MARCH1-mediated MHCII ubiquitination promotes dendritic cell selection of natural regulatory T cells

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Membrane-associated RING-CH1 (MARCH1) is an E3 ubiquitin ligase that mediates ubiquitination of MHCII in dendritic cells (DCs). MARCH1-mediated MHCII ubiquitination in DCs is known to regulate MHCII surface expression, thereby controlling DC-mediated T cell activation in vitro. However, its role at steady state or in vivo is not clearly understood. Here, we show that MARCH1 deficiency resulted in a substantial reduction in the number of thymus-derived regulatory T cells (T reg cells) in mice. A specific ablation of MHCII ubiquitination also significantly reduced the number of thymic T reg cells. Indeed, DCs deficient in MARCH1 or MHCII ubiquitination both failed to generate antigen-specific T reg cells in vivo and in vitro, although both exhibited an increased capacity for antigen presentation in parallel with the increased surface MHCII. Thus, MARCH1-mediated MHCII ubiquitination in DCs is required for proper production of naturally occurring T reg cells, suggesting a role in balancing immunogenic and regulatory T cell development.

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Abbreviations used: cDC, conventional DC; MARCH1, membrane-associated RING-CH1; mTEC, medullary TEC; PDC, plasmacytoid DC; TEC, thymic epithelial cell; T reg cell, regulatory T cell.

Membrane-associated RING-CH1 (MARCH1) is an E3 ubiquitin ligase that mediates ubiquitination of MHCII and CD86 in DCs (Matsuki et al., 2007; Baravalle et al., 2011). This ubiquitination induces MHCII and CD86 endocytosis, lysosomal transport, and degradation (Shin et al., 2006; van Niel et al., 2006; Baravalle et al., 2011). The functional role of MARCH1 has been studied mainly in the context of DC maturation and T cell activation or regulation in vitro. When DCs are exposed to maturation stimuli, MARCH1 is rapidly down-regulated (De Gassart et al., 2008; Walseng et al., 2010). This down-regulation leads to an increase in MHCII and CD86 on the DC surface, which enhances the ability of the cell to stimulate antigen-specific T cells (Baravalle et al., 2011). In contrast, when DCs are exposed to the immune suppressive cytokine IL-10, MARCH1 is up-regulated (Tze et al., 2011; Baravalle et al., 2011). This up-regulation results in a reduction of MHCII and CD86 surface levels, and diminishes the DC's ability to

activate T cells (Baravalle et al., 2011). These studies suggest that MARCH1 plays a regulatory role in T cell activation during immune responses. However, the role of MARCH1 at steady state or in vivo is not well understood although a recent study has suggested that MARCH1 might be involved in splenic DC homeostasis (Ohmura-Hoshino et al., 2009).

At steady state, MHCII plays an important role in CD4 T cell development in the thymus (Laufer et al., 1996). Furthermore, MHCII critically impacts the development of natural regulatory T cells (T reg cells), a unique CD4 T cell subset equipped with potent immune suppressive capacity (Hsieh et al., 2012). Co-stimulatory molecules, including CD86, also mediate T reg cell and NKT cell development (Salomon et al., 2000; Williams et al., 2008). Thus, given the function of MARCH1 in controlling MHCII

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and CD86 expression, together with the role of MHCII and CD86 in T cell development, we hypothesized that MARCH1 might be an important regulator of T cell development in the thymus. To test this hypothesis, we examined MARCH1 expression in the thymus and further examined whether its expression plays an important role in the development of specific T cell subsets.

RESULTS AND DISCUSSION

To determine MARCH1 expression in thymic APCs, we isolated thymic epithelial cells (TECs), conventional DCs (cDCs; CD8$^+$ DCs and Sirpα^+ DCs), plasmacytoid DCs (PDCs), and B cells using FACS (Fig. S1), and determined MARCH1 mRNA levels by quantitative RT-PCR. For comparison, we analyzed thymocytes, which have been shown to lack MARCH1 (Matsuki et al., 2007). We found that thymocytes and TECs hardly expressed MARCH1, whereas cDCs, PDCs, and B cells expressed relatively high levels (Fig. 1 A). Thus, MARCH1 is specifically expressed by hematopoietic APCs in the thymus.

Next, we asked if MARCH1 plays a regulatory role in the surface expression of MHCII and CD86 in thymic hematopoietic APCs. Surface levels of MHCII and CD86 were determined by flow cytometry in WT and MARCH1 KO mice. We found that MHCII and CD86 were both markedly increased in all hematopoietic APCs in MARCH1 KO mice but not in TECs (Fig. 1 B). In contrast, no changes were observed in the surface expression of other co-stimulatory molecules such as CD80 and CD40 (unpublished data). Thus, MARCH1 appears to play a regulatory role in the surface expression of MHCII and CD86 in hematopoietic thymic APCs. Interestingly, we found that the number of PDCs was slightly reduced in MARCH1 KO mouse thymus, but the number of TECs, cDCs, and B cells did not change (Fig. 1 C).

To determine the role of MARCH1 in T cell development, we determined thymocyte number, maturation status, and composition in WT and MARCH1 KO mice. No significant differences were observed in total thymocyte numbers, or in the percentage of mature thymocytes as measured by CD3 and CD24 expression (Fig. 1, D and E). The percentage of γδT cells was slightly reduced in MARCH1 KO mice, but that of natural killer T (NKT) cells did not change (Fig. 1 F). No differences were observed in the expression levels of CD4 and CD8, the percentages of CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$CD8$^-$ (CD4 SP), and CD4$^-$CD8$^+$ (CD8 SP) subsets, or in the percentages of Annexin V$^+$ apoptotic cells (Fig. 1, G–I). We also examined the TCR repertoire of CD4 SP and CD8 SP subsets using a panel of TCRV$_\beta$-specific antibodies, but no differences were detected except that V$_\beta$3$^+$ CD8 SP cells were slightly more abundant in MARCH1 KO mice (Fig. 1, J and K). However, when we examined T reg cells, we found an ∼50% reduction both in frequency (as a percentage of CD4 SP thymocytes) and absolute number in MARCH1 KO mice, although levels of Foxp3 and CD25 expression were unchanged (Fig. 1, L–N). No significant difference was observed in the percentages

of either Ki-67$^+$ or Annexin V$^+$ T reg cells (Fig. 1 O), suggesting T reg cell reduction in MARCH1 KO mice was caused by neither reduced T reg cell proliferation nor increased apoptosis. Notably, T reg cell frequency in the lymph node was also significantly reduced in MARCH1 KO mice (unpublished data). These findings demonstrate that MARCH1 deficiency results in a reduction in T reg cell number, but this reduction is not associated with a defect in T reg cell proliferation or survival, implicating the role of MARCH1 in T reg cell development.

