Ubiquitin ligase MARCH 8 cooperates with CD83 to control surface MHC II expression in thymic epithelium and CD4 T cell selection

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INTRODUCTION

MHC II antigen presentation plays a critical role in CD4+ T cell function and hence regulation of adaptive immunity. In the thymus, presentation of self-antigens by thymic epithelial cells (TECs) and DCs is responsible for the positive and negative selection of CD4+ T cells (Klein et al., 2014). Minor alterations in MHC II expression or the repertoire of self-antigens presented by TECs or DCs can profoundly affect the capacity of the immune system to respond to infection or susceptibility to infection (Nakagawa et al., 1998; Peterson et al., 2008; Mohan and Unanue, 2012). Characterization of the mechanisms that regulate the generation, surface expression, and turnover of MHC II in TECs and DCs is thereby required to fully understand how a healthy CD4+ T cell repertoire develops.

Ubiquitination is a critical posttranslational mechanism that regulates MHC II trafficking and levels at the cell surface (Cho and Roche, 2013; Moffat et al., 2013). This has primarily been studied in DCs, where in resting cells, MHC II is trafficked to the surface but then delivered to lysosomes and degraded, whereas DC activation results in reduced MHC II delivery to lysosomes and accumulation at high levels on the cell surface (Villadangos et al., 2005). This pattern of MHC II trafficking is regulated in mouse and human DCs via the oligoubiquitination of a single conserved lysine (K225) in the C-terminal cytosolic tail of the MHC II β chain (Ohmura-Hoshino et al., 2006b; Shin et al., 2006; van Niel et al., 2006; De Gassart et al., 2008). Ubiquitination of MHC II is performed by members of the membrane-associated RING-CH (MARCH) family of E3 ubiquitin ligases. MARCH proteins were originally identified as the mammalian homologues of the viral immunosuppressive membrane ubiquitin ligases K3 and K5 (Goto et al., 2002; Bartee et al., 2004). 11 members have been described (Ohmura-Hoshino et al., 2006a), with MARCH 1 being responsible for ubiquitinating MHC II. This function was initially described in an overexpression study (Ohmura-Hoshino et al., 2006b) and confirmed with analysis of MARCH 1-deficient mice. 

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DCs (Young et al., 2008; Walseng et al., 2010; Oh et al., 2013) and B cells (Matsuki et al., 2007; Oh et al., 2013). Whether MARCH 1–mediated ubiquitination also regulates MHC II trafficking in TECs and whether this contributes to CD4+ T cell selection is unclear. Studies in mice lacking functional CD83 suggested this to be the case. Mice that express functionally impaired CD83 or lack CD83 altogether exhibit dramatically impaired CD4+ T cell selection (Fujimoto et al., 2002; Tze et al., 2011). The mechanism involved is proposed to involve CD83-dependent regulation of MHC II ubiquitination (Tze et al., 2011). CD83 was proposed to sequester MARCH 1 in TECs, preventing it from ubiquitinating MHC II and thereby promoting high MHC II expression at the cell surface. Although an attractive hypothesis, this mechanism has not been validated in vivo. MARCH 1 is not active in TECs (Oh et al., 2013), and therefore, the target for CD83 remains undefined, as is the role of MHC II ubiquitination in antigen presentation mediated by TECs.

Herein, we have identified MARCH 8, the most closely related family member of MARCH 1 (Bartee et al., 2004), as the major E3 ubiquitin ligase responsible for MHC II trafficking in TECs, a process that is regulated by CD83, with important consequences for thymic CD4+ T cell selection.

RESULTS AND DISCUSSION

MARCH 8 controls MHC II trafficking in TECs

MARCH 1–mediated MHC II ubiquitination accelerates MHC II internalization and/or delivery to lysosomal compartments in DCs, and as a consequence, the absence of MARCH 1 increases the surface expression of MHC II in splenic or thymic DCs (Fig. 1 A; Walseng et al., 2010; Oh et al., 2013). The absence of MARCH 1 did not affect MHC II expression in TECs (Fig. 1 A; Oh et al., 2013), suggesting that either ubiquitination does not play a role in regulating MHC II expression in these cells or another E3 ubiquitin ligase is active in TECs. If the latter, a potential candidate is MARCH 8, which is closely related to MARCH 1 and is capable of MHC II ubiquitination in transfected cells (Ohmurra-Hoshino et al., 2006b; Lapaque et al., 2009; Tze et al., 2011). To investigate this, we generated March8−/− mice (Fig. 1 B; refer to the Mice section of Materials and methods for details) and analyzed MHC II expression in DCs and TECs. MARCH 8 deficiency caused increased MHC II expression in TECs but not in DCs (Fig. 1 C). TECs and DCs lacking both MARCH 1 and MARCH 8 expressed similar MHC II levels as their counterparts, lacking only MARCH 1 or MARCH 8, respectively (Fig. 1 C and not depicted), indicating the two E3 ligases play nonredundant roles. Neither MARCH 1 nor MARCH 8 deficiency impacted MHC I expression in DCs and TECs, respectively (Fig. 1 D).

