Major histocompatibility complex class II molecules (MHC-II) on antigen presenting cells (APCs) engage the TCR on antigen-specific CD4 T cells, thereby providing the specificity required for T cell priming and the induction of an effective immune response. In this study, we have asked whether antigen-loaded dendritic cells (DCs) that have been in contact with antigen-specific CD4 T cells retain the ability to stimulate additional naïve T cells. We show that encounter with antigen-specific primed CD4 T cells induces the degradation of surface MHC-II in antigen-loaded DCs and inhibits the ability of these DCs to stimulate additional naïve CD4 T cells. Cross-linking with MHC-II mAb as a surrogate for T-cell engagement also inhibits APC function and induces MHC-II degradation by promoting the clustering of MHC-II present in lipid raft membrane microdomains, a process that leads to MHC-II endocytosis and degradation in lysosomes. Encounter of DCs with antigen-specific primed T cells or engagement of MHC-II with antibodies promotes the degradation of both immunologically relevant and irrelevant MHC-II molecules. These data demonstrate that engagement of MHC-II on DCs after encounter with antigen-specific primed CD4 T cells promotes the down-regulation of cell surface MHC-II in DCs, thereby attenuating additional activation of naïve CD4 T cells by these APCs.

**Results and Discussion**

**Encounter with Antigen-Specific CD4 T Cells Inhibits Subsequent MHC-II–Restricted APC Function of DCs.** Although it is well known that DCs can efficiently prime naïve CD4 T cells, it remains to be seen whether these DCs are capable of activating additional naïve CD4 T cells of the same (or differing) specificity after they have completed their task of T-cell priming. To examine this question, we have pretreated HEL-pulsed DCs with I-A<sup>b</sup>-HEL<sub>46–61</sub>–restricted 3A9 CD4 T cells before adding additional carbosulfan sulfoxide-modified CD8 T cells. Preincubation of the DCs with naive control or 3A9 T cells had no effect on the ability of the DCs to stimulate additional naïve 3A9 T cells (Fig. 1A). Surprisingly, preincubation of the DCs with previously primed 3A9 CD4 T cells, but not control T cells, almost completely prevented the subsequent proliferation of CFSE-labeled naïve 3A9 T cells by these DCs (Fig. 1B and Fig. S1A). To rule out the possibility that the proliferation of the T cells in the preculture affected naïve CD4 T-cell proliferation, the entire preculture was irradiated.

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**Abstract**

Major histocompatibility complex class II molecules (MHC-II) on antigen presenting cells (APCs) engage the TCR on antigen-specific CD4 T cells, thereby providing the specificity required for T cell priming and the induction of an effective immune response. In this study, we have asked whether antigen-loaded dendritic cells (DCs) that have been in contact with antigen-specific CD4 T cells retain the ability to stimulate additional naïve T cells. We show that encounter with antigen-specific primed CD4 T cells induces the degradation of surface MHC-II in antigen-loaded DCs and inhibits the ability of these DCs to stimulate additional naïve CD4 T cells. Cross-linking with MHC-II mAb as a surrogate for T-cell engagement also inhibits APC function and induces MHC-II degradation by promoting the clustering of MHC-II present in lipid raft membrane microdomains, a process that leads to MHC-II endocytosis and degradation in lysosomes. Encounter of DCs with antigen-specific primed T cells or engagement of MHC-II with antibodies promotes the degradation of both immunologically relevant and irrelevant MHC-II molecules. These data demonstrate that engagement of MHC-II on DCs after encounter with antigen-specific primed CD4 T cells promotes the down-regulation of cell surface MHC-II in DCs, thereby attenuating additional activation of naïve CD4 T cells by these APCs.
Antigen-Specific T Cells Stimulate the Loss of MHC-II from the DC Surface. In an attempt to identify the mechanism of APC inactivation by antigen-specific T-cell interactions, we monitored expression of MHC-II–peptide complexes on these DCs. By using the I-A\(^{\alpha}\)-HEL\(_{66-61}\)–specific mAb Aw3.18.14 (18), we found that pretreatment of HEL-pulsed DCs with primed, but not naive, I-A\(^{\alpha}\)-HEL–restricted T cells dramatically reduced expression of I-A\(^{\alpha}\)-HEL\(_{66-61}\) complexes on these DCs (Fig. 2 A) and inhibited CD69 up-regulation of naive 3A9 T cells by these DCs (Fig. 2 B). The reduced APC function observed by T-cell pretreatment was not irreversible, because addition of HEL\(_{66-61}\) peptide restores I-A\(^{\alpha}\)-HEL\(_{66-61}\) complex expression and APC function by these DCs. Like the loss of APC function, the loss of MHC-II from the surface of DCs was antigen-specific, because preincubation of DCs with control CD4 T cells did not affect expression of I-A\(^{\alpha}\)-HEL\(_{66-61}\) complexes or CD69 up-regulation of naive 3A9 T cells.

