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21. Yeast strains used were BYB69 (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 lys2Δ::hisG trp1-1 ura3-1 ste5Δ::LYS2*), BYB84 (*MAT***a** *gal2 leu2 prb1-1122 pep4-3 prc1-407 trp1 ura3-52 ste5Δ*), BYB88 (*MAT***a** *ade2-101^{oc} his3-Δ200 leu2Δ-1 lys2-801^{am} trp1-Δ63 ura3-52 ste4Δ::TRP1 ste5Δ::LYS2*), and DC17 (MATα *his1*).
22. The *CEN* plasmid (pCJ117) expressing $(His)_6$- and Myc epitope–tagged *STE5*$^+$ from the *GAL1* promoter is described in detail elsewhere (*15*). A multicopy plasmid (pCJ6) expressing the same construct was produced by inserting the 3.3-kb Bam HI–Bam HI *STE5*-containing fragment from pCJ117 into the Bam HI site in the vector, YEp352Gal (*19*). pCJ93 expresses the same construct from the authentic *STE5* promoter in the vector, YCplac33 (*20*), and was engineered with polymerase chain reaction (PCR) to have the sequence, 5′-CATATGATG-3′, immediately upstream and in-frame with the first codon of the $(His)_6$ tag. The double mutant allele, *ste5(C177A C180A)*, used in most of the experiments presented here was produced by PCR amplification using a primer encoding the sequence 5′-AA**C**-GC**G**TCTGC-TACGTTAGCT-3′, in which the indicated bases (underlined) were altered to convert the Cys codons at positions 177 and 180 to Ala codons (and in which the silent mutations, indicated by boldface, were introduced to create an Mlu I site). The mutated segment was then used to replace the corresponding fragment in the other *STE5*-containing plasmids to generate constructs in which Ste5(C177A C180A) was expressed from the *STE5* promoter on a *CEN* plasmid (pCJ70), from the *GAL1* promoter on a *CEN* plasmid (pCJ119), and from the *GAL1* promoter on a multicopy plasmid (pCJ48).
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24. Protease-deficient strain BYB84 (*21*), carrying vector alone or expressing from the *GAL1* promoter NH_2-terminally c-Myc–tagged versions of either normal Ste5 or the RING-H2 domain mutant (*22*), and also carrying either a *CEN* vector alone, or the same vector expressing *STE11*, *STE7*, or *STE4* from the *GAL1* promoter, or expressing *FUS3* from the endogenous *FUS3* promoter on a 2 μm DNA-based vector, were grown under selection in SCRaf medium (*15*) to $A_{600\ nm}$ = 0.6. The cultures were induced by the addition of Gal to a final concentration of 2% and incubated for an additional 2 to 3 hours. The cells were collected, lysed, clarified, and the resulting extracts were subjected to immunoprecipitation (*15*). The resulting immune complexes were resuspended in 1× SDS-PAGE sample buffer, boiled for 5 min, and then resolved by SDS-PAGE. After transfer onto Immobilon-P membranes (Millipore) using semidry transfer apparatus (Bio-Rad), proteins were detected by immunoblotting with rabbit polyclonal antisera to Ste11, Ste7, Fus3, Ste4, and Ste5, as appropriate.
25. Qualitative mating tests were performed by patching the *MAT***a** strains to be tested on appropriate selective medium, and then replica-plating onto a lawn of DC17 on YP medium containing either 2% Gal/0.2% Suc or 2% Glc (depending on the promoter used for *STE5* expression) at 30°C overnight. The resulting mating plates then were replica-plated onto a minimal medium [synthetic complete (SC)] (*23*) selective for diploids and further incubated at 30°C overnight.
26. The GST fusions were generated as follows. A 640-bp fragment encoding *S. japonicum* GST was generated by PCR with a pGEX vector (Pharmacia) as the template and appropriate primers to install Ase I sites at both the 5′ and 3′ ends of the product, and inserted into the Nde I site in-frame and downstream of codon 913 of the *STE5* coding sequence in pCJ117 (*22*), creating a junction (5′-CATAATATGTCC-3′) encoding $His_{912}Asn_{913}$ Met-Ser (where Met is the first residue of GST), thus yielding pCJ148. pCJ149, expressing the Ste5(C177A C180A)-GST fusion, was created from pCJ119 in an analogous fashion. The translation stop codon in both pCJ148 and pCJ149 is provided by the natural TAG at the end of the *STE5* coding sequence.
27. *MAT***a** strains to be tested were grown in an appropriate selective medium (SC) (*23*) containing either 2% glucose (Glc) or 2% raffinose (Raf), depending on the promoter regulating *STE5* expression. Strains carrying plasmids expressing *STE5* constructs from the *GAL1* promoter were induced by addition of galactose (Gal) to a final concentration of 2% and incubation for 60 min before dilution and were plated on a medium containing 2% Gal and 0.2% sucrose (Suc). Samples (0.2 ml) of serial dilutions of the *MAT***a** strains were mixed, in triplicate, with 0.6 ml of a culture of a *MAT*α tester strain (DC17) that had been grown to midexponential phase in yeast extract–peptone (YP) (*23*) medium. Portions (0.4 ml) of these mixtures were plated on a medium lacking the appropriate supplements to select for diploids and incubated at 30°C for 36 to 40 hours. Corresponding dilutions of the *MAT***a** strains were also plated to determine the total number of viable haploids. Mating efficiencies were calculated as the ratio of diploid cells formed to the total number of input *MAT***a** haploids.
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Negative Regulation by HLA-DO of MHC Class II–Restricted Antigen Processing

