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Discrete Domains of MARCH1 Mediate Its Localization, Functional Interactions, and Posttranscriptional Control of Expression

Maurice Jabbour,* Erin M. Campbell,† Hanna Fares,† and Lonnie Lybarger2*‡

Within APCs, ubiquitination regulates the trafficking of immune modulators such as MHC class II and CD86 (B7.2) molecules. MARCH1 (membrane-associated RING-CH), a newly identified ubiquitin E3 ligase expressed in APCs, ubiquitinates MHC class II, thereby reducing its surface expression. Following LPS-induced maturation of dendritic cells, MARCH1 mRNA is down-regulated and MHC class II is redistributed to the cell surface from endosomal compartments. Here, we show that MARCH1 expression is also regulated at the posttranscriptional level. In primary dendritic cell and APC cell lines of murine origin, MARCH1 had a half-life of <30 min. MARCH1 degradation appears to occur partly in lysosomes, since inhibiting lysosomal activity stabilized MARCH1. Similar stabilization was observed when MARCH1-expressing cells were treated with cysteine protease inhibitors. Mutational analyses of MARCH1 defined discrete domains required for stabilization, proper localization, and functional interaction with substrates. Taken together, these data suggest that MARCH1 expression is regulated at a posttranscriptional level by trafficking within the endolysosomal pathway where MARCH1 is proteolyzed. The short half-life of MARCH1 permits very rapid changes in the levels of the protein in response to changes in the mRNA, resulting in efficient induction of Ag presentation once APCs receive maturational signals. The Journal of Immunology, 2009, 183: 6500 – 6512.

A ntigen presentation is strictly regulated to ensure immune priming only under the appropriate circumstances. This is true of both the MHC class I and class II presentation pathways, which share a requirement for costimulation to efficiently activate naive CD8 and CD4 T cells, respectively. In the case of MHC class II-expressing professional APCs, such as dendritic cells (DCs), macrophages, and B cells, the ability to prime CD4 T cells is coupled to their maturation status. Immature APCs are characterized by relatively low levels of MHC class I and class II, and costimulatory molecules including CD80 (B7.1) and CD86 (B7.2). Various stimuli, which include TLR ligands such as LPS, induce rapid changes in APCs that result in enhanced priming capacity. Although these changes are manifold, notable among them is a substantial increase in MHC class II and CD80/86 levels (1, 2). Consequently, matured APCs are much more potent in their T cell-activating ability (2). In large part, the rapid, maturation-induced changes in MHC class II (and probably CD86) levels are the result of changes in intracellular trafficking pathways (3, 4).

Extensive work has shown that in immature APCs, MHC class II molecules are sorted into the endolysosomal system, either directly from the trans-Golgi network and/or after transient appearance at the plasma membrane (5, 6). This sorting process requires specific information in the cytosolic tail of the MHC class II “chaperone”, the invariant chain (3). In immature DCs, MHC class II molecules are primarily found in late endosomes containing internal vesicles (7). When DCs are matured with stimuli such as LPS, MHC class II molecules leave endosomes and traffic to the cell surface (6, 8, 9), where they maintain high levels of expression. Interestingly, in immature DCs, MHC class II β-chains are constitutively ubiquitinated on their cytosolic tails, causing MHC class II to be retained within the endosomolysosomal system; ubiquitination is lost once DCs mature (10–13). MHC class II β-chains that cannot be ubiquitinated are expressed at high levels at the cell surface even in immature DCs (11, 12). It has recently become clear that membrane-associated RING-CH protein 1 (MARCH1) is the E3 ligase responsible for ubiquitinating MHC class II in immature APCs (13, 14). Maturation of APCs results in a decrease in MARCH1 mRNA and redistribution of MHC class II to the cell surface (13, 15). Thus, MARCH1 appears to function as a negative regulator of Ag presentation. In addition to affecting Ag display (MHC class II), MARCH1 also regulates the expression of the costimulatory molecule CD86 (13, 16).

MARCH1 is a member of a family of RING domain-containing E3 ligases, which were identified by virtue of their relatedness to viral immune evasion molecules (16, 17). Like most of its cellular relatives, MARCH1 is membrane-anchored and possesses a RING domain of the RING-CH subtype (18, 19). MARCH1 and its closest homolog, MARCH8 (c-MIR), regulate the surface expression of MHC class II and CD86 through ubiquitin-dependent mechanisms (10, 14). While MARCH8 is broadly expressed (16, 20), MARCH1 expression is highly enriched in lymphoid tissues (16).
Indeed, MARCH1 hemizygous mice (13) were shown that MARCH1 mRNA levels dropped substantially 16 h after LPS treatment of human DCs. However, a decrease was also evident as early as 4 h post-treatment. Although relatively slight at 4 h, this decrease could be well meaningful to the biology of MARCH1. Indeed, MARCH1 hemizygous mice (MARCH1+/−), which are presumed to have a 2-fold reduction in MARCH1 protein levels, have significantly increased surface MHC class II levels on immature APCs (14). Thus, the amount of MARCH1 protein that is normally present in immature APCs is just sufficient to affect MHC class II expression. Even modest decreases in MARCH1 protein levels could shift the balance to higher MHC class II and CD86 expression. By extension, modest changes in MARCH1 mRNA levels could produce biologically relevant changes in MARCH1 protein levels. In order for this mechanism to account for the rapid induction kinetics of MHC class II and CD86 on DCs following maturation, additional requirements must be met. Foremost among these is that MARCH1 protein levels must track closely with its mRNA levels. This would be the case if MARCH1 protein was unstable. Furthermore, this could help explain the fact that it has been difficult to detect endogenous MARCH1. Here, we have explored the posttranscriptional expression of MARCH1. Our results show that MARCH1 turns over in APCs with rapid kinetics, and this process is partly dependent on lysosomal acidity and cysteine proteases. Furthermore, we identify regions of MARCH1 that regulate its turnover, function, and localization. Overall, the data support a model wherein the levels of MARCH1 are tuned, through its inherent instability, to provide the minimal levels of E3 ligase function to suppress Ag presentation in immature APCs through down-regulation of MHC class II and CD86, and also permit rapid induction of Ag presentation following maturation.