To investigate the fate of MHC-II after interaction with T cells by using a different TCR transgenic mouse system, we monitored the expression of biotinylated cell surface MHC-II after incubating OVA-pulsed DCs with either naive or primed OVA–specific OT-II T cells. Pretreatment with primed, but not naive, OT-II T cells resulted in the loss of MHC-II from the DC surface (Fig. 2 C). In these experiments, even the OT-II T cells present in the pretreatment culture were present during the cell lysis procedure, ruling out the possibility that intact surface MHC-II was merely transferred from the DCs to T cells. The loss of MHC-II from the surface of DCs was antigen dependent, because OT-II CD4 T cells did not affect expression of cell surface MHC-II when the DCs were not loaded with OVA\(_{323-339}\) peptide. The ability of primed, but not naive, CD4 T cells to reduce MHC-II expression in DCs is likely due to enhanced conjugate formation between DCs and primed, but not naive, T cells (Fig. S2). This result is attributable to increased expression of adhesion molecules on primed T cells that enhance even antigen-independent T-cell adhesion (19). These results demonstrate that primed T cells induce the degradation of MHC-II from the DC surface in an antigen-dependent manner.

Before the addition of CFSE-labeled naive 3A9 T cells. Once again, preculture with antigen-specific T cells prevented subsequent naive CD4 T-cell proliferation, whereas preculture with nonspecific T cells did not (Fig. 1 B). Lastly, we purified the DCs after the preculture period with nonspecific or antigen-specific T cells, and even these purified DCs were unable to stimulate naive 3A9 T-cell proliferation (Fig. S1 B), demonstrating that the encounter of DCs bearing specific MHC-II–peptide complexes with antigen-specific primed CD4 T cells inhibits the ability of the DCs to serve as APCs for subsequent naive CD4 T-cell activation.

Cross-Linking of MHC-II Promotes Endocytosis and Lysosomal Degradation of Surface MHC-II in DCs. The APC-mediated down-regulation of the TCR upon T-cell:APC conjugate formation can be mimicked by TCR cross-linking using anti-TCR mAb (13). To mimic the engagement of MHC-II with ligand(s) on T cells, we cross-linked MHC-II on the surface of DCs by using MHC-II mAb. Like preincubation of DCs with antigen-specific primed T cells, cross-linking of I-A molecules on the surface of HEL-pulsed DCs for only 4 h dramatically reduced the ability of the DCs to stimulate antigen-specific 3A9 T cells (Fig. 3 A).

