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Ubiquitination of CD86 Is a Key Mechanism in Regulating Antigen Presentation by Dendritic Cells

Günther Baravalle,* Hyesuk Park,* Megan McSweeney,* Mari Ohmura-Hoshino,† Yohei Matsuki,‡ Satoshi Ishido,‡ and Jeoung-Sook Shin*

Dendritic cells (DCs) require costimulatory molecules such as CD86 to efficiently activate T cells for the induction of adaptive immunity. DCs maintain minimal levels of CD86 expression at rest, but upregulate levels upon LPS stimulation. LPS-stimulated DCs produce the immune suppressive cytokine IL-10 that acts in an autocrine manner to regulate CD86 levels. Interestingly, the underlying molecular mechanism behind the tight control of CD86 is not completely understood. In this study, we report that CD86 is ubiquitinated in DCs via MARCH1 E3 ubiquitin ligase and that this ubiquitination plays a key role in CD86 regulation. Ubiquitination at lysine 267 played the most critical role for this regulation. CD86 is ubiquitinated in MARCH1-deficient DCs to a much lesser degree than in wild-type DCs, which also correlated with a significant increase in CD86 expression. Importantly, CD86 is continuously ubiquitinated in DCs following activation by LPS, and this was due to the autocrine IL-10 inhibition of MARCH1 downregulation. Accordingly, DCs lacking MARCH1 and DCs expressing ubiquitination-resistant mutant CD86 both failed to regulate CD86 in response to autocrine IL-10. DCs expressing ubiquitination-resistant mutant CD86 failed to control their T cell-activating abilities at rest as well as in response to autocrine IL-10. These studies suggest that ubiquitination serves as an important mechanism by which DCs control CD86 expression and regulate their Ag-presenting functions. *The Journal of Immunology, 2011, 187: 2966–2973.

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D86 is a costimulatory molecule that is mainly expressed on APCs such as dendritic cells (DCs) (1, 2). CD86 on DCs engages with CD28 on T cells, which provides T cells with the costimulatory signals that significantly lower the activation threshold and allow naive T cells to be readily activated (1, 2). DCs dynamically regulate CD86 levels depending on their need to harness or reduce their T cell-activating ability. DCs upregulate CD86 expression following their contact with microbial Ags (3), which presumably facilitates activation of microbe-specific T cells capable of combating infection. DCs downregulate CD86 expression following exposure to IL-10, an immune-suppressive cytokine produced by various immune cells (4–6). Importantly, DCs themselves produce IL-10 following stimulation by LPS (4, 5). This DC-produced IL-10 inhibits LPS-induced CD86 increase in DCs by acting in an autocrine manner, thus preventing excessive CD86 expression.

The molecular mechanism underlying CD86 regulation has begun to emerge. A recent study demonstrated that DCs of MARCH1-deficient mice expressed significantly elevated CD86 levels, suggesting a role for MARCH1 in CD86 regulation (7). MARCH1 is a membrane-anchored E3 ubiquitin ligase that has previously been shown to ubiquitinate MHC class II mediating its lysosomal degradation (8, 9). A more recent study has shown that activated human monocytes dramatically upregulate MARCH1 expression upon incubation with recombinant IL-10 (10). This MARCH1 upregulation inversely correlated with CD86 expression (10). Most recently, Tze et al. (11) have demonstrated that mouse bone marrow-derived DCs (BMDCs) also upregulated MARCH1 when they were incubated with IL-10. Furthermore, pretreatment of BMDCs with IL-10 effectively suppressed LPS-induced CD86 increase only when DCs expressed MARCH1 (11). This study suggested that MARCH1 plays an important role in IL-10-mediated regulation of CD86 in DCs, but the specific mechanism by which MARCH1 regulates CD86 was not clearly defined. None of the studies thus far have demonstrated whether CD86 is ubiquitinated and, if so, whether this ubiquitination is achieved by MARCH1 in DCs.

MARCH1’s only physiologic substrate identified thus far is MHC class II (8, 12). In this study, we demonstrate that CD86 is ubiquitinated in DCs and that this ubiquitination is also mediated by MARCH1. We further demonstrate that CD86 ubiquitination plays an important role in DCs to regulate their CD86 expression and their T cell-activating abilities both at rest and in response to autocrine IL-10.