**Materials and Methods**

**Mice**

Three- to 5-wk-old C57BL/6 mice were obtained from Charles River Laboratories or The Jackson Laboratory and housed in the animal facility at the University of Arizona. All procedures involving mice were done according to protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

**Abs and other reagents**

Mouse anti-HA (influenza hemagglutinin epitope) Ab (clone 6E2) and mouse anti-myc Ab (clone 9B11) were purchased from Cell Signaling Technology. Rat anti-CD86 (clone GL1) and goat anti-mouse cathepsin L were obtained from R&D Systems. Rat anti-mouse MHC class II Ab (clone M5/114.15.2), rat anti-mouse invariant chain (In-1), hamster anti-CD80 (16-10A1), and hamster anti-CD1c (clone N418) were obtained from BD Biosciences. Goat anti-mouse cathepsin S (M19) was purchased from Santa Cruz Biotechnology. Mouse anti-chicken actin Ab (ACTN05), rabbit anti-human EEA-1, and rabbit anti-LAMP-1 were purchased from Abcam. DEC205 was purchased from Cedarlane Laboratories. Rabbit anti-human Derlin-1 was obtained from Medical and Biological Laboratories. Rabbit anti-human furin convertase was obtained from Thermo Scientific. Mouse anti-GFP mAb was obtained from Covance. Cycloheximide (used at 12.5 μg/ml) was purchased from Sigma-Aldrich. Bafilomycin A (used at 0.32 μM) was obtained from BIOMOL. Inhibitors for cathepsins L (Z-FF-AMK) and S (Z-FL-COCHO) were purchased from Calbiochem and each were used at 40 μM.

**Cell lines and cell culture**

DC2.4 is a DC-like cell line (22) derived from C57BL/6 mice and was provided by Dr. K. Rock (University of Massachusetts Medical School). WT3 is a C57BL/6-derived mouse embryo fibroblast cell line (23) and was obtained from Dr. T. Hansen (Washington University School of Medicine). The RAW264.7 macrophage cell line (24) and the A20 B cell lymphoma cell line (25) were obtained from the American Type Culture Collection. Bone marrow-derived DC (BMDCs) were generated in a manner similar to that described (26, 27) by culture of C57BL/6 bone marrow cells for 6 days in the presence of 10 ng/ml IL-4 and 10 ng/ml GM-CSF (PeproTech). Non-adherent cells were collected postculture, and phenotypic analysis revealed purity that was consistently ≥80% (CD11c+; see Fig. 1). The MDCC cell line was generated using C57BL/6 BMDC cultures (as above) infected with the J2 retrovirus encoding the v-myec and v-raf oncogenes (28, 29), in a manner similar to that described for DC2.4. A20 cells were infected with the retrovirus produced by 6CREJ2 cells (obtained from Dr. H. Young, National Cancer Institute) was used to infect day 2 BMDC cultures. Cultures were maintained for 30 days in the presence of GM-CSF and IL-4. During this time, both adherent and nonadherent cells expanded. However, only the nonadherent fraction was collected at each passage for further expansion. Transformed cells were then cloned by limiting dilution, and clones were screened based on low adherence and expression of marker characteristics of DCs. During the cloning steps and all subsequent propagation, only GM-CSF (5 ng/ml) was included in the medium. Clone 2B1 expresses CD11c, DEC205, MHC class II, and CD86, and it was selected for these studies (see supplemental Fig. 1), although the expression of MHC class II was relatively low and could be substantially increased following treatment with IFN-γ. All cells were cultured in complete RPMI (Mediatech) supplemented with 10% FCS (HyClone), 1 mM HEPES (Invitrogen), 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (all from Mediatech).

**DNA constructs**

The mouse MARCH1 cDNA was obtained by RT-PCR from splenocyte cDNA. A clone was obtained that encoded a protein that matched a MARCH1 sequence within GenBank (BAC92449). This clone was used as a template for subsequent constructs. A 5’-terminal HA tag (influenza hemagglutinin epitope) was added by PCR, encoding the sequence MAPPYDPVDYAPGQFVS, immediately upstream of the MARCH1 start codon. Depictions of the MARCH1 mutant constructs generated for this study, as well as the specific residues mutated in each case, are provided in the relevant figures. The murine CD86 (B7.2) cDNA was generated by RT-PCR from C57BL/6 BMDC cDNA. Site-specific mutants were produced using QuickChange XL mutagenesis kit (Stratagene) according to the supplier’s instructions. Gene knockdown was accomplished using the lentiviral small hairpin RNA (shRNA) vector pLentiLox 3.7 (30) (American Type Culture Collection). Multiple shRNA oligos were designed for each target using Oligoengine or obtained from the RNAi Consortium at the Broad Institute (www.broad.mit.edu/node/563). The sequences of the shRNA oligos (sense strands) are given below, with the 19-mer sense sequence complementary to the target gene undefined. The following sequence fragments create the hairpin and flanking sequences for ligation into HpaII/XhoI-digested pLentiLox 3.7 as described (30): cethspsin (CalL) no. 1, TGGTTTTCCAGTACTATTAAGGTTAGCAGGCTGTTTTCC; CalL no. 2, TGTGAGCGTAAAGGCTTGCATTTTTCA; CalS no. 1, TGAGACCTGCAAGGCTCGGAATTTTGA; CalS no. 2, CATGTGAGCCTGAAGGCTCGGAATTTTGA; CTTC no. 1, TGCAGACCGCTTTACATTTT; CTTC no. 2, TGGAGAGCTTTACATTTT; GAGGCGCCTACTCTTTTTC. This design included homology sequences for unwanted similarity to other genes. cDNA constructs were expressed from either retroviral or lentiviral bicistronic vectors. The retroviral vectors were all murine stem cell virus-derived nonreplicating vectors. pMIG is bicistronic, with the

4 The online version of this article contains supplemental material.
gene of interest upstream of an IRES (internal ribosome entry site) element that precedes cGFP (pMSCV-IRES-GFP). pMIB is derived from pMIG, wherein the GFP cDNA was replaced with the bleomycin (zeocin) resistance gene (31). pMIB has been described (32) and encodes a hygromycin resistance gene as the second cistron. pMIB was used to express CD86. pCIG is a lentiviral vector derived from pCDH-CMV-MCS-EF1-copGFP (System Biosciences). It was generated by replacing the EF1 promoter-copGFP cassette with an IRES-cGFP cassette downstream from the CMV promoter. The correct sequence of all constructs was confirmed by DNA sequence analysis.