By using antibody cross-linking as a surrogate for T-cell engagement, we were able to study the mechanism of MHC-II down-regulation after MHC-II ligation. Approximately 40% of cell surface I-A was spontaneously internalized after 30 min in mock–cross-linked DCs, and cross-linking dramatically enhanced the extent of MHC-II endocytosis in DCs (Fig. 3 B). By following the fate of surface biotinylated MHC-II, we found that ~50% of cell surface MHC-II I-A was degraded in DC after 8 h of culture in medium alone (Fig. 3 C). When cell surface MHC-II was cross-linked, however, the extent of MHC-II degradation was dramatically enhanced. The effect of cross-linking on MHC-II expression did not depend on the specific mAb used to cross-link MHC-II, because identical results were obtained when MHC-II I-A was cross-linked by using the I-A\(^{\alpha}\)-specific mAbs 11-5.2 or 10-3.6 (Fig. S3 A) or when MHC-II I-E was cross-linked by using the I-E\(^{\alpha}\)-specific mAbs 14-4-4S or 17-3-3 (Fig. S3 B). Control studies using anti–MHC-I mAb revealed that cross-linking of surface MHC-I had no effect on the turnover of MHC-II (Fig. S3 A). The disappearance of MHC-II after cross-linking was due to lysosomal proteolysis, because neutralizing lysosomal proteinases activity by treating cells with the vacuolar H\(^{+}\) ATPase inhibitor Bafilomycin A1 or the weak-base chloroquine completely blocked the cross-linking–induced loss of MHC-II (Fig. 3 D), demonstrating that cross-linking promotes lysosomal degradation of internalized MHC-II. Curiously, the cross-linking–induced loss of MHC-II did not depend on MHC-II ubiquitination, because cross-linking-stimulated endocytosis and degradation of MHC-II similarly in wild-type DCs, March-I deficient DCs, and MHC-II I-A\(^{\alpha}\)K\(_{221}\)R ubiquitination-deficient DCs (Fig. S4).
cross-linking resulted in profound MHC-II clustering in nearly all cells examined (Fig. 4A). Because MHC-II has been found to be associated with cholesterol-dependent "lipid raft" membrane microdomains in both human and mouse APCs (23, 24), we explored the possibility that the integrity of these domains is important for cross-linking–induced MHC-II clustering and endocytosis. Cholesterol depletion (and lipid raft disruption) using the cyclic carbohydrate MβCD almost completely prevented the cross-linking–induced clustering of MHC-II on the surface of DCs (Fig. 4A). Although cholesterol depletion had no effect on the spontaneous internalization of surface-tagged MHC-II, cross-linking–enhanced MHC-II endocytosis was nearly blocked by MβCD treatment (Fig. 4B). These results demonstrate that MHC-II cross-linking leads to MHC-II clustering and that cross-linking–induced MHC-II clustering and endocytosis are cholesterol dependent. Unfortunately, disruption of lipid rafts on living cells prevents APC:T-cell interactions (20), so extending this approach to investigate whether APC raft integrity is required for T-cell–induced down-regulation of MHC-II is not possible.

**Cross-Linking of MHC-II Induces Internalization of both Relevant and Irrelevant MHC-II in DCs.** If anti–MHC-II mAb were able to physically cross-link surface MHC-II molecules present in discrete membrane microdomains, we would predict that cross-linking I-A would also promote the endocytosis and degradation of different MHC-II molecules present in these same domains. We tested this prediction by cross-linking surface MHC-II on DCs using either I-A–specific or I-E–specific mAb and F(ab')2 antibodies. Cross-linking either surface I-A or surface I-E molecules each promoted I-A degradation (Fig. 5A). To examine the effect of MHC-II cross-linking on APC function, we pretreated HEL-pulsed DCs with anti-I-A or anti-I-E mAb under cross-linking (F(ab')2) and non-cross-linking conditions. Mock cross-linking did not affect the proliferation of naïve 3A9 T cells by these DCs (Fig. 5B). By contrast, cross-linking of DC surface bound I-A or I-E mAb inhibited their ability to stimulate I-A^k–HEL/66,61–specific 3A9 T cells. This result is in agreement with our observation that either I-A or I-E cross-linking promotes the down-regulation of I-A and demonstrates that cross-linking–mediated down-regulation of MHC-II results in decreased antigen presentation by DCs.

