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**[*]

[*]Department of Microbiology and Immunology, Sandler Asthma Basic Research Center, University of California, San Francisco, San Francisco, CA 94143

[†]Laboratory for Infectious Immunity, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230-0045, Japan

Abstract

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.

CD86 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$^{-/-}$ mice (The Jackson Laboratory), and MARCH1$^{-/-}$ mice (8) were grown as previously described (13). B blasts from CD86$^{-/-}$ mice were generated by treating splenic B cells with 25 μg/ml LPS overnight. CD8$^+$ T cells from OT-I 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 fluorophore-conjugated Abs and analyzed on an FACS LSRII system (BD Biosciences). All transduced BMDCs were presorted on an FACSAria cell sorter (BD Biosciences). Only GFP[low] DCs were used in the experiments because the levels of CD86 expressed on GFP[low] DCs approximated levels of endogenous CD86 expression. BMDCs and splenic DCs used to immunoprecipitate endogenous CD86 were prepurified 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 $CD8^+$ OT-I T cells at a 1:10 DC/T cell ratio. After overnight culture, IL-2 concentrations in the supernatant were determined by ELISA.

Quantitative RT-PCR analysis

Total RNA was isolated by TRIzol (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. 1*A*, *right panel*). The anti-ubiquitin Ab detected proteins ranging from 100–150 kDa (Fig. 1*A*, *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. 1*A*, *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. 1*A*, *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. 1*B*, *right panel*). Accordingly, the ubiquitin ladder also shifted upwards, indicating that the ubiquitinated species is indeed CD86 (Fig. 1*B*, *left panel*). Lastly, we also verified ubiquitination of endogenous CD86 in splenic DCs (Fig. 1*C*). 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. 2*A*), similar to endogenous CD86 (Fig. 1*A*, 1*C*). However, ubiquitinated species were absent in the immunoprecipitate of CD86 (K > R) mutant (Fig. 2*A*), confirming that CD86 (K > R) mutant is resistant to ubiquitination. Concurrently, we found that total CD86 (Fig. 2*A*) and surface CD86 (Fig. 2*B*) 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. 2*B*). 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. 3*A*). 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 (K) were sequentially replaced with arginines (R). As shown in Fig. 3*B*, 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. 3*C*, 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. 3*C*). 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. 4*A*). 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. 4*B*), whereas overall ubiquitination was not affected (data not shown). Reduced CD86 ubiquitination was accompanied by elevated total CD86 (Fig. 4*B*). 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. 4*C*). 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. 4*B*). 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. 5*A*) unless MARCH1, MARCH2, or MARCH8 were coexpressed (Fig. 5*B*–*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 CD86 levels were markedly reduced in cells expressing wild-type CD86 (KKKKK), but not in cells expressing cytoplasmic lysine-free CD86 (RRRRR) (Fig. 5*B*), similarly to what we observed in DCs. Subsequent analysis of other CD86 mutant-expressing cells revealed that lysine 267 was critical for MARCH1 to fully downregulate surface CD86 to wild-type CD86 levels (see RRKKK and KRKKK). Lysine 267 was also sufficient for MARCH1 to reduce CD86 to its full degree (see RKRRR). Interestingly, however, lysine 267 was neither necessary nor sufficient for MARCH2 to reduce CD86 (compare KKKKK with RKRRR) (Fig. 5*C*). MARCH2-mediated regulation was critically dependent on lysine 280 (see KKRRR and RKRRR). MARCH8, similar to MARCH1, could sufficiently regulate CD86 via lysine 267

alone (Fig. 5*D*, compare KKKKK with RKRRR). However, MARCH8 effectively reduced the surface expression of all other lysine mutants except for lysine-free CD86. But notably, even lysine-free CD86 was appreciably reduced by MARCH8 (see RRRRR) (Fig. 5*D*). Taken together, the lysine 267 sufficiency observed in DCs was recapitulated in B blasts expressing MARCH1 or MARCH8. However, the lysine specificity observed in DCs was most similarly reproduced in B blasts expressing MARCH1, implicating a major role of MARCH1 in CD86 regulation in DCs.