Materials and Methods

Mice and cells

BMDCs from C57/BL6 mice (The Jackson Laboratory), CD86<sup>−/−</sup> mice (The Jackson Laboratory), and MARCH1<sup>−/−</sup> mice (8) were grown as previously described (13). B blasts from CD86<sup>−/−</sup> mice were generated by treating splenic B cells with 25 μg/ml LPS overnight. CD8<sup>+</sup> T cells from OT-1 mice (The Jackson Laboratory) were isolated from lymph nodes using a T cell isolation kit (StemCell Technologies).

Plasmids and retroviral transduction

The cDNA of mouse CD86 and MARCH1 was cloned into the LZRS-pBMN plasmid using EcoRI and XhoI sites. Internal ribosome entry site-enhanced GFP (EGFP) was previously inserted using NotI sites of the LZRS vector.
(13). All CD86 mutant constructs and the mutant MARCH1 (I66A, W97A) construct were generated by site-directed mutagenesis. The CD86-EGFP construct was generated by PCR, eliminating the stop codon of CD86, and cloning into LZRS-EGFP (where EGFP was inserted between the BamHI and EcoRI sites) using a BamHI site. mCherry-T2A–MARCH constructs were generated by linking the cDNAs of mCherry and MARCH with the self-cleaving 2A peptide (14). Generation of retrovirus and subsequent DC transduction was performed as previously described (13).

Flow cytometry and cell sorting
Cells were incubated with indicated fluorochrome-conjugated Abs and analyzed on an FACS LSRII system (BD Biosciences). All transduced BMDCs were sorted on a FACS Aria cell sorter (BD Biosciences). Only GFP<sup>+</sup> DCs were used in the experiments because the levels of CD86 expressed on GFP<sup>−</sup> DCs approximated levels of endogenous CD86 expression. BMDCs and splenic DCs used to immunoprecipitate endogenous CD86 were prepared with a murine DC isolation kit (Miltenyi Biotec).

Immunoprecipitation and Western blot
BMDCs or splenic DCs were solubilized in Triton-lysis buffer as described (13). The soluble cell lysates were mixed with CD86 Ab (GL-1) and incubated for 2 h at 4°C. Protein G-Sepharose beads were added, and samples were incubated for an additional 2 h at 4°C. The beads were washed three times with Triton-lysis buffer prior to Peptide-N-Glycosidase F treatment. Thereafter, samples were run for SDS-PAGE, blotted to polyvinylidene difluoride membranes, and developed with the indicated Abs.

Surface biotinylation
Plasma membrane proteins of CD11c bead-purified DCs pretreated with LPS in the presence or absence of anti-IL-10R Ab were biotinylated by incubating cells with the membrane-impermeable biotinylation reagent sulfo-NHS–biotin according to the manufacturer’s protocol (Pierce).

In vitro T cell activation assay
BMDCs were incubated with increasing amounts of SIINFEKL peptide and analyzed on an FACS LSRII system. Samples were normalized for β-actin expression.

Quantitative RT-PCR analysis
Total RNA was isolated by TRZol (Invitrogen) extraction, and first-strand cDNA was generated using the SuperScript III system (Invitrogen) and oligo (dT) as primers. Quantitative PCR was performed using SYBR Green reagents (5-prime) on an Eppendorf realplex system. Samples were normalized for β-actin expression.

Results
CD86 is ubiquitinated in DCs
To determine if CD86 is ubiquitinated in DCs, we immunoprecipitated CD86 from BMDCs and performed Western blot analysis. The anti-CD86 Ab reacted with proteins ranging from 70–80 kDa, consistent with glycosylated forms of CD86 (Fig. 1A, right panel). The anti-ubiquitin Ab detected proteins ranging from 100–150 kDa (Fig. 1A, left panel), consistent with glycosylated CD86 that has multiple ubiquitin molecules attached. Treatment of the immunoprecipitates with a deglycosylating enzyme, PNGase F, resulted in an expected downward shift of CD86 to a single band at ~32 kDa (Fig. 1A, right panel). Immunoblotting for ubiquitinated proteins demonstrated a similar downward shift of the ubiquitinated species, which formed a ladder at increments of ~8 kDa, consistent with the addition of ubiquitin monomers (Fig. 1A, left panel). The fusion of EGFP to CD86 increased the m.w. of CD86, as shown by the upward shift of the anti-CD86 Ab-reactive protein band (Fig. 1B, right panel). Accordingly, the ubiquitin ladder also shifted upwards, indicating that the ubiquitinated species is indeed CD86 (Fig. 1B, left panel). Lastly, we also verified ubiquitination of endogenous CD86 in splenic DCs (Fig. 1C). Taken together, these data demonstrate CD86 ubiquitination in DCs.