Transfections and transfections

Transient transfections were performed using FuGene 6 reagent (Roche Diagnostics) according to the supplier’s instructions. For retroviral vector production, PlatE cells (ecotropic) (33) and Phoenix cells (amphotropic) (34) were used to generate replication-defective viral particles following transient transfection, as described (35). Ecotropic virus was used to infect DC2.4, RAW 264.7, and WT-3 cells; amphotropic virus was used to infect A20 and MJDc cells. For stable lines, drug selection was done for >1 wk and maintained continuously during cell passage. For lentiviral vector infection of BMDCs, replication-defective virus was packaged by transient transfection of 293T cells (36) with the ViraPower packaging plasmids (Invitrogen). Two and 3 days posttransfection, culture supernatants were harvested, centrifuged briefly at 200 × g, and filtered through a 0.2-μm filter. Then, virus-containing supernatants were used directly for infection, or the virus particles were concentrated by ultracentrifugation. This was done as described (37) using a Beckman XL-70 centrifuge. Supernatants were centrifuged in OptiSeal tubes using an SW28 rotor for 2 h at 4°C at 50,000 × g. Pellets were resuspended in complete medium at 1/10 the volume of the starting supernatant. For infection, bone marrow cultures were established as described above. After 3 days, virus was added along with 8 μg/ml hexadimethrine bromide (Polybrene; Sigma-Aldrich) and returned to culture for expansion.

Flow cytometry

The surface expression of CD80, CD86, and MHC class II was monitored on cells by flow cytometry. Cell staining was performed in staining buffer (1% BSA, 0.1% azide in Dulbecco’s PBS; D-PBS). Fc receptors were blocked using Fc block (anti-CD16/32) (clone 2.4G2; BD Biosciences). Primary Abs were diluted in staining buffer and incubated with cells for at least 30 min on ice. Then, cells were washed, resuspended in 1× D-PBS, and fixed with an equal volume of 1% paraformaldehyde (in D-PBS). For intracellular staining, cells were fixed and permeabilized with 1% paraformaldehyde plus 0.5% saponin (Calbiochem) in D-PBS for 20 min on ice. Cells were then washed twice and stained with the primary Ab for at least 30 min on ice. After two washes, cells were probed with fluorescein-conjugated secondary Abs on ice for 30 min, followed by washing. Cells were then fixed as above and samples collected using a FACSCalibur cytometer (BD Biosciences) and data was analyzed using either CellQuest Pro software (BD Biosciences) or WinMidi (freeware available through the Scripps Research Institute).

Immunofluorescence

DC2.4 cells with or without MARCH1 and MARCH1 mutants (stable transfectants) were stained for MARCH1 (HA tag) and various Abs to define distinct cellular compartments. Staining was performed in suspension using a modified version of the intracellular flow cytometry protocol described above. After the final wash, nuclei were stained with Hoechst 33242 (Invitrogen) for 10 min at 4°C. After the final staining step, cells were suspended in D-PBS and air dried on microscope slides using a Shandon Cytospin cytocentrifuge (Thermo Scientific) at 800 rpm for 2–3 min. Mounting medium was added (Prolong Gold; Invitrogen) and pre-cleaned coverslips were placed and sealed with fingernail polish. Images were taken using a Zeiss 510 Meta confocal microscope using an NA1.4 aperture objective with ×63 magnification. Image analysis was done with Imaris (freeware available from http://www.bitplane.com/), and the overlap between the MARCH1 signal and the various markers was estimated using the JACoP plug-in (38). Additional details are provided in the legend to Fig. 6.

Results

MARCH1 protein stability

During the course of LPS-induced DC maturation, it has been shown that MARCH1 mRNA levels decrease as soon as 4 h postinduction, and protein levels are also reduced at this time point (13). These relatively rapid changes suggest that MARCH1 protein levels closely mirror those of its mRNA, and this could occur if the MARCH1 protein is relatively unstable. To explore the properties of the MARCH1 protein, including stability, we began by expressing it in a variety of murine cell lines, as well as primary BMDCs. Fig. 1, A and B, shows the expression of CD86 at the cell surface of cell lines retrovirally transduced with vectors encoding an epitope-tagged version of MARCH1. CD86 expression levels were used as an indicator of MARCH1 expression and function since it is a natural target of MARCH1 (13) and is expressed in the cell lines used here. In a DC-like cell line (DC2.4), expression of MARCH1 resulted in a significant decrease in CD86 expression (Fig. 1A). Although these cells are derived from BMDCs, they do not express endogenous MARCH1 as determined by quantitative RTPCR (not shown). As a control, we expressed a mutant version of MARCH1 harboring a point mutation within the RING-CH domain (39, 40). This RING-CH domain mutant was incapable of down-regulating surface CD86 expression, as expected (Fig. 1A), even though it was expressed at comparable levels to wild-type MARCH1 (see below). Similar to the DC2.4 cells, transduction of CD86-expressing fibroblasts with MARCH1 caused a drop in surface CD86 expression (Fig. 1B). Thus, as has been reported previously in human cells (13, 14, 16), MARCH1 is functional in non-APCs, indicating that no additional APC-specific factors are strictly required for its function.

We next examined MARCH1 protein levels in these cells lines over time following inhibition of translation using cycloheximide (cycloheximide chase). Fig. 1C shows an immunoblot for epitope-tagged MARCH1 from lysates of DC2.4 cells treated with cycloheximide for various times. We observed a rapid decrease in the steady-state levels of MARCH1 with an estimated half-life of <30 min. Similar decay rates were found in other APC-derived cell lines (A20 B cells and RAW 264.7 macrophages; data not shown), as well as a fibroblast line (B6/WT3; Fig. 1D). Placing the epitope

