ones. This degradation is achieved via ubiquitination of the cytoplasmic tail of the I-Aβ chain of MHC II (2–4).

In this regard, we and several groups have discovered the membrane-associated RING-CH I (MARCH-I) molecule, which contributes to the ubiquitination-mediated degradation of MHC II in APCs (5–7). The generation and analysis of MARCH-I knockout (KO) mice revealed that MARCH-I is a physiological E3 ubiquitin ligase for MHC II (5). In addition, the knockdown of MARCH-I in immature human DCs led to a remarkable up-regulation of the surface expression of B7-2, which is a costimulatory molecule. Thus, MARCH-I is suggested to be a physiological E3 ubiquitin ligase for both MHC II and B7-2. However, the biological relevance of MARCH-I-mediated regulation of MHC II and B7-2 remains unknown. In this report, we show that MARCH-I maintains DC function in the steady state via MHC II ubiquitination.

Materials and Methods

Mice

MARCH-I KO mice were backcrossed to the C57BL/6 background at least 10 times before use in this study. MHC II KO mice (8) were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions according to RIKEN guidelines and were used for analysis at 7 to 8 wk of age.

Cell preparation

To purify conventional splenic DCs (cDCs), a DC-enriched, low-density cell fraction from the spleen was prepared by centrifugation of collagenase-digested cells through the density medium Nycodenz (Accurate Chemical). Non-DCs were depleted from the purified, DC-enriched, low-density cell fraction using immunomagnetic beads. cDCs were isolated as CD11c+ cells from the cell fraction described above using a FACSVantage SE Turbo apparatus and were analyzed further. CD8+ or CD8−cDCs were isolated from CD11c+ cells using the FACSVantage SE Turbo apparatus. DCs were generated in vitro by culturing bone marrow (BM) cells obtained from each mouse with Flt-3L (100 ng/ml) (PeproTech) for 6 or 8 days.

Flow cytometry analysis and antibodies

Flow cytometry analysis was performed using a FACSCalibur HG or a FACSAria Turbo apparatus (BD Biosciences). The M1/69 Ab for CD24, 30-F11 Ab for CD45RA, M5/114.15.2 Ab for MHC II, 53–6.7 Ab for CD8, HI3 Ab for CD11c, 116-10A1 Ab for B7-1, L3T3 Ab for CD4, 1C10 Ab for CD40, M1/70 Ab for CD11b, 194 Ab for Ly5.2, and Y-Ae Ab for the complex of I-Aβ/MHC II presenting the I-Eα peptide 52–68 used for FACS analysis were obtained from BD Biosciences or from eBioscience.

Analysis of DC functions

To analyze the cytokine-producing ability of splenic cDCs, sorted splenic cDCs (1 × 10⁶) were stimulated with LPS (1 µg/ml) or anti-CD40 Ab (10 µg/ml) for
48 h, and the amount of IL-12 and TNF-α in the culture supernatants was measured by ELISA kit (R&D Systems). To analyze the Ag-specific presentation ability of splenic cDCs, 3 h after i.v. injection with 3 mg of OVA per mouse, splenic cDCs were purified and the indicated numbers of irradiated splenic cDCs were cultured with CD4 T cells (2 × 10⁵) from OT-II transgenic mice for 3 days. Proliferation was measured by [3H]thymidine incorporation (Amersham/GE Healthcare). In another case, irradiated cDCs (2 × 10⁵) were cultured with CD4 T cells (4 × 10⁵) obtained from OT-II transgenic mice in the presence of the OVA polypeptide for 3 days. Proliferation was measured as described above. In addition, isolated cDCs were incubated with a GFP-fused I-E αβ polypeptide (50 μg/ml) overnight and stained with the Y-Aε Ab together with anti-CD11c, anti-CD4, and anti-CD8 Abs.

Statistics
ELISA and T cell proliferation data were analyzed using the unpaired, two-tailed Student’s t test. Significance was set at \( p < 0.05 \).

Results and Discussion
Abnormality of splenic DCs in MARCH-I KO mice
Given its expression in immature but not in activated/mature cDCs (9, 10), MARCH-I is thought to play an important role in immature cDCs. Therefore, we examined the effect of its deletion in steady-state splenic DCs. As expected, the surface expression of MHC II and B7-2 was increased on CD11c<sup>high</sup> cells, which correspond to cDCs (Fig. 1A and supplemental Fig. 1).<sup>5</sup>

The number of splenocytes and the percentage of CD11c<sup>high</sup> cells were not significantly different in MARCH-I-deficient cDCs (Fig. 1A). However, the expression levels of IL-12 and TNF-α upon stimulation with LPS or anti-CD40 Ab were significantly impaired in both CD8<sup>-</sup> and CD8<sup>+</sup> cDCs derived from MARCH-I KO animals (Fig. 1B). Similar abnormalities were observed in the DC population of lymph nodes; CD8 expression was reduced in CD8<sup>-</sup> cDCs of MARCH-I KO animals (supplemental Fig. 2).