To determine if hematopoietic or nonhematopoietic cells were responsible for the T reg cell reduction in MARCH1 KO mice, four groups of chimeric mice were generated by injecting lethally irradiated WT and MARCH1 KO mice with BM isolated from either WT or MARCH1 KO mice. We found that MARCH1 KO mice that received MARCH1-deficient BM exhibited ∼50% reduction in T reg cells compared with WT mice that received MARCH1-sufficient BM (Fig. 2, A and B). Importantly, WT mice that received MARCH1-deficient BM also had ∼50% reduction in T reg cells compared with WT mice that received MARCH1-sufficient BM (Fig. 2, A and B). However, MARCH1 KO mice that received MARCH1-sufficient BM had the same number of T reg cells as compared with the control chimeric mice (Fig. 2, A and B). These findings indicate that T reg cell reduction in MARCH1 KO mice was caused by MARCH1 deficiency in hematopoietic cells.

Next, we examined whether the role of MARCH1 in T reg cell development is mediated by cell-intrinsic or extrinsic mechanisms. For this study, we generated a large cohort of mixed chimeric mice by injecting lethally irradiated WT mice with different ratios of BM mixtures isolated from WT and MARCH1 KO mice, and examined T reg cell numbers by flow cytometry. We found that T reg cell abundance, both as a frequency and absolute number, was inversely related to the percentage of MARCH1 KO BM-derived cells (Fig. 2, C and D). This finding indicates that MARCH1 contributes to T reg cell development via a cell-intrinsic mechanism.

We also examined whether MARCH1 expression in thymocytes or T reg cells contributes to T reg cell development. To address this, we determined the frequency of T reg cell derived from WT and MARCH1 KO in 1:1 mixed BM chimeric mice. We found that the mice had T reg cells derived from WT and MARCH1 KO at similar frequencies, indicating that MARCH1 expression in thymocytes or T reg cells does not contribute to T reg cell development. Thus, the role of MARCH1 in T reg cell development involves a T reg cell–extrinsic mechanism.

To verify the role of MARCH1 in T reg cell development, we used two mouse models in which T reg cells are generated by a specific antigen and the generation can be quantitatively determined. The first was the OT-II/Rip-mOVA mouse model. The Rip-mOVA mice express membrane-bound OVA under the control of the rat insulin promoter, which results in medullary TEC (mTEC) expression of OVA owing to the ability of these cells to promiscuously express tissue-restricted
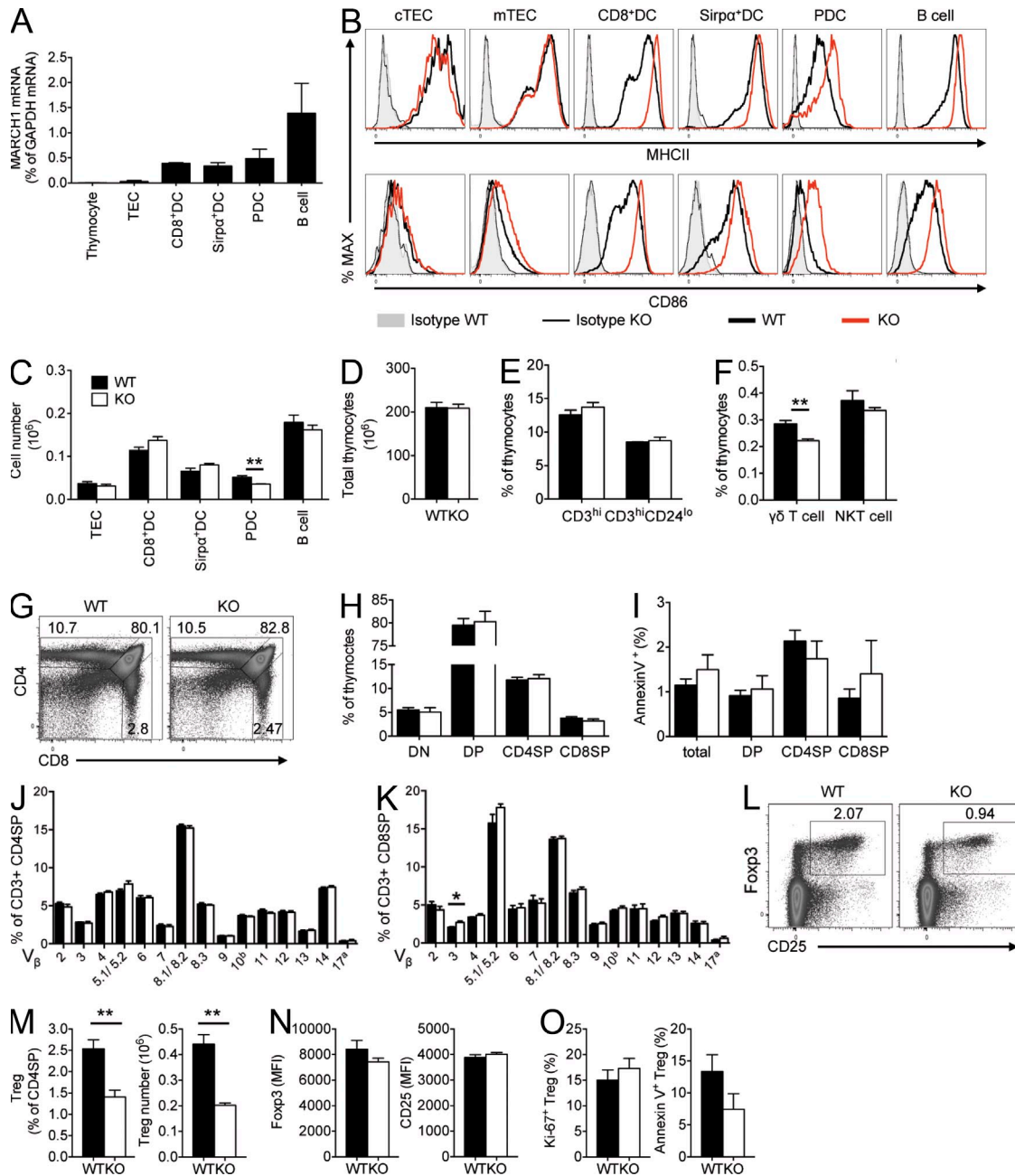
Figure 1.   MARCH1 is expressed in hematopoietic thymic APCs but not in TECs, and is required for thymic T reg cell differentiation. (A) Thymic APCs were enriched, the indicated cell types were isolated by FACS (Fig. S1 A), and MARCH1 mRNA levels were assessed by quantitative RT-PCR. Each bar represents mean $\pm$ SEM of at least three independent measurements. (B) Surface expression of MHCII and CD86 in thymic APCs from WT and MARCH1 KO mice was measured by flow cytometry using anti-MHCII antibody, anti-CD86 antibody, and corresponding isotype control antibodies. Representative data of at least two independent experiments are shown. (C) Thymic APCs from WT and KO mice were quantitated. Each bar represents mean $\pm$ SEM of measurements made from five mice. **, $P < 0.01$. (D–F) WT and MARCH1 KO (KO) mouse total thymocytes (D), percentage of the indicated populations among CD3^{hi} and CD3^{hi}CD24^{lo} thymocytes (E), of $\gamma\delta$ T cells ($\gamma\delta$TCR$^+$; F) and of NKT cells (PBS57-loaded CD1d tetramer$^+$; F) were quantitated. Each bar represents mean $\pm$ SEM of measurements made from 3–10 mice. **, $P < 0.01$. (G–I) WT and MARCH1 KO mouse thymocytes were analyzed for CD4 and CD8 expression by flow cytometry. (G) Representative flow cytometry plots. (H) Percentage of each thymocyte subset presented by mean $\pm$ SEM of measurements made from 10 mice. (I) Percentage of Annexin V$^+$ cells in each subset presented by mean $\pm$ SEM of measurements made from three to seven mice. (J and K) TCR V$_\beta$ repertoire of CD4 SP and CD8 SP thymocytes was determined by flow cytometry. Each bar represents mean $\pm$ SEM of measurements made from four to five mice. *, $P < 0.05$. (L and M) Foxp3 and CD25 expression in CD4 SP cells was determined by flow cytometry. (L) Representative flow cytometry plots showing T reg cell gating. (M) Percentage and total number of T reg cells; data presented as mean $\pm$ SEM of measurements made from 10 (left) or 5 (right) mice. **, $P < 0.01$ (N) Mean fluorescence intensity (MFI) of Foxp3 (left) and CD25 (right) on T reg cells was measured. Each bar represents mean $\pm$ SEM of measurements made from five mice. (O) Percentage of Ki-67$^+$ (left) and Annexin V$^+$ (right) T reg cells in T reg cells was assessed by flow cytometry. Each bar represents mean $\pm$ SEM of measurements made from three mice.

