HHS Public Access

Author manuscript

Immunol Rev. Author manuscript; available in PMC 2016 July 01.

Published in final edited form as:

Immunol Rev. 2015 July ; 266(1): 134–144. doi:10.1111/imr.12303.

Molecular mechanism and cellular function of MHCII ubiquitination

Jaehak Oh and **Jeoung-Sook Shin**

Department of Microbiology and Immunology, Sandler Asthma Basic Research Center, University of California San Francisco, San Francisco, CA, USA

Summary

The major histocompatibility complex class II (MHCII) is ubiquitinated via the evolutionally conserved lysine in the cytoplasmic tail of the β chain in dendritic cells (DCs) and B cells. The ubiquitination is mediated by the membrane-associated RING-CH1 (MARCH1) ubiquitin ligase although it can be also mediated by the homolog ligase MARCH8 in model cell lines. The ubiquitination promotes MHCII endocytosis and lysosomal sorting that results in a reduction in the level of MHCII at cell surface. Functionally, MHCII ubiquitination serves as a means by which DCs suppress MHCII expression and reduce antigen presentation in response to the immune-regulatory cytokine IL-10 and regulatory T cells. Recently, additional roles of MHCII ubiquitination have begun to emerge. MHCII ubiquitination promoted DC production of inflammatory cytokines in response to the Toll-like receptor ligands. It also potentiated DC ability to activate antigen-specific naïve $CD4^+$ T cells while limiting the amount of antigens presented at cell surface. Similarly, MHCII ubiquitination promoted DC activation of $CD4^+$ thymocytes supporting regulatory T-cell development independent of its effect of limiting antigen presentation. Thus, ubiquitination appears to confer MHCII a function independent of presenting antigens by a mechanism yet to be identified.

Keywords

MHCII; ubiquitination; MARCH; dendritic cell; TLR; regulatory T cell

Introduction

The major histocompatibility complex class II (MHCII) is an antigen-presenting molecule playing an important role in both the development and activation of $CD4^+$ T cells. Being highly expressed in epithelial cells and dendritic cells (DCs), MHCII molecules present a broad array of self-antigens in the thymus to developing thymocytes, and mediate their maturation into $CD4^+$ T cells (1). MHCII molecules are also highly expressed in DCs in the periphery, which continuously sample environment and monitor the presence of microbial antigens (2–4). Upon encounter of such antigens, DCs internalize and process them into

Correspondence to: Jeoung-Sook Shin, 513 Parnassus Avenue, HSE-201, San Francisco, CA 94143, Tel: 415-476-5451, jeoung-sook.shin@ucsf.edu.

There are no conflicts of interest associated with this review.

small peptides, and load the peptides onto MHCII molecules. Concurrently, DCs migrate to the draining lymph nodes where they present MHCII-loaded antigens to antigen-specific naïve $CD4^+$ T cells, which in turn differentiate to effector T cells capable of producing inflammatory cytokines and combating infection. Many other cell types including non-hematopoietic cells express MHCII under inflammatory conditions (5). These cells present antigens to memory or effector $CD4^+$ T cells contributing to the amplification of T-cell immunity at sites of inflammation.

MHCII-mediated antigen presentation is critically dependent on proper intracellular transport of MHCII (6, 7). MHCII synthesized in the endoplasmic reticulum (ER) traffics to lysosomes where it loads antigenic peptides prior to traveling to the plasma membranes. In 2006, we found that MHCII is post-translationally modified by ubiquitination and that this ubiquitination plays a significant role in controlling MHCII intracellular transport (8). This finding has prompted active investigation on the specific mechanisms by which MHCII is ubiquitinated as well as the ubiquitination regulates MHCII transport. The functional role of MHCII ubiquitination has also been investigated by generating cells or animals in which MHCII ubiquitination was specifically abolished. In this review, we summarize some of these studies and update our current understanding of the molecular mechanism and cellular function of MHCII ubiquitination.

Molecular mechanism of MHCII ubiquitination

MHCII is composed of an α and a β subunit and is ubiquitinated via the cytoplasmic lysine of the β subunit (8, 9). This lysine modified by ubiquitination is strictly conserved throughout the isotypes of MHCII and the species of vertebrate (Table 1), implicating the ubiquitination in an MHCII-mediated function important to vertebrates.

Two ubiquitin ligases have been identified capable of mediating ubiquitination of MHCII, both of which belong to the membrane-associated RING-CH (MARCH) family. MARCH protein family was originally identified as the mammalian homologs of the Kaposi's sarcoma virus ubiquitin ligases K3, K5, and mK3 (10, 11). These viral ligases have RING-CH domain and are constitutively associated with membranes via the transmembrane domains. They ubiquitinate MHCI and other immune-associated membrane proteins, and induce rapid endocytosis and degradation of the molecules (12–17). Thus, expression of these ligases was considered as an immune evasion strategy that the virus developed to manipulate host immune system for their survival. However, virus often pirate from host, which led to a postulation that mammalian cells express similar types of ligases that are associated with membranes and target immune-associated membrane proteins. This postulation prompted a bioinformatics search for mammalian proteins that possess the RING-CH domain and transmembrane domain(s), which led to the identification of MARCH family proteins (10). Eleven members have been identified so far, many of which indeed ubiquitinate and downregulate the surface expression of immune-associated membrane proteins (10, 18, 19). Among those, MARCH1 and MARCH8 were shown to be capable of ubiquitinating MHCII.