Next, we examined CD8<sup>-</sup> and CD8<sup>+</sup> DCs from the spleen of each type of mouse. As shown in Fig. 1B, the production of IL-12 and TNF-α upon stimulation with LPS or the anti-CD40 Ab was significantly impaired in both CD8<sup>-</sup> and CD8<sup>+</sup> DCs derived from MARCH-I KO animals. We also examined the ability of cDCs to present Ags via MHC II. First, we used the GFP-fused I-Eαβ polypeptide as an Ag, and its presentation

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<sup>5</sup> The online version of this article contains supplemental material.
by MHC II was monitored using the Y-Ae Ab, which specifically recognizes the complex of I-A<sup>+</sup> MHC II presenting the I-Eα peptide 52–68 (11, 12). As shown in Fig. 1C, cDCs from MARCH-I KO mice were unable to present the GFP-fused I-Eα polypeptide efficiently. Finally, we examined the ability of cDCs to present the Ags using naive CD4<sup>+</sup> T cells. As shown in Fig. 1D, the Ag-presenting ability of cDCs was significantly inhibited in MARCH-I KO mice.

Possible requirement of splenic microenvironment for DC abnormality

We examined how MARCH-I deficiency caused DC abnormality in vivo. Because MARCH-I expression was restricted to APCs in the spleen (5, 13), we assumed that this abnormality was due to intrinsic defects in DCs. However, we could not observe any abnormalities in DCs generated from MARCH-I-deficient BM cells using Flt-3L in vitro, even though the expression of MHC II and B7-2 was significantly increased (supplemental Fig. 3). These results suggest that the incidence of the DC abnormality observed in MARCH-I KO mice was not caused by intrinsic defects in DCs but required unidentified factors in the spleen. DCs generated from BM cells using Flt-3L in vitro (Flt-3L BMDCs) reported into CD8<sup>+</sup> cDCs in the spleen after in vivo transplantation (14); therefore, we transfected Flt-3L BMDCs into nonirradiated Ly5.1 mice and examined the surface expression of CD8 on Ly5.2<sup>+</sup>CDC11c<sup>+</sup>B220<sup>−</sup>CD24<sup>+</sup> cDCs in the spleen. As shown in Fig. 2A, CD8 expression was inhibited on Flt-3L BMDCs from MARCH-I KO mice. Moreover, we examined the cytokine-producing ability of Flt-3L BMDCs transferred into Ly5.1 mice. Each type of transferred Flt-3L BMDC was collected using an anti-Ly5.2 Ab from the spleen of Ly5.1 recipient mice and stimulated with LPS in vitro. As shown in Fig. 2B, the production of IL-12 was inhibited in transferred MARCH-I-deficient Flt-3L BMDCs.

**FIGURE 2.** Possible requirement of an in vivo microenvironment for DC abnormality. A. Each type of Flt-3L BMDC was transferred into nonirradiated Ly5.1 mice i.v. Three days after transplantation, the surface expression of CD8 on the Ly5.2<sup>+</sup>CD45RA<sup>−</sup>CD11c<sup>+</sup>CD11b<sup>−</sup>CD24<sup>+</sup> population was analyzed in the recipient spleen by FACS. The shaded histograms and dotted lines show the results of CD8 and isotype control (Cont) staining, respectively. B. Flt-3L BMDCs transferred into Ly5.1 mice were purified and stimulated with LPS, and the production of IL-12 was measured by ELISA. Data are expressed as means ± SD (n = 4), * p < 0.05 (n = 4).

**FIGURE 3.** Requirement of MHC II (A) but not of B7-2 (B) for DC abnormality. Isolated CD11c<sup>high</sup> population from indicated mice were stimulated with LPS or anti-CD40 (α-CD40) Ab, and cytokine production was examined by ELISA. Data are means ± SD (n = 4); * p < 0.05 (n = 4).