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. 6*A*). Notably, this enhanced surface CD86 expression did not involve any increase in CD86 transcription (Fig. 6*B*), but involved a decrease in CD86 ubiquitination and an increase in total CD86 (Fig. 6*C*, 6*D*). 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. 6*E*, 6*F*), 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. 7*A*). This reduction correlated well with the reduction in CD86 ubiquitination (Fig. 6*C*, 6*D*). 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. 7*B*). MARCH1-deficient DCs expressed CD86 at consistently high levels regardless of IL-10 signaling (Fig. 7*B*). 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 signaling. LPS markedly increased CD80 expression, and the increase was enhanced by the blockade of IL-10 signaling both in wild-type and MARCH1-deficient DCs (Fig. 7*B*). 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. 7*C*, polyubiquitinated CD86 mostly disappeared in MARCH1-deficient DCs compared with wild-type DCs (Fig. 6*C*). 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. 8*A*, 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. 8*B*). Furthermore, this defective CD86 regulation was completely rescued by the presence of lysine 267 (Fig. 8*C*). 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. 8*D*, 8*F*), whereas expression of CD86 (K > R) mutant did not (Fig. 8*E*). 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 (Fig. 9*A*). 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. 9*B*, 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, MARCH1-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 downregulation.

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 CD8 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this article

**BMDC**   bone marrow-derived dendritic cell

DC   dendritic cell

EGFP   enhanced GFP

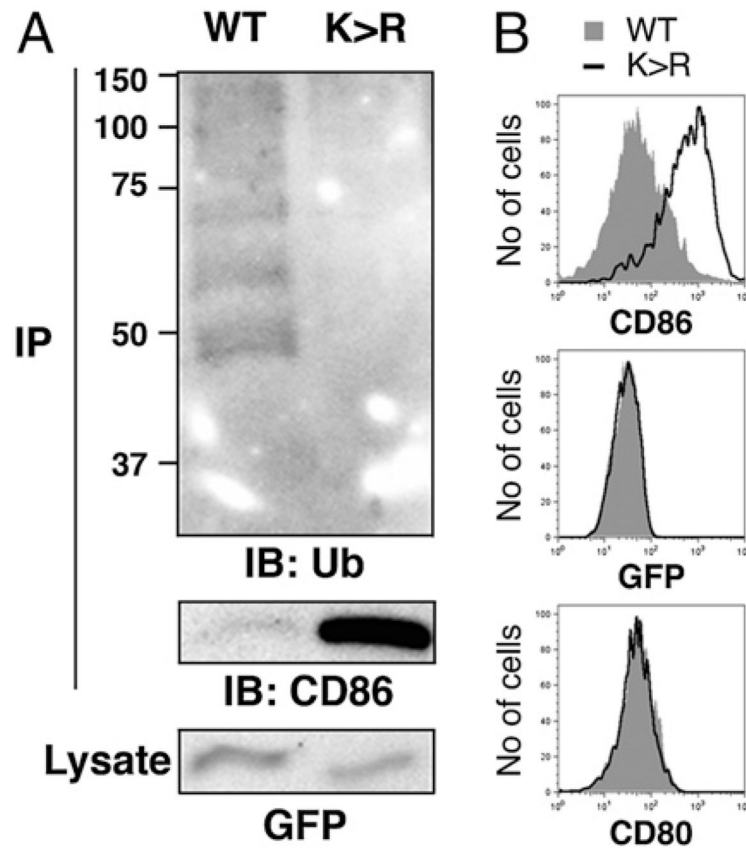**K**   lysine

R   arginine

References

1. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol. 1996; 14:233–258. [PubMed: 8717514]

2. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005; 23:515–548. [PubMed: 15771580]

3. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell. 2001; 106:255–258. [PubMed: 11509172]

4. Haase C, Jørgensen TN, Michelsen BK. Both exogenous and endogenous interleukin-10 affects the maturation of bone-marrow-derived dendritic cells in vitro and strongly influences T-cell priming in vivo. Immunology. 2002; 107:489–499. [PubMed: 12460194]

5. Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. J Immunol. 2001; 166:4312–4318. [PubMed: 11254683]

6. De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. Eur J Immunol. 1997; 27:1229–1235. [PubMed: 9174615]

7. Ohmura-Hoshino M, Matsuki Y, Mito-Yoshida M, Goto E, Aoki-Kawasumi M, Nakayama M, Ohara O, Ishido S. Cutting edge: requirement of MARCH-I-mediated MHC II ubiquitination for the maintenance of conventional dendritic cells. J Immunol. 2009; 183:6893–6897. [PubMed: 19917682]