antigens (Kurts et al., 1996). When we crossed these mice to OT-II mice that express a TCR specific for OVA peptide (323–339) loaded onto IA_b MHCII molecule, we found that T reg cell numbers greatly increased (Fig. 3, A [top] and B [black bars]), whereas CD4 SP cell numbers dramatically decreased compared with those found in OT-II mice (Fig. 3 C, black bars). To verify that generated T reg cells were specific for OVA, we stained thymocytes with the anti-$V_\alpha 2$ antibody, which recognizes the transgenic TCR of OT-II mice. Over 95% of newly generated T reg cells were strongly labeled by this antibody (unpublished data), indicating that the generated T reg cells are specific for OVA. We also observed increased T reg cell numbers when we crossed Rip-mOVA mice to OT-II; MARCH1 KO mice, but to a much lesser degree than that observed in MARCH1-sufficient OT-II mice (Fig. 3, A [bottom] and B [blue bars]). CD4 SP cells were largely deleted, although slightly less efficiently than in MARCH1-sufficient OT-II mice (Fig. 3 C, blue
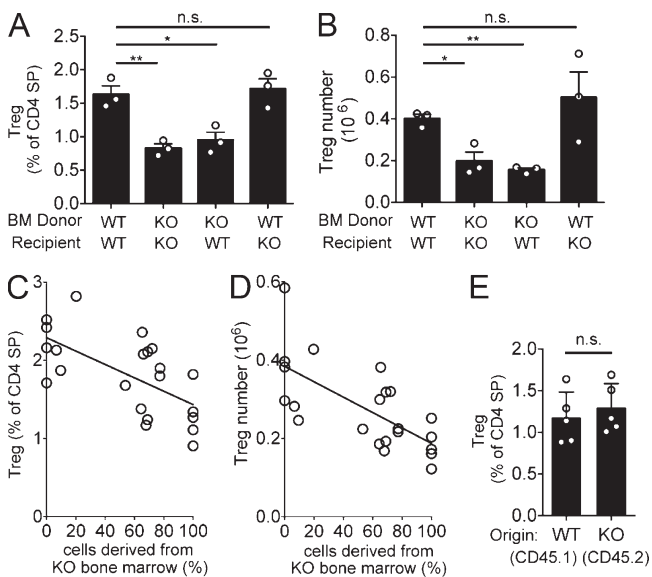
bars). This finding suggests that MARCH1 plays a significant role in T reg cell development against mTEC-driven antigens.

