MARCH1 protects the lipid raft and tetraspanin web from MHCII proteotoxicity in dendritic cells

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Dendritic cells (DCs) produce major histocompatibility complex II (MHCII) in large amounts to function as professional antigen presenting cells. Paradoxically, DCs also ubiquitinate and degrade MHCII in a constitutive manner. Mice deficient in the MHCII-ubiquitinating enzyme membrane-anchored RING-CH1, or the ubiquitin-acceptor lysine of MHCII, exhibit a substantial reduction in the number of regulatory T (Treg) cells, but the underlying mechanism was unclear. Here we report that ubiquitin-dependent MHCII turnover is critical to maintain homeostasis of lipid rafts and the tetraspanin web in DCs. Lack of MHCII ubiquitination results in the accumulation of excessive quantities of MHCII in the plasma membrane, and the resulting disruption to lipid rafts and the tetraspanin web leads to significant impairment in the ability of DCs to engage and activate thymocytes for Treg cell differentiation. Thus, ubiquitin-dependent MHCII turnover represents a novel quality-control mechanism by which DCs maintain homeostasis of membrane domains that support DC’s Treg cell-selecting function.

Introduction

Membrane-anchored RING-CH1 (MARCH1) is a membrane-anchored ubiquitin ligase expressed in hematopoietic cells, particularly antigen presenting cells (Matsuki et al., 2007). It is composed of an N-terminal cytoplasmic tail that possesses a catalytic RING domain, two transmembrane domains that interact with a specific substrate, and a C-terminal cytoplasmic tail. Upon recognition of substrate, MARCH1 brings a ubiquitinated E2 ubiquitin-conjugating enzyme into close proximity of its RING domain and substrate and catalyzes ubiquitin transfer from E2 to substrate. Transferred ubiquitin molecules serve as a signaling motif for endocytosis and lysosomal sorting, resulting in internalization and lysosomal degradation of the substrate (Lehner et al., 2005; Ohmura-Hoshino et al., 2006).

Several immune-associated molecules have been shown to be endocytosed and degraded in cells overexpressing MAR CH1 (Bartee et al., 2004). However, major histocompatibility complex II (MHCII) and CD86 are the only molecules shown to be ubiquitinated by MARCH1 under physiological conditions (Matsuki et al., 2007; De Gassart et al., 2008; Baravalle et al., 2011). MHCII has an evolutionally conserved lysine in the cytoplasmic tail of its β-chain, and this lysine is targeted for ubiquitination (Shin et al., 2006; van Niel et al., 2006; Oh and Shin, 2015). CD86 has multiple lysines in the cytoplasmic tails, and many of these lysines can be ubiquitinated (Baravalle et al., 2011; Corcoran et al., 2011). In accordance with the role of MARCH1 in mediating ubiquitination and endocytosis of MHC II and CD86, MARCH1 ablation resulted in a marked increase in the surface expression of these two molecules in dendritic cells (DCs) in mice. Interestingly, these mice exhibited a significant reduction in the number of regulatory T (Treg) cells in the thymus (Oh et al., 2013). More interestingly, mice deficient in the cytoplasmic lysine (K) of MHCII (called “MHCII K” here) exhibited a similar deficiency in thymic Treg cells (Oh et al., 2013). Furthermore, DCs deficient in MARCH1 or MHC II K were defective at differentiating immature thymocytes to Treg cells in vitro (Oh et al., 2013). This finding suggests that MHCII ubiquitination plays an important role in DC function of selecting Treg cells. However, the underlying mechanisms have not been identified.

Treg cells are selected through a cognate interaction of CD4+ thymocytes with thymic antigen-presenting cells, and the strength of this interaction is one of the key determinants for Treg cell selection (Hsieh et al., 2012; Stritesky et al., 2012; Klein et al., 2014). Low-avidity interaction does not relay sufficient signal to interacting thymocytes for expression of foxp3, the key transcription factor that guides Treg cell differentiation, whereas high-avidity interaction triggers apoptotic cell death resulting in negative selection of the interacting thymocytes.
Only the intermediate-avidity interaction delivers a signal appropriate for Treg cell differentiation.

DCs deficient in MARCH1 or the MHCII K display peptide-loaded MHCII (pMHCII) at much larger amounts than WT DCs on the surface (Walseng et al., 2010; Oh et al., 2013). Because pMHCII is the molecule that mediates a cognate interaction of DCs with CD4+ thymocytes, an increase in pMHCII in DCs will increase DC avidity for antigen-specific thymocytes. The increased avidity is then likely to drive the thymocytes to apoptotic cell death while repressing differentiation into Treg cells. However, the mice deficient in MARCH1 or MHCII K did not show any increase in apoptotic cell death of CD4+ thymocytes or Treg cells (Oh et al., 2013). Furthermore, lowering the amount of the peptide loaded onto MHCII did not restore the development of Treg cells in MARCH1 or MHCII K−/− deficient mice (Oh et al., 2013). This finding suggests that the role of MARCH1 in supporting DC function of selecting Treg cells is independent of controlling surface expression of pMHCII. In this study, we have investigated the specific mechanism by which MARCH1-deficient MHCII ubiquitination supports DC selection of Treg cells.

Results

DC expression of MARCH1 is important for Treg cell development in the thymus

To determine the extent to which DCs contribute to the role of MARCH1 in promoting Treg cell development in vivo, we generated mice with loxP-flanked alleles of the RING domain of MARCH1 and bred them to CD11c-CRE transgenic mice expressing CRE recombinase under the control of the CD11c promoter (MARCH1fl/fl CD11c-CRE mice, Fig. S1). The loxP-flanked region of MARCH1 was efficiently and specifically deleted in DCs of MARCH1fl/fl CD11c-CRE mice (Fig. 1A). DCs in these mice expressed MHCII at markedly elevated levels, similar to DCs from MARCH1-deficient mice (MARCH1−/−) or MHCII K−/− deficient mice (MHCIIK<sub>ΔR</sub>K<sub>ΔK</sub>), the mice in which the ubiquitin acceptor lysine K of MHCII was replaced with arginine [R]; Fig. 1B). MARCH1Δ<sub>R</sub>CD11c-CRE mice had a normal number of total, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>−</sup>, CD4<sup>−</sup>CD8<sup>−</sup>, and CD4<sup>−</sup>CD8<sup>+</sup> thymocytes, but the number of Treg cells was reduced by approximately half (Fig. 1C), similarly to what has been observed in MARCH1−/− mice and MHCIIΔR/K<sub>ΔK</sub> mice (Oh et al., 2013). Thus, the role of MARCH1 in promoting Treg cell development is critically dependent on MARCH1 expressed in DCs.