SDS-PAGE and immunoblotting

Cell lysates were generated by lysis in 1% Igepal CA-630 (Nonidet P-40) (from Sigma-Aldrich) dissolved in 50 mM Tris, 150 mM NaCl (pH 7.4) buffer (TBS), supplemented with 0.3 mM PMSF, 20 mM iodacetamide, 10 μM MG132 (all from Sigma-Aldrich), and protease inhibitor cocktail III (Calbiochem). Postnuclear lysates were mixed with LDS sample buffer (Invitrogen) and 2-ME (1% final concentration). Protein content was determined using the BCA protein assay from Thermo Scientific. Samples were separated by electrophoresis on 4–12% or 12% Nu-PAGE SDS polyacrylamide gels (Invitrogen). Proteins were then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Blocking of membranes was done for 1 h with 5% dried milk, 0.1% Tween 20 (Sigma-Aldrich), and 0.1% SDS in D-PBS. After washing three times with 0.1% Tween 20, 0.01% SDS in D-PBS, membranes were incubated with the appropriate dilution of primary Ab for >1 h, washed three times, incubated with appropriate biotin-conjugated secondary Abs for 1 h, followed by incubation with streptavidin-conjugated HRP (Zymed Laboratories) for 1 h. Membranes were incubated with ECL chemiluminescent substrate (GE Healthcare) and visualized using Blue Ultra Autorad film (from ISc Bio-Express) or were incubated with SuperSignal West Femto (Thermo Scientific) and visualized using a ChemiDoc XRS (Bio-Rad) digital imaging system. Determination of the band intensities from immunoblots was done using Quantity One software (Bio-Rad), and plots were generated using GraphPad Prism software.
tag at either end of MARCH1 did not make a difference in terms of stability (not shown). To determine whether these results are representative of MARCH1 in primary APCs, we examined MARCH1 turnover in BMDCs. As mentioned above, endogenous MARCH1 is quite difficult to detect. Therefore, we used lentiviral vectors to transduce BMDC with an HA-tagged version of MARCH1. In these cells, MARCH1 expression decreased precipitously within the first 30 min after the addition of cycloheximide, confirming that MARCH1 is highly unstable in DCs (Fig. 1).

These findings with MARCH1 are somewhat similar to MARCH7, which is also unstable. In the case of MARCH7, the instability is related to its autoubiquitination (41). We examined the ligase-deficient mutant of MARCH1 (W104A) and found that while its steady-state expression levels were comparable to wild-type MARCH1, it did exhibit a modest, but significant increase in stability (Fig. 1F). Thus, the ubiquitin ligase activity of MARCH1 does make a minor contribution to its instability, which could reflect autoubiquitination or an indirect effect on stability through a ubiquitin-dependent process initiated by MARCH1. It is also possible that MARCH1 could be ubiquitinated by another E3 ligase.

Curiously, we did notice a band in our anti-MARCH1 immunoblots that runs above the predicted full-length MARCH1, and its

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** MARCH1 is unstable in multiple cell types. A, CD86 expression on DC2.4 cells stably transduced with a lentiviral vector encoding either wild-type N-terminal HA-tagged MARCH1 (WT) or a RING-CH mutant (W104A). The thin gray peak indicates staining with an irrelevant isotype control Ab. B, CD86-expressing WT3 fibroblasts were stably transduced with retroviral vectors encoding MARCH1 with an N-terminal HA tag. The thin gray peak indicates staining with an irrelevant isotype control Ab. C, DC2.4 cells stably expressing N-terminal HA-tagged MARCH1 were incubated for the indicated times with cycloheximide, then lysates were blotted for MARCH1 (HA tag). Incubation in diluent (DMSO) was used as a control, and actin blots were performed as loading controls. Graphs show the turnover of MARCH1 from three independent experiments. The signal intensity of the MARCH1 and actin bands was estimated using Quantity One software. For each time point, the actin-normalized values were averaged between the experiments and graphed as the percentage remaining relative to time 0 (DMSO-treated samples) ± SEM. D, Immunoblot for MARCH1 (HA tag) and actin was performed from lysates of WT3 fibroblasts incubated with cycloheximide for the indicated times. E, Mouse BMDCs were generated as described in Materials and Methods and infected with a lentiviral vector encoding HA-MARCH1. Experiments were performed 3 days postinfection. Top panel, Cychoheximide-chase and immunoblot of MARCH1 in BMDCs, performed as described above. Immunoblot for GFP was also included; GFP is encoded by the lentivirus. Bottom panel, Purity of the BMDC preparations (MHC class II vs CD11c staining, and isotype-matched control staining). F, DC2.4 cells stably expressing HA-tagged MARCH1 or the W104A mutant were incubated for the indicated times with cycloheximide and treated as in C. Full-length MARCH1 is denoted with an asterisk. Graphs show the turnover of MARCH1 from three independent experiments. For each time point, the actin-normalized values were averaged between the experiments and plotted as the percentage remaining relative to time 0 (DMSO-treated samples) ± SEM. ***, p < 0.01.
levels seemed to increase at later time points of the cycloheximide chase (see Fig. 1, D and F, for example). The size difference between this band and the full-length version of MARCH1 is not large enough to be explained by monoubiquitination, and we have been unable to detect ubiquitination of MARCH1 by immunoprecipitation and anti-ubiquitin blotting (not shown). The nature of this larger band is presently unclear, but it could represent another type of posttranslational modification such as phosphorylation; N-linked glycosylation is excluded since no potential glycosylation sites exist in the MARCH1 luminal domain. Regardless, these results indicate that additional factors besides ubiquitination are likely involved in the regulation of MARCH1 levels.

**MARCH1 turnover requires lysosome function**

Having determined that MARCH1 is rapidly degraded, we wanted to define the mechanisms responsible for this regulation. Given that MHC class II molecules are degraded in a lysosome-dependent manner following MARCH1-mediated ubiquitination (13, 14), and the reported presence of MARCH1 in lysosomes (16), we tested the requirement for lysosome function in MARCH1 degradation. DC2.4 cells expressing MARCH1 were treated with bafilomycin A (Baf A), which blocks lysosome acidification (42), and steady-state levels of MARCH1 were analyzed by immunoblot. Treatment of cells with Baf A led to an increase in the MARCH1 signal over time (Fig. 2A). To determine whether the increase in MARCH1 levels after Baf A treatment was due to stabilization of MARCH1, an experiment was performed in which DC2.4 cells expressing MARCH1 were treated with Baf A and cycloheximide to follow the turnover of MARCH1 over time. Fig. 2B shows that Baf A does stabilize MARCH1 in cells treated with cycloheximide. Although stabilization was not complete, MARCH1 turnover was more stable when lysosomal pH was increased. The fact that MARCH1 turnover was not completely abolished by Baf A treatment suggested an alternative mechanism for the turnover of a fraction of MARCH1 within the cells. Indeed, we found that MARCH1 could be partially stabilized by treatment of cells with a proteasome inhibitor (MG132), indicating that some MARCH1 turnover was mediated by proteasomes (data not shown). Proteasome-dependent turnover of membrane proteins is typically a result of endoplasmic reticulum-associated degradation during initial biogenesis (43, 44), and a portion of MARCH1 may be degraded via this pathway.