The ability of MARCH8 to ubiquitinate MHCII was found somewhat unexpectedly. It had been known that MARCH8 ubiquitinates the costimulatory molecule CD86 and induces endocytosis and lysosomal degradation (20). When MARCH8-transgenic mice were generated however, the mice exhibited a substantial defect in $CD4^+$ T-cell development (21). Remarkably, the level of MHCII expression in thymic epithelial cells was markedly reduced, and MHCII in other MHCII-expressing cells was also reduced. When MHCII-transfected 293 T-cell lines were co-transfected with MARCH8, MHCII surface levels were reduced, and MHCII was ubiquitinated via the β chain cytoplasmic lysine.

The role of MARCH1 to mediate MHCII ubiquitination was found from MARCH1-deficient mice, which were generated by disrupting the locus of the RING domain critical for the catalytic activity of the ubiquitin ligase. These mice exhibited in an increase in the level of MHCII expression in B cells and DCs (22). This increase was accompanied by a near absence of ubiquitinated MHCII in these cells, evidencing that MARCH1 is the ubiquitin ligase that mediates MHCII ubiquitination *in vivo* (22). Treatment of human monocyte-derived DCs with shRNA that specifically downregulates the expression of MARCH1 resulted in a substantial increase in the level of MHCII expression in the cells and a marked decrease in the level of ubiquitinated MHCII evidencing that MARCH1 also mediates ubiquitination of MHCII in humans. Later, MARCH8-deficient mice were also generated to determine the role of MARCH8 in ubiquitinating MHCII under physiologic condition. These mice, however, expressed normal levels of MHCII in DCs and B cells, and MHCII ubiquitination was also comparable to that in cells from wildtype mice (authors' unpublished data). Thus, MARCH1 is necessary for MHCII ubiquitination in DCs and B cells, while MARCH8 is not.

Although MARCH1 and MARCH8 are both classified as the RING ubiquitin ligase, they are distinct from most other RING ligases in following two structural traits. Firstly, they are transmembrane proteins while others are cytosolic. MARCH1 and MARCH8 both have two transmembrane domains linked by a short peptide exposed to the lumen. These transmembrane domains are 100% identical in two proteins (10). Secondly, the RING domain of MARCH1 protein family has a cystein at the fourth coordinating position and a histidine at the fifth (RING-CH), while the conventional RING domain has a histidine residue in the fourth position and a cystein in the fifth (RING-HC), (23, 24). Although this difference generates a significant change in the structure of the RING domain, this changes does not make a substantial alteration to the binding site for the E2 ubiquitin conjugating enzymes (24). The RING domain of the viral ubiquitin ligase K3 has been shown to bind the UbcH5 and Ubc13 E2 enzymes (24, 25). Whether these enzymes also bind to MARCH1 or MARCH8 is not known.

The substrate specificity of MARCH1 and MARCH8 appears to be determined by transmembrane domains. When the transmembrane domains of MARCH1/8 were swapped with those of MARCH9, the chimeric protein failed to reduce MHCII surface expression in transfected cells (26). The transmembrane domains of MHCII were also critical for the recognition by MARCH1/8, while the interface between the transmembrane domain and the cytosol controls efficiency of the recognition (27). In the cytosolic domain of MHCII β chain, the lysine at the fourth amino acid from the membrane is the only key single residue;

other residues could be either removed or substituted with negligible effect (28). MARCH1 also ubiquitinates CD86 in DCs inducing endocytosis and intracellular degradation (29). MARCH1 recognition of CD86 is also mediated by the transmembrane domains. Interestingly, MARCH1 also interacts with CD83 through the transmembrane domains, but does not ubiquitinate it (30). Owing to this pseudo-substrate-like property, CD83 can act as a competitive inhibitor of MARCH1. When CD83 was over-expressed in cells that express MARCH1, MHCII, and CD86, MARCH1 bound CD83 over MHCII or CD86, and this competitive binding resulted in a decrease in MHCII and CD86 ubiquitination (30).