**Antigen-Specific T Cells Promote Loss of Relevant and Irrelevant MHC-II from DCs.** To determine whether exposure of an APC to an antigen-specific T-cell can inhibit the ability of the APC to stimulate unrelated T cells, we simultaneously pulsed DCs with preprocessed HEL and PCC peptides. In agreement with our previous results (Fig. 1), we found that preincubation with primed I-A^k–HEL/66,61–specific 3A9 T cells almost completely prevented the DCs from stimulating additional naïve 3A9 T cells (Fig. 6A). We also found that pretreatment with primed 3A9 T cells prevented the DCs from stimulating naïve I-E^k–PCC/81,104–specific AND T cells (Fig. 6B). The inhibition of AND T-cell activation by DCs was antigen-specific, because pretreatment of HEL/PCC-pulsed DCs with control T cells had no effect on AND T-cell proliferation. Taken together with the results of antibody cross-linking experiments, these data demonstrate that the interaction of antigen-specific primed T cells with DCs leads to the internalization and degradation of both immunologically "relevant" and "irrelevant" MHC-II from the DC surface.

In this study, we found that the encounter of antigen-loaded DCs with previously primed antigen-specific CD4 T cells leads to MHC-II loss from the APC that limits its ability to activate either immunologically relevant or irrelevant naïve CD4 T cells. In our attempt to identify a molecular mechanism for this phenomenon, we examined the importance of MHC-II organization on the APC surface in this process. MHC-II molecules on the surface of APCs are present in cholesterol-rich membrane microdomains termed lipid rafts, and the integrity of APCs raids is essential for efficient CD4 T-cell activation (23). We found that cross-linking of MHC-II promotes the aggregation of surface MHC-II into
present on the cell surface at time 0. The data shown are the mean expressed as a fraction of the percentage of amount of tagged MHC-II from three independent experiments. *<P<0.05 (relative to control). (C) Surface proteins of DCs were biotinylated on ice by using sulfo-NHS-biotin, and one aliquot of cells was immediately harvested (control, t=0). The remaining cells were incubated with an isotype control mouse mAb (mock) or MHC-II I-A mouse mAb 11-5.2 for 30 min on ice before the addition of anti-mouse IgG F(ab')2 at 37 °C for the indicated times. The cells were harvested, lysed, and biotinylated proteins were isolated by using streptavidin-Sepharose beads. The amount of biotinylated MHC-II present in the mock cross-linked samples (squares) or MHC-II cross-linked samples (circles) was analyzed by immunoblotting using the indicated antibodies and quantitative densitometry of the blots. The amount of surface MHC-II present at each time point was expressed as a percentage of the total amount of surface MHC-II present on biotinylated cells at control time 0. The data shown are the mean ± SD of three independent experiments. *<P<0.05 (relative to mock). (D) Surface proteins of DCs were biotinylated on ice by using sulfo-NHS-biotin, and one aliquot of cells was immediately harvested (control, t=0). The remaining cells were incubated with mock Ab (mouse IgG) or mouse anti-MHC-II mAb 11-5.2 for 2 h at 37 °C. The cells were lysed, biotinylated proteins were isolated with streptavidin-Sepharose beads, and the amount of biotinylated MHC-II present in each sample as well as MHC-II survival was determined as described above. The data shown are the mean ± SD of three independent experiments. *<P<0.05 (relative to control).