MARCH1-mediated MHCII ubiquitination is required for DCs to effectively engage and activate thymocytes

We have previously shown that DCs deficient in MARCH1 were poor at differentiating immature thymocytes into Treg cells in vitro. To determine how MARCH1 deficiency impairs DC function of developing Treg cells, we examined whether MARCH1 is important for DCs to stably engage thymocytes and provide sufficient stimulatory signal for activation. DCs were isolated from thymi of MARCH1-deficient mice, loaded with ovalbumin, and co-cultured with CD4<sup>+</sup>CD8<sup>+</sup> (DP), CD4<sup>−</sup>CD8<sup>+</sup> (CD4SP), and CD4<sup>−</sup>CD8<sup>−</sup> (CD8SP) thymocytes and Treg cells (CD4<sup>+</sup>CD8<sup>+</sup> Foxp3<sup>+</sup>CD25<sup>+</sup>) in MARCH1fl/fl and MARCH1fl/fl CD11c-CRE mice. Each circle represents an individual mouse. Error bars represent SEM. **, P < 0.01.

Figure 1. DC expression of MARCH1 is important for Treg cell development in the thymus. (A) The amount of loxP-flanked MARCH1 genomic DNA (gDNA) in Sirpα− thymic DCs, Sirpα− thymic DCs, thymic B cells, and thymic epithelial cells (TECs) in MARCH1fl/fl CD11c-CRE mice relative to MARCH1fl/fl mice. Each cell type was isolated by FACS as described previously (Oh et al., 2013). Data are averaged from three individual mice. (B) Surface expression of MHCII in Sirpα− and Sirpα+ thymic DCs of MARCH1fl/fl, MARCH1Δ<sub>R</sub> CD11c-CRE, MARCH1−/−, and MHCIIΔR/K<sub>ΔK</sub> mice. Data represent three independent experiments. (C) Numbers of total, CD4<sup>+</sup>CD8<sup>+</sup> (DN), CD4<sup>−</sup>CD8<sup>+</sup> (DP), CD4<sup>−</sup>CD8<sup>−</sup> (CD4SP), and CD4<sup>−</sup>CD8<sup>+</sup> (CD8SP) thymocytes and Treg cells (CD4<sup>+</sup>CD8<sup>+</sup> Foxp3<sup>+</sup>CD25<sup>+</sup>) in MARCH1fl/fl and MARCH1fl/fl CD11c-CRE mice. Each circle represents an individual mouse. Error bars represent SEM. **, P < 0.01.
DCs were not much different from DCs of WT mice for the level of expression of the costimulatory molecule CD80 and the adhesion molecule ICAM1 (Fig. S2 A).

Next, we examined whether the role of MARC1 in promoting DC engagement and activation of thymocytes depends on its activity of mediating ubiquitination of MHC II. For this examination, DCs were isolated from thymi of MHC IIK>R/K>R mice and analyzed as described earlier. Similar to MARC1-deficient DCs, MHC II K–deficient DCs engaged and activated thymocytes less efficiently than WT DCs (Fig. 2, F–H). MHC II K–deficient DCs were not altered for the expression of CD86, CD80, and ICAM-1 compared with WT DCs (Fig. S2 B). This finding indicates that the effect of MARC1 in promoting DC engagement and activation of thymocytes is largely dependent on its ability to ubiquitinate MHCII.

The presence of too many MHCII molecules, not the absence of ubiquitinated MHCII, is responsible for poor engagement and activation of thymocytes by MHCII K–deficient DCs

To identify the specific mechanism by which MHCII ubiquitination supports DC engagement and activation of thymocytes, we used bone marrow–derived DCs (BMDCs), which showed a similar dependence on MHCII ubiquitination to engage and activate thymocytes (Fig. 3, B–D). We first tested whether too-high expression of MHCII or the absence of ubiquitinated MHCII is responsible for the poor engagement and activation of thymocytes by MHCII K–deficient DCs. To reduce surface MHC II levels without introducing MHC II ubiquitination, we crossed MHCIIK>R/K>R mice to MHCII−/− mice and derived BMDCs from...
resulting MHCII<sup>K>R−</sup> mice. These BMDCs expressed MHCII at slightly lower levels than BMDCs from MHCII<sup>K>R−</sup> mice on the surface (Fig. 3 A). Interestingly, these DCs engaged thymocytes significantly better than DCs derived from MHCII<sup>K>R−</sup> mice, suggesting that too-high MHCII, not the absence of ubiquitinated MHCII, exerted a negative impact on the ability of DCs to engage thymocytes (Fig. 3 B). The level of CD25 expressed in CD69<sup>+</sup> thymocytes is shown in D. (E and F) BMDCs were derived from MHCII<sup>K>R−</sup> mice and transduced with retrovirus encoding MHCII<sup>K>R−</sup> mutant along with cytosolic GFP. The efficiency of transduction and the expression of MHCII in transduced cells were determined by flow cytometry. The representative dot plots are shown in E (cells transduced with retrovirus encoding MHCII WT and MHCII [K>R] mutant are shown in left and right, respectively). The expression level of GFP and MHCII in transduced cells is shown in F (left and right panel, respectively). The level of CD25 expressed in DCs derived from WT mice was overlaid for comparison (blue in the right panel of F). The MFI of the MHCII stain in DCs transduced with retrovirus encoding MHCII WT and DCs derived from WT mice is indicated in F. (G–I) BMDCs were derived from MHCII<sup>K>R−</sup> mice and transduced with retrovirus encoding MHCII<sup>K>R−</sup> mutant along with cytosolic GFP. Transduced cells (GFP<sup>+</sup> cells) were isolated by FACS and cultured with CD4<sup>+</CD8</sup>− thymocytes isolated from OT-II mice in the presence of increasing concentrations of OVA<sub>323-339</sub> peptide. The percentage of DCs that formed stable conjugates with thymocytes is shown in G. The percentage of thymocytes that expressed CD69 is shown in H. The level of CD25 expressed in CD69<sup>+</sup> thymocytes is shown in I. Data represent at least three independent experiments with triplicates for each condition. Error bars represent SEM. *, P < 0.05; **, P < 0.01.
This finding provides definitive evidence that the failure of MHCII K–deficient DCs to effectively engage and activate thymocytes was because of the presence of too many MHCII molecules and not the absence of ubiquitinated MHCII in the cells.