Role of ubiquitination in MHCII intracellular transport

The intracellular pathway of MHCII transport has been extensively studied (6, 7, 31). MHCII is synthesized in the ER as a heterodimer and associates with an accessory molecule named invariant chain. The invariant chain functions a chaperone molecule that assists MHCII folding (32, 33), but it also acts as a molecular vehicle that carries the MHCII to the lysosomes through endosomal trafficking pathways (34, 35). During this trafficking, the invariant chain is degraded stepwise by multiple proteases, only leaving the small peptide fragment named CLIP that occupies MHCII's peptide-binding groove (36–41). The CLIP:MHCII complex then interacts with the peptide-exchange factor named DM (H2M in mice), which facilitates MHCII to dislodge CLIP and load peptides derived from endocytosed antigens (42–44). These peptide-loaded MHCII molecules then travel to the plasma membranes (45–48).

While the aforementioned pathway of antigen presentation is led by newly synthesized MHCII, there is another pathway of antigen presentation, which is led by recycling MHCII (49–51). Upon arrival to the plasma membrane, the peptide:MHCII (pMHCII) complexes re-enter cells and reach the early endosomes. Early endosomes have endosomal proteases, which can process endocytosed antigens and generate antigenic peptides. These peptides are loaded onto MHCII molecules that just entered the early endosomes. The pMHCII complex formed in this early endosome then transport back to the plasma membrane through recycling endosomes. This recycling pathway of antigen presentation has been shown to be indispensable for presentation of some virus-driven antigens, implicating a significant contribution of this pathway to proper immune responses against viral infection (52).

The role of ubiquitination in MHCII intracellular transport was first implicated in a microscopy study (8). Subcellular distribution of MHCII was examined in mouse bone marrow-derived DCs (BMDCs) that express MHCII wildtype versus MHCII (K>R) mutant in which the lysine of MHCII β chain cytoplasmic tail was replaced with arginine, and thus resistant to ubiquitination. While MHCII wildtype was primarily localized in the lysosomes, MHCII (K>R) mutant was largely absent in the lysosomes but was heavily accumulated in the plasma membranes. This dramatic change in the subcellular localization suggested that ubiquitination plays an important role in the control of MHCII intracellular transport, particularly transport from the lysosomes to the plasma membranes. To test this possibility, it was examined whether MHCII ubiquitination occurs before or after MHCII dissociates from the invariant chain (9). It was found that ubiquitination was selectively associated with MHCII free of invariant chain, suggesting that the ubiquitination occurs after MHCII

dissociates from the invariant chain. Subsequently examined was whether the rate of newly synthesized MHCII to reach the cell surface slows down when MHCII ubiquitination was inhibited in cells (22). The rate of newly synthesized MHCII to reach cell surface did not significantly differ whether the ubiquitination was inhibited or not. These findings suggest that MHCII transport from ER to the plasma membrane is independent of MHCII ubiquitination.

The role of ubiquitination in MHCII endocytosis was examined by several research groups including ours. Two groups independently measured the rate of MHCII endocytosis in mouse BMDCs that express MHCII wildtype or MHCII (K>R) mutant. Both groups showed that MHCII (K>R) mutant enters DCs at a significantly slower rate than MHCII wildtype, indicating that ubiquitination promotes MHCII endocytosis (8, 9). The role of ubiquitination in MHCII endocytosis was also examined in human DCs. Human monocyte-derived DCs treated with MARCH1-specific shRNA internalized MHCII at a much slower rate than DCs treated with control shRNA, supporting the role of ubiquitination in MHCII endocytosis (53). In a more recent study, we showed that MHCII in B cells is internalized at significantly slower rate than MHCII in DCs although MHCII is ubiquitinated in both cell types. Interestingly, MHCII was attached by 1~3 ubiquitin molecules in B cells while it was attached by up to 8 in DCs (54). By generating the cDNA constructs that encode a series of MHCII-ubiquitin fusion proteins attached by an increasing number of ubiquitin molecule(s), we found that MHCII endocytosis was not enhanced by mono-ubiquitination at all while it was improved by oligo-ubiquitination slightly and by poly-ubiquitination more markedly (54). Thus, the extent to which ubiquitination contributes to MHCII endocytosis varies by the length of ubiquitin chain attached. A study has shown that MHCII ubiquitination does not make a significant contribution to MHCII endocytosis in B cells (22). This result could be attributed to the length of ubiquitin chain attached to MHCII significantly short in B cells compared to DCs.

However, the role of ubiquitination in MHCII endocytosis remains somewhat controversial. Walseng *et al.* examined MHCII endocytosis in mouse BMDCs cultured from wildtype mice, MARCH1-deficient mice, and MHCII (K>R) knockin (KI) mice, which express MHCII (K>R) mutant instead of MHCII wildtype. No significant difference was observed in MHCII endocytosis among the cells, and authors concluded that ubiquitination does not promote MHCII endocytosis (55). This conclusion is apparently contradictory to ours and some others, and underlying reason is not clear at the moment. One consideration worth taking is that each laboratory measured MHCII endocytosis by different assays. One group labeled surface proteins with biotin and measured the rate of biotinylated MHCII appearing inside cells (22). We labeled surface MHCII with fluorescently-tagged MHCII antibody and measured the rate of the antibody appearing inside the cells (54). Other groups labeled surface MHCII with a fluorescently tagged antibody but measured the rate of the antibody disappearing from cell surface (53, 55). Depending on assays, the kinetics of MHCII endocytosis appeared significantly different, even when the cells of examination were derived from the same tissue of the same species of animal (22, 54). Another consideration to take is the ratio of MHCII to MARCH1 in cells of examination. The BMDCs examined by Walseng *et al.* expressed MHCII under the control of the MHCII endogenous promoter