8. Matsuki Y, Ohmura-Hoshino M, Goto E, Aoki M, Mito-Yoshida M, Uematsu M, Hasegawa T, Koseki H, Ohara O, Nakayama M, et al. Novel regulation of MHC class II function in B cells. EMBO J. 2007; 26:846–854. [PubMed: 17255932]

9. Ohmura-Hoshino M, Goto E, Matsuki Y, Aoki M, Mito M, Uematsu M, Hotta H, Ishido S. A novel family of membrane-bound E3 ubiquitin ligases. J Biochem. 2006; 140:147v154. [PubMed: 16954532]

10. Thibodeau J, Bourgeois-Daigneault MC, Huppé G, Tremblay J, Aumont A, Houde M, Bartee E, Brunet A, Gauvreau ME, de Gassart A, et al. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. Eur J Immunol. 2008; 38:1225–1230. [PubMed: 18389477]

11. Tze LE, Horikawa K, Domaschenz H, Howard DR, Roots CM, Rigby RJ, Way DA, Ohmura-Hoshino M, Ishido S, Andoniou CE, et al. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. J Exp Med. 2011; 208:149–165. [PubMed: 21220452]

12. De Gassart A, Camosseto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, Gatti E. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc Natl Acad Sci USA. 2008; 105:3491–3496. [PubMed: 18305173]

13. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, Mellman I. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. Nature. 2006; 444:115–118. [PubMed: 17051151]

14. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol. 2004; 22:589–594. [PubMed: 15064769]

15. Goto E, Ishido S, Sato Y, Ohgimoto S, Ohgimoto K, Nagano-Fujii M, Hotta H. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. J Biol Chem. 2003; 278:14657–14668. [PubMed: 12582153]

16. Bartee E, Mansouri M, Hovey Nerenberg BT, Gouveia K, Früh K. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J Virol. 2004; 78:1109–1120. [PubMed: 14722266]

17. Raiborg C, Rusten TE, Stenmark H. Protein sorting into multivesicular endosomes. Curr Opin Cell Biol. 2003; 15:446–455. [PubMed: 12892785]

18. Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu Rev Cell Dev Biol. 2003; 19:141–172. [PubMed: 14570567]

19. Ishido S, Choi JK, Lee BS, Wang C, DeMaria M, Johnson RP, Cohen GB, Jung JU. Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. Immunity. 2000; 13:365–374. [PubMed: 11021534]

20. Coscoy L, Ganem D. A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. J Clin Invest. 2001; 107:1599–1606. [PubMed: 11413168]

21. Coscoy L, Sanchez DJ, Ganem D. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. J Cell Biol. 2001; 155:1265–1273. [PubMed: 11756476]

22. Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol. 2008; 9:944–957. [PubMed: 19002207]

23. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998; 67:425–479. [PubMed: 9759494]

24. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. EMBO J. 2000; 19:94–102. [PubMed: 10619848]

25. Barriere H, Nemes C, Du K, Lukacs GL. Plasticity of poly-ubiquitin recognition as lysosomal targeting signals by the endosomal sorting machinery. Mol Biol Cell. 2007; 18:3952–3965. [PubMed: 17686993]

26. Ishido S, Matsuki Y, Goto E, Kajikawa M, Ohmura-Hoshino M. MARCH-I: a new regulator of dendritic cell function. Mol Cells. 2010; 29:229–232. [PubMed: 20213309]

27. Wilson NS, El-Sukkari D, Villadangos JA. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. Blood. 2004; 103:2187–2195. [PubMed: 14604956]

28. Young LJ, Wilson NS, Schnorrer P, Proietto A, ten Broeke T, Matsuki Y, Mount AM, Belz GT, O'Keeffe M, Ohmura-Hoshino M, et al. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. Nat Immunol. 2008; 9:1244–1252. [PubMed: 18849989]

29. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. Immunol Rev. 2008; 226:205–218. [PubMed: 19161426]

30. Chang J, Kunkel SL, Chang CH. Negative regulation of MyD88-dependent signaling by IL-10 in dendritic cells. Proc Natl Acad Sci USA. 2009; 106:18327–18332. [PubMed: 19815506]