**MHCII K deficiency alters the lipid raft and tetraspanin web in the plasma membrane of DCs**

We sought to identify the specific mechanism by which too many MHCII molecules impair DC ability to engage and activate thymocytes. MHCII is not randomly scattered in the plasma membrane of DCs but is associated with a distinct membrane domain named lipid raft, an ordered membrane structure composed of discrete species of lipids, including cholesterol, sphingolipid, and glycolipid (Anderson et al., 2000; Anderson and Roche, 2015). The lipid raft also encompasses a specific set of proteins including glycosylphosphatidylinositol (GPI)-anchored molecules (Simons and Sampaio, 2011). Although the mechanism by which MHCII associates with the lipid raft is not clearly understood, this association increases the local concentration of MHCII loaded with antigens lowering the threshold of antigen-specific T cell activation (Anderson et al., 2000). We found that MHCII in MHCII K–deficient DCs was fractionated into the light-buoyant density fractions at degrees similar to MHCII in WT DCs (Fig. S3), indicating that ubiquitination is not required for MHCII to associate with the lipid raft. However, we noticed that the absolute amount of MHCII associated with the lipid raft was markedly high in MHCII K–deficient DCs, although the amount associated with nonlipid raft was also high (Fig. S3). Because the lipid raft is an ordered structure made by tight interactions between lipids and proteins, we speculated that excessive accumulation of MHCII in the lipid raft might have altered the integrity of the structure, which in turn negatively affected the ability of MHCII K–deficient DCs to engage and activate thymocytes. Interestingly, we found that the antibodies raised against the GPI-anchored molecule CD48 or CD24 poorly bound to MHCII K–deficient DCs compared with WT DCs (Fig. 4 A). Cholera toxin B (CTxB), a specific ligand of the sphingolipid GM1, also poorly bound to MHCII K–deficient DCs (Fig. 4 A). However, the antibodies raised against the nonraft molecules CD45 and CD71 bound to MHCII K–deficient DCs similarly to WT DCs (Fig. 4 A).

In addition to the lipid raft, another membrane domain named tetraspanin web associates MHCII in DCs (Kropshofer et al., 2000). We found that MHCII in MHCII K–deficient DCs was fractionated into the light-buoyant density fractions at degrees similar to MHCII in WT DCs (Fig. S3), indicating that ubiquitination is not required for MHCII to associate with the lipid raft. However, we noticed that the absolute amount of MHCII associated with the lipid raft was markedly high in MHCII K–deficient DCs, although the amount associated with nonlipid raft was also high (Fig. S3). Because the lipid raft is an ordered structure made by tight interactions between lipids and proteins, we speculated that excessive accumulation of MHCII in the lipid raft might have altered the integrity of the structure, which in turn negatively affected the ability of MHCII K–deficient DCs to engage and activate thymocytes. Interestingly, we found that the antibodies raised against the GPI-anchored molecule CD48 or CD24 poorly bound to MHCII K–deficient DCs compared with WT DCs (Fig. 4 A). Cholera toxin B (CTxB), a specific ligand of the sphingolipid GM1, also poorly bound to MHCII K–deficient DCs (Fig. 4 A). However, the antibodies raised against the nonraft molecules CD45 and CD71 bound to MHCII K–deficient DCs similarly to WT DCs (Fig. 4 A).

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et al., 2002; Unternaehrer et al., 2007). This membrane domain consists of tetraspanin molecules and a limited number of partner proteins and is enriched with cholesterol and the sphingolipid ganglioside similarly to the lipid raft (Charrin et al., 2014). We found that the antibodies raised against the tetraspanin molecule CD9 or CD81 also poorly bound to MHCI K–deficient DCs compared with WT DCs (Fig. 4 B). Antibodies raised against CD63, CD37, or CD54 did not bind to either WT or MHCI K–deficient DCs possibly by little expression of these tetraspanins in the DC surface (unpublished data). These findings suggest that MHCI K deficiency induces substantial alteration in the composition or structure of the lipid raft and tetraspanin web in DC plasma membrane.

To better characterize alteration of the lipid raft and tetraspanin web in MHCI K–deficient DCs, we examined whether the alteration was restricted to the plasma membrane or also made in the intracellular compartments. First, we examined whether the aforementioned antibodies or CTxB bind more poorly to MHCI K–deficient DCs than WT DCs after fixation. As shown in Fig. 4 C, the antibodies and CTxB poorly bound to fixed MHCI K (K>R) DCs compared with fixed WT DCs. Then, we permeabilized the fixed DCs and stained them with the antibodies and CTxB. Permeabilization substantially increased the binding of anti-CD48 antibody, anti-CD24 antibody, and CTxB, implicating the presence of these molecules in intracellular compartments (Fig. 4 C). Notably, we found that permeabilization markedly reduced the difference in binding of the antibodies or CTxB between MHCI K–deficient DCs and WT DCs (Fig. 4 C), suggesting that the lipid raft in the intracellular compartments of MHCI K–deficient DCs is not altered as much as that in the plasma membrane.

CD9 appeared mostly localized in the plasma membrane in DCs because permeabilization increased anti-CD9 antibody binding only to a marginal level (Fig. 4 C). The difference in binding of anti-CD9 antibody between WT and MHCI II K–deficient DCs did not look much different after permeabilization. Binding of anti-CD81 antibody was substantially increased after permeabilization, implicating the presence of a substantial amount of intracellular CD81 (Fig. 4 D). The degree of binding difference between WT and MHCI K–deficient DCs also increased after permeabilization indicating that there might be substantial alteration in CD81-associated tetraspanin web in the intracellular compartments of MHCI K–deficient DCs (Fig. 4 D).

Next, we examined whether MHCI K–deficient DCs expressed lipid raft or tetraspanin molecules at lower levels than WT DCs. First, we determined the level of mRNA by quantitative RT-PCR. We did not find any significant reduction in the amount of transcript of CD48, CD24, CD9, or Sptlc2, the key enzyme in sphingolipid biosynthesis, in MHCI K–deficient DCs compared with WT DCs (Fig. 4 E). Second, we examined the protein amount by Western blot analysis. No difference was observed in the amount of CD48, CD9, or CD81, although the amount of CD24 appeared slightly lower in MHCI K–deficient DCs (Fig. 4 F). Thus, MHCI K–deficient DCs express CD48, CD9, and CD81 at levels similar to WT DCs whereas CD24 might be expressed at slightly lower levels. Collectively, we conclude that that MHCI K deficiency in DCs is accompanied by substantial changes in the conformation and stability of the molecules associated with the lipid raft and tetraspanin web in the plasma membrane.