We next sought to confirm our findings using primary APCs. Immature BMDCs were transduced with MARCH1-expressing lentiviral vectors. Treatment of these cells with Baf A led to increased levels of MARCH1, similar to the results obtained above with the cell lines. This was the case for MARCH1 with either an N-terminal (HA) or C-terminal (myc) epitope tag (Fig. 2C). As a control for the effects of Baf A treatment, we examined the processing of the MHC class II invariant chain (Ii), where it is known that effective inhibition of lysosomal peptidases leads to the accumulation of the invariant-chain fragments (3); Baf A treatment led to the expected accumulation of p31 and p10 invariant-chain fragments in BMDCs (Fig. 2C). Overall, these experiments demonstrate that MARCH1 is stabilized when lysosome function is inhibited, and this holds true in multiple cell types, including BMDCs. Additionally, proteasome activity also regulates turnover of a fraction of MARCH1. Taken together, these two activities (lysosome- and proteasome-dependent degradation) limit the levels of MARCH1 that are present at steady-state.

**Proteolytic processing of MARCH1**

The stabilizing effect of inhibitors of lysosome acidification on MARCH1 suggested a role for lysosomal hydrolases in MARCH1...
In fact, we noted the presence of apparent breakdown fragments of MARCH1 in some cells, consistent with proteolysis (data not shown). We reasoned that cysteine proteases, and cathepsins in particular, were good candidates to affect MARCH1, since these proteases are directly involved in the processing of invariant chain during MHC class II biogenesis within APCs (3, 45). The cathepsin family contains many members that exhibit cell type-specific expression and activity (45, 46), as well as cross-regulation (47, 48). We focused on the leupeptin-sensitive cathepsins, including cathepsin S and cathepsin L, which are important in MHC class II Ag presentation (46, 49).

**FIGURE 3.** Lysosomal proteases affect MARCH1 turnover. A, DC2.4, WT3, and RAW 264.7 (macrophage-like) cells were incubated for 5 h with the indicated inhibitors: leupeptin, cathepsin L inhibitor (Z-FF-FMK), and Baf A. Cell lysates were then blotted for MARCH1 and actin. The band intensities were determined, normalized to actin, and displayed below the blots as fold increase over control (DMSO). B, DC2.4 and RAW cells with or without HA-MARCH1 were incubated for 5 h with the indicated cathepsin inhibitors and then cell lysates were blotted for MARCH1 and the invariant-chain (p31 and p10 fragments indicated). C, Cycloheximide chase in HA-MARCH1-expressing DC2.4 cells with or without the cathepsin L inhibitor. Graphs show the turnover of MARCH1 from three independent experiments, where the signal intensity of the MARCH1 bands was quantified, normalized to actin, and graphed as the percentage remaining relative to time 0 (DMSO-treated samples) ± SEM. The inset graph shows quantitation of the same samples, except that it begins with the 30 min chase point, with all samples normalized to the 30 min value so that turnover of the MARCH1 remaining at 30 min could be evaluated. *, p < 0.05 and **, p < 0.01. D, DC2.4 cells plus HA-MARCH1 were infected with shRNA lentiviral vectors to knockdown expression of cathepsin S (left panel) or cathepsin L (right panel). For each gene, two different shRNA oligos were designed. At least 3 days or more after infection, lysates from the different cell lines were blotted with the indicated Abs.

We next sought to determine which of the leupeptin-sensitive proteases(s) are involved in MARCH1 regulation. Here, we took a candidate approach and tested inhibitors specific for different cathepsins; results for cathepsin L and S inhibitors are shown in Fig. 3, A and B. We consistently observed an increase in the steady-state levels of MARCH1 when using an inhibitor of cathepsin L in APC cell lines. This was due to an increase in the stability of MARCH1 as determined by cycloheximide chase in the presence of the inhibitor (Fig. 3C). This stabilization was similar to what we observed when MARCH1-expressing cells were treated with Baf A (see Fig. 2B), being most apparent at the later chase time points. In contrast, drug inhibition of cathepsin S did not increase MARCH1 levels in any of the cell lines. Invariant-chain blots were performed to confirm that the drug treatments were effective, since invariant-chain processing involves both cathepsins L and S (Fig. 3B). This analysis showed a consistent increase in the p10 fragment and in full-length invariant chain using the cathepsin L inhibitor, as expected (50). The anticipated effect of cathepsin S inhibition on invariant-chain processing was more difficult to show, raising the possibility that the drug inhibition was ineffective. Therefore, we utilized a complementary approach to assess the possible role of cathepsin S in MARCH1 regulation. RNA interference was employed to knock down the expression of cathepsins S and L. In this case, lentiviral shRNA vectors were
designed encoding oligos specific for both cathepsins S and L. As shown in Fig. 3D, knockdown of cathepsin S, while effective in reducing cathepsin S levels (with oligo no. 2), did not stabilize MARCH1 in DC2.4 cells. In contrast, knockdown of cathepsin L did lead to an increase in MARCH1 steady-state levels (Fig. 3D). For cathepsin L, the efficiency of knockdown was not as great, explaining the relatively modest increase in MARCH1 protein (with oligo no. 1; Fig. 3D). However, when considered along with the inhibitor experiments, it seems that cathepsin L does contribute, in part, to the inherent instability of MARCH1. Cathepsin L inhibition had no obvious effect on MARCH1 in the fibroblast line (Fig. 3A), even though these cells express cathepsin L (not shown) and MARCH1 is unstable in these cells (Fig. 1D). Additionally, the cathepsin L inhibitor did not consistently increase MARCH1 levels to the same extent as Baf A in each of the cell types tested, so we conclude that multiple lysosomal proteases contribute to the turnover of MARCH1, in addition to cathepsin L. The combined findings for treatment with Baf A, leupeptin, and the cathepsin L inhibitor argue that endolysosomes are an important site of MARCH1 turnover.