(55). The BMDCs examined by our group expressed MHCII under the control of a retroviral promoter, the activity of which is approximately 5% over that of MHCII endogenous promoter (54). Consequently, the amount of MHCII in DCs of our experiment is substantially lower than that of the other group, but the amount of MARCH1 is not, resulting in the ratio of MHCII to MARCH1 substantially lower in cells of our experiment. Because the ratio of MHCII to MARCH1 is lower, the fraction of MHCII interacting with and ubiquitinated by MARCH1 at a given moment will be higher, and thus the impact that this ubiquitination makes on a total MHCII pool will be larger. This may explain why the role of ubiquitination in MHCII endocytosis was obvious in our study but not in the others. This possibility is further supported by the finding that the effect of MARCH family ubiquitin ligases in downregulating their substrates is only obvious when the level of MARCH expression reaches a certain threshold (10, 29).

The role of ubiquitination in MHCII lysosomal sorting and degradation was also examined by many investigators. It had been known for a while that the lysosomes in DCs exhibit the morphology of multivesicular body (MVB) and that the internal vesicles of MVBs are enriched with MHCII molecules (46). We examined whether ubiquitination is involved in MHCII sorting to MVB internal vesicles. By employing the immunogold electron microscopy, we found that MHCII (K>R) mutant was sorted to the internal vesicles of MVB at a significantly smaller fraction than MHCII wildtype (8). A similar observation was made in a study independently performed by van Neil *et al.* (9). We also examined whether ubiquitin-mediated MVB sorting associates with lysosomal degradation of MHCII. Drugs that inhibit lysosomes but not proteasomes resulted in a huge accumulation of MHCII in DCs, similarly to what has happened when MHCII ubiquitination was inhibited. Others also showed that MHCII (K>R) mutant turns over at a significantly slower rate than MHCII wildtype (55, 56). Thus, ubiquitination promotes MHCII sorting to the internal vesicles of MVB and subsequent degradation by lysosomal proteases. Notably, recent studies have identified a ubiquitin-independent mechanism by which MHCII is sorted to MVB of a distinct destination (57). This mechanism of MVB sorting appears to lead MHCII to extracellular secretion via exosomes.

Functional role of MHCII ubiquitination

Suppression of APC antigen presentation by IL-10 or regulatory T cells

Microbial stimuli including Toll-like receptor (TLR) ligands dramatically increase the surface expression of MHCII in DCs. However, this increase is not accompanied by an increase in the synthesis of MHCII (58, 59). Instead, DCs exposed to microbes downregulate the expression of MARCH1 (53, 60). As a consequence, MHCII ubiquitination is downregulated and it results in an increase in MHCII surface levels.

Based on this inverse relationship between MHCII ubiquitination and MHCII surface expression in DCs, one can presume that MHCII ubiquitination could be utilized for the purpose of suppressing antigen presentation by DCs. In agreement with this reasoning, IL-10, a potent immune regulatory cytokine, was found to upregulate the expression of MARCH1 in DCs (30). This upregulation increases MHCII ubiquitination, which consequently results in a reduction in MHCII surface levels. Importantly, MARCH1-

deficient DCs is completely refractory to IL-10, maintaining MHCII surface levels persistently high. IL-10 exerts a similar effect on human monocytes; human blood monocytes exposed to IL-10 upregulated the expression of MARCH1, increasing MHCII ubiquitination (61). These studies provide strong evidence that IL-10-mediated suppression of MHCII expression in DCs and monocytes is dependent on MHCII ubiquitination.

Recently, regulatory T cells (Tregs) were also found to exploit MHCII ubiquitination to suppress DC expression of MHCII (62). Incubation of DCs with induced Treg (iTreg) cells, which were differentiated *in vitro* from naïve $CD4^+$ T cells, resulted in a decrease in the surface levels of MHCII in DCs, and this decrease was accompanied by an increase in the levels of MARCH1. In addition, iTreg cells decreased the expression of CD83 in DCs. CD83 acts as an inhibitor of MHCII ubiquitination by binding to MARCH1 and sequestering it away from its substrates. Thus, iTreg cells appear to suppress DC expression of MHCII by employing two mechanisms, one that upregulates the expression of MARCH1 and the other that increases the accessibility of MARCH1 to MHCII. Essentially, these mechanisms are both dependent on IL-10 derived from iTreg cells (62).