MHCI overcrowding is responsible for the alteration of the lipid raft and tetraspanin web in MHCI K–deficient DCs

Next, we determined whether the alteration of the lipid raft and tetraspanin web in MHCI K–deficient DCs was caused by MHCI II overcrowding. DCs were cultured from MHCI K–deficient mice and examined whether these DCs are better bound by anti-CD48, -CD24, or -CD9 antibodies and CTxB than DCs derived from MHCI K–deficient mice. We found that all of these antibodies and CTxB bound better to DCs derived from MHCI K–deficient mice (Fig. 5 A). We also examined binding of these antibodies and CTxB to DCs that expressed the MHCI K (K>R) mutant and WT under the retroviral promoter described in Fig. 3. All the antibodies and CTxB bound to DCs that expressed MHCI K WT or the MHCI K (K>R) mutant at similar levels (Fig. 5 B). These findings strongly support the hypothesis MHCI overcrowding is responsible for the alteration of the lipid raft and tetraspanin web in MHCI K–deficient DCs.

To further confirm that the alteration of the lipid raft and tetraspanin web in MHCI K–deficient DCs is attributed directly to MHCI II overcrowding, we ablated MHCI ubiquitination in DCs in an inducible manner. The MARCH1 WT mice described in Fig. 1 were crossed with ERT-Cre mice, which expressed estrogen receptor–Cre fusion protein under the human ubiquitin promoter. BMDCs were cultured from these mice and treated with the ligand of the estrogen receptor tamoxifen. Tamoxifen was expected to enter DCs and trigger cre-dependent recombination of the MARCH1 gene. We found that tamoxifen treatment resulted in an increase in the surface level of MHCI in as early as 24 h (Fig. 5 C). Importantly, this increase was accompanied by a reduction in the binding of anti-CD48, -CD24, or -CD9 antibodies but not CTxB (Fig. 5 C). On day 3 to 5 after tamoxifen treatment, the level of surface MHCI further increased whereas the binding of anti-CD48, -CD24, or -CD9 antibodies further decreased, and CTxB binding also decreased (Fig. 5 C). These data demonstrate a strong correlation between the degree of MHCI overcrowding and the degree of alteration of the lipid raft and tetraspanin web, further supporting that MHCI overcrowding is the direct cause of alteration in these membrane domains. In addition, this finding suggests that GPI-anchored molecules and tetraspanin molecules are more readily altered than GM1 by MHCI overcrowding.

Because MARCH1 ablation increases not only MHCI but also CD86, we tested whether CD86 accumulation may also induce similar alteration in the lipid raft and tetraspanin web. DCs derived from WT mice were transduced with the retrovirus encoding CD86 (K>R) mutant in which the cytoplasmic lysines of CD86 were replaced with arginines (Baravalle et al., 2011). We found that the cells transduced with this retrovirus expressed >50 times as many CD86 molecules as untransduced cells. However, the transduced and untransduced cells were bound by anti-CD48, -CD24, or -CD9 antibody and CTxB similarly (Fig. S4), indicating that CD86 overcrowding does not make an alteration similar to what MHCI overcrowding makes.

Lipid raft and tetraspanin web support DC engagement and activation of thymocytes

We examined whether intact lipid raft or tetraspanin web is required for DCs to stably engage and sufficiently activate thymocytes. First, we disturbed the organization of these membrane domains by using methyl β-cyclodextrin (MβCD), a...
cholesterol-chelating agent (Charrin et al., 2003). MβCD treatment diminished DC binding by anti-CD48, -CD24, or -CD9 antibodies, and CTxB (Fig. 6 A), similarly to what MHCII K deficiency did. MβCD treatment also impaired DC ability to engage and activate thymocytes (Fig. 6, B–D). Next, we treated BMDCs with myriocin, a potent and specific inhibitor of spltc2 (Hanada, 2003). Myriocin did not impair DC binding by anti-CD48 or -CD24 antibodies although it modestly impaired binding by CTxB and anti-CD9 or -CD81 antibodies (Fig. 6 E). Myriocin treatment significantly reduced the efficiency of DCs to engage and activate thymocytes (Fig. 6, F–H). Notably however, myriocin treatment markedly reduced the surface expression of MHCII in DCs (unpublished data). Because surface MHCII makes a direct impact on the efficiency of DC activation of antigen-specific thymocytes, it is unclear whether the reduced engagement and activation of thymocytes by myriocin-treated DCs is the result of the perturbation of the lipid raft or tetraspanin web.

Then, we examined whether any specific molecules associated with the lipid raft or tetraspanin web are important for DCs to stably engage and sufficiently activate thymocytes. To this end, we pretreated DCs with anti-CD48, -CD24, -CD9, or -CD81 antibodies before culturing with OT-II thymocytes. We found that all of the antibodies appreciably reduced DC engagement with thymocytes compared with the control antibody (anti-TNP), although the statistically significant difference was reached only by anti-CD48 antibody and the KMC8 anti-CD9 antibody (Fig. 6 I). These two antibodies also reduced DC engagement and activation of thymocytes at significant levels (Fig. 6, J and K). This finding implicates the role of CD48 and CD9 in DC engagement and activation of thymocytes and suggests that alteration of these two molecules may be the primary cause for diminished engagement and activation of thymocytes by MHCII K–deficient DCs.

Sphingomyelin supplementation lessens the alteration of the lipid raft and tetraspanin web in MHCII K–deficient DCs and improves the ability of the DCs to engage and activate thymocytes and drive Treg cell differentiation