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HLA-DM is a major histocompatibility complex (MHC) class II–like molecule that facilitates antigen processing by catalyzing the exchange of invariant chain–derived peptides (CLIP) from class II molecules for antigenic peptides. HLA-DO is a second class II–like molecule that physically associates with HLA-DM in B cells. HLA-DO was shown to block HLA-DM function. Purified HLA-DM-DO complexes could not promote peptide exchange in vitro. Expression of HLA-DO in a class II$^+$ and DM$^+$, DO$^-$ human T cell line caused the accumulation of class II–CLIP complexes, indicating that HLA-DO blocked DM function in vivo and suggesting that HLA-DO is an important modulator of class II–restricted antigen processing.

MHC class II molecules assemble in the endoplasmic reticulum (ER) as a nonameric complex consisting of an invariant chain trimer associated with three class II αβ dimers (*1*). Signals in the invariant chain cytoplasmic domain direct the complex into the endocytic pathway, where invariant chain degradation results in the transient formation of a class II αβ dimer with a residual fragment of the invariant chain, CLIP (class II–associated invariant chain peptides), in the peptide-binding groove (*2*). The interaction of the αβ-CLIP complex in the antigen-processing compartment, or MHC class II compartment (MIIC) (*3*), with a second class II–like molecule, called HLA-DM in humans and H-2M in mice, induces CLIP dissociation (*4*, *5*). Association of empty αβ dimers with DM stabilizes them until high-affinity peptides derived from internalized proteins can bind (*6*, *7*). Mature αβ-peptide complexes then leave the endocytic pathway and are expressed on the cell surface.

Another class II–like molecule, HLA-DO, expressed only in B cells and thymic epithelium (*8*–*12*), physically associates with DM in the ER and during and after transport to the MIIC (*8*). We therefore investigated what the effect of DO association might be on the ability of DM to catalyze CLIP dissociation and peptide loading. HLA-DM was affinity-purified (*4*) from the Burkitt's lymphoma B cell line Raji and from the DO-negative DM-transfectant T2/DM (*13*). Protein immunoblotting with rabbit antisera specific for the DM and DO-β chain cytoplasmic domains (*14*) showed that the purified material from Raji contained DO, whereas that from T2/DM did not (Fig. 1A). DM-DO association was maintained through extensive washing of the monoclonal antibody (mAb) MaP.DMB/c affinity column with sodium deoxycholate. Thus, the interaction is qualitatively different from that of DM with conventional class II molecules, which is retained only in mild detergents such as CHAPS or digitonin