Cholesterol-dependent MHC-II clustering is necessary for cross-linking–induced MHC-II endocytosis and degradation. (A) DCs were treated (or not) with MjCD (10 mM) for 20 min at 37 °C. The cells were incubated with biotinylated MHC-II I-A mouse mAb 11-5.2 for 30 min on ice, washed, and incubated with or without goat anti-mouse IgG F(ab')2 (10 μg/mL) for 5 min at 37 °C. The cells were fixed and immunolabeled cell surface MHC-II was visualized by confocal microscopy using Alexa Fluor 546-conjugated streptavidin. A representative image of I-A distribution on mock cross-linked or MHC-II cross-linked DCs is shown. At least 20 individual cells in each condition were examined in each experiment. The percentage of the cells that have clustered cell surface MHC-II were quantified. The data shown are the mean ± SD from three independent experiments. *<P<0.05 (relative to control). (B) DCs were treated (or not) with MjCD (10 mM) for 20 min at 37 °C. The cells were incubated with MHC-II I-A mouse mAb 11-5.2 for 30 min on ice, washed, and incubated with or without anti-mouse IgG F(ab')2 for 30 min at 37 °C. The remaining cell-surface MHC-II antibodies were detected by incubating with fluorescently labeled streptavidin on ice, and the cells were analyzed by FACS analysis. The mean fluorescent intensity after 30 min of mock- or MHC-II cross-linking was expressed as a percentage of the amount of tagged MHC-II present on the cell surface at time 0. The data shown are the mean ± SD from three independent experiments. *<P<0.05 (relative to control).
are pretreated with naive antigen-specific T cells and that nothing in this model precludes additional DCs (that have not yet encountered antigen-specific T cells) from acquiring antigen and stimulating additional relevant (or irrelevant) T cells.

Although our study clearly shows that the primed CD4 T-cell-dependent down-regulation of MHC-II on DCs is both TCR and MHC-II–peptide dependent, it does not unambiguously identify the T-cell ligand that engages MHC-II on the DC. Because the TCR on antigen-specific T cells is actually down-regulated after activation (27), it is possible that other MHC-II interacting molecules are involved in the MHC-II cross-linking. Surface expression of the MHC-II binding protein LAG-3 is dramatically enhanced during CD4 T-cell activation (28, 29); however, preliminary experiments revealed that LAG-3–deficient OT-I T cells also promoted MHC-II degradation in DCs. The TCR and CD4 are also likely candidates for MHC-II ligands; however, analysis of conjugates between MHC-II–antigen-specific T cells lacking CD4 and antigen-loaded DCs is not technically possible. It is also possible that signaling in the DC, either by MHC-II itself or by an additional DC receptor, leads to T-cell–dependent, antigen-specific MHC-II clustering and endocytosis. Although determining the precise mechanism leading to pMHC-II loss after primed T-cell engagement will require additional study, our data showing that antigen-loaded DCs can be rendered nonfunctional by antigen-specific primed T cells provides a cellular mechanism to limit naïve T-cell activation by APCs during the course of an immune response.

Materials and Methods

Mice. B10.BR mice (H-2b), C57BL/6 mice (H-2b), 3A9 TCR transgenic mice, OT-I TCR transgenic mice, and AND TCR transgenic mice were purchased from Jackson Laboratory. March-I knockout mice and MHC-II Kβ−/−ubiquitin mutant knock-in mice on the C57BL/6 background have been described (9, 30). All mice were cared for in accordance with National Institutes of Health guidelines with the approval of the National Cancer Institute Animal Care and Use Committee.

Antibodies and Reagents. Anti-mouse I-A mAb (clone 11-5-2) and anti-mouse I- E mAb (clone 14-4-4S) was from BioLegend. Anti-FcγRI/II (clone 2.4G2), anti-TCRγδ (clone H57-597), anti-CD28 (clone 37.51), anti-CD9 (KMC8), and anti-CD11c-PE (HL3) antibody were obtained from BD Biosciences. The I-Ak- Hel46–specific mAb Abw3.18.14 (18) was a gift from Emil Unanue (Washington University School of Medicine, St. Louis, MO). The anti-I-α/β–chain rabbit serum has been described (31), and rabbit anti-mouse MHC-I serum was a gift from Jon Yewdell (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Sulfo-NHS-biotin was from Thermo Scientific. Streptavidin-Sepharose beads, methyl β-cyclodextrin (MβCD), Bafilomycin A1, chloroquine, and HEL protein were purchased from Sigma-Aldrich. Alexa Fluor-conjugated antibodies, CFSE, CellTracker Green, and CellTracker Red were obtained from Invitrogen. HRP-conjugated antibodies were obtained from Southern Biotech. Goat anti-mouse IgG F(ab′)2 antibody was purchased from Jackson ImmunoResearch. CD4 T-cell isolation Kits, CD11c MicroBeads, CD4 MicroBeads, and CD90.2 MicroBeads were purchased from Miltenyi Biotech. Peptides corresponding to HEL46–61, OVA253–269, and PCC were synthesized at the Center for Biologics Development, National Institutes of Health, Bethesda, MD. The anti-human CD4 T-cell Isolation antibody was purchased from Jackson ImmunoResearch.