We sought to determine the specific mechanism by which MHC II overcrowding alters the lipid raft or tetraspanin web and determine whether this alteration is responsible for poor engagement and activation of thymocytes by MHCII K–deficient DCs. It has been suggested that MHCII binds cholesterol and sphingolipid through its transmembrane domain (Roy et al., 2013; Björkholm et al., 2014). We postulated that cholesterol and sphingolipid in the lipid raft and tetraspanin web may be sequestered by MHCII because MHCII accumulates in these membrane domains. This sequestration would make other molecules associated with the lipid raft or tetraspanin web deprived of cholesterol or sphingolipid, resulting in alteration in the conformation or stability of the molecules. To test this hypothesis, we supplemented MHC II K–deficient DC culture with cholesterol or sphingomyelin and examined whether this supplementation makes the DCs better bound by anti-CD48, -CD24, -CD9, or -CD81 antibodies or CTxB. We found that sphingomyelin significantly improved DC binding by all these reagents (Fig. 7 A) although cholesterol did not (not depicted). Importantly, sphingomyelin also significantly improved the ability of MHCII K–deficient DCs to engage and activate thymocytes (Fig. 7, B–D). Then, we examined whether sphingomyelin treatment also improves the ability of DCs to engage and activate thymocytes and drive Treg cell differentiation.
the ability of MHCII K−deficient DCs to drive Treg cell development. Immature non-Treg CD4+ thymocytes were isolated from FoxpGFPOT-II mice, which report Foxp3 by GFP expression. The isolated thymocytes were co-cultured with MHC II K−deficient DCs that had been supplemented with sphingomyelin in the presence of increasing concentrations of ovalbumin peptide. 2 d later, the expression of GFP and CD25 in thymocytes was determined by flow cytometry. We found that the thymocytes cultured with sphingomyelin-treated DCs expressed GFP and CD25, the markers of Treg cells, at a significantly higher frequency than those cultured with vehicle-treated DCs (Fig. 7, E and F). Thus, sphingomyelin supplementation significantly restores the ability of MHCII K−deficient DCs to drive Treg cell development. Collectively, these findings suggest that the alteration of the lipid raft and tetraspanin web was responsible for poor engagement, activation, and Treg cell differentiation.
of thymocytes by MHCIi K–deficient DCs and suggest that sphingolipid may be one of the limiting factors in MHCIi K–deficient DCs for maintaining homeostasis of the lipid raft and tetraspanin web against MHCIi overcrowding.

The degree of Treg cell deficiency in MAR CH1- or MHCIi K–deficient mice correlates with the degree of alteration in the lipid raft and tetraspanin web in thymic DCs

The data described so far led to the hypothesis that the failure of MHCIi K–R/K–R or MAR CH1−/− mice to generate Treg cells to a sufficient number is attributed to the failure of thymic DCs of these mice to maintain homeostasis of the lipid raft and/or tetraspanin web. To test this hypothesis, we first examined thymic DCs derived from WT, MHCIi K–R/K–R, and MHCIi K–R/+ mice for binding by anti-CD48, -CD24, or -CD9 antibodies and CTxB. Similar to what was observed with BMDCs (Fig. 5 A), these antibodies and reagent bound to DCs derived from WT mice the most, and DCs derived from MHCIi K–R/K–R mice the least (Fig. 8 A). Then, we compared the ability of these mice to generate antigen-specific Treg cells. The generation of OVA-specific Treg cells was examined in conditions where OVA was expressed in the thymus under the control of a rat insulin promoter (Fig. 8 B) or administered to the circulation (Fig. 8 C; Oh et al., 2013). Although MHCIIP−R/K−R mice completely failed to generate OVA-specific Treg cells in both conditions, MHCIIP−R/K−R mice generated them to an appreciable amount; however, it did not reach the amount generated by WT mice (Fig. 8, B and C). Lastly, we compared the amount of total Treg cells in WT, MHCIIP−R/K−R, and MHCIIP−R/+ mice. Treg cell frequency was reduced by half in MHCIIP−R/K−R mice compared with WT mice, and it remained similarly low in MHCIIP−R/+ mice (Fig. 8 D).

We performed similar experiments using MAR CH1−/− and MAR CH1+/− mice. Thymic DCs of MAR CH1−/− mice were most poorly bound by anti-CD48, -CD24, or -CD9 antibodies and CTxB, and DCs of MAR CH1−/− mice showed markedly improved binding (Fig. 8 E). OVA-specific Treg cells were not generated at all in MAR CH1−/− mice but were generated in MAR CH1+/− mice as efficiently as in WT mice both when OVA was expressed in the thymus and when administered to the circulation (Fig. 8, F and G). The total Treg cell amount was also significantly restored in MAR CH1−/− mice, although it did not reach the level of WT mice (Fig. 8 H). Collectively, Treg development in the thymus decreased in proportion to the degree to

Figure 7. Sphingomyelin supplementation lessens alteration of the lipid raft and tetraspanin web in MHCIi K–deficient DCs and improves the ability of the DCs to engage and activate thymocytes and drive Treg cell differentiation. (A) BMDCs were derived from MHCIi K–R/K–R mice, treated with ethanol (EtOH) or sphingomyelin (SM; 200 µM) for 3 d and stained by using monoclonal antibodies raised against indicated molecules except that CTxB was used to detect GM1. (B–D) BMDCs derived from MHCIi K–R/K–R mice were treated with EtOH or SM and cultured with CD4−CD8+ thymocytes isolated from OT-II mice in the presence of increasing concentrations of OVA323-339 peptide. The percentage of DCs that formed stable conjugates with thymocytes is shown in B. The percentage of thymocytes that expressed CD69 is shown in C. The level of CD25 expressed in CD69+ thymocytes is shown in D. (E and F) BMDCs derived from MHCIi K–R/K–R mice were treated with EtOH or SM and cultured with GFP−CD4+CD8− thymocytes isolated from Foxp3GFPOT-II mice in the presence of increasing concentrations of OVA323-339 peptide. 2 d later, the expression of GFP and CD25 in the thymocytes was determined by flow cytometry. Representative dot plots are shown in E. The percentage of GFP+CD25+ cells among thymocytes (Treg cells) is shown in F. Data represent two to four independent experiments. Error bars represent SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.005.
which the lipid raft and tetraspanin web were altered in thymic DCs. These data suggest the important contribution of the DC lipid raft and tetraspanin web to Treg cell development.

**MARCH1 significantly contributes to the diversification of Treg cell repertoire and function**

Having found the mechanism by which MARCH1 supports DC selection of Treg cells, we explored the functional significance of this mechanism. Specifically, we determined the contribution that MARCH1 may make to the diversity of Treg cells. MARCH1−/− mice were crossed to transgenic mice with a fixed TCRβ chain (Hsieh et al., 2004). Treg cells were isolated, and the variable TCRα chains were sequenced and analyzed at the individual TCR level (Fig. S5, A and B). We found a substantial loss of Treg cell TCR clones in MARCH1-deficient mice, estimated to comprise ~33% of the Treg cell TCR clones present in WT mice (Fig. 9, A and B). Remarkably, most of them completely disappeared in MARCH1−/− mice (Fig. 9 A).

Some clones were found enriched, although the frequency was as low as 2.3% (Fig. 9, A and B). This finding indicates that MARCH1 plays a significant role in diversifying Treg cell TCR repertoire. We also examined the TCR repertoire of non-Treg CD4+ thymocytes and found that 8.4% of the analyzed TCRs were enriched in MARCH1−/− mice (Figs. S5, C and D). Thus, MARCH1 appears to also play some role in negatively selecting CD4+ thymocytes.