TECs can be divided into two major types: cortical (cTEC) and medullary (mTEC). In turn, mTECs can be subdivided into immature cells that express low levels of MHC II (mTEClow) and more mature cells that express higher MHC II levels (mTEChigh; Gray et al., 2007; Rossi et al., 2007). A subtype of mTEChigh expresses autoimmune regulator (AIRE), a transcription factor that induces ectopic gene expression, required for effective negative selection of tissue-reactive thymocytes (Mathis and Benoist, 2009). Analysis of TEC subsets showed that MARCH 8 regulates surface MHC II expression in both cTECs and AIRE+ mTECs but not in AIRE− mTEC high (Fig. 1 E). Given that overexpressed MARCH 8 can ubiquitinate CD86 in HeLa cells (Bartee et al., 2004), we also assessed whether this molecule was a physiological substrate of MARCH 8. This was not the case as CD86, in addition to closely related CD80, was unaltered on the surface of March8−/− TECs (Fig. 1 F).

Ubiquitination of surface MHC II is responsible for controlling its surface turnover. We therefore monitored the rate of surface MHC II turnover in cells lacking MARCH 8 ligases. To do this, we used a specific hybridization internalization probe (SHIP) assay that we have previously used to monitor the internalization of surface receptors (Reuter et al., 2015). Measurement of surface MHC II turnover in wild-type DCs by SHIP showed that these cells internalized ~30% of their surface MHC II molecules within 30 min (Fig. 2 A). Internalization was significantly reduced for March1−/− DCs (Fig. 2 A). This supports observations that ubiquitinated MHC II molecules are more readily lost from the cell surface, either because of their enhanced endocytosis from the plasma membrane or reduced recycling back to the cell surface (Cho and Roche, 2013; Moffat et al., 2013). Examination of MHC II turnover in TECs showed that surface MHC II is more stable in these cells than in DCs but nevertheless was regulated by MARCH 8 ubiquitination, as its deficiency in TECs caused significantly reduced MHC II internalization (Fig. 2 B). Therefore, MARCH 8, but not MARCH 1, is active in TECs and is the E3 ubiquitin ligase responsible for the posttranslational regulation of surface MHC II trafficking in these cells.

The absence of MARCH 8 does not significantly alter the CD4+ T cell repertoire

To examine the consequence of MARCH 8 deficiency and elevated MHC II TECLes levels on thymic function, we assessed CD4+ T cell selection in March8−/− mice. The proportion and number of CD4+ single-positive (SP) thymocytes (Fig. 3 A) as well as the proportion and number of CD4+ splenic T cells (Fig. 3 B) were unaltered in the absence of MARCH 8. In addition, CD4+ SP thymocytes from March8−/− mice displayed no evidence of an altered phenotype, with normal levels of CD3 and CD4 and the activation markers CD3, CD69, Nur77, CD25, and Qa-2 (Fig. 3 C). This was also the case for splenic CD4+ T cells (Fig. 3 D). The proportion of FoxP3+ regulatory T cells (T reg cells) in the thymus and spleen were similar in wild-type and March8−/− mice (Fig. 3 E). Finally, the lack of MARCH 8 had no impact on MHC II–mediated selection of OT-II CD4+ OVA-specific TCR transgenic T cells (Fig. 3 F).