**Requirement of MHC II, but not of B7-2, for DC abnormality in MARCH-I KO mice**

Given that the DC abnormality presumably requires the microenvironment of the spleen, molecules able to associate with the splenic microenvironment may be involved in this process. MHC II and B7-2 are candidate molecules, as they show increased surface expression on MARCH-I-deficient DCs and can associate with the microenvironment of the spleen (e.g., with splenic CD4<sup>+</sup> T cells). To test this hypothesis, MARCH-I KO animals were crossed to MHC II- or B7-2-deficient mice. Interestingly, cDCs from MARCH-I<sup>−/−</sup>/MHC II<sup>−/−</sup> (MARCH-I/MHC II double KO (dKO)) and MARCH-I<sup>−/−</sup>/B7-2<sup>−/−</sup> (MHC II KO) animals produced IL-12 and TNF-α at equal levels upon stimulation, even though the surface expression of B7-2 was increased in MARCH-I/MHC II dKO animals (Fig. 3A and supplemental Fig. 4). Furthermore, these mice exhibited the same expression levels of CD4 and CD8 on cDCs (supplemental Fig. 5A). This was not the case in MARCH-I<sup>−/−</sup>/B7-2<sup>−/−</sup> (MARCH-I/B7-2 dKO) animals (Fig. 3B and supplemental Fig. 5B).

**DC abnormality via loss of MHC II ubiquitination**

Our results strongly suggest that MARCH-I maintains the functions of cDCs through ubiquitination-mediated degradation of MHC II but not through that of B7-2. This idea is also supported by the fact that we found similar abnormalities of cDCs in a different genetic MHC II background (supplemental Fig. 6). To confirm this hypothesis, we generated MHC II knockin mice (MHC II KI), which accumulated MHC II via the loss of ubiquitination-mediated degradation. We replaced the I-A β-chain with a mutant I-A β-chain in which the K225 residue was mutated to R225 (see supplemental Fig. 7), because the K225 of the I-A β-chain is responsible for the ubiquitination-mediated degradation of MHC II molecules (2–4). As
production of IL-12 was measured by ELISA. Data are means ± SD (n = 4); *, p < 0.05 (n = 4). B, Indicated cDCs were stimulated as in A. The production of IL-12 was measured by ELISA. Data are means ± SD (n = 4); *, p < 0.05 (n = 4). C, Indicated CD11c+ cells from the indicated mice were irradiated and cultured with OT-II CD4 T cells in the presence of the OVA polypeptide. The proliferation of OT-II CD4 T cells was measured. Data are expressed as means ± SD of triplicate samples; *, p < 0.05, compared with control (Cont). OVA conc, OVA concentration.

expected, the surface expression of MHC II was significantly increased due to the loss of ubiquitinated I-A β protein in homozygous KI animals (supplemental Fig. 7, C and D). As shown in Fig. 4A, the ability of cDCs to produce cytokines upon stimulation with LPS was impaired in homozygous MHC II KI mice. Both CD8− and CD8+ DCs exhibited low cytokine-producing ability in MHC II KI mice (Fig. 4B). The expression levels of CD4 and CD8 in MHC II KI mice were reduced significantly on cDCs (supplemental Fig. 8). In addition, the ability of cDCs to present model Ags through MHC II was impaired in MHC II KI animals (Fig. 4C and supplemental Fig. 9). Taken together, MARCH-I-mediated MHC II ubiquitination is required for the maintenance of cDCs in vivo.

Lack of enhancement of MHC II signaling in MARCH-I-deficient DCs

Why does the stabilized MHC II cause DC abnormalities? Our results suggest that the interaction between the stabilized MHC II and certain factors in the spleen causes DC abnormalities in the steady state, either directly or indirectly. Because the crosslinking of surface MHC II molecules with an anti-MHC II Ab suppresses the functions of BMDCs through MHC II signaling in vitro (15, 16), we examined whether the crosslinking of MHC II molecules induced strong suppression in MARCH-I-deficient BMDCs. As shown in supplemental Fig. 10, the suppressive effect of MHC II crosslinking was not enhanced in MARCH-I-deficient BMDCs compared with control BMDCs. This result suggests that stabilized MHC II may contribute indirectly to the DC abnormality in vivo.

Implications of DC abnormality in MARCH-I KO mice

Recently, we and other groups demonstrated that the stimuli that induce DC maturation stabilize surface MHC II molecules by inhibiting the ubiquitination of the I-A β-chain (3, 4). Furthermore, loss of MHC II ubiquitination was accompanied by the down-regulation of MARCH-I expression; therefore, MARCH-I is thought to be an important regulatory molecule of DC activation, and loss of MARCH-I may be a triggering signal for immunity (10). As these events are similar to those that take place in MARCH-I KO mice, we speculate that matured/activated DCs receive signals that cause DC abnormalities indirectly via stabilized MHC II. If this is the case, such an inhibitory signal may prevent excessive immune reactions. In other words, this may prevent detrimental immunity, such as autoimmune diseases. Consistent with this idea, we observed a down-regulation of CD4 and CD8 on LPS-stimulated splenic cDCs in vivo (data not shown), and several groups have reported LPS-induced DC malfunction in vivo (17). The elucidation of the molecular basis of the DC abnormality induced by the loss of MHC II ubiquitination is required to test this important hypothesis.

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Disclosures

The authors have no financial conflict of interest.

References