CD86 ubiquitination limits CD86 expression in DCs
We then examined the specific role of CD86 ubiquitination in DCs. To create an ubiquitination-resistant CD86, we generated a retroviral construct in which all five cytoplasmic lysines (K) of CD86 were replaced by arginines (R). We also generated a retrovirus encoding wild-type CD86 for a comparison. Both retroviral constructs additionally encoded GFP bicistronically. Each retrovirus was transduced into DCs cultured from CD86-deficient mice. When the wild-type CD86 immunoprecipitate was analyzed, ubiquitinated species appeared with a ladder-like pattern (Fig. 2A), similar to endogenous CD86 (Fig. 1A, 1C). However, ubiquitinated species were absent in the immunoprecipitate of CD86 (K>R) mutant (Fig. 2A), confirming that CD86 (K>R) mutant is resistant to ubiquitination. Concurrently, we found that total CD86 (Fig. 2A) and surface CD86 (Fig. 2B) levels were both elevated in DCs expressing CD86 (K>R) mutant. However, GFP and CD80 expression levels were similar in DCs expressing wild-type and mutant CD86, indicating comparable efficiencies of transduction and levels of maturation in these cells (Fig. 2B). These findings indicate that DCs regulate CD86 expression via ubiquitination.

Ubiquitination at lysine 267 plays the primary role in regulating CD86
CD86 has five cytoplasmic lysines, which can all be potential targets of ubiquitination (Fig. 3A). We examined whether all five of the cytoplasmic lysines are equally important for the ubiquitination and regulation of CD86 or whether a specific lysine is more critical than others. We made retroviral constructs encoding a series of CD86 mutants, in which the cytoplasmic lysines were sequentially replaced with arginines (R). As shown in Fig. 3B, lysine 267 was found to be most critical and also sufficient for the regulation of CD86 surface expression (see KRKKK and RKRRR). To determine whether lysine 267 is the specific target of ubiquitination, we expressed wild-type CD86, CD86 (RRRRR), CD86 (RKRRR), and CD86 (KRKKK) in CD86-deficient DCs and analyzed the ubiquitination of CD86. As shown in Fig. 3C, the CD86 (RKRRR) mutant was ubiquitinated to the same degree as wild-type CD86. Its total CD86 levels were comparably low.
Interestingly, CD86 (KRKKK) was also ubiquitinated to a similar degree as wild-type CD86. However, its total levels were much higher compared with wild-type CD86, but still lower than CD86 (RRRRR) (Fig. 3C). These findings indicate that multiple lysines can be ubiquitinated and involved in CD86 regulation in DCs. However, lysine 267 ubiquitination exerts the most potent and sufficient effect for CD86 regulation.

**CD86 is ubiquitinated by MARCH1**

Having found that CD86 is ubiquitinated in DCs, we examined whether the ubiquitination is mediated by MARCH1. As shown previously (7), BMDCs cultured from MARCH1-deficient mice expressed much higher levels of CD86 than BMDCs cultured from wild-type mice (Fig. 4A). Notably, other hematopoietic cells also expressed elevated levels of CD86 in MARCH1-deficient mice (Supplemental Fig. 1). The expression of other costimulatory molecules such as CD80, CD83, and CD40 and adhesion molecules such as ICAM-1 remained identical (data not shown). To determine the requirement of MARCH1 in CD86 ubiquitination, we performed the same immunoprecipitation experiment as described earlier using MARCH1-deficient BMDCs. We found a marked reduction in CD86 ubiquitination in MARCH1-deficient DCs (Fig. 4B), whereas overall ubiquitination was not affected (data not shown). Reduced CD86 ubiquitination was accompanied by elevated total CD86 (Fig. 4B). Importantly, CD86 increases due to MARCH1 deficiency could be rescued by the retroviral expression of wild-type MARCH1, but not by the expression of E3 ligase mutant MARCH1 (Fig. 4C). This finding shows that MARCH1 regulates CD86 expression via its ubiquitin ligase activity, but not via any other accessory function. Taken together, these data demonstrate the important role of MARCH1 in mediating CD86 ubiquitination in DCs.

