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Supporting Information

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SI Materials and Methods

The following antibodies and reagents were used in this study: anti–peptide–MHC class II complex (pMHC-II) mAb (L243), anti–HLA-DR mAb (Tu36), anti-CD74 mAb (M-B741), anti-EEA1 mAb (14/EEA1), and anti–Lamp-1 mAb (H4A3) were obtained from BD Biosciences. Anti-actin antibody was from Millipore. Biotinylated anti-ubiquitin antibody (P4D1) was from eBiosciences. Anti-V5 epitope tag antibody was from Abcam. Anti-invariant chain (Ii) mAb (LL1) and anti-DR β-chain mAb (XD5.A11) were described previously (1). HRP-conjugated antibodies were obtained from Southern Biotech.

Plasmids encoding human March-I and C-terminal V5-tagged March-I were provided by Satoshi Ishido (RIKEN, Yokohama, Japan). A plasmid encoding GFP-CD63 was provided by Juan Bonifacino (National Institutes of Health, Bethesda, MD). Plasmids encoding HLA-DRα, HLA-DRβ, Ii p33 (Ii WT), Ii p33 Δ20 (Ii Δtail), and Ii p33 $L_7A/L_{17}A$ (Ii dileucine motif mutant [LL mutant]) were described previously (2–4).

1. Walseng E, Bakke O, Roche PA (2008) Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. *J Biol Chem* 283(21):14717–14727.
2. Roche PA, Teletski CL, Stang E, Bakke O, Long EO (1993) Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc Natl Acad Sci USA* 90(18):8581–8585.
3. Pieters J, Bakke O, Dobberstein B (1993) The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J Cell Sci* 106(pt 3): 831–846.
4. Bremnes B, Madsen T, Gedde-Dahl M, Bakke O (1994) An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalization. *J Cell Sci* 107(pt 7):2021–2032.

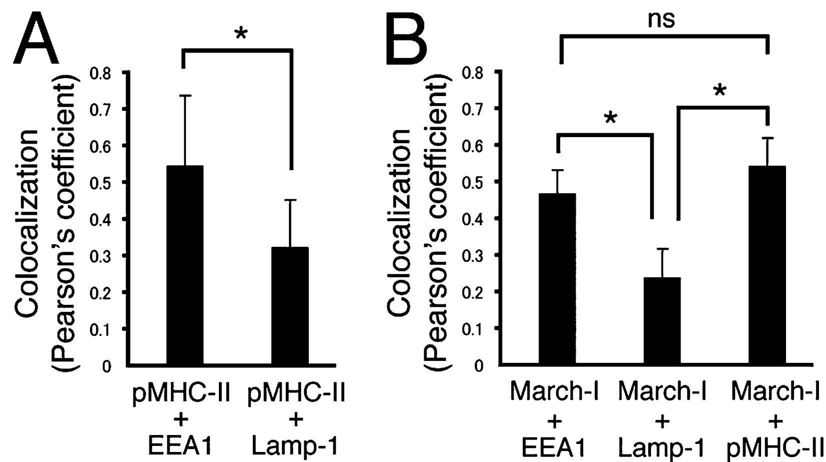

Fig. S1. Peptide-loaded MHC-II is ubiquitinated on the plasma membrane and in early endosomes. Confocal immunofluorescence microscopy images were analyzed by using National Institutes of Health (NIH) ImageJ software and colocalization of the indicated proteins was determined by using Pearson coefficient analysis. (*A*) Human DCs were analyzed for colocalization of internalized pMHC-II (10 min) with EEA1 or Lamp-1 (as in Fig. 1*B*). (*B*) HeLa-CIITA cells were transfected with V5 epitope-tagged March-I and analyzed for colocalization of March-I with EEA1, Lamp-1, or internalized pMHC-II (10 min; as in Fig. 1*C*). The data shown are mean ± SD obtained from at least 10 different cells (*$P < 0.05$). ns, not significant.