Next, we examined whether the restriction in the repertoire of Treg cells in MARCH1-deficient mice is accompanied by the restriction in their functional capacity. Treg cells isolated from MARCH1-deficient mice suppressed proliferation of CD4+ T cells as effectively as those from WT mice in vitro (Fig. 9 C). To assess Treg cell functionality in physiologically relevant settings, we used two in vivo assays. First, we transferred naive CD4+ T cells to lymphopenic mice (Powrie et al., 1994), which develop wasting disease and inflammatory bowel disease (IBD), which are ameliorated by cotransfer of Treg cells (Mottet et al., 2003). We found that Treg cells derived from MARCH1−/− mice suppressed both wasting disease and IBD as well as or more effectively than those from WT mice (Fig. 9 D and Fig. S5 E).

Second, we used a mouse model of lethal acute graft-versus-host disease (aGVHD) preventable by cotransfer of Treg cells derived from the graft donor (Hoffmann et al., 2002). Transplantation of irradiated BALB/c mice with C57BL/6 mouse bone marrow cells that included CD4+ T cells but not Treg cells resulted in a precipitous death leaving no mice surviving on day 22 after transplantation (Fig. 9 E). However, mice cotransplanted with Treg cells derived from WT C57BL/6 mice were substantially protected from death, resulting in about two thirds of the mice surviving over 36 d after transplantation (Fig. 9 E). In contrast, mice
cotransplanted with Treg cells derived from MARCH1-deficient C57BL/6 mice died as rapidly as those transplanted with CD4+ T cells alone, although some mice managed to live (Fig. 9E). Thus, Treg cells generated in the absence of MARCH1 are incompetent in suppressing lethal aGVHD. These findings suggest that MARCH1 contributes to the diversification of the immunosuppressive function of Treg cells as well as TCR repertoire of the cells.

**Discussion**

The aim of this study was to define the mechanism by which MARCH1-mediated ubiquitination of MHCII supports DC function of selecting natural Treg cells. The presented study indicates that ubiquitin-dependent MHCII turnover is required for DCs to maintain homeostasis of the lipid raft and tetraspanin web in the plasma membrane and that DC ability to engage and activate thymocytes is critically dependent on integrity of these membrane domains. Thus, MARCH1-mediated MHCII ubiquitination represents a novel quality-control mechanism by which DCs maintain homeostasis of membrane domains that supports Treg cell–selecting function.

Ubiquitination induces endocytosis and lysosomal sorting and degradation of MHCII. Accordingly, when the ubiquitin acceptor lysine of MHCII was mutated in mice, the level of MHCII molecules, including the antigen-loaded MHCII, markedly
increased in DC surface. Yet, the DCs were less efficient at activating antigen-specific thymocytes than WT DCs. By using two independent methods, we showed that this reduced efficiency is attributed not to the absence of ubiquitinated MHCII molecules in the cells but to the presence of too many MHCII molecules. First, we found that the efficiency of MHCII K−deficient DCs at engaging and activating thymocytes was significantly improved when the cells were manipulated to express only one allele of the MHCII. Second, DCs that expressed MHCII via an exogenous promoter that drove MHCII expression much more weakly compared with the endogenous promoter were able to stably engage and strongly activate thymocytes in the absence of the ubiquitin acceptor lysine in MHCII.

We found that MHCII K deficiency resulted in distortion of the lipid raft and tetraspanin web in the plasma membrane of DCs. This distortion was manifested by poor binding of the antibodies or ligands directed to GPI-anchored molecules, GM1, or tetraspanin molecules. This poor binding appears to be largely attributed to conformational changes of the individual molecules driven by MHCII overcrowding in the membrane domains. A recent study has shown that the fraction of GM1 molecules available for CTxB binding changes as the property of GM1-associating membrane changes (Sachl et al., 2015). Another recent study has shown that tetraspanin molecules contain a large intramembrane cavity occupied by cholesterol and that the occupancy of this cavity modulates conformation of tetraspanins (Zimmerman et al., 2016). Possibly, the lipid raft and tetraspanin web in MHCII K−deficient DCs were altered in membrane property by MHCII overcrowding, which resulted in conformational changes in the associated molecules including GM1. Notably, we found that the amount of CD24 present in MHCII K−deficient DCs was slightly lower than that in WT DCs, although the amount of CD24 transcript was identical. We speculate that some of the CD24 molecules in MHCII K−deficient DCs might have been cleaved from the cell surface because phosphatidylinositol-phospholipase C and the angiotensin-converting enzyme, the enzymes capable of cleaving GPI anchor, are activated upon destabilization of the lipid raft (Metz et al., 1994; Sharom and Lehto, 2002; Kondoh et al., 2005). Additional studies using high-resolution microscopy and biophysical experiments would help in understanding the molecular changes occurring in the plasma membrane of MHCII-overcrowded DCs.

The lipid raft and tetraspanin web both promote DC activity of engaging and activating mature CD4+ T cells (Anderson et al., 2000; Kropshofer et al., 2002; Hilbold et al., 2003). However, the role of these membrane domains in DC activity of engaging, activating, and differentiating CD4+ thymocytes has not been investigated. Cholesterol and sphingolipid both play an important role in maintaining structural integrity of the lipid raft as well as the tetraspanin web (Odintsova et al., 2006). We found that DCs treated with the reagents that reduce cellular raft as well as the tetraspanin web (Odintsova et al., 2006). We found that DCs treated with these reagents also poorly engaged and activated thymocytes compared with untreated DCs. This finding supports that the lipid raft and tetraspanin web were disrupted in MHCII K−deficient DCs and that these membrane domains play an important role in DCs to stably engage and strongly activate thymocytes.