**Efficient CD86 regulation by MARCH1 requires lysine 267**

A small but appreciable amount of ubiquitinated CD86 was detected in MARCH1-deficient DCs, implying that other ubiquitin ligases could also mediate CD86 ubiquitination in DCs (Fig. 4B).

Previous studies have shown that forced expression of MARCH1, MARCH2, and MARCH8 resulted in a marked reduction of surface CD86 in transfected HeLa cells and other cell lines (15, 16). This finding implies that MARCH2 and MARCH8 could also ubiquitinate CD86, thus participating in its regulation. To better...
characterize the role of these MARCH proteins, we examined their specificity for lysines of CD86. Specifically, we wished to determine whether MARCH1, MARCH2, or MARCH8 can adequately regulate CD86 via lysine 267.

As a model, we employed mouse B blasts, in which CD86 surface expression was not affected by the mutation of CD86 lysines (Fig. 5A) unless MARCH1, MARCH2, or MARCH8 were coexpressed (Fig. 5B–D). We transduced CD86-deficient B blasts using two retroviral constructs. One virus encoded for GFP and CD86, either wild-type or lysine mutant, and the other virus encoded for mCherry, a red fluorescent protein, and a MARCH ubiquitin ligase. Following transduction, we examined CD86 surface levels in mCherry+GFP+ cells. Upon MARCH1 coexpression, we found that surface expression of mCherry+GFP+ cells are shown as mean fluorescence intensities (MFI). Data are representative of two independent experiments.

CD86 ubiquitination in DCs is facilitated by autocrine IL-10

A recent study has implicated an important role of MARCH1 in IL–10–mediated suppression of CD86 in DCs (11). However, whether this suppression is mediated by CD86 ubiquitination is not clear. DCs not only react to exogenous IL-10. Upon activation, DCs endogenously produce IL-10, which can then act in an autocrine manner to prevent excessive CD86 expression (4, 5). However, the underlying mechanism has not been elucidated. Therefore, we investigated whether the regulation of CD86 by autocrine IL-10 in DCs involves CD86 ubiquitination and, if so, whether this ubiquitination is mediated by MARCH1.

Consistent with previous reports (4, 5), blockade of autocrine IL-10 signaling by adding an anti–IL-10R Ab markedly enhanced the LPS-induced increase of surface CD86 in DCs (Fig. 6A). Notably, this enhanced surface CD86 expression did not involve any increase in CD86 transcription (Fig. 6B), but involved a decrease in CD86 ubiquitination and an increase in total CD86 (Fig. 6C, 6D). Because ubiquitination mediates endocytosis and subsequent degradation of many membrane proteins (17, 18), we asked whether the reduced CD86 ubiquitination following anti–IL-10R Ab treatment involves a reduced turnover of surface CD86. To test this, we incubated DCs with membrane-impermeable biotinylating agents at 4°C to label cell-surface proteins. Half of the cells were lysed immediately, and the other half was further incubated at 37°C for 16 h prior to lysis. Each cell lysate was immunoprecipitated using anti-CD86 Ab, and the precipitates were blotted with HRP-conjugated streptavidin to determine the levels of biotinylated CD86. We found ~80% reduction of biotinylated CD86 within 16 h in LPS-stimulated DCs (Fig. 6E, 6F), indicating that 80% of CD86 molecules that were on the surface have been degraded during this 16 h chase. However, the degradation was markedly delayed when DCs had been treated with anti–IL-10R Ab. Taken together, IL-10 produced by LPS-stimulated DCs exerts an inhibitory effect on CD86 expression by promoting CD86 ubiquitination and turnover and not by reducing CD86 transcription.