Although it is clear that MHCII ubiquitination serves as an important means by which IL-10 suppresses DC expression of MHCII, its functional significance has not been firmly established. The importance of IL-10 in immune suppression is well manifested in IL-10-deficient mice and in mice treated with IL-10 receptor neutralizing antibodies (63–65). These mice were killed by ~20 fold lower doses of LPS than wildtype mice (64, 66). They also developed many murine models of autoimmune diseases including experimental autoimmune encephalitis in suboptimal conditions, and diseases were often more severe than in wildtype mice (67–69). IL-10-deficient mice also spontaneously developed enterocolitis (70, 71). It is not known whether any of these phenotypes develop in mice deficient in MARCH1 or MHCII ubiquitination. MARCH1 KO mice and MHCII (K>R) KI mice are both fertile and show no signs of diseases in specific pathogen-free (SPF) animal facilities. It is possible that IL-10's immune regulatory roles are mainly mediated by its inhibitory effects on proinflammatory cytokines rather than MHC II. Indeed, the protective role of IL-10 in endotoxemia is attributed to its inhibitory effect on the production of TNFα (72). Alternatively, MHCII ubiquitination is important not only for IL-10-mediated immune regulation but also for the activation and/or expansion of innate or adaptive immunity. This possibility has been raised by some of recent studies, which are described below.

Cytokine production in response to TLR ligands

A recent study has shown that DCs derived from MARCH1 KO mice or MHCII (K>R) KI mice produced significantly lesser amounts of IL-12 and TNFα in response to the TLR4 ligand LPS than those derived from wildtype mice (73). The whole splenocytes isolated from MARCH1 KO mice also produced lesser amounts of TNFα in response to LPS or poly (I:C), a ligand of TLR3 (74). These findings suggest that MHCII ubiquitination may play a significant role in TLR signaling. Although the underlying mechanism remains to be defined, it may be pertinent to the role of intracellular MHCII in promoting TLR signaling proposed previously. It has been shown that the engagement of TLR4, 3, or 9 in DCs and macrophages induced the formation of a protein complex composed of CD40, the tyrosine

kinase Btk, and intracellular but not surface MHCII (75). This protein complex led to sustained phosphorylation of Btk that interacts with MyD88 and TRIF supporting TLR signaling. MHCII-deficient DCs or macrophages produced lesser amounts of inflammatory cytokines in response to TLR engagement, similarly to DCs deficient in MHCII ubiquitination. Considering that MHCII ubiquitination promotes MHCII endocytosis, MHCII ubiquitination may enhance the amounts of intracellular MHCII, which in turn would promote TLR signaling and increase cytokine production.

$CD4^+$ T-cell activation

As the major role of MHCII is to present antigens, the role of ubiquitination in antigen presentation has been examined. An initial study showed that DCs from MARCH1 KO or MHCII (K>R) KI mice presented lesser amounts of antigens than those from wildtype mice (73). However, subsequent studies by the same as well as others laboratories corrected that DCs deficient in MHCII ubiquitination present a more amount of antigens, corresponding to a higher level of MHCII expressed at cell surface (55, 76, 77). Surprisingly however, the enhanced antigen presentation did not result in an enhanced activation of antigen-specific naïve $CD4^+$ T cells. Instead, it resulted in less proliferation and less production of effector cytokines including IL-2, IFN-γ, and IL-17 (76). This finding raises a possibility that MHCII ubiquitination plays an important role in DC-mediated T-cell activation.

It has been previously examined whether MHCII ubiquitination plays a significant role in antibody production, which depends on T-cell activation. In this study, antibody response was examined in a knockin mouse strain that express MHCII (K>R)-EGFP fusion protein, in comparison to the control knockin mouse strain that express MHCII-EGFP fusion protein (78). No significant difference was observed between the mice, and the authors concluded that MHCII ubiquitination is dispensable for antibody production. However, this study includes an important caveat. MHCII (K>R)-EGFP protein could be ubiquitinated via lysine residues present in EGFP attached to MHCII. Alternatively, but not exclusively, the attached EGFP could interfere with the ubiquitination resulting in MHCII-EGFP to be ubiquitinated not as efficiently as MHCII wildtype or ubiquitinated to a fashion different from physiologic one. Thus, whether MHCII ubiquitination plays a significant role in antibody production remains as an unresolved issue.

We recently examined the phenotypes of T cells present in MARCH1 KO or MHCII (K>R) KI mice in comparison to wildtype mice. We did not find any significant differences in the number of naïve and memory $CD4^+$ T cells as well as $CD8^+$ T cells in the spleen and lymph nodes (unpublished data).