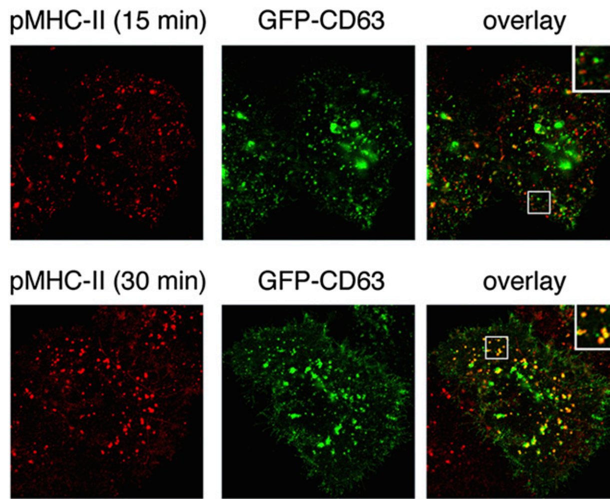

Fig. S2. Internalized pMHC-II moves from early endosomes to late endosomes/lysosomes within 30 min. HeLa-CIITA cells were transfected with a plasmids encoding V5 epitope-tagged March-I and the lysosomal marker GFP-CD63. The cells were incubated with anti–pMHC-II antibody (L243) on ice for 30 min, washed, and recultured for 15 min or 30 min at 37 °C. The cells were fixed, permeabilized, and stained with a secondary antibody allowing detection of internalized pMHC-II mAb L243. The distribution of internalized pMHC-II (red) and GFP-CD63 (green) are shown. (*Insets*, *Right*) Enlarged region of the cell.

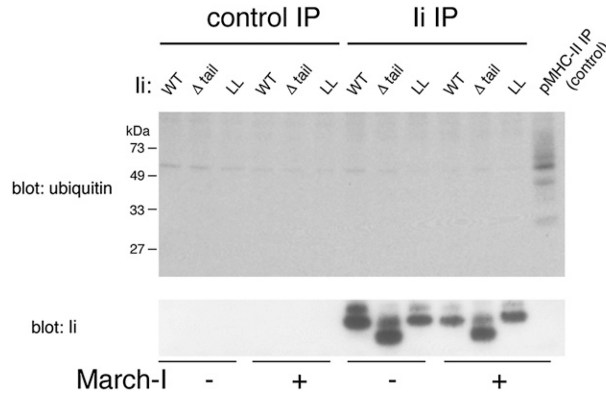

Fig. S3. Free Ii is not ubiquitinated by March-I. HEK-293T cells were transfected with plasmids encoding WT Ii, Ii Δtail, or Ii LL mutant alone or together with March-I. The cells were lysed in Triton X-100, and aliquots of each lysate were subjected to immunoprecipitation by using control IgG or anti-Ii antibody. The immunoprecipitates were analyzed by immunoblotting by using anti-ubiquitin or anti-Ii antibodies. A lysate of HEK-293T cells expressing WT Ii, March-I, and MHC-II α- and β-chains was subjected to immunoprecipitation by using anti-pMHC-II mAb L243 antibody and served as a positive control on the ubiquitin immunoblot.