Regulation of CD86 in DCs via autocrine IL-10 is dependent on MARCH1

To determine the role of MARCH1 in autocrine IL-10–mediated regulation of CD86, we first examined the expression of MARCH1 in DCs in response to autocrine IL-10. We found that blockade of IL-10 signaling resulted in a significant reduction in MARCH1 expression in DCs (Fig. 7A). This reduction correlated well with the reduction in CD86 ubiquitination (Fig. 6C, 6D). Next, we determined whether MARCH1 is required for autocrine IL-10–mediated CD86 regulation. Blockade of IL-10 signaling markedly enhanced the LPS-induced CD86 increase in MARCH1-expressing wild-type DCs, but not in MARCH1-deficient DCs (Fig. 7B). MARCH1-deficient DCs expressed CD86 at consistently high levels regardless of IL-10 signaling (Fig. 7B). To verify that MARCH1-deficient DCs are not defective in IL-10 signaling per se, we examined CD80 expression, which is known to be downregulated by IL-10 signal-
ing. LPS markedly increased CD86 expression, and the increase was enhanced by the blockade of IL-10 signaling both in wild-type and MARCH1-deficient DCs (Fig. 7B). These findings indicate that MARCH1 is specifically required for autocrine IL-10 regulation of CD86 in DCs. Lastly, we determined whether MARCH1 mediates CD86 ubiquitination in DCs in response to autocrine IL-10. To this end, we examined CD86 ubiquitination in LPS-activated MARCH1-deficient DCs. As shown in Fig. 7C, polyubiquitinated CD86 mostly disappeared in MARCH1-deficient DCs compared with wild-type DCs (Fig. 6C). However, oligoubiquitinated CD86, which had approximately two to three ubiquitin monomers attached, was still detected, indicating that those ubiquitinated CD86 species were formed by other ubiquitin ligase(s). This finding suggests that MARCH1 mainly mediates polyubiquitination of CD86 and that this polyubiquitination is involved in regulating CD86 levels in DCs in response to autocrine IL-10.

**Autocrine IL-10–mediated CD86 regulation in DCs requires CD86 ubiquitination**

To further prove that CD86 ubiquitination is required for CD86 regulation by autocrine IL-10, we retrovirally expressed ubiquitination-resistant CD86 in DCs and examined how these cells control CD86 levels in response to autocrine IL-10. As shown in Fig. 8A, DCs expressing wild-type CD86 markedly enhanced CD86 expression when IL-10 signaling was blocked. However, DCs expressing CD86 (K>R) mutant constantly expressed high CD86 levels regardless of IL-10 signaling (Fig. 8B). Furthermore, this defective CD86 regulation was completely rescued by the presence of lysis 267 (Fig. 8C). We also examined the effects of autocrine IL-10 on total CD86. Expression of wild-type CD86 and CD86 (RKRRR) mutant increased upon blockade of IL-10 signaling (Fig. 8D, 8F), whereas expression of CD86 (K>R) mutant did not (Fig. 8E). These findings demonstrate that autocrine IL-10–mediated regulation of CD86 expression is dependent on its ubiquitination.

**CD86 ubiquitination plays a significant role in controlling DC-mediated Ag presentation**

Thus far, our findings indicate that CD86 ubiquitination serves as an important mechanism by which DCs express minimal levels of CD86 at rest and by which DCs express increased but controlled levels of CD86 following LPS stimulation. Therefore, we wished to see whether CD86 ubiquitination also plays an important role in controlling T cell-activating abilities of DCs. Through our studies, we have developed two ways to interfere with CD86 ubiquitination in DCs. One is to ablate MARCH1 expression, and the other is to replace wild-type CD86 with ubiquitination-resistant mutant CD86 (K>R). Because the former may abolish the ubiquitination of so far unknown MARCH1 substrates in addition to abolishing CD86 ubiquitination, we chose the latter to specifically interfere with CD86 ubiquitination and determine its consequences on DC-mediated Ag presentation.