Fig. 5. Cross-linking of MHC-II induces degradation of both relevant and irrelevant MHC-II in DCs. (A) Surface proteins of DCs were biotinylated on ice by using sulfo-NHS-biotin, and the cells were incubated with isotype control mouse mAb (mock), MHC-II I- E mouse mAbs 14–4–4S, or MHC-II I-A mouse mAbs 11–5–2 for 30 min at 37 °C and cross-linked by using anti-mouse IgG F(ab′)2 at 37 °C for 4 h. The cells were harvested, lysed, and biotinylated proteins were isolated by using streptavidin-Sepharose beads. The amount of biotinylated MHC-II present in each sample was analyzed by immunoblotting using the indicated antibody. The amount of surface MHC-II remaining after cross-linking was expressed relative to that present in mock cross-linked cells. The data shown are the mean ± SD of three independent experiments. *P < 0.05 (relative to mock). (B) HEL-loaded DCs were incubated with isotype control mouse mAb, MHC-II I- E mAb 14–4–4S, or MHC-II I-A mAb 11-5-2 for 30 min at 37 °C. The cells were incubated alone (mock cross-link) or with anti-mouse IgG F(ab′)2, F(ab′)2 cross-link) at 37 °C for 3 h. The pretreated DCs were isolated with naïve CFSE-labeled 3A9 DC T cells at 1:10 ratio. CFSE dilution was examined 48 h later by FACS analysis. The naïve CD4 T-cell division index was calculated by using FlowJo software. The division index under each condition was determined and was expressed relative to that of cells dividing under mock cross-linking conditions. The data shown are the mean ± SD from three independent experiments. *P < 0.05 (relative to isotype control).

Fig. 6. Preculture of HEL/PCC-loaded DCs with HEL-specific T cells inhibits activation of both HEL- and PCC-specific T cells. DCs loaded with both HEL and PCC were preincubated alone (no T cells) or with primed 3A9 tg+ or primed 3A9 tg+ T cells for 4 h at a 1:1 ratio. The pretreated culture was irradiated, washed, and either CFSE-labeled naïve I-A+HEL46– 61–specific 3A9 T cells (A) or CFSE-labeled naïve I-E– PCC81–104–specific AND T cells (B) were added to the culture at a 1:10 ratio (DC: naïve T-cell). CD4 T-cell proliferation was measured 48 h later by FACS analysis. The division index under each condition was expressed relative to that culture condition in which CD4 T cells were not added. The data shown are the mean ± SD from three independent experiments. *P < 0.05 (relative to no T cells control).
DC and T-Cell Isolation. Dendritic cells were prepared by differentiating mouse bone marrow cells in medium containing 20 ng/ml GM-CSF for 7 d by using standard protocols (32). The cells were routinely 90% CD11c⁺, MHC-II⁺, CD86⁺, CD40⁺ after 7 d of culture. Naïve CD4 T cells were obtained from isolated lymph nodes by negative selection using a MACS CD4 T Cell isolation Kit according to the manufacturer’s specifications. To generate primed CD4 T cells, naïve CD4 T cells cultured in 24-well plates coated with 5 μg/ml anti-CD3 and 5 μg/ml anti-CD28 mAb for 3 d. The activation status of T cells was confirmed by FACS analysis of cell scatter plots, increased expression of the activation marker CD69, and antibody-induced TCR down-regulation.