Treg cell development

$CD4^+$ T cells develop in the thymus through the interaction of T-cell precursor thymocytes with MHCII-expressing APCs in the thymus (1, 79, 80). Among thymic APCs, cortical thymic epithelial cells (cTECs) play an important role for positive selection of $CD4^+$ thymocytes by providing a survival signal to thymocytes that bind pMHCII molecules to a relatively low affinity. Medullary thymic epithelial cells (mTECs) mediate negative selection by providing a death signal to thymocytes that bind pMHCII molecules to a strong

affinity. As mTECs present various self-antigens including those restrictedly expressed in peripheral tissues, mTEC-mediated negative selection plays an important role in preventing organ-specific autoimmune diseases (81). mTEC also plays an important role in the development of Treg cells, which are believed to develop from positively selected $CD4^+$ thymocytes binding pMHCII molecules to an intermediate affinity (82). DCs in the thymus also play a significant role in negative selection of $CD4^+$ T cells and Treg cell differentiation (83–86). They present mTEC-driven tissue-restricted antigens as well as blood-borne self-antigens, playing a non-redundant role from mTECs for negative selection and Treg differentiation. B cells in the thymus also have been shown capable of deleting self-reactive $CD4^+$ thymocytes contributing to the negative selection (87).

Given the important role of ubiquitination in controlling MHCII transport and the important role of MHCII-mediated antigen presentation in $CD4^+$ T-cell development, we examined whether MHCII ubiquitination plays any role in $CD4^+$ T-cell development (77). Firstly, we determined MHCII ubiquitination in mouse thymic APCs. The MHCII ubiquitinating enzyme MARCH1 was expressed in APCs of bone marrow origin but not in TECs. Accordingly, DCs and B cells but neither cTECs nor mTECs expressed an increased level of MHCII in MARCH1 KO mice and MHCII (K>R) KI mice compared to wildtype mice. This finding suggests that MHCII is ubiquitinated in DCs and B cells but not TECs in the thymus. Secondly, we characterized the profile of developing $CD4^+$ thymocytes in MARCH1 KO and MHCII (K>R) KI mice compared to wildtype mice. No significant alteration was observed in either the number, maturation status, or T-cell receptor (TCR) repertoire. However, the frequency of Treg cells was reduced by half in both MARCH1 KO and MHCII (K>R) KI mice. This reduction accompanied neither increased apoptosis nor decreased proliferation of Treg cells.

To test whether MHCII ubiquitination plays a role in Treg cell development, we employed two mouse models in which antigen-specific Treg cells develop by thymic presentation of the specific antigens. In one model, antigen was expressed by mTEC but was transferred to and presented by DCs. In the other model, antigen was specifically taken up by DCs from the circulation and presented by DCs. In both models, development of antigen-specific Treg cells was critically dependent on MHCII ubiquitination as it was largely abolished in MARCH1 KO, and MHCII (K>R) KI mice. Notably, deletion of antigen-specific $CD4^+$ thymocytes was mostly intact. This finding indicates that MHCII ubiquitination plays a significant and specific role in Treg development in the thymus. Lastly, we compared the ability of DCs derived from wildtype, MARCH1 KO, and MHCII (K>R) KI mice to generate Treg cells. DCs derived from MARCH1 KO and MHCII (K>R) mice generated Treg cells at significantly reduced levels compared with DCs from WT mice. Taken together, these findings strongly suggest that MHCII ubiquitination plays an important and specific role in DC-mediated Treg development in the thymus.

The specific mechanism by which MHCII ubiquitination promotes DC generation of Treg cells remains to be identified. A prevailing dogma is that strong avidity interaction between APCs and thymocytes mediates clonal deletion while intermediate avidity mediates Treg differentiation (82, 88). By this theory, one can envision that DCs of MARCH1 KO or MHCII (K>R) KI mice interacted with thymocytes with strong avidity by increased pMHCII

levels and that this high avidity interaction led the interacting thymocytes to a death rather than Treg cell differentiation. However, we did not find any increase in the deletion of $CD4^+$ thymocytes in either MARCH1 KO mice or MHCII (K>R) KI mice (77). In addition, reducing a dose of antigens, which would reduce the number of pMHCII on DCs and thus the avidity of these cells to interacting thymocytes, did not increase Treg cell differentiation both *in vivo* and *in vitro* (77). Thus, it is not likely to be the increased pMHCII that resulted MARCH1 KO and MHCII (K>R) KI mice to produce Treg cells at lower numbers. In addition to MHCII, B7 (CD86/CD80) costimulatory molecules play an essential role for Treg development by interacting with CD28 on thymocytes; mice deficient in B7 in APCs and mice deficient CD28 in T cells both exhibited a marked reduction in the number of Treg cells in the thymus (89–91). A recent study has shown that strong costimulation exerts a negative effect in Treg cell differentiation in the periphery (92). We found that CD86 level was very high in DCs in MARCH1 KO mice. Although this increase in CD86 expression could have exerted a negative effect on Treg development in these mice, the same reasoning cannot be applied to MHCII (K>R) KI mice as these mice express CD86 at a normal level. Thus, CD86 level does not seem to be relevant to the unsuccessful Treg development observed in MHCII (K>R) KI mice.