31. Ejrnaes M, Filippi CM, Martinic MM, Ling EM, Togher LM, Crotty S, von Herrath MG. Resolution of a chronic viral infection after interleukin-10 receptor blockade. J Exp Med. 2006; 203:2461–2472. [PubMed: 17030951]

32. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB. Interleukin-10 determines viral clearance or persistence in vivo. Nat Med. 2006; 12:1301–1309. [PubMed: 17041596]
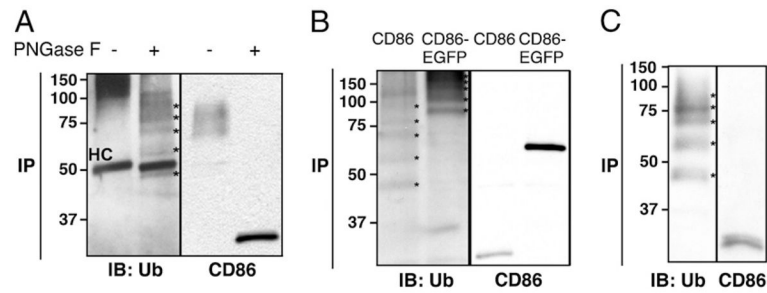
FIGURE 1.
CD86 is ubiquitinated in DCs. *A*, CD86 was immunoprecipitated (IP) from mouse BMDCs, treated with or without PNGase F, and immunoblotted (IB). HC indicates the H chain of anti-CD86 Ab. *B*, CD86$^{-/-}$ BMDCs were retrovirally transduced to express wild-type CD86 or CD86-EGFP fusion proteins. CD86 was immunoprecipitated (IP), treated with PNGase F, and immunoblotted (IB). *C*, Splenic DCs were isolated using CD11c magnetic beads. CD86 immunoprecipitates were treated and immunoblotted as described. Asterisk indicates a ladder of proteins reacting with anti-Ub Ab.