The second mouse model was the OT-II/OVA injection model. Previous studies have shown that when OVA was i.v. injected into OT-II mice, the OVA was specifically taken up and presented by Sirpα^+ DCs in the thymus, and the presentation resulted in the development of T reg cells and the deletion of CD4 SP thymocytes (Baba et al., 2009, 2012). Consistent with these findings, we found that OVA injected i.v. was specifically taken up by Sirpα^+ DCs in the thymus (Fig. 3 D). OVA uptake efficiency was not different between DCs of WT and MARCH1 KO mice (Fig. 3 E). To determine the role of MARCH1 in T reg cell development in this model, we injected increasing doses of OVA into OT-II and OT-II; MARCH1 KO mice. When thymocytes were analyzed 3 d later, we found that T reg cell numbers had increased in OT-II mice but not in OT-II; MARCH1 KO mice (Fig. 3, F and G). In contrast to T reg cells, the number of CD4 SP thymocytes were reduced both in OT-II and OT-II; MARCH1 KO mice although to a slightly lesser degree in OT-II; MARCH1 KO mice (Fig. 3 H). These findings suggest that MARCH1 is not critical for the deletion of blood-borne, antigen-specific CD4 SP thymocytes but is essential for the development of T reg cells.

To identify the specific mechanism by which MARCH1 contributes to T reg cell development, we examined the role of MARCH1-mediated MHCII ubiquitination. For this study, we generated MHCII (K > R) knock-in (KI) mice in which the MHCII cytoplasmic lysine (K) was replaced by arginine (R), and thus could not be modified by ubiquitination (Fig. 4, A and B). Similarly to what was observed in MARCH1 KO mice, an increase in MHCII surface levels was observed in hematopoietic APCs of MHCII (K > R) KI mice, most markedly in DCs (Fig. 4 C, top). No increase was observed in co-stimulatory molecules, including CD86, CD80, and CD40 (Fig. 4 C, bottom; and not depicted). Remarkably, we found that this mouse strain also exhibited a reduction (∼30%) in both the frequency and number of T reg cells (Fig. 4 E), whereas no significant changes were observed in thymocyte numbers or composition (Fig. 4 D and not depicted). We also examined the development of antigen-specific T reg cells in these mice by using the OT-II/OVA-injection model. OVA uptake efficiency was similar between DCs of WT and MHCII (K > R) KI mice (Fig. 4 F). However, T reg cell generation was completely abolished in OT-II; MHCII (K > R) KI mice, whereas CD4 SP thymocyte deletion was largely intact (Fig. 4, G and H). These findings suggest that MARCH1 contributes to T reg cell development in part by mediating MHCII ubiquitination and indicate that MHCII ubiquitination alone plays a significant role in T reg cell development.

Having found that defective MHCII ubiquitination leads to a marked increase in MHCII surface levels in hematopoietic APCs, we examined whether it results in an increase in peptide-loaded MHCII (pMHCII) surface levels. We first determined pMHCII loaded with endogenous antigens. For this study, two F1 hybrid mice were generated by crossing a WT or a MHCII (K > R) KI mouse, both of which are on the C57BL/6
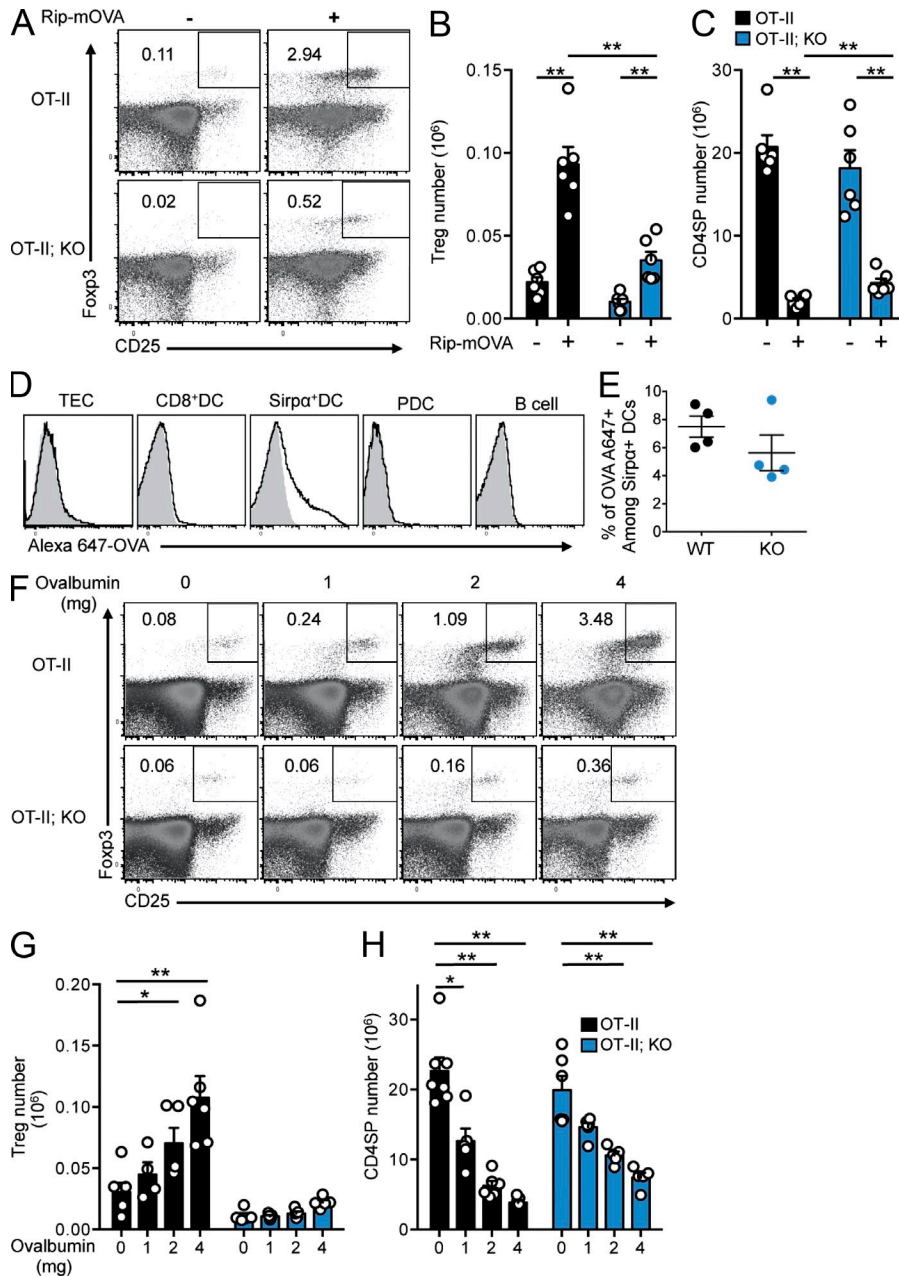