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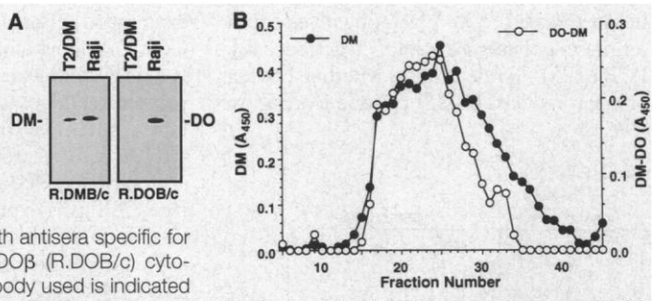

Fig. 1. Fractionation of DM-DO complexes. (**A**) Affinity-purified DM complexes from the Burkitt's lymphoma, Raji, and T2/DM cells were purified as described (*4*), then subjected to SDS-PAGE and protein immunoblotting (*31*) for DM and DO with antisera specific for the DMβ (R.DMB/c) and DOβ (R.DOB/c) cytoplasmic domains. The antibody used is indicated at the bottom and the reactive component on the side of each gel. (**B**) ELISA (*14*) of DM and DM-DO complexes from Raji cells fractionated by gel filtration to enrich for DM-DO complexes. Fractions 19 to 21 were pooled and used in subsequent experiments as DM-DO complexes.

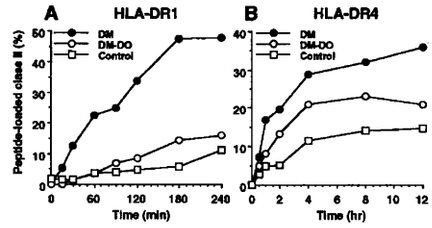

Fig. 3. The inhibitory effect of DO is not restricted to DR3 αβ-CLIP complexes. Affinity-purified radiolabeled HLA-DR1 (**A**) or HLA-DR4 (**B**) αβ-CLIP complexes were incubated with the DR1- or DR4-specific biotinylated-HAp peptide and either DM or DM-DO complexes (matched for a content of 20 ng of DM) for the indicated times at pH 5.0. As a control for spontaneous peptide loading, no DM or DM-DO complexes were added to the samples labeled Control. After neutralization, DR-HAp complexes were immunoprecipitated with streptavidin-agarose beads, analyzed by SDS-PAGE, and quantitated by image analysis.

(*6*, *15*). The DM-DO complex was further purified by gel filtration, and the fractions were assayed independently for DM and DM-DO by specific enzyme-linked immunoabsorption assays (ELISAs) (*14*). Although the resolution of the column was limited, DM-DO complexes eluted earlier than free DM molecules (Fig. 1B). By pooling a small number of fractions, we obtained complexes that were 83% DM-DO (with 17% free DM), as determined by ELISA. Although we were unable to analyze the complexes for DOα, Liljedahl *et al.* (*8*) showed that the formation and transport of DM-DO complexes require both DOα and DOβ subunits.

The functional activities of DM-DO and DM in an MHC class II peptide-loading assay (*4*) were compared with DM purified from T2/DM cells, although similar results were obtained with the residual DM from the Raji preparation (*16*). ^{35}S-Methionine–labeled HLA-DR3-CLIP complexes, purified from the DM-negative cell line T2.DR3 (*13*), were incubated at 37°C, pH 5.0, for various times with a DR3-binding peptide (MOMP) (*13*) and DM or DM-DO complexes. Exchange of CLIP for MOMP was assayed by SDS–polyacrylamide gel electrophoresis (PAGE); DR3-MOMP complexes are stable in SDS unless heated, whereas DR3-CLIP complexes are not (*4*). The generation of SDS-stable dimers was catalyzed more efficiently by DM than by DM-DO (Fig. 2A), as quantitated by image analysis (Fig. 2B). The DM and DM-DO preparations were matched for DM content (Fig. 2C). In titration experiments (Fig. 2D), the activity of DM at a dilution of 1/8 approximated that of DM-DO matched for DM content with the undiluted DM-DO preparation (Fig. 2E). Thus, the activity of the DM-DO preparation is ~13% that of the pure DM, close to the estimated contamination with free DM molecules. This suggests that DM-DO complexes are completely inactive. Additional experiments showed that the addition of