FIGURE 2.
CD86 ubiquitination regulates CD86 expression in steady-state DCs. *A*, BMDCs cultured from CD86$^{-/-}$ mice were retrovirally transduced to express wild-type CD86 (WT) and CD86 mutant in which all of the five cytoplasmic lysines of CD86 were replaced with arginines (K > R). Each retroviral construct included internal ribosome entry site-EGFP cDNA downstream of the CD86 sequence. CD86 immunoprecipitates (IP) were treated with PNGase F and then immunoblotted (IB). Cell lysates were also immunoblotted. *B*, Flow cytometry analysis of DCs expressing WT CD86 and CD86 (K > R) mutant.
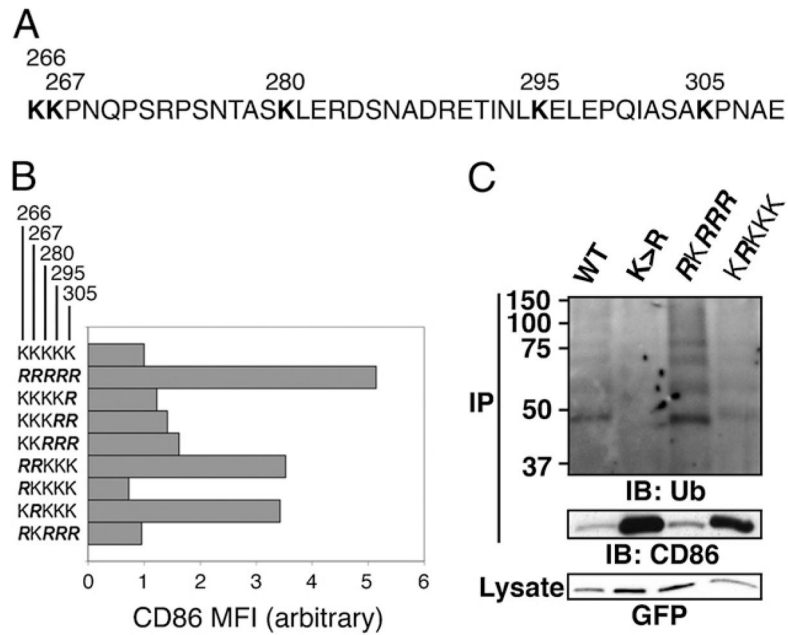
FIGURE 3.
Ubiquitination via lysine 267 is sufficient for the regulation of CD86 in DCs. *A*, Amino acid sequence of the cytoplasmic domain of mouse CD86. Lysine residues (K) are numbered and highlighted in bold. *B*, Flow cytometry analysis of CD86$^{-/-}$ BMDCs expressing wild-type CD86 and a series of CD86 mutants. Wild-type CD86 is designated as KKKKK to indicate five lysines at the cytoplasmic sites numbered. Each of CD86 mutants is designated as a combination of K and R to indicate the specific site(s) where lysine is replaced with arginine (*R*). The relative surface expression of CD86 in the cells expressing CD86 wild-type and mutants is shown as mean fluorescence intensity (MFI). Data are representative of three independent experiments. *C*, Western blot analysis of CD86 immunoprecipitates and lysates prepared from DCs expressing wild-type CD86 (WT) or CD86 (K > R), CD86 (RKRRR), and CD86 (KRKKK) mutants.
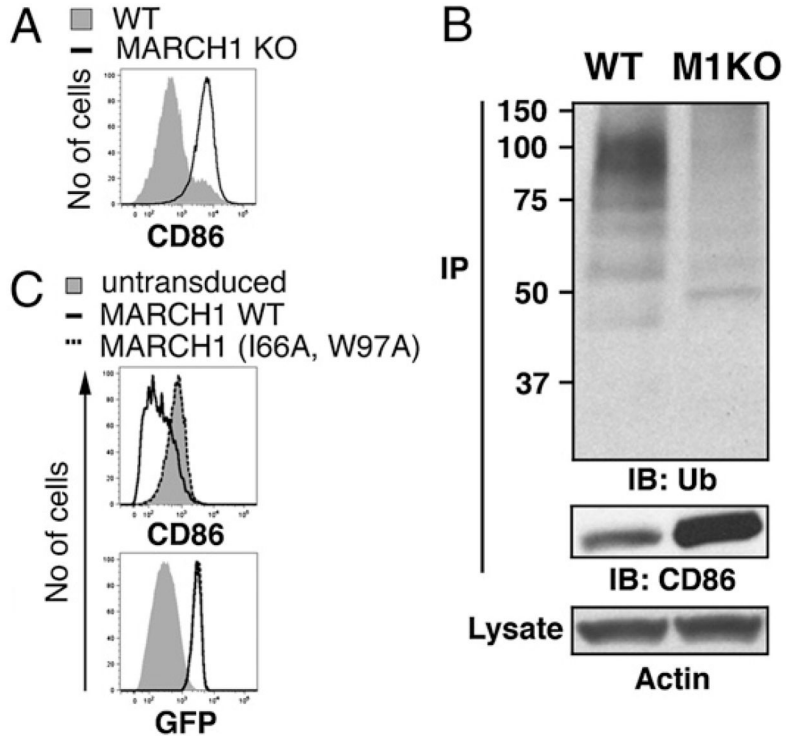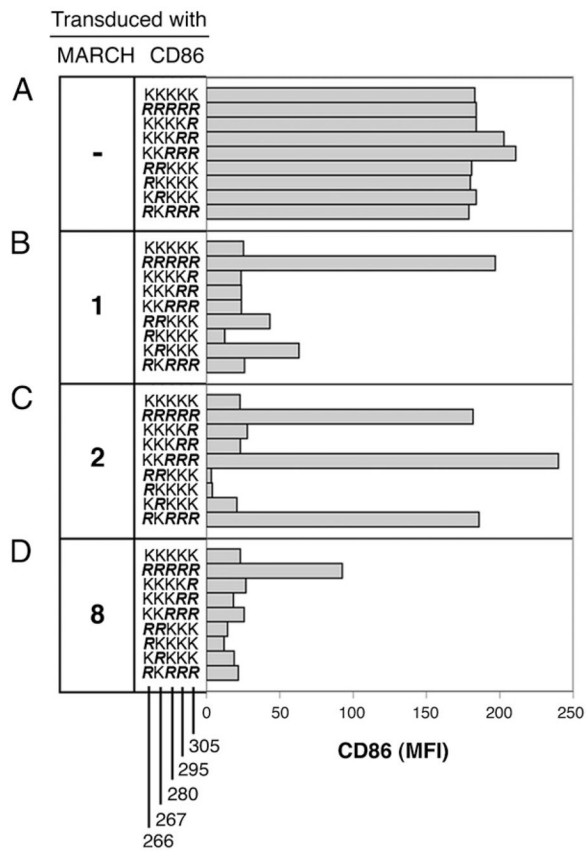
FIGURE 4.
MARCH1-mediated ubiquitination regulates CD86 in DCs. *A*, Flow cytometry analysis of BMDCs generated from wild-type (WT) mice (solid gray) or MARCH1$^{-/-}$ mice (full line). *B*, BMDCs cultured from WT or MARCH1$^{-/-}$ mice (M1KO) were processed for immunoprecipitation of CD86 followed by Western blot analysis. *C*, Flow cytometry analysis of surface CD86 on MARCH1$^{-/-}$ BMDCs transduced or untransduced with retrovirus encoding wild-type MARCH1 or mutant MARCH1 (I66A, W97A).