The specific mechanisms by which the lipid raft or tetraspanin web supports DC ability to conjugate and activate thymocytes remain to be further defined. We found that pretreatment with anti-CD48 antibody resulted in a significant reduction in DC ability to engage and activate thymocytes, implicating a role of CD48. CD48 has been shown to bind the adhesion molecule CD2 and facilitate activation of T cells (Kato et al., 1992). A similar mechanism may explain the contribution of CD48 to DC engagement and activation of thymocytes. Pretreatment of DCs with anti-CD9 antibody also resulted in a reduction in DC engagement and activation of thymocytes, although only the KMC8 antibody but not the MZ3 antibody did so. Although the specific epitope recognized by the KMC8 antibody is not known, that epitope may mediate CD9 interaction with some other molecules that promote DC engagement and activation of thymocytes. CD9 and CD81 can interact with multiple integrin molecules in the plasma membrane through their ectodomains (Berdichevski, 2001). CD9 and CD81 are also connected to actin cytoskeleton involving ezrin-radixin-moesin proteins (Sala-Valdés et al., 2006). Thus, these tetraspanin molecules may serve as a linker between adhesion molecules on the surface of DCs and the cytoskeleton in the cortex and promote stable DC interaction with thymocytes. The co-stimulatory molecule CD86 has been shown to be recruited to the lipid raft upon T cell–DC interaction, implicating the role of the lipid raft in T cell stimulatory activity of CD86. Although we did not examine it in this study, MHCII K deficiency may have caused CD86 to poorly associate with the lipid raft in MHCII (K>R) DCs making it unable to provide sufficient co-stimulatory signal to the interacting thymocytes.

We found that MARCH1 deficiency reduced not only the number of Treg cells but also repertoire of the cells. Approximately 33% of Treg TCR clones diminished in MARCH1-deficient mice, and the majority of them were undetectable. We also found some clones of non-Treg CD4+ thymocytes were enriched in MARCH1-deficient mice. However, the frequency of these enriched clones comprised only 8% of total non-Treg CD4+ thymocyte repertoire, implicating a relatively minor role of MARCH1 in negative selection over Treg cell development. Both negative selection and Treg cell development are promoted by high-affinity interaction between DCs and thymocytes. However, a recent study has revealed that the TCR affinity threshold to elicit negative selection is ∼100-fold higher than that of Treg cell development. This study also showed that Treg cells can develop in a broad ∼1,000-fold range of TCR affinity. This extremely broad range of Treg cell affinity may be attributed to the role of the lipid raft and tetraspanin web in promoting DC engagement and activation of thymocytes. We found that the Treg cells generated in the absence of MARCH1 sufficiently suppressed wasting disease and IB but not aGVHD. Previous studies have shown that the alloantigen-specific Treg cells suppressed alloreactive effector T cells more effectively than polyclonal Treg cells in vitro (Chen et al., 2009; Veerapathran et al., 2013). Alloantigen-specific Treg cells also suppressed aGVHD more effectively than polyclonal Treg cells when transferred to mice that received allograft transplantation (Yamazaki et al., 2006; Golshayan et al., 2007). Thus, MARCH1 may play a particularly important role in the development of Treg cells reactive to alloantigens.

In conclusion, this study reveals an unexpected role of MARCH1 and MHCII ubiquitination in DC membrane homeostasis and Treg cell–selecting function. The dependency of MARCH1 and MHCII ubiquitination in other DC functions, such as development of effector T cells, would be an important remaining topic to be studied.
Materials and methods

Mice
C57BL/6, BALB/c, B6.PL-Thy1.1/CyJ (Thy1.1), B6.129S2-H2Ab+1-E/J (MHCII−/−), B6.129S2-Tcrααmonom/J(TCRα−/−), B6.129S7-Rag1−/−/Monom/J (Rag1−/−), B6.Cg-Tg(Igax-cre-1)1-Heiz/J (CD11c-CRE), B6.Cg-Tg(UBC-cre/ERT2)1Heiz/J (ERT-CRE), B6.SJL-Tg(ACTFLPe)9205-Dym/J (ACT-FLP), B6.Cg-Tg(TcrαTcrβ)25Cbn/J (OT-II), and B6.SJL-Ptprc−/−/Boy/J (Boy) mice were purchased from Jackson Laboratories. B6.129-H2A1bi6141GruN12 (MHCII−/−) mice were purchased from Taconic. MHCII−/−/R–R– (Oh et al., 2013), MARCH1−/− (Matsuki et al., 2007), Rip-mOVA (Kurts et al., 1996), foxp3−/− (Haribhai et al., 2007), and TcIβ transgenic (Hsieh et al., 2004) mice were described previously. All mice were maintained in the University of California, San Francisco (UCSF), mouse facility, and all animal studies were performed according to protocols approved by the UCSF Institutional Animal Ethics Committee.

Antibodies and reagents
The following antibodies were purchased from BioLegend, eBioscience, or BD Biosciences and used for flow cytometry or enrichment: FITC-Sirpa, PE/Cy7-Sirpa, PerCP/Cy5.5-B220, APC-EpCAM, PE-CD11c, PE/Cy7-CD11c, Brilliant Violet 605-CD11c, Pacific Blue-AI/E, FITC-CD8α, Alexa700-CD8α, APC-V-2, APC-V-5, PE-V-6, PE-Foxp3, Pacific Blue-Foxp3, PerCP/Cy5.5-Thy1.1, APC-TCRβ, PE-CD69, PE/Cy7-CD69, Brilliant Violet 605-CD45, APC-CD45, Brilliant Violet 605-25, Brilliant Violet 785-CD25, FITC-CD45RB, APC-ICAM1, PE-CD25, PE-CD10, PE-CD48, PE/CD11c, PE-CD61, PE/Cy7-CD61, Brilliant Violet 605-CD25, Brilliant Violet 605-25, Brilliant Violet 785-CD25, FITC-CD45RB, APC-ICAM1, PR-CD9, PE-CD81, TP-CD71, PE/Cy7-CD45, APC-streptavidin, Biotin-Thy1.2, Biotin-CD8β, Biotin-CD25, and Biotin-B220. Alexa647-CTxB subunit was purchased from Molecular Probes. For Western blotting, the following clones were used: MHC II (K>R) mutant, CD86 (K>R) mutant was generated as previously described (Shin et al., 2006; Baravalle et al., 2011). The generated virus was mixed with polybrene (10 μg/ml) and added to BMDCs on the second day of culture. The BMDCs were spun at 2,500 rpm for 2 h at RT, and the supernatant was replaced with the Flt3-ligand-containing media. Cells were cultured for an additional 10 to 12 d.

Quantitative PCR
Genomic DNA was purified using the PureLink genomic DNA Mini kit (Invitrogen) from Sirpα+ DCs (CD11c+MHCII−/−-autofluorescence-Sirpα-). Sirpα+ DCs (CD11c+MHCII−/−-autofluorescence-Sirpα-) B cells (CD20+B220−MHCII−/−), and thymic epithelial cells (EpCAM−MHCII−/−B220−) isolated from the thymi of MARCH1−/− and MARCH1−/− CD11c-CRE mice. Quantitate PCR was performed on an Eppendorf realeplex system by using SYBR Green reagents and the primer pair spanning 3′ Loxp site (5′ primer, AGCTGTAAGAACCTGACTCAA; 3′ primer, GAGGAGG TCGTTGTCGTTGAAA). See also Fig. S1. Total RNAs were isolated from sorted cDCs by TRizol (Invitrogen). The first-strand cDNA was generated by using SuperScript III system (Invitrogen) with the use of oligo dT as primer. Quantitative PCR was performed by using primers specific for CD48, CD24, Splt2, CD9, and HPRT (https://pga.mgh .harvard.edu/primerebank/).