Cell Surface Biotinylation. Plasma membrane proteins of DCs were biotinylated by incubating CD86low, CD40low after 7 d of culture. Naïve CD4 T cells were cultured in 24-well plates coated with 5 μM antigenic peptide and, in some experiments, cell surface proteins were biotinylated as described above. Antigen-pulsed DCs were incubated with bio- tinylated reagent sialu-HHS-biotin (1 mg/ml) in HBSS for 30 min on ice according to the manufacturer’s protocol. After surface biotinylation, free biotin was quenched by washing the cells twice with ice-cold HBSS containing 50 mM glycine. The cells were extensively washed in ice cold HBSS and resuspended in complete medium before use. At the conclusion of each indicated incubation, the cells were solubilized in lysis buffer (10 mM Tris HCl at pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mg/ml BSA, 50 mM PMSF, 0.1% TLCK, and 5 mM CaCl₂) and biotin-labeled proteins were iso- lated by using streptavidin-Sepharose beads. Biotinylated proteins were detected by SDS/PAGE and immunoblotting using protein-specific antibodies by using protocols described (33).

DC Pretreatment and T-Cell Proliferation. Day 6 DCs were pulsed overnight with or without 5 μM antigenic peptide and, in some experiments, cell surface proteins were biotinylated as described above. Antigen-pulsed DCs were preincubated at a 1:1 ratio with naive or primed CD4 T cells for 4 h. For MHC-II cross-linking studies, DCs were incubated with anti-MHC-II antibody (5 μg/ml) in the presence of the Fc receptor blocking antibody 2.4G2 anti-body (5 μg/ml) at 37 °C for 30 min and cross-linked with goat anti-mouse IgG F(ab')2 (10 μg/ml) at 37 °C for indicated times.

13. San José E, Borroto A, Niedergang F, Alcover A, Alarcón B (2000) Triggering the TCR MHC-II antibody (5 μg/ml) in complete media at 37 °C for 5 min. The cells were fixed in PBS containing 4% PFA and plated on poly-L-lysine-coated coverslips. Surface MHC-II was visualized by immunofluorescence microscopy using Alexa Fluor 546-conjugated streptavidin. All cells were imaged by using a Zeiss LSM 510 META confocal microscope using a 63× oilimmersion objective lens (N.A. 1.4), and the cells were scored as having clustered or “nonclustered” MHC-II by a blind observer.

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FACS-Based MHC-II Internalization Assay. DCs were incubated with bio- tinylated anti–MHC-II antibody (5 μg/ml) in the presence of the Fc receptor blocking antibody 2.4G2 anti-body (5 μg/ml) on ice 30 min and washed twice with FACS staining buffer (PBS containing 2% (vol/vol) FBS). Cell surface MHC-II antibodies were cross-linked with goat anti-mouse IgG (Fab')2 (10 μg/ml) in complete media at 37 °C for various times. After two washes with FACS buffer, the amount of primary antibody remaining on the cell surface was identified by staining using Alexa-conjugated streptavidin on ice. The cells were washed in ice-cold FACS staining buffer and were then fixed in 1% PFA in PBS. Expression of each antibody was determined by flow cy- tometry using a FACSCalibur (Becton Dickinson). The mean fluorescence intensity was determined for each FACS profile and expressed as a per- centage of the value present on cells kept on ice for the duration of the internalization assay.

Cross-Linking and Cell Surface MHC-II Staining. DCs were incubated with the biotinylated anti–MHC–II mAb 11-5.2 (5 μg/ml) on ice for 30 min and washed twice with FACS staining buffer. Cell surface MHC-II antibodies were cross- linked with goat anti-mouse IgG F(ab')2 (10 μg/ml) in complete media at 37 °C for 5 min. The cells were fixed in PBS containing 4% PFA and plated on poly-L-lysine-coated coverslips. Surface MHC-II was visualized by immunofluorescence microscopy using Alexa Fluor 546-conjugated streptavidin. All cells were imaged by using a Zeiss LSM 510 META confocal microscope using a 63× oilimmersion objective lens (N.A. 1.4), and the cells were scored as having clustered or “nonclustered” MHC-II by a blind observer.