While pMHCII-TCR and B7-CD28 interactions are indispensable for Treg development, there are other interactions that make a significant contribution to it. One such interaction is the interaction between TNF receptor superfamily (TNFRSF) such as GITR, Ox40, and TNFR2 expressed on thymocytes and its ligands expressed on APCs (93). Another important interaction is the interaction between cytokine receptors IL-2R, IL-15R, and IL-7R expressed on thymocytes and each of specific cytokines (94). Both of these interactions potentiate TCR signaling in thymocytes, and thus provide additive signals to thymocytes with lower TCR affinity enriching for such cells in the repertoire of Treg cells. Perhaps, MHCII ubiquitination may play an important role for DCs to express ligands of TNFRSF, IL-2, IL-15, or receptors capable of trans-presenting these cytokines (95, 96). This scenario brings a possibility that MHCII ubiquitination is not absolutely required for DCs to select Treg cells, but equip DCs with the ability to differentiate thymocytes with low TCR affinity into Treg cells, thus contributing to the breadth of Treg cell repertoire (97). It will be interesting to examine Treg cell repertoire in MARCH1 KO or MHCII (K>R) KI mice and see whether a specific group of TCR Treg cells are missing in their repertoire, and if so, whether they represent the whole cohort or a subgroup of Treg cells generated by DCs, some of which have been recently identified (98).

The functional significance to the role of MHCII ubiquitination in Treg development remains to be seen. As stated early, neither MARCH1 KO nor MHCII (K>R) KI mice develop any obvious diseases under SPF condition. It may suggest that Treg cell compartment developing in a MHCII ubiquitination-dependent manner may be dispensable for mice to maintain immune homeostasis under this condition. Alternatively, these mice may be incompetent to properly respond to immune stimuli considering the possibility DCs in these mice do not produce inflammatory cytokines at sufficient levels and do not activate T cells efficiently. A new strategy appears to be in need by which the functionality of Treg

cells generated in the absence of MHCII ubiquitination can be examined in isolation from all the rest of negative effects that MHCII ubiquitination makes in animals.

Future direction

Although it is clear that MHCII ubiquitination controls MHCII intracellular transport, its impact in antigen presentation is not clearly established. A couple of peptide antigens tested so far were presented at higher levels in cells that express ubiquitination-resistant MHCII. These peptides were mostly derived from proteins endocytosed by DCs or from those expressed by and localized in the lysosomes in DCs (55). If these peptides were generated in the lysosomes, they are likely to be presented by newly synthesized MHCII, which come to lysosomes via the invariant chain, load lysosomally processed peptides, and travel to cell surface. As MHCII is ubiquitinated after it reaches the plasma membranes, the antigen presentation mediated by the newly synthesized MHCII is not likely to be influenced by the lack of MHCII ubiquitination. However, antigen presentation mediated by the recycling MHCII could be affected by the lack of MHCII ubiquitination, because MHCII recycling is dependent on MHCII endocytosis and post-endocytic sorting, the intracellular pathways controlled by ubiquitination. A previous study has shown that a few specific peptides derived from the influenza hemagglutinin are presented only by recycling MHCII (52). Whether presentation of these peptides is affected by the lack of MHCII ubiquitination will be an interesting question to ask. One could also compare the repertoire of whole peptides presented by MHCII in the presence or absence of ubiquitination. This analysis will help better assessing the impact of MHCII ubiquitination in presentation of diverse peptides, which is important for establishing tolerance as well as immunity.

The role of MHCII ubiquitination in functions independent of antigen presentation begins to emerge (Fig. 1). We speculate that some, if not all, may be mediated by the ubiquitin chain attached to MHCII acting as an intracellular signaling molecule. A previous study has shown that intracellular MHCII plays an important role in TLR signaling by interacting with CD40 and Btk. Possibly, this interaction may be mediated by the ubiquitin chain attached to MHCII. Some other signaling may also involve the ubiquitin chain on MHCII. When antigen-presenting APCs interact with antigen-specific $CD4^+$ T cells or thymocytes, this interaction induces signaling not only to T cells but also to APCs (99). Such signaling is mediated by MHCII engaged with TCR or by CD40 engaged with CD40L (100, 101). MHCII engagement has been shown to trigger calcium mobilization and cAMP production that stimulates protein tyrosine kinase C translocation to the nucleus in B cells (102, 103). CD40 engagement triggers NF-κB signaling in DCs as well as B cells (104–106). The outcome of this signaling is likely to influence interacting T cells, potentially regulating their activation or differentiation. It will be interesting to test whether DCs lacking ubiquitinated MHCII respond to T cells differently.