Domains of MARCH1 involved in stability and function

To define important functional domains of MARCH1, including domains that might influence stability, we generated a series of MARCH1 mutants. Several MARCH1 variants were created, deleting either N-terminal or C-terminal sequences, as well as point mutations (depicted in Fig. 4A). The various mutants were expressed in APC cell lines following transduction with retroviral vectors. Initially, the expression of each mutant was characterized by immunoblot for the epitope tag. Fig. 4B reveals that the steady-state levels of mutants with N-terminal truncations were significantly higher than wild-type MARCH1. By contrast, the C-terminal deletion mutants were expressed at slightly lower levels than wild-type MARCH1, and the internal deletion mutant (ΔN120–141) was barely detectable. The increased levels of the N-terminal truncation mutants correlated with an increase in the half-life of these mutants as determined by cycloheximide chase (Fig. 4C and data not shown). Additionally, intracellular staining/flow cytometry was used to compare the expression levels of each mutant construct, and these results correlated well with the immunoblot results. Specifically, truncations of the N terminus resulted in higher expression than did wild-type MARCH1. We noted that the staining peaks for the N-terminal truncations /ΔH9004N1–40 and /ΔH9004N1–66 were biphasic, suggesting loss of these constructs within some cells with continued cell passage (discussed below). The C-terminal truncations were similar in expression to wild type (Fig. 4D). These results indicate the presence of a stability determinant within the N-terminus of MARCH1, and in its absence, MARCH1 is expressed at higher levels.

Function of MARCH1 mutants

We next evaluated the function of these MARCH1 mutants, with function defined as the ability to regulate the surface expression of MARCH1 targets. To this end, we transiently transfected each mutant into WT3 fibroblasts (that express CD86) to assess the ability of the mutants to regulate the surface expression of CD86. Fig. 5A shows flow cytometric analysis of each transfected. Transient transfection permits the analysis of function within a cohort of cells that express a range of different levels of MARCH1. Here, a GFP-expressing plasmid was cotransfected to denote transfected

FIGURE 4. Expression and stability of MARCH1 mutants. A, Depiction of the various mutations made within MARCH1. All of the mutants possess an N-terminal HA tag. B, Immunoblot of DC2.4 cell lines stably expressing the indicated MARCH1 constructs following retroviral transduction. C, Cycloheximide chase of DC2.4 cells expressing wild-type MARCH1 or the ΔN120–141 mutant. Two exposures of the MARCH1 (HA) blot are shown. The band intensities were quantified, normalized to actin, and graphed as the percentage remaining from time 0 (DMSO). D, Intracellular staining and flow cytometry of DC2.4 cells expressing the indicated MARCH1 constructs. Cells were stained for MARCH1 (HA tag). The gray peak in each histogram represents staining of the parental DC2.4 cells, which represents background staining.
cells. The shorter C-terminal truncation ($\Delta C_{257-279}$) retained its ability to decrease CD86 at the cell surface, whereas the larger deletion ($\Delta C_{222-279}$) did not, although both mutants had comparable expression to wild-type MARCH1 (as seen in Fig. 4, B and D). This result is consistent with studies of viral E3 RING-CH ligases such as mK3, kK3, and kK5, where the C-terminal domain is critical for function (51–56) and, where examined, for substrate interaction (52, 54). Somewhat surprisingly, deletion of the first 40, or first 66, residues from the N terminus of MARCH1 did not abolish function toward CD86. This was only accomplished by deletion of the entire N terminus, including the RING-CH domain ($\Delta N_{1-121}$). We also tested the activity of MARCH1 variants with mutations in potential tyrosine-based sorting motifs, which are typically involved in trafficking within the endolysosomal system ($YXX\phi$) (57, 58). Three such motifs exist in MARCH1: one in the N terminus and two in the C terminus. Simultaneous mutation of both motifs in the C terminus ($L215S$, $Y222F$) eliminated most of the ability of MARCH1 to regulate CD86 surface levels (Fig. 5A), without affecting steady-state levels (Fig. 4D). Mutation of each motif, separately, revealed that the $L215S$ motif was required for full activity of MARCH1 (data not shown). Mutation of the N-terminal motif ($Y118F$) had no effect on MARCH1’s ability to down-regulate CD86 (Fig. 5A).

To complement these data, we also expressed MARCH1 mutants stably in APC cell lines. This was done in part to determine whether each mutant could regulate MHC class II surface expression, and if this correlated with the ability of that particular mutant to also regulate CD86. In other words, did mutation of certain regions affect MARCH1’s function equally toward both targets? Additionally, this approach provides a means to evaluate the function of each mutant when expressed at comparable levels (comparable transcription) following retroviral transduction. First, we stably transduced MARCH1 variants into DC2.4 cells and found that each mutant that failed to regulate surface CD86 levels in fibroblasts also failed to regulate its expression in DC2.4 cells (Fig. 5B). To assess the ability of the mutants to regulate MHC class II, we employed an additional cell line, since the levels of MHC class II were quite low on the DC2.4 parental cells, despite the fact that they do not express endogenous MARCH1. To circumvent this problem, we generated a “DC-like” cell line (MJDC) by immortalization of BMDCs with the J2 retrovirus (28, 29). Subclones of the transduced line were screened for a DC-like phenotype, which included expression of DEC205, CD11c, costimulatory molecules, and, most importantly for our analyses, MHC class II (see supplemental Fig. 1). This cell line permits simultaneous analysis of MARCH1 function against CD86 and MHC class II. As shown in Fig. 5C, MARCH1 mutants that failed to regulate CD86 surface expression also failed to regulate its expression in DC2.4 cells (Fig. 5B).

**FIGURE 5.** Comparison of the activity of the MARCH1 mutants. A, WT3 fibroblasts that stably express CD86 were transiently cotransfected with MARCH1-expressing constructs and a GFP-expressing plasmid. Flow cytometry was performed to analyze surface CD86 levels 2 days posttransfection. The shaded peak indicates staining of cells transfected with the GFP plasmid alone, gated on the GFP-positive fraction. The heavy trace represents CD86 staining of the MARCH1 cotransfected cells (gated on GFP-positive cells). B, DC2.4 cell lines stably transduced with the indicated MARCH1 mutants were analyzed for surface CD80 and CD86 levels. C, MJDC cells were analyzed for CD86, MHC class II, and CD80 levels after stable retroviral transduction with the indicated MARCH1 mutant constructs. Cells were treated with 100 U/ml mouse IFN-γ for 18 h before analysis to increase transcription of MHC class II genes. The gray peaks in B and C represent staining of the respective parental cell lines (lacking MARCH1) and the shaded peaks represent staining of parental cells with an isotype control Ab.
Governed toward CD86 were also able to down-regulate MHC class II (Fig. 5C and data not shown). None of the variants of MARCH1, including wild-type, affected CD80 (B7.1) cell surface expression, as reported (16).