Evaluation and Research, Food and Drug Administration, and purified by reverse-phase HPLC.

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Activated T cells stimulate the loss of MHC-II from the surface of DCs. HEL-loaded DCs were preincubated alone (no T cells) or with primed 3A9 tg− or primed 3A9 tg+ T cells for 4 h. (A) The pretreated DC culture was added directly to CFSE-labeled 3A9 T cells. (B) The CD4 T cells from the preculture were depleted by MACS sorting using a mixture of anti-CD4 and anti-CD90.2 mAb. The purified DCs were then added to CFSE-labeled 3A9 T cells. In each experiment the division index was calculated by using FlowJo software. The division index under each condition was expressed relative to that culture condition in which CD4 T cells were not added. The data shown are the mean ± SD from three independent experiments. *P < 0.05 (relative to no T cells control).

Primed CD4 T cells interact efficiently with antigen-loaded DCs. Equal numbers of OVA323-339 peptide-pulsed DCs (red) and either naive or primed OT-II CD4 T cells (green) were incubated together for 4 h, and the association of CD4 T cells with DCs was examined by confocal immunofluorescence microscopy.
Cross-linking promotes degradation of surface MHC-II I-A and I-E in DCs. Surface proteins of immature DCs were biotinylated on ice by using sulfo-NHS-biotin, and the cells were incubated with control mouse Ab, MHC-II I-A mouse mAbs 11-5.2 or 10-6.3, or MHC-I mouse mAb (A) or MHC-II I-E mouse mAbs 14-4-4S or 17-3-3 (B) for 30 min at 37 °C and cross-linked by using anti-mouse IgG Fab, at 37 °C for 4 h. The cells were harvested, lysed, and biotinylated proteins were isolated by using streptavidin-Sepharose beads. The amount of biotinylated MHC-II present in each sample was analyzed by immunoblotting using an I-A β-chain specific rabbit serum (A) or an I-E α-chain rabbit serum (B). The amount of surface MHC-II was expressed as a percentage of mock cross-link. The data shown are the mean ± SD of three independent experiments. *P < 0.05 (relative to mock).

Ubiquitination of MHC-II is not required for cross-linking induced endocytosis and degradation. DCs from wild-type mice (filled symbols), March-I− deficient mice (open symbols; A) or MHC-II K225R ubiquitination mutant mice (open symbols; B) were incubated with biotinylated MHC-II I-A mouse mAb AF6-120.1 for 30 min on ice. The cells were washed and incubated with (circles) or without (squares) anti-mouse IgG Fab, at 37 °C for the indicated times. The remaining cell-surface MHC-II antibodies were determined by incubating with fluorescently labeled streptavidin on ice and FACS analysis. (C and D). Surface proteins of DCs from wild-type or March-I− deficient mice (C) or MHC-II K225R ubiquitination mutant mice (D) were biotinylated on ice by using sulfo-NHS-biotin. The cells were incubated with control Ab (mouse IgG) or MHC-II I-A mouse mAb AF-6.120.1 for 30 min at 37 °C and cross-linked with anti-mouse IgG F(ab')2 for 6 h at 37 °C. The cells were lysed, biotinylated proteins were isolated by using streptavidin-Sepharose beads, and biotinylated MHC-II was analyzed by immunoblotting using MHC-II β-chain antibodies. The amount of surface MHC-II present at each time point was expressed as a percentage of the total amount of surface MHC-II present on biotinylated cells that remained on ice (t = 0 h). The data shown are the mean ± SD of three independent experiments. *P < 0.05 (relative to mock).