Figure 2. **T reg cell reduction in MARCH1 KO mice is caused by MARCH1 deficiency in hematopoietic cells, but not T reg cells themselves.** (A and B) BM was isolated from WT and MARCH1 KO (KO) mice and injected into lethally irradiated WT and KO mice. A shows the percentage of T reg cells among CD4 SP cells, and B shows the T reg cell number. Each bar represents mean ± SEM of measurements made from three mice. Data from one representative experiment out of two independent experiments are shown. *, P < 0.05; **, P < 0.01; n.s., not significant. (C and D) WT and MARCH1 KO BM were isolated, mixed at various ratios, and injected into lethally irradiated WT mice. 8 wk later, thymocytes were analyzed by flow cytometry. T reg cell frequency (C) and number (D) were plotted as a function of the increasing percentage of cells derived from KO BM. Each circle represents a measurement made from a single chimeric mouse. Best-fit lines determined by the linear regression analysis are shown. Data are from three independent experiments. (E) WT (CD45.1) and KO (CD45.2)-derived thymocytes were gated from 1:1 mixed chimeric mice, and the frequency of T reg cells from each gated population was determined. Each bar represents mean ± SEM of measurements made from five mice. Data are from two independent experiments. Paired two-tailed Student's t test was performed. n.s., not significant.

Figure 3. MARCH1 plays a significant role in T reg cell development driven by both mTEC– and blood–borne antigens. (A–C) OT-II and OT-II; MARCH1 KO (OT-II; KO) mice were crossed to Rip-mOVA mice (+) or not (−). Thymocytes were stained and analyzed by flow cytometry to determine the number of T reg cells and CD4 SP cells. (A) Representative flow cytometry data with numbers indicating the frequency of T reg cells among CD4 SP cells. (B and C) Total number of T reg cells and CD4 SP cells. Data are from five independent experiments. Each bar represents mean ± SEM of measurements made from 6 mice. **, P < 0.01 (D) WT mice were injected with PBS (filled histograms) or 50 µg Alexa Fluor 647–conjugated OVA (open histograms). After 1 h, thymi were isolated and analyzed by flow cytometry. Representative data of four independent experiments are shown. (E) WT and MARCH1 KO (KO) mice were injected i.v. with Alexa Fluor 647–conjugated OVA, and the percentage of Alexa Fluor 647+ Sirpα+ DCs was determined by flow cytometry. Data are from four independent experiments. Data represent mean ± SEM of measurements made from four mice. (F–H) OT-II (black) and OT-II; MARCH1 KO (OT-II; KO, blue) mice were injected i.v. with indicated amounts of OVA. 3 d later, thymi were isolated and analyzed by flow cytometry. (F) Representative flow cytometry data with numbers indicating the frequency of T reg cells among CD4 SP cells. (G and H) Total number of T reg cells and CD4 SP cells. Data are from eight independent experiments. Each bar represents mean ± SEM of measurements made from five to seven mice. *, P < 0.05; **, P < 0.01.

background, with WT BALB/c mice that express I-E MHCII molecules. The surface level of I-E$_\alpha$(52–68)–IA$_b$ complexes was determined by flow cytometry using the specific antibody, YAe (Rudensky et al., 1991). We found that cDCs of the (KI x BALB/c) F1 mice expressed I-E$_\alpha$(52–68)–IA$_b$ complexes at higher levels than cDCs of the (WT x BALB/c) F1 mice, whereas no or only a modest increase was observed in the TEC, PDC, or B cell compartments (Fig. 5 A). We also generated (MARCH1 KO × BALB/c) F1 mice and performed similar experiments and analyses. cDCs of these mice also expressed higher levels of I-E$_\alpha$(52–68)–IA$_b$ complexes than cDCs of the (WT x BALB/c) F1 mice (Fig. 5 B). Next, we examined pMHCII loaded with exogenous antigens.

Thymic cDCs were isolated from WT, MHCII (K > R) KI, and MARCH1 KO mice, incubated with the I-E$_\alpha$ recombinant protein, and the surface expression of I-E$_\alpha$(52–68)–IA$_b$ complexes was determined by flow cytometry. We found that thymic DCs from MHCII (K > R) KI and MARCH1 KO mice expressed I-E$_\alpha$(52–68)–I-A$_b$ at higher levels than those from WT mice (Fig. 5 C). These findings indicate that thymic DCs of MHCII (K > R) KI and MARCH1 KO mice present higher levels of endogenous and exogenous antigens.