Flow cytometry and cell sorting
Cells were incubated with fluorophore-conjugated antibodies in FACS buffer (1% BSA in PBS) containing propidium iodide solution (BioLegend) for 30 min on ice. When staining DCs, cells were preincubated with CD16/32 Fc block antibody (UCSF Cell Culture Facility). Foxp3 was stained by using Foxp3/Transcription Factor Staining Buffer Set as instructed (eBioscience). Samples were analyzed and sorted by the FACS LSRII system (BD Biosciences) and BD FACS Aria3 Cell Sorter (BD Biosciences), respectively.

Thymocyte conjugation and activation assay
CD4+ thymocytes were isolated through negative selection using biotin-CD8β and biotin-CD25 antibodies and streptavidin-conjugated magnetic beads (Stemcell Technologies) from Thy1.1 OT-II mice. Isolated cells (1 × 105) were co-cultured with thymic DCs (2 × 104) or BMDCs (2 × 104) in the presence of increasing amounts of OVA protein or OVA323-339 peptide. Thymocyte conjugation was assayed after 3 h by centrifuging cell mixtures and vortexing pellets for 5 s at the highest speed before fixation and antibody staining. The percentage of Thy1.1+ Vβ55 cells among CD11c+B220+ population was determined by flow cytometry. Thymocyte activation was assayed after 24 h by determining the percentages of CD69+ and the geometric mean fluorescence intensity of CD25 of CD69+ among Thy1.1+ Vβ55 population by flow cytometry.
Western blotting
CD4+CD8− conventional CD4+ T cells (2.5 × 10⁴ cells) sorted from BoyJ (CD45.1+) mice spleens were labeled with CellTrace Violet (CTV; Invitrogen) and mixed with Treg cells (CD25−CD4+CD8−) sorted from thymi of WT or MARCH1−/− mice to various ratios. The cell mixtures were added with 10⁴ beads conjugated with anti-CD3 and anti-CD28 antibodies and cultured in 37°C for 3 d. The percentages of CD45.1+ conventional CD4+ T cells with their CTV diluted were determined by flow cytometry.

Treg cell–dependent suppression of wasting disease and IBD
Naive CD4+ T cells (CD45RB−CD25−CD4+CD8−) were isolated from WT mouse spleens and lymph nodes and injected into 9- to 12-wk-old Rag1−/− mice (4–5 × 10⁴ cells per mouse). Some mice were also injected with Treg cells isolated from WT or MARCH1−/− mouse thymi (2–2.5 × 10⁵ cells per mouse). Mice were weighed every 2–3 d. Mice were euthanized on day 56, and distal colons were stained by using hematoxylin and eosin. The pathology was scored as follows: mucosal damage (0, none; 1, discrete lymphoepithelial lesions; 2, diffuse crypt elongation; or 3, extensive crypt elongation or mucosal ulceration), immune cell infiltration (0, none; 1, widely scattered leukocytes or focal aggregates; 2, confluent leukocytes extending into submucosa with focal effacement of the mucosa; or 3, leukocyte infiltration with transmural extension), and crypt abscess (0, absent; or 1, present; Yamaji et al., 2012). Values for each slide were summed for a total score of 0–7.
Stritesky, G.L., S.C. Jameson, and K.A. Hogquist. 2012. Selection of self-reactive T cells in the thymus. Annu. Rev. Immunol. 30:95–114. https://doi.org/10.1146/annurev-immunol-020711-075035

Unternährer, J.J., A. Chow, M. Pypaert, K. Inaba, and I. Mellman. 2007. The tetraspanin CD9 mediates lateral association of MHC class II molecules on the dendritic cell surface. Proc. Natl. Acad. Sci. USA. 104:234–239. https://doi.org/10.1073/pnas.0609665104

van Niel, G., R. Wubbolts, T. Ten Broeke, S.I. Buschow, F.A. Ossendorp, C.J. Melief, G. Raposo, B.W. van Balkom, and W. Stoorvogel. 2006. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. Immunity. 25:885–894. https://doi.org/10.1016/j.immuni.2006.11.001

Veerapathran, A., J. Pidala, F. Beato, B. Betts, J. Kim, J.G. Turner, M.K. Hellerstein, X.Z. Yu, W. Janssen, and C. Anasetti. 2013. Human regulatory T cells against minor histocompatibility antigens: ex vivo expansion for prevention of graft-versus-host disease. Blood. 122:2251–2261. https://doi.org/10.1182/blood-2013-03-492397

Walseng, E., K. Furuta, B. Bosch, K.A. Weih, Y. Matsuki, O. Bakke, S. Ishido, and P.A. Roche. 2010. Ubiquitination regulates MHC class II-peptide complex retention and degradation in dendritic cells. Proc. Natl. Acad. Sci. USA. 107:20465–20470. https://doi.org/10.1073/pnas.1010990107

Yamaji, O., T. Nagaishi, T. Totsuka, M. Onizawa, M. Suzuki, N. Tsuge, A. Hasegawa, R. Okamoto, K. Tsuchiya, T. Nakamura, et al. 2012. The development of colitogenic CD4(+) T cells is regulated by IL-7 in collaboration with NK cell function in a murine model of colitis. J. Immunol. 188:2524–2536. https://doi.org/10.4049/jimmunol.1100371

Yamazaki, S., M. Patel, A. Harper, A. Bonito, H. Fukuyama, M. Pack, K.V. Tarbell, M. Talmor, J.V. Ravetch, K. Inaba, and R.M. Steinman. 2006. Effective expansion of alloantigen-specific Foxp3+ CD25+ CD4+ regulatory T cells by dendritic cells during the mixed leukocyte reaction. Proc. Natl. Acad. Sci. USA. 103:2758–2763. https://doi.org/10.1073/pnas.0510606103

Zimmerman, B., B. Kelly, B.J. McMillan, T.C. Seegar, R.O. Dror, A.C. Kruse, and S.C. Blacklow. 2016. Crystal structure of a full-length human tetraspanin reveals a cholesterol-binding pocket. Cell. 167:1041–1051.