First, we determined whether resting DCs that fail to ubiquitinate CD86 can activate T cells more efficiently. We employed an in vitro Ag presentation assay in which peptide-loaded DCs were cocultured with peptide-specific naive CD8+ T cells. T cell activation was then determined by measuring the production of IL-2 by ELISA. We found that DCs expressing CD86 (K>R) mutant activated T cells to a much greater degree than DCs expressing wild-type CD86.
expressing wild-type CD86 (\(\text{RKRRR}\)) treated as indicated. Western blots of CD86-\(\text{WT}\) BMDCs expressing wild-type CD86 (D), CD86 (K > R) (E), or CD86 (RKRRR) (F) treated as indicated.

FIGURE 8. Control of CD86 expression by autocrine IL-10 requires CD86 ubiquitination. Flow cytometry of CD86 \(-/-\) BMDCs transduced with retrovirus encoding wild-type CD86 (A), CD86 (K > R) (B), or CD86 (RKRRR) (C) and treated as indicated. Western blots of CD86-\(\text{WT}\) BMDCs expressing wild-type CD86 (D), CD86 (K > R) (E), or CD86 (RKRRR) (F) treated as indicated.

Notably, MHC class I expression was identical in DCs expressing wild-type or mutant CD86 (Supplemental Fig. 2). This finding indicates that CD86 ubiquitination plays a significant role in DCs to regulate their T cell-activating abilities in the resting state. Next, we determined whether activated DCs that fail to ubiquitinate CD86 also fail to control their T cell-activating ability in response to autocrine IL-10. As shown in Fig. 9B, blockade of IL-10 signaling by anti–IL-10R Ab markedly increased the ability of DCs to activate T cells only when DCs expressed wild-type CD86 but not when DCs expressed CD86 (K > R) mutant. In addition, CD86 (K > R)-expressing DCs activated T cells better than wild-type CD86-expressing DCs regardless of IL-10 signaling. These findings indicate that CD86 ubiquitination in regulating T cell-activating abilities of DCs in response to autocrine IL-10.

Discussion

In conclusion, our study demonstrates that DCs ubiquitinate CD86 and that its ubiquitination plays an important role in regulating CD86 in DCs both at rest and upon activation in response to autocrine IL-10. Our study also demonstrates that CD86 ubiquitination plays a significant role in DCs to regulate their T cell-activating ability.

Ubiquitination has been implicated in CD86 regulation for a decade. Early studies demonstrated that CD86 is downregulated in human B cell lines expressing MIR2, a viral RING E3 ubiquitin ligase (19, 20). A subsequent study revealed that MIR2 ubiquitinates CD86 and induces CD86 endocytosis and subsequent degradation (21). Given the fact that viruses often usurp host physiology for their advantages, it was postulated that CD86 is ubiquitinated and thus regulated by mammalian E3 ubiquitin ligases. Soon afterward, the MARCH protein family was identified in mammalian cells (9, 15). Among nine family members examined, forced expression of MARCH1, -2, and -8 induced a significant reduction of surface CD86 in several cell lines (16). Furthermore, ubiquitinated CD86 was detected in MARCH8-transfected cell lines (15). More recently, MARCHI-deficient mice were developed (8). These mice were found to express significantly elevated CD86 levels in DCs, suggesting a role for MARCH1 in regulating CD86 in DCs (7). However, evidence that CD86 is ubiquitinated in a physiological setting and that this ubiquitination is mediated by MARCH1 has been missing until now.

Our study demonstrates for the first time, to our knowledge, that CD86 is ubiquitinated in DCs and that this ubiquitination is mediated by MARCH1. MARCH1 appears to polyubiquitinate CD86, which then is required for the regulation of CD86 levels in DCs. The specific mechanism by which this ubiquitination controls CD86 expression is not clear. Ubiquitination mediates proteasomal degradation of proteins misfolded in the endoplasmic reticulum (22). Ubiquitination mediates endocytosis and subsequent lysosomal or proteasomal degradation of many transmembrane proteins (17, 18, 23). The specific involvement of endoplasmic reticulum, proteasomes, and lysosomes in ubiquitin-mediated CD86 regulation remains to be determined.