To better define the functional role of MHCII ubiquitination, it will be important to identify the specific proteins that interact with MHCII-attached ubiquitin chains and the specific linkage that elongates the chain. Over 20 ubiquitin-binding domains (UBDs) are present in mammals (107). They are frequently found in molecules that mediate intracellular signaling including TLR signaling, and exist at multiple copies, either of the same or a different

structure (108–110). Some UBD binds ubiquitin chains in a linkage-specific manner, and this binding often leads to a specific outcome including kinase activation, protein interaction, and protein transport (111). Ubiquitin linkage alone also associates with functional outcomes of ubiquitination; the K48 linkage has been associated with proteasomal degradation, the K63 linkage with membrane trafficking and signaling, and the most recently characterized N-terminal amino group linkage with signaling (107, 112). Continuing development of new methodologies promise to identify in near future these two effector components associated with ubiquitinated MHCII, which may have a direct relevance to the cellular function of MHCII ubiquitination.

Acknowledgments

This work is supported by the UCSF Sandler Asthma Basic Research Center and the National Institute of Health, USA, Grant R01 GM105800.

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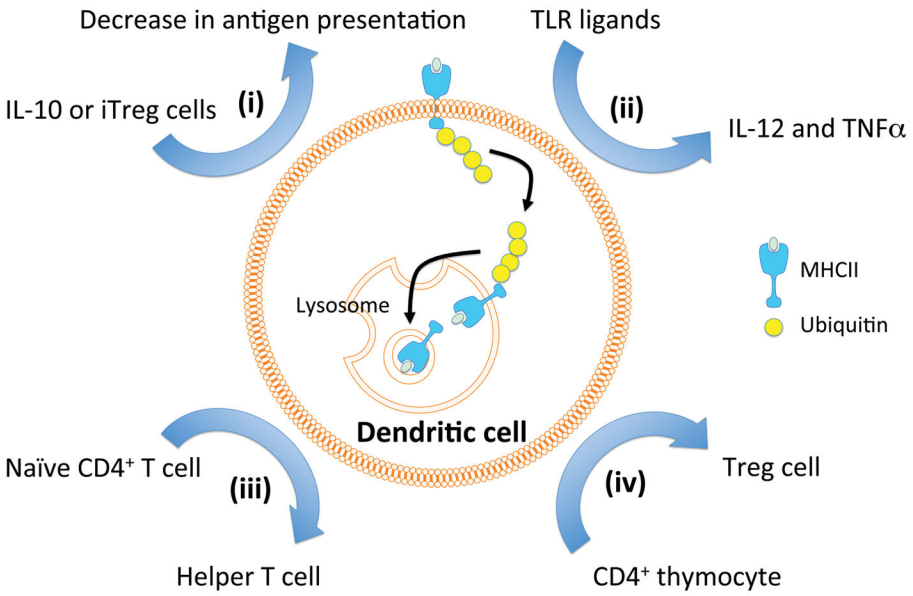

Fig. 1. Functional role of MHCII ubiquitination

(i) MHCII ubiquitination implements the immune regulatory effect of IL-10 or induced Treg (iTreg) cells by suppressing DC/monocyte surface expression of MHCII (30, 61, 62). This role is attributed to the effect of ubiquitination in promoting MHCII endocytosis and lysosomal degradation. (ii) MHCII ubiquitination promotes TLR-mediated inflammatory cytokine production in DCs and possibly B cells (73, 74). (iii–iv) MHCII ubiquitination potentiates the ability of DCs to activate antigen-specific naive $CD4^+$ T cells (76) and differentiate $CD4^+$ thymocytes into regulatory T cells (77). The specific mechanisms are not clearly understood but appear to be independent of the antigens that MHCII molecules present.

Table 1

Conservation of a lysine in the cytoplasmic tail of MHCII beta chains

Species	MHCII beta	Cytoplasmic tail amino acid	Accession number
Human	DRB1	RNQ**K**GHSGLQPTGFLS	NP_002115.2
	DRB5	KNQ**K**GHSGLHPTGLVS	NP_002116.2
	DPB1	RSK**K**VQRGSA	NP_002112.3
	DQB1	RSQ**K**GPQGPPPAGLLH	NP_001230890.1
Chimpanzee	DRB5	KNQ**K**GHSGLHPTGLVS	XP_001165809.3
	DPB1	RSK**K**VQRGSA	XP_518391.3
	DQB	RSQ**K**GLLH	XP_003339166.1
Monkey	DRB1	RNQ**K**GHSGLQPTGLLS	NP_001037726.1
	DPB	RSK**K**VQRGSV	NP_001127765.1
	DQB1	RSQ**K**GLPH	NP_001127769.1
Mouse	H2-Ab1	RSQ**K**GPRGPPPAGLLQ	NP_996988.2
	H2-Eb1	RNQ**K**GQSGLQPTGLLS	NP_034512.2
Dog	DRB1	RNQ**K**GHSGLQPTGLLS	NP_001014768.1
	DQB1	RSQ**K**GSRGSPPAGLLH	NP_001014403.1
Cow	DRB	RNQ**K**GHPTLQPTGLLS	XP_005201033.1
	DQB	RSQ**K**GLMR	NP_001074392.1
Chicken		CSK**K**GQPDPTSPGILN	XP_423039.4
Frog		RSK**K**TMTRFSVVQNENLMS	NP_001039259.1