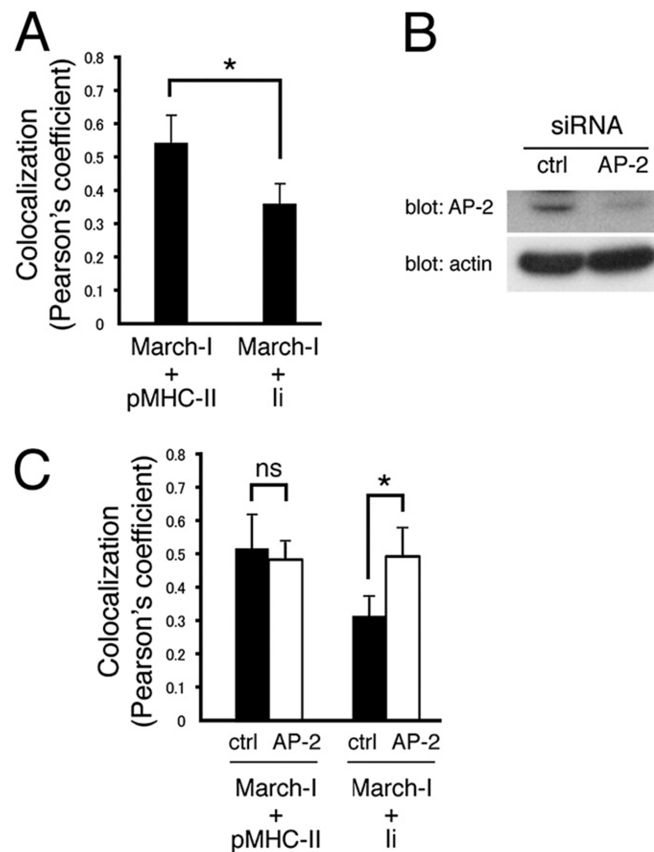

Fig. S4. Disruption of clathrin-mediated endocytosis promotes early endosomal localization of MHC-II–Ii complexes. (*A*) HeLa-CIITA cells were transfected with V5 epitope-tagged March-I and analyzed for colocalization of March-I with internalized pMHC-II (10 min) or Ii (10 min; as in Fig. 4*A*). Confocal immunofluorescence microscopy images were analyzed by using NIH ImageJ software and colocalization of the indicated proteins was determined by using Pearson coefficient analysis. The data shown are the mean ± SD obtained from at least 10 different cells (*$P < 0.05$). (*B*–*D*) HeLa-CIITA cells were transfected with control siRNA or siRNA targeting the μ2 subunit of AP-2. After 2 d, the cells were supertransfected with a plasmid encoding V5 epitope-tagged March-I. (*B*) Equal portions of each lysate were assayed for the present of AP-2 μ2 subunit or actin by immunoblot analysis. (*C*) March-I expressing HeLa-CIITA cells treated with control siRNA or AP-2 siRNA were examined for colocalization of March-I with internalized pMHC-II (10 min) or Ii (10 min) (as in Fig. 4*C*). Confocal immunofluorescence microscopy images were analyzed by using NIH ImageJ software, and colocalization of the indicated proteins was determined by using Pearson coefficient analysis. The data shown are mean ± SD obtained from at least 10 different cells (*$P < 0.05$).

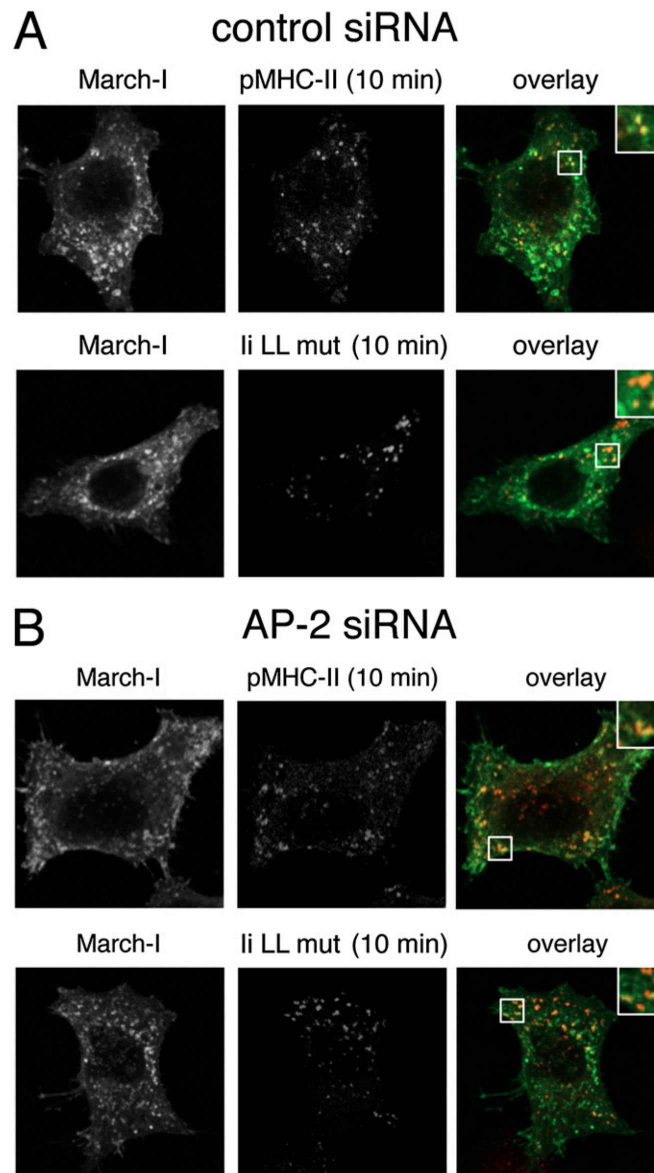

Fig. S5. Disruption of clathrin-mediated endocytosis does not affect localization of internalized pMHC-II and MHC-II–Ii LL mutant complexes with March-I. HeLa cells were transfected with control siRNA or siRNA targeting the μ2 subunit of AP-2. After 2 d, the cells were supertransfected with a plasmid encoding V5 epitope-tagged March-I together with plasmids encoding MHC-II α-chain, MHC-II β-chain, and the Ii LL mutant. The distribution of March-I with internalized pMHC-II (10 min) or Ii LL mutant (10 min) in control siRNA-treated HeLa-CIITA cells (*A*) or AP-2 siRNA-treated cells (*B*) was analyzed by confocal immunofluorescence microscopy. (*Insets*, *Right*) Enlarged region of the cell.