In the course of our characterization of the N-terminal truncation mutants lacking either the first 40 or 66 N-terminal residues, we noted that stable cell lines lost protein expression over several cell passages, even in the presence of selecting antibiotics. This is evident from our data, where intracellular MARCH1 staining and flow cytometry revealed two distinct peaks of expression with these mutants (Fig. 4D). Similarly, surface CD86 expression in cells expressing these mutants also showed a bimodal pattern wherein a fraction of the stable transductants express wild-type levels of CD86 (Fig. 5, B and C). In these cells, the levels of the MARCH1 mutants continue to drop over time. This pattern was reproducible in independent transductions of multiple cell lines, and argues that these MARCH1 variants are detrimental to the cells, probably by affecting transport pathways. Nonetheless, we can conclude that the N-terminal deletion that retains the RING-CH domain (∆N1–66) retains its ability to down-regulate CD86 and MHC class II surface expression, a fact that is confirmed by the short-term transient transfection assays with this construct where toxicity is not an issue (Fig. 5A). On the basis of these results, we conclude that the N-terminal region of MARCH1 contains sequence elements that affect the stability of the protein, but do not directly affect function. Therefore, this N-terminal region is not involved in substrate recognition or recruitment of effector molecules. Conversely, the C-terminal 50 residues of MARCH1 are required for function, but do not influence the stability of MARCH1. A summary of the properties of each mutant is provided in Table I.

### Subcellular distribution of MARCH1 mutants

Analysis of the MARCH1 mutants defined regions of MARCH1 that affect its expression levels and function. To what extent can these changes be explained by alterations in the trafficking of the MARCH1 mutants? For example, could the increase in the steady-state levels of the MARCH1 N-terminal truncation mutants be explained by redistribution of these mutants to different cellular compartments? To address these questions, we used confocal microscopy to examine the localization of wild-type MARCH1 and the mutants. It has been reported that human MARCH1 resides, in part, in late endosome/lysosomes, due to its partial colocalization with LAMP-1 in HeLa cells (16). Additionally, a GFP-fused form of human MARCH1 in HeLa cells was detected in early endosomes (transferrin receptor-positive) (13). Fig. 6A shows the distribution of mouse MARCH1 in DC2.4 cells, where MARCH1 staining is compared with that of representative markers for various cellular compartments, including endoplasmic reticulum (Derlin-1), transf-Golgi (furin), early endosomes (EEA-1), and late endosomes/lysosomes (LAMP-1).

### Table I. Properties of the MARCH1 mutants

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<td>Stable state levels</td>
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<td>Function (CD86, MHC II)</td>
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<td>Cellular localization</td>
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<td>Furin+, LAMP-1+, LAMP-1+, and other</td>
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* Function: determined by analyzing the surface expression of either CD86 or MHC class II in the presence of each MARCH1 construct using flow cytometry (+ indicates a functional MARCH1 construct; −, a nonfunctional MARCH1 construct; see Fig. 5).  
* Localization: determined by costaining with HA antibody against MARCH1 and either Derlin-1, LAMP-1, Furin, or EEA-1 (see Fig. 6 and supplemental Fig. S2 for complete immunofluorescence analysis).  

Not available.
both variants are expressed at higher levels than wild-type MARCH1 (see Fig. 4, B and D), it is possible that the altered distribution of these proteins prevents their rapid degradation. Regardless, it is notable that the ΔN1-66 mutant (but not the RING domain-deleted ΔN1-121 mutant) retains the ability to efficiently down-regulate both CD86 and MHC class II (Fig. 5). Thus, the

FIGURE 6. Distribution of wild-type vs mutant forms of MARCH1 within cells. A, Immunofluorescence and confocal microscopy were performed with HA-MARCH1-expressing cells or nonexpressing parental cells. Cells were costained for MARCH1 vs the indicated molecules and representative images are provided. The left panels show staining of DC2.4 cells, and right panels show staining of MJDC cells. In each set of images, staining of the indicated marker is shown as green in the merged image, and MARCH1 (HA) staining is shown in red in the merged image. Staining of the parental (no MARCH1) cell line is shown on the bottom left. B, Staining of each indicated HA-tagged MARCH1 mutant is shown vs furin and LAMP-1. Staining of additional markers (EEA-1 and Derlin-1) is provided for each mutant in supplemental Fig. 2. C, The relative distribution of each mutant as compared with wild-type MARCH1. Images representing single cells (n = 15) taken from multiple fields were analyzed for each mutant stained together with furin or LAMP-1. The extent of overlap between the MARCH1 signal and the marker protein was determined using the JACoP (38) plug-in for National Institutes of Health ImageJ with the default settings. Specifically, JACoP was used to determine the Manders coefficient (64) for MARCH1-positive staining, which was also positive for the marker in question. This coefficient was given a value of 1 for wild-type MARCH1, and then the values for each mutant were normalized to wild type. The relative Manders coefficients are plotted ± SEM. Paired t tests were used to determine whether the mutants were different from wild type for each marker. *, p < 0.01 and **, p < 0.0001. Note that for all images, a lower threshold was applied uniformly to each set of samples (imaged for a given marker) based on negative controls (background subtraction). The signal for MARCH1 (HA) and the marker protein in each set of images was normalized by setting the brightest pixel in each image (for each fluorescence channel) as the maximum signal (histogram stretching) to facilitate visualization and comparison across fields. This was necessary to permit comparisons between wild-type and mutant MARCH1 constructs, which are expressed at different levels (see Fig. 4).
altered localization of MARCH1 ΔN1–66 does not dramatically affect its basic function.

**Discussion**
MARCH1 has emerged as a critical regulator of Ag presentation, serving to suppress the ability of immature APCs to activate T cells through its effects on MHC and costimulatory molecules. The transition of APCs from the immature to the mature state is essential for effective T cell priming by APCs and is necessarily subject to tight regulation. Although MARCH1 is clearly a component of this regulatory system, much remains to be determined regarding its involvement and control. MARCH1 transcription decreases in response to DC maturation with LPS (13) and increases after stimulation of monocytes with the antiinflammatory cytokine IL-10 (21). Thus, MARCH1 activity is regulated, at least in part, through changes in gene transcription. Since MARCH1 protein levels appear to be balanced such that relatively small changes significantly alter the cell surface display of MHC class II, then the instability of MARCH1 is an essential component of its regulation. Even in immature DCs where MARCH1 is active, MARCH1 turns over quite rapidly. Note that we have not observed any appreciable increase in MARCH1 turnover following maturation with LPS (data not shown), which is known to enhance lysosomal activity in DCs (60). It is interesting that the more stable N-terminal mutants appear to be somewhat more potent than wild-type MARCH1 at down-regulating CD86 (Fig. 5B), consistent with the idea that the inherent stability of MARCH1 maintains its levels at a critical functional threshold.