FIGURE 5.
Lysine 267 is sufficient for the MARCH1-mediated regulation of CD86. *A*, Splenic B blasts generated from CD86$^{-/-}$ mice were transduced with two groups of retroviruses. One retroviral group encodes MARCH1, -2, or -8 (*B–D*) along with mCherry. The other group encodes wild-type CD86 or various CD86 mutants along with GFP. Surface levels of CD86 expression of mCherry$^+$GFP$^+$ cells are shown as mean fluorescence intensities (MFI). Data are representative of two independent experiments.
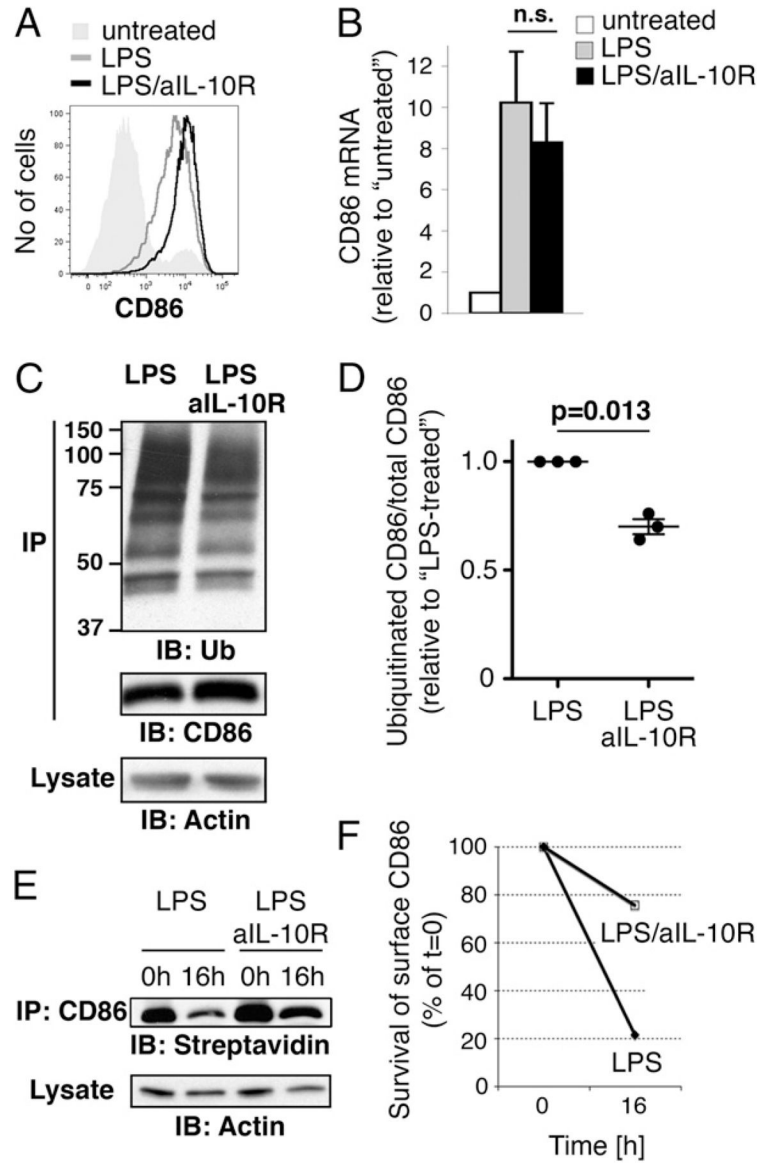
FIGURE 6.