Next, we examined the ability of DCs from WT, MHCII (K > R) KI, and MARCH1 KO mice to induce T reg cells and to delete thymocytes in vitro. We treated thymic cDCs from WT, MHCII (K > R) KI, and MARCH1 KO mice
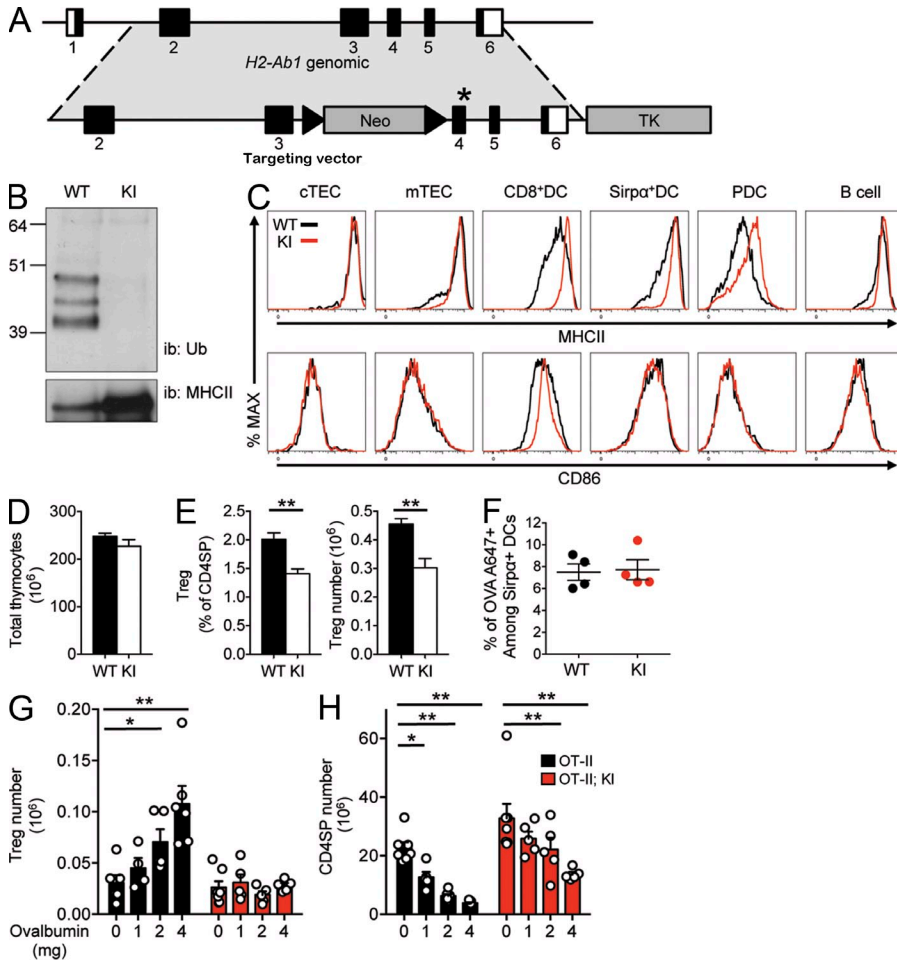
Figure 4. MHCII ubiquitination plays a significant role in T reg cell development. (A) Targeting construct used to generate MHCII (K > R) KI mice. Numbered rectangles indicate exons. The loxP (black arrowhead)-flanked neomycin (neo) cassette was inserted for positive selection of transfected ES cells and the thymidine kinase (TK) cassette was inserted for negative selection. Exon 4 was modified to change the cytoplasmic Lys to an Arginine (*). (B) MHCII was immunoprecipitated from splenic DCs of WT and KI mice, and immunoblotted (ib) using anti-ubiquitin (Ub) and anti-MHCII antibodies. Representative data of at least three independent experiments are shown. (C) Surface expression of MHCII and CD86 in thymic APCs of WT and KI mice was determined by flow cytometry. Representative data of at least three independent experiments are shown. (D–E) WT and MHCII (K > R) KI (KI) mouse thymocyte number (D) and T reg cell frequency and number (E) were quantitated. Each bar represents mean ± SEM of measurements made from 6–10 mice. (F) WT and MHCII (K > R) KI (KI) mice were injected i.v. with Alexa Fluor 647–conjugated OVA, and the percentage of Alexa Fluor 647+Sirpα+ DCs was determined by flow cytometry. Data represent mean ± SEM of measurements made from four mice. (G and H) OT-II (black) and OT-II; MHCII (K > R) KI (OT-II; KI, red) mice were injected i.v. with indicated amounts of OVA. 3 d later, thymi were isolated and analyzed by flow cytometry. T reg cells (G) and CD4 SP cells (H) were quantitated. Data are from eight independent experiments. Each bar represents mean ± SEM of measurements made from five to seven mice. *, P < 0.05; **, P < 0.01.

with OVA and cultured them with immature CD4 SP thymocytes isolated from Thy1.1; Foxp3-EGFP; OT-II mice. T reg cell generation and thymocyte deletion were determined 24 h later. We found that thymic cDCs from MHCII (K > R) KI and MARCH1 KO mice generated T reg cells at significantly reduced levels compared with WT DCs (Fig. 5 D, left graph, and Fig. S1 B), but induced thymocyte death as efficiently as WT DCs (Fig. 5 D, right graph). To confirm that the defect in T reg cell generation of thymic cDCs of MHCII (K > R) KI and MARCH1 KO mice was attributable to a DC-intrinsic defect, we performed the same experiment using DCs derived from BM (BMDCs) of these mice. Similarly to what was found with thymic cDCs, BMDCs from MHCII (K > R) KI and MARCH1 KO mice generated T reg cells less efficiently than BMDCs derived from WT mice (Fig. 5 E, left graph, and Fig. S1 C), but induced thymocyte death with a similar efficiency (Fig. 5 E, right graph). Collectively, these findings indicate that the ability of DCs to differentiate immature

thymocytes into T reg cell is dependent on MARCH1-mediated MHCII ubiquitination.

In this study, we have found that MARCH1 is specifically expressed by hematopoietic APCs in the thymus and that MARCH1 plays an important role in T reg cell development, in part by mediating MHCII ubiquitination in DCs. This study provides a significantly improved understanding of the functional role of MARCH1 and MHCII ubiquitination, the contribution of DCs to thymic T reg cell development, and the underlying mechanism.