Lysosomal acidification significantly affects the levels of MARCH1, and this implies proteolysis of MARCH1 within endolysosomal compartments, where MHC class II ubiquitination appears to occur (11, 12). However, the topology of MARCH1 presents a potential obstacle to this model. MARCH1 possesses two transmembrane domains, with a short, connecting lumenal domain; the bulk of the molecule resides on the cytosolic face of the membrane (16). How could lysosomal enzymes access MARCH1? It is possible that the lumenal domain is targeted, but replacement of this domain with unrelated sequences did not stabilize MARCH1 (not shown). Furthermore, the size of the fragments of MARCH1 that we detect is not consistent with cleavage within the lumenal domain (data not shown). The most likely explanation is that the cytosolic domains of MARCH1 are exposed to proteases within multivesicular bodies (61). Indeed, electron microscopy has shown an abundance of MHC class II molecules within these structures (3), and this requires ubiquitination of the MHC class II β-chain (11, 12). Therefore, it is probable that MARCH1 also traffics through multivesicular bodies where it is exposed to the myriad proteases present in this compartment. Among these proteases, cathepsins are likely candidates to affect MARCH1, given their established roles in Ag processing (3, 46). Most of the cathepsins are cysteine proteases (46, 49), and we found that the cysteine/serine protease inhibitor leupeptin was able to stabilize MARCH1. Furthermore, inhibition of cathepsin L increased MARCH1 levels through stabilization, but not in all cell types. We failed to observe any stabilizing effect of cathepsin S inhibition/knockdown on MARCH1. From our results, we conclude that MARCH1 protein levels are regulated by multiple, redundant proteases within lysosomes, including cathepsin L. A similar finding has emerged for the processing of TLR9, which must be cleaved within endolysosomes before signaling, and this cleavage event can be mediated by different proteases (62, 63).

Our characterization of mutant forms of MARCH1 complemented our studies of the factors that affect MARCH1 protein levels. In particular, we identified a region of MARCH1 that influenced its stability. Removal of as few as the first 40 residues of the N terminus affected the stability of MARCH1, without compromising activity. The increased stability of the N-terminal truncations was correlated with a change in the subcellular distribution of the mutants relative to wild type. Most notably, in addition to the Golgi and lysosomal staining seen with wild-type MARCH1, these mutants exhibited abundant vesicular staining throughout the cell. The distribution of MARCH1 raises questions about its subcellular site of action. At steady-state, we observed the most pronounced colocalization between MARCH1 and a trans-Golgi marker, with relatively little MARCH1 present within endolysosomes. However, since MARCH1 turnover requires lysosomal activity, it must traffic through this compartment. In the case of MHC class II, it seems that the effects of MARCH1 are manifested in a post-Golgi compartment, after processing of the invariant chain (11, 13), resulting in rapid endocytosis of MHC class II from the cell surface (12–14). Consistent with this finding, it was shown that human MARCH1 can be detected in early endosomes (transferring receptor-positive), but not later, HLA-DM-positive compartments (13). In this instance, MARCH1 was expressed as a fusion with GFP, which could affect its localization, perhaps causing some accumulation in early endosomes where it could continue to ubiquitinate MHC class II. Although MHC class II molecules are quickly internalized in the presence of MARCH1, it is possible that ubiquitination occurs before the initial arrival of MHC class II at the cell surface or during recycling within early endosomes. Upon reaching the cell surface, ubiquitin could then exert its effects on MHC class II. Whatever the case, MARCH1 itself appears to traffic through the late endocytic compartment, at least transiently, to be degraded.

Collectively, the available data suggest a dynamic pattern of trafficking along the endocytic pathway for MARCH1, between the trans-Golgi, early endosomes, and late endosomes. Our results indicate that this trafficking requires information within the N terminus. However, disruption of this pathway does not necessarily abolish function. Rather, it affects the stability of MARCH1, which appears to be important for maintaining the proper levels of MARCH1. Deletion of the C-terminal 50 residues (ΔC229–279) did not dramatically affect localization or stability of MARCH1. Notably, this mutant retains both of the C-terminal YXXΦ motifs (Fig. 4A). However, mutation of these motifs affected MARCH1 function, but not expression levels, arguing that they are not required for trafficking into endolysosomes. Rather, these residues may be important for substrate interaction or recruitment of downstream effector molecules. While it remains to be determined exactly where and when MHC class II and CD86 encounter MARCH1 during biogenesis, and how this process is influenced by APC maturation, it seems likely that these two targets of MARCH1 share some common steps in their trafficking. This may help explain how these two unrelated proteins can both be targeted by the same E3 ligase. A curious feature of the viral RING-CH E3 ligases is the ability of some to target multiple, unrelated substrates (18). As a whole, the basis of substrate recognition by viral RING-CH molecules is not well understood. Our characterization of the viral mK3 protein has demonstrated a clear requirement for “adapter-type” proteins in substrate recruitment (31, 52). MARCH1 may also require cofactors to assist in recruitment of its distinct substrates, and such cofactors would likely tie into the trafficking pathways utilized by MARCH1 and its substrates. Elucidation of the full spectrum of molecules involved in MARCH1-dependent regulation of Ag presentation will be essential to understand how MARCH1 may contribute to both immune activation and tolerance.
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Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Figure 1.  *Phenotypic characterization of MJDC, a DC-like cell line.* A. This cell line (clone 2B1) was derived as described in Materials and Methods. Cells were analyzed for the expression of molecules that are characteristic of DC, including DEC205, CD11c, MHC class II, MHC class I, CD80, CD86. The thin line in each histogram represents isotype-control background staining. Where indicated, cells were treated with 100 U/ml interferon-γ for 18 hours.

Supplemental Figure 2.  *Localization of MARCH1 mutants.* Confocal microscopic analysis of DC2.4 cells stably expressing the indicated mutants. MARCH1 staining is shown versus the indicated markers: EEA-1 (early endosome) and Derlin-1 (ER). Note that a lower threshold was applied uniformly to each set of samples (imaged for a given marker) based on negative controls (background subtraction). The signal for MARCH1 (HA) and the marker protein in each set of images was normalized by setting the brightest pixel in each image (for each fluorescence channel) as the maximum signal (histogram-stretching) in order to facilitate visualization and comparison across fields.