To analyze the potential impact of MARCH 8 deficiency on the CD4+ T cell repertoire in more detail, we
Figure 1. **MARCH 8 controls surface MHC II expression in TECs.** (A) Spleen and thymus DCs or TECs were isolated from wild-type or March1−/− mice, and surface MHC II was examined by flow cytometry. Data are representative of at least two independent experiments. (B) Diagram illustrating generation of March8−/− mice. TGA indicates a stop codon. (C) Spleen and thymus DCs or TECs were isolated from wild-type, March8−/−, or March1−/− March8−/− mice, and surface MHC II was analyzed by flow cytometry. Data are representative of at least two independent experiments. (D) MHC I expression by wild-type and March8−/− DCs and March8−/− TECs. (E) MHC II expression by wild-type and March8−/− cTECs and AIRE− and AIRE+ mTECs. Data are pooled from two to five independent experiments and are mean ± SEM. ***, P < 0.0001, unpaired Student’s t test. MFI, mean fluorescence intensity; n.s., not significant. (F) Expression of CD80 and CD86 by wild-type and March8−/− TECs. Data are representative of at least two independent experiments.
examined antigen-specific CD4+ T cell populations in the lymph nodes and spleen of naive mice. To do this, CD4+ T cells were enriched from wild-type or March8−/− mice using different MHC II–peptide tetramers and enumerated (Moon et al., 2007). Similar numbers of naive CD4+ T cells specific for MHC II–restricted peptides derived from I-E α chain, ribosomal protein L6 (AasF peptide), calnexin, or STM1540 proteins of Salmonella enterica serovar Typhimurium were detected in the two groups of mice (Fig. 3 G). Single-cell analysis of the TCR repertoire of I-E α–specific CD4+ T cells revealed no major differences in the preferences for TCRβ usage by cells isolated from wild-type and March8−/− mice (Fig. 3 H).

Our data shows no major perturbation in CD4+ T cell differentiation, selection, and/or maintenance of CD4+ T cells in the periphery in the absence of MARCh8. This is surprising because CD4+ T cell selection is critically sensitive to minor reductions in MHC II levels observed in Cd83-deficient mice (Kuwano et al., 2007; Tze et al., 2011). At face value, this suggests that mechanisms of CD4+ T cell selection can tolerate significant increases in MHC II levels more readily than subtle reductions. This notion is consistent with the observation that increased MHC II in thymic DCs that lack MARCh1 does not dramatically alter CD4+ negative selection in March1−/− mice (Oh et al., 2013). In contrast, reduced MHC II expression alone does not appear to affect CD4+ T cell selection because mice deficient in CD83 or expressing only one MHC II allele display similarly low surface MHC II; yet, only CD83-deficient mice have a defect in CD4+ T cell selection (Kuwano et al., 2007). This suggests that altered MHC II turnover on the TEC surface, rather than reduced expression level, may be the major factor responsible for impaired CD4+ T cell selection in CD83-deficient mice. If so, counteracting the effect of the lack of CD83 on MHC II turnover in TECs should restore CD4+ T cell selection. We tested this hypothesis in the following set of experiments.

CD83 regulation of MARCH8 activity is critical for CD4+ T selection

CD83 has been proposed to sequester the E3 ligase that ubiquitinates MHC II in TECs (Tze et al., 2011). In the absence of CD83, the ligase would over-ubiquitinate MHC II, causing increased internalization and reduced surface expression of the molecule. MARCH1 was proposed as the ligase involved, but as we show here, the ligase that regulates MHC II turnover and surface expression in TECs is MARCH8, not MARCH1. Therefore, we examined whether MARCH8 was responsible for the reduction in TEC MHC II expression and impaired CD4+ T cell selection in mice lacking functional CD83.

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Figure 3. The absence of MARCH 8 does not significantly alter the CD4+ T cell repertoire. (A and B) Frequency and number of CD4+ CD3+ thymocytes (A) or splenic CD4+ T cells (B) from wild-type or March8−/− mice. The graphs display data pooled from a minimum of three independent experiments with symbols representing individual mice. Bars designate the mean. (C and D) Summary of the fold difference in geometric mean fluorescence intensity for
the substrate specificity of the two ligases that may underpin specialized roles in distinct antigen-presenting cell types. Our results do not exclude another possibility, namely that MARCH 8 ubiquitinates another receptor that, if not ubiquitinated, can compensate for the lack of CD83. However, replacement of the Lys residue that becomes ubiquitinated in the MHC II β chain with an Arg is sufficient to compensate for the effect of CD83 deficiency (see von Rohrscheidt et al. in this issue), an observation that discards this possibility.

It will be interesting to examine whether the specialization of MARCH 1 and 8, and indeed other members of the MARCH family, extends to other antigen-presenting cell types. The substrate specificity of each MARCH may be mediated by differential transmembrane region interactions (Bartee et al., 2004) or by the participation of distinct accessory proteins involved in MARCH function (e.g., the E2 ligases and/or deubiquitinating enzymes). These specializations may affect not just which substrates are ubiquitinated, but also the type of ubiquitin chains incorporated into the same substrate in different cell types, as has been shown to be the case for MHC II in DCs and B cells (Ma et al., 2012). Differential use of ubiquitination machinery opens the prospects of exerting fine control of MHC II antigen presentation in specific cell types with important consequences for the manipulation of immune outcomes.