Many previous studies have implicated DCs in the development of thymic T reg cells (Proietto et al., 2008; Atibalentja et al., 2009, 2011; Baba et al., 2012). Our study reinforces this role by demonstrating that the mechanism involves MARCH1-mediated MHCII ubiquitination. One supporting evidence is that the development of T reg cells against i.v.-injected OVA, which was taken up only by DCs, was completely abolished in mice deficient in MARCH1 or MHCII ubiquitination.
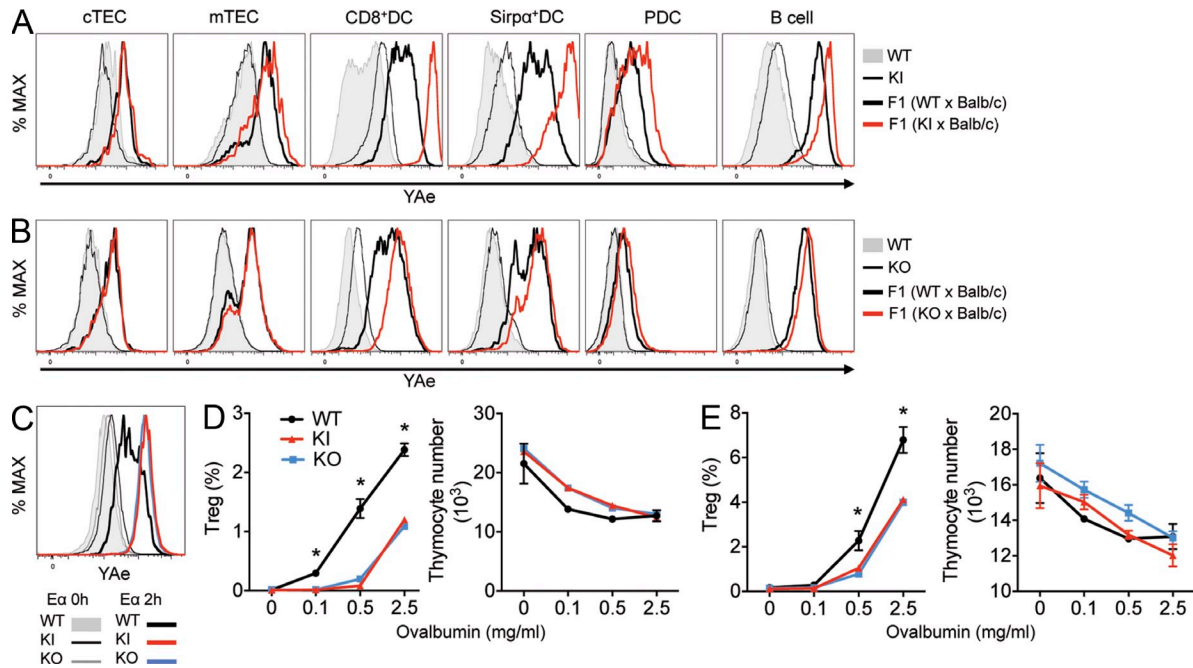
Figure 5. DCs deficient in MHCII ubiquitination or MARCH1 exhibit increased antigen presentation but generate fewer T reg cells. (A and B) The surface expression of I-E$_\alpha$(52–68)–I-A$_b$ complexes in thymi of (WT x BALB/c) F1, (MHCII [K > R] KI x BALB/c) F1 (A), and (MARCH1 KO x BALB/c) F1 (B) was determined by flow cytometry using the YAe antibody. APCs of WT, MHCII (K > R) KI (KI), and MARCH1 KO (KO) mice, which do not express I-E$_\alpha$, were used as controls. (C) Isolated thymic cDCs were incubated with 20 µg of I-E$_\alpha$ recombinant protein for 2 h. The surface expression of I-E$_\alpha$(52–68)–I-A$_b$ complexes was determined before and after incubation. (D) Thymic cDCs were isolated from WT, MHCII (K > R) KI (KI), and MARCH1 KO (KO) mice, incubated for 2 h with increasing concentrations of OVA, and cultured for 24 h with CD4+CD8−CD24+EGFP− V$_\alpha$2+ thymocytes isolated from Thy1.1; Foxp3-EGFP KI; OT-II mice before flow cytometry analysis. (E) BMDCs derived from WT, KI, and KO mice were treated as in D, except that OVA was left in culture. Percentage of EGFP+CD25+ cells (T reg cells) among Thy1.1+MHCII− thymocytes and total number of Thy1.1+MHCII− thymocytes that remained in culture are presented. Data represent mean ± SEM of measurements from two independent experiments, performed in duplicate (D) and triplicate (E). *, P < 0.01.

The other evidence is that the ability of DCs to differentiate immature thymocytes into T reg cells in vitro was markedly reduced in the absence of MARCH1 or MHCII ubiquitination. Interestingly, we found that MARCH1 was not expressed in TECs, yet the development of T reg cells against mTEC-driven antigen was dependent on MARCH1. Previous studies have shown that antigens expressed by mTECs are transferred to and presented by DCs and that these DCs mediate central tolerance against the antigens (Gallegos and Bevan, 2004; Millet et al., 2008; Koble and Kyewski, 2009). Our finding suggests that the development of T reg cells against mTEC-driven antigens is also mediated by DCs and that MARCH1-mediated MHCII ubiquitination plays an important role in this process.

MHCII ubiquitination promotes T reg cell development via a mechanism distinct from avidity control. Previous studies have proposed that avidity is a critical determinant for thymocyte fate decision; thymocytes that interact with APCs with strong avidity are deleted, whereas those interacting with intermediate avidity differentiate into T reg cells (Wirnsberger et al., 2011; Hsieh et al., 2012). In accordance with this view, a recent study has shown that miRNA mediated suppression of MHCII expression in mTEC resulted in an increase in T reg cell development and a decrease in CD4 SP thymocyte

deletion (Hinterberger et al., 2010). However, the avidity model does not explain the results of our study. First, we did not find any evidence that CD4 SP thymocyte deletion was enhanced in MARCH1 KO or MHCII (K > R) KI mice, although these mice expressed higher levels of MHCII. Specifically, no changes were observed in the number, apoptotic rate, or repertoire of CD4 SP thymocytes in these mice. Second, despite increased MHCII expression and antigen presentation, DCs deficient in MARCH1 or MHCII ubiquitination were not any better at deleting CD4 SP thymocytes in vitro or in vivo. Third, the T reg cell–generating defect of MHCII ubiquitination-deficient DCs was not relieved by reducing antigen presentation by reducing antigen doses. Collectively, these findings suggest that it is not the avidity control but rather some feature uniquely accompanied by MHCII ubiquitination that positively acts for T reg cell development. One possible mechanism is that ubiquitination-mediated MHCII endocytosis may generate distinct MHCII surface dynamics in DCs, which may provide thymocytes with a discrete TCR signaling that acts for T reg cell differentiation. Alternatively, ubiquitinated MHCII may serve as a signaling molecule in DCs, and this signaling may condition DCs for T reg cell generation.

In conclusion, we have shown that MARCH1-mediated MHCII ubiquitination plays an important and specific role in DC-mediated T reg cell development. By using this distinct mechanism, DCs may generate a unique cohort of T reg cells that perhaps play unique roles in immune tolerance.

MATERIALS AND METHODS

Mice. C57BL/6, B6.SJL-*Ptprª Pepcᵇ*/BoyJ, BALB/c, B6.PL-*Thy1ª*/CyJ, and OT-II mice were purchased from The Jackson Laboratory. MARCH1 KO (Matsuki et al., 2007), Rip-mOVA (Kurts et al., 1996), and Foxp3-EGFP KI mice (Haribhai et al., 2007) were previously described. All mice were maintained in the University of California San Francisco (UCSF) mouse facility and analyzed at the age of 4–8 wk except BM chimeric mice. All experiments were performed according to protocols approved by the UCSF Institutional Animal Ethics Committee.