LPS-induced autocrine IL-10 facilitates CD86 ubiquitination and turnover in DCs. *A*, Flow cytometry of wild-type BMDCs following indicated treatment. *B*, Transcripts of CD86 in BMDCs following indicated treatments. Values are relative to actin mRNA, and data are depicted as fold difference of that observed in untreated BMDCs. Data represent the mean ± SEM of three independent experiments. *C*, Western blot analysis of CD86 immunoprecipitates and cell lysates generated from wild-type mice. *D*, The relative amounts of ubiquitinated CD86 are expressed as fold difference of that observed in LPS-treated BMDCs. Data represent individual values of three independent experiments and mean values. *E*, BMDCs were first treated as indicated and then surface biotinylated followed by a chase for 16 h at 37°C. CD86 was immunoprecipitated and analyzed as described earlier. *F*,

The relative amounts of biotinylated CD86 are expressed as a percentage of the total amount of biotinylated CD86 at $t = 0$. Data are representative of two independent experiments.
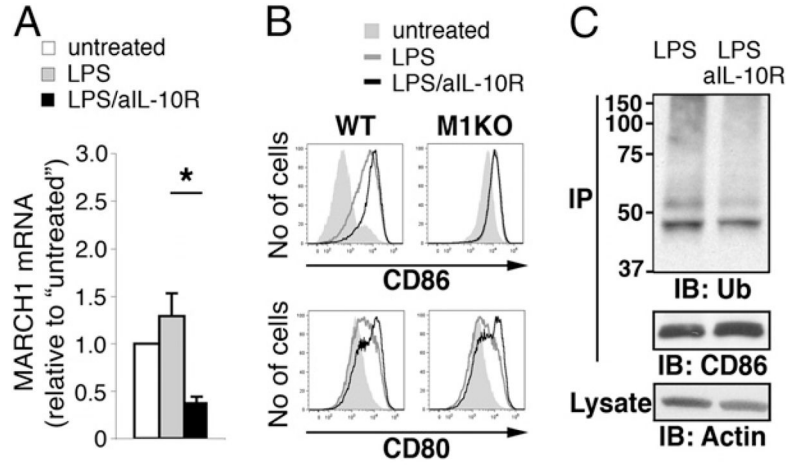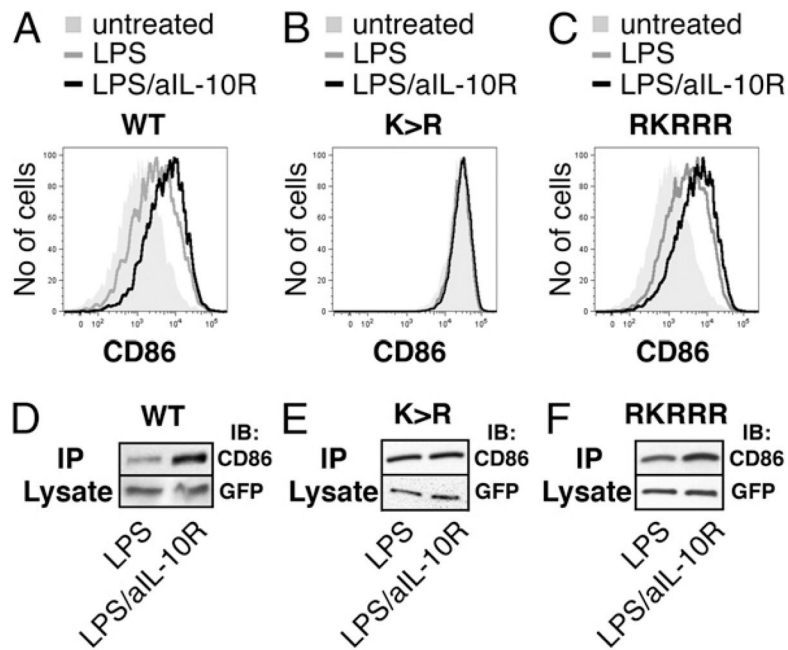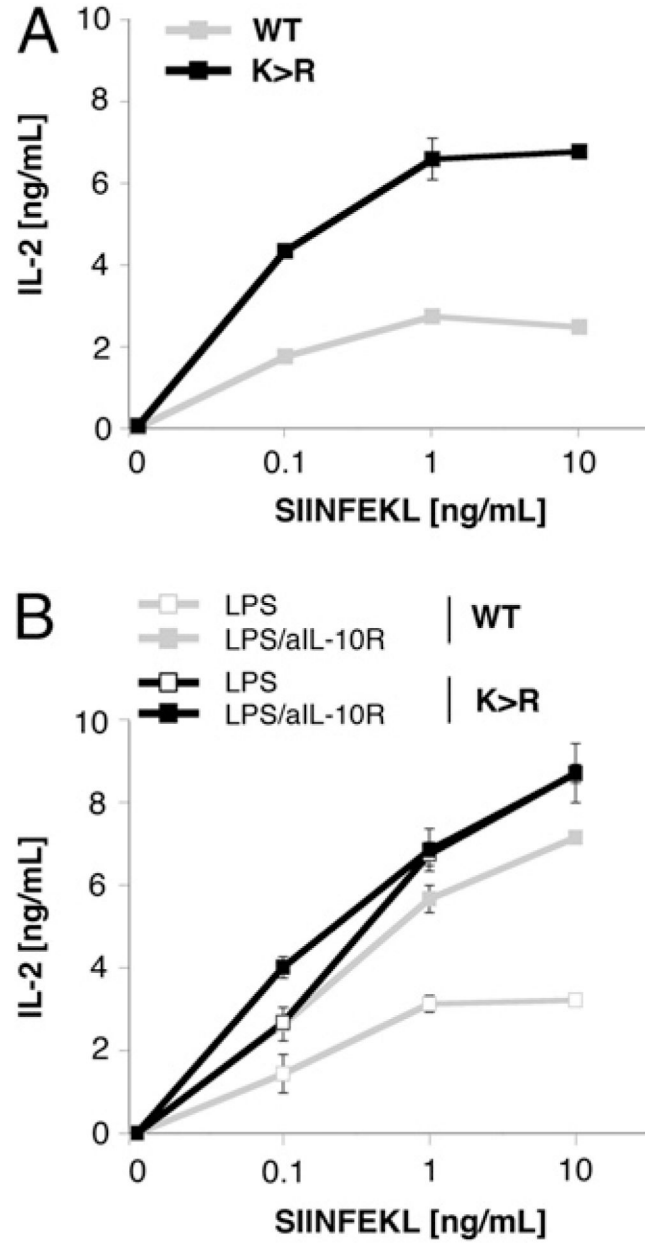
FIGURE 7.