Our data indicate that additional E3 ligase(s) can ubiquitinate CD86 in DCs. Appreciable amounts of ubiquitinated CD86 were detected in MARCH1-deficient DCs, in which the major ubiquitinated species were oligoubiquitinated. The functional role of this oligoubiquitination is not clear. It may also contribute to CD86 regulation but to a lesser degree than MARCH1-mediated polyubiquitination. Oligoubiquitinated cargo is much less receptive to both proteasomal and lysosomal degradation than is polyubiquitinated cargo (24, 25). MARCH2 or MARCH8 might be involved in this MARCH1-independent oligoubiquitination. MARCH2 seems to specifically target lysine 280, whereas MARCH8 does not require a specific lysine to reduce CD86 levels. Nevertheless, neither MARCH2 nor MARCH8 seem to play a critical role in regulating CD86 in DCs in the resting state. DCs of MARCH2- and MARCH8-deficient mice expressed CD86 at comparable levels to wild-type mouse DCs (G. Baravalle, S. Ishido, and J.-S. Shin, unpublished observations) (26). The contribution of MARCH2 or MARCH8 for CD86 regulation may only become significant in the absence of MARCH1 or upon MARCH1 down-regulation.
We confirmed that MARCH1-deficient DCs expressed significantly elevated CD86 levels at rest. This finding indicates an important role for MARCH1-mediated ubiquitination in DCs to actively suppress CD86 expression. However, the functional role for this active CD86 regulation in resting DCs is not clear. DCs continuously present self-Ags under steady-state conditions (27). These self-Ags could be recognized by T cells, which then might lead to the activation of self-reactive T cells. DCs may prevent this unnecessary and potentially harmful T cell activation by actively suppressing CD86 expression. Notably, our in vitro Ag presentation assay indicates that resting DCs can activate Ag-specific T cells more efficiently when CD86 ubiquitination was limited. We also found that DCs continue to ubiquitinate CD86 following activation by LPS and that this ubiquitination was in part mediated by autocrine IL-10. LPS-stimulated DCs produce IL-10, which acts in an autocrine manner, inhibiting the excessive increase of CD86 in DCs (4). However, the mechanism underlying this CD86 regulation by autocrine IL-10 has not been defined. Our study indicates that autocrine IL-10 counteracts the LPS-induced downregulation of MARCH1 in DCs. This allows DCs to continuously ubiquitinate CD86 upon activation and helps to regulate CD86 expression. The mechanism by which IL-10 counteracts the effects of LPS is not clear. One potential mechanism is that IL-10 may inhibit LPS signaling, which downregulates MARCH1 expression in DCs (12, 28). Alternatively but not exclusively, IL-10 may upregulate MARCH1 expression independently of LPS signaling. By any means, MARCH1-mediated ubiquitination plays an essential role for DCs to regulate CD86 in their response to autocrine IL-10. This finding is consistent with the recent report that MARCH1 is important for DCs to suppress CD86 expression in response to recombinant IL-10 (11).

IL-10 produced by LPS-stimulated DCs also exerts a regulatory effect on Ag-presenting functions of DCs, which is likely to include the regulation of inflammatory cytokine production and the regulation of Ag-presenting molecules and costimulatory molecules (29, 30). Our study indicates that this regulatory effect of autocrine IL-10 in MHC class I-mediated DC Ag presentation is largely dependent on CD86 ubiquitination. Especially when DCs presented high doses of Ags, the regulation of T cell activation by DCs was critically dependent on CD86 ubiquitination (Supplemental Fig. 3). This finding implies that CD86 ubiquitination may play a significant role in IL-10–mediated immune regulation, perhaps in the regulation of CD86 T cell immunity. Previous studies have demonstrated that lymphocytic choriomeningitis virus chronic mouse infection model involves diminished CTL responses and hyperproduction of IL-10 by DCs (31, 32). CD86 ubiquitination in DCs may be a significant contributing factor to this infection.

In summary, our study indicates that CD86 is ubiquitinated in DCs and that this ubiquitination plays an important role not only in regulating CD86 expression but also by regulating Ag-presenting functions of DCs. Our study also demonstrates that CD86 ubiquitination is one of the important mechanisms underlying IL-10–mediated DC regulation. We speculate that CD86 ubiquitination may play additional important functional roles in the steady state as well as during immune responses, which remain to be explored.

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Disclosures
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