Generation of MHCII (K > R) KI mice. The construct for targeting the C57BL/6 *H2-Ab1* locus in ES cells was made using a combination of recombineering, DNA synthesis and standard molecular cloning techniques. First, a mouse BAC (RP23-444j20) containing the *H2-Ab1* gene was modified using the galK method (Warming et al., 2005) to replace the sequence 5′-CACAGGAGTCAGAAA-3′ within exon 4 with 5′-CACAGATCT-CAAAGG-3′, thus changing Lysine 253 into an Arginine and introducing a diagnostic BglII site. Second, a 5,320-bp fragment (assembly NCBI37/mm9, chr17:34,401,196-34,406,515) from the modified BAC was retrieved into plasmid pBlight-TK (Warming et al., 2006). Next, a loxP-Pgk-em7-Neo-loxP cassette was inserted in intron 3 between position chr17: 34,404,665 and 34,404,666. The final vector was confirmed by DNA sequencing. The *H2-Ab1* knock-in vector was linearized with NotI and C57BL/6 C2 ES cells were targeted using standard methods (G418-positive and ganciclovir-negative selection). Positive clones were identified using PCR and TaqMan analysis, and confirmed by sequencing of the modified locus. Correctly targeted ES cells were then transfected with a Cre expression vector to remove the selection marker, and subsequently injected into blastocysts using standard techniques. Germline transmission was obtained after crossing resulting chimeras with C57BL/6 females.

Quantitation of thymocytes and enrichment of thymic APCs. Each mouse thymus was harvested, and a single-cell suspension was prepared as previously described (Williams et al., 2009). Cell numbers were determined by using the Z2 analyzer (Beckman Coulter). To enrich thymic APCs, thymic digests were filtered through a 70-µm filter cap strainer (Falcon; BD), resuspended in 4 ml of high-density (1.115 g/ml) Percoll solution, and subsequently layered with 2 ml of low-density (1.065 g/ml) Percoll solution, followed by 2 ml of PBS. This Percoll gradient was centrifuged at 2,700 rpm for 30 min at 4°C to enrich APCs between the PBS and low-density Percoll layers. For in vitro E_α presentation assay, thymic DCs were isolated using CD11c-magnetic beads (Miltenyi Biotec).

Antibodies and reagents. The following antibodies were purchased from BioLegend, eBioscience, or BD and used for flow cytometry: PE/Cy7-CD11c, Pacific blue-IA/IE class II, Pacific blue-Rat IgG2, PE-Sirpα, Pacific blue-mPDCA1, Alexa Fluor 647-mPDCA1, Ly51-FITC, PE-CD86, PE-Rat IgG2a, Alexa Fluor 700-CD86, PE/Cy5-CD4, Alexa Fluor 700-CD4, FITC-CD8α, Alexa Fluor 700-CD8α, APC/Cy7-CD8α, PE/Cy7-CD11c, PerCP/Cy5.5-CD80, CD11b-FITC, Alexa Fluor 700-CD45, Pacific blue-CD45.1, Alexa Fluor 647-CD45.2, PE-CD3ε, Alexa Fluor 647-γδTCR, PE/Cy7-CD3ε, PE/Cy7-CD24, APC-CD25, V_α2-FITC, APC-Epcam, PE-Foxp3, FITC-YAe, PE/Cy7-Ki-67, Alexa Fluor 700-CD19, PerCP/Cy5.5-Thy1.1, and the TCR V_β screening panel. PE-empty CD1d tetramer and PE-PBS57-loaded CD1d tetramer were provided by National Institutes of Health tetramer core facility. Pacific blue-Annexin V (BioLegend) and Fixable Viability Dye eFluor780 (eBioscience) were also used for flow cytometry.

Flow cytometry and cell sorting. For surface staining, cells were incubated on ice for 30 min with fluorophore-conjugated antibodies in MACS buffer (Miltenyi Biotec) containing propidium iodide solution (BioLegend) and CD16/32 Fc block antibody (UCSF CCF). Intracellular Foxp3 was stained using Foxp3/Transcription Factor Staining Buffer Set (eBioscience). All samples were analyzed and sorted by the FACS LSRII system (BD) and BD FACS Aria3 Cell Sorter (BD), respectively.

Quantitative RT-PCR. Total RNAs were isolated by TRIzol (Invitrogen) extraction. The first-strand cDNA were generated using SuperScript III system (Invitrogen) and using Oligo(dT) as primers. Quantitative PCR was performed on an Eppendorf realplex system using SYBR Green reagents (5 Prime) and primers specific for MARCH1 and GAPDH.

BM chimeric mice. For the WT/MARCH1 KO BM chimera experiment, WT or MARCH1 KO BM cells were isolated from femurs and/or tibiae, and subjected to RBC lysis. Lethally irradiated recipient mice of WT or MARCH1 KO genotype were i.v. injected with 1×10^6 BM cells. For the mixed BM chimera experiment, BM cells were isolated from CD45.1⁺ BoyJ mice and CD45.2⁺ MARCH1 KO mice, mixed at 1:0, 9:1, 1:1, or 0:1, and injected into lethally irradiated WT mice expressing both CD45.1 and CD45.2. Mice were examined 8 wk later.

In vitro thymocyte T reg cell differentiation and deletion assay. CD4⁺ CD8⁻CD24⁺TCRV$_\alpha$2⁺EGFP⁻ thymocytes (5×10^4) isolated from Thy1.1; Foxp3-EGFP KI; OT-II mice were co-cultured with CD11c⁺mPDCA1⁻ thymic cDCs (1×10^4) or CD11c⁺CD11b⁺BMDCs (1×10^4) incubated with increasing amounts of OVA. After 24-h culture, Thy1.1⁺MHCII⁻ total thymocyte numbers and the percentage of CD25⁺EGFP⁺ cells among Thy1.1⁺ MHCII⁻ cells was determined by flow cytometry.

Statistical analyses. Unpaired two-tailed Student's *t* test was performed to assess the significance, unless stated otherwise.

Online supplemental material. Fig. S1 shows flow cytometry gating strategy. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122695/DC1.

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