Regulation of CD86 by autocrine IL-10 depends on MARCH1 expression. *A*, Transcripts of MARCH1 in BMDCs following indicated treatments. Values are relative to actin mRNA, and data are depicted as fold difference of that observed in untreated BMDCs. Data represent the mean ± SEM of three independent experiments. *B*, Flow cytometry of wild-type and MARCH1$^{-/-}$ BMDCs following indicated treatment. *C*, Western blot analysis of CD86 immunoprecipitates and cell lysates generated from MARCH1-deficient mice. *$p <$ 0.05.

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$^{-/-}$ BMDCs expressing wild-type CD86 (*D*), CD86 (K > R) (*E*), or CD86 (RKRRR) (*F*) treated as indicated.

FIGURE 9.
CD86 ubiquitination plays a significant role in controlling DC Ag-presenting functions. In vitro T cell activation assay using immature BMDCs (*A*) or BMDCs treated as indicated (*B*). BMDCs cultured from CD86$^{-/-}$ mice were retrovirally transduced to express wild-type CD86 or CD86 (K > R) mutant. DCs were loaded with increasing concentrations of the OVA-specific peptide, SIINFEKL, and cocultured with naive CD8$^+$ T cells isolated from OT-I transgenic mice. Sixteen to 18 h later, IL-2 in the supernatant was determined by ELISA. Data, expressed as the mean ± SEM, are representative of five independent experiments.