MATERIALS AND METHODS

Mice

C57BL/6, March1−/− (Matsuki et al., 2007), March8−/−, OT-II, and Cd83−/−/− (Tze et al., 2011) mice were bred and maintained in specific pathogen–free conditions at the Bio21 Molecular Science and Biotechnology Institute. In brief, March8−/− mice were generated by the insertion of loxp sites flanking exon 5 that encodes the RING-variant domain. Mice were crossed with CAG-Cre mice to delete loxp sites flanking exon 5 that encodes the RING-variant domain. Mice were crossed with CAG-Cre mice to delete loxp sites flanking exon 5 that encodes the RING-variant domain.

Isolation of DCs

Organs were finely chopped and digested with 140 µg/ml DNase I (Roche) and 1 mg/ml collagenase type III (Worthington Biochemical Corporation). Cell clusters were further dissociated by treatment with 10 mM EDTA. Light-density dissociation was followed by two washes and incubation of FIP-anti–MHC II antibody (clone M5114) was functionalized with Click-IT Cy5′ Fluorescence internalization probe (FIP)–azide (5′-Cy5′-TCAGTTCAGGACCCCTGCGT-N3′) and quencher (Q; 5′-AGCCAGGGTCTGTAAGTCGTA-BHQ2′) were purchased from Integrated DNA Technologies. Anti–MHC II antibody (clone M5114) was functionalized with Click-IT succinimidyl ester DIBO alkyne (Thermo Fisher Scientific) by incubating with a 10-fold molar excess of succinimidyl ester DIBO alkyne for 2 h at 4°C. Functionalized antibody was purified using a Zeba spin desalting column (Thermo Fisher Scientific) and incubated with a twofold molecular excess of FIP-azide at 4°C overnight. FIP-labeled antibody was purified using a 30-kD Amicon filter (EMD Millipore), and the degree of functionalization was measured with a spectrophotometer (1000 UV-Vis; NanoDrop). SHIP internalization assays were performed by staining cells on ice for 30 min with FIP-Cy5–conjugated anti–MHC II antibody in the presence of anti-CD16/32 antibody (BioLegend) to block nonspecific binding to Fc receptors. Staining was followed by two washes and incubation of FIP–anti–MHC II–bound cells in complete RPMI 1640 at 37°C and
10% CO₂. Cells were removed at specific time points and placed on ice. Cells were washed, phenotyped for surface markers, and resuspended in media containing propidium iodide with or without 1 mM Q. Cells were analyzed using a flow cytometer (LSRFortessa). Data were analyzed with FlowJo software (Tree Star).

**T cell analysis of spleen and thymus**

Spleen or thymus single-cell suspensions were depleted of red blood cells and stained with antibodies specific for CD3 (KT3-1.1; WEHI antibody facility) and CD4 (RM4-4) and CD8α (53-6.7; both BioLegend). For Foxp3+ T reg cell analysis, samples were stained with CD25 (PC61; WEHI antibody facility), fixed, and permeabilized with transcription factor staining buffer (eBioscience) and stained with anti-FoxP3 (FJK-16s; eBioscience) according to the manufacturer’s instructions. Samples were analyzed on a flow cytometer (LSRFortessa) and FlowJo software.

**Enumeration and isolation of naive epitope-specific CD4+ T cells**

Tetramer-based magnetic enrichment was used for the identification and isolation of naive epitope-specific CD4+ T cells, as has been described previously (Moon et al., 2007). In brief,
The entire enriched sample was acquired on an LSR II flow cytometer with FACSDiva software (BD), and data were analyzed with FlowJo software.

TCR CDR3β usage

Individual I-Eα-specific CD4+ T cells were sorted into wells of a 96-well plate using a flow cytometer (FACSAria; BD). Single-cell reverse transcription and nested, multiplexed PCR amplification of TCR CDR3β regions were performed. Second-round PCR products were purified and used as a template in sequencing reactions with internal TRBC antisense primers, as previously described (Dash et al., 2011). TCR gene segments were assigned using the International ImMunoGeneTics database (Lefranc, 2001).

Online supplemental material

Fig. S1 shows flow cytometry gating strategy. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20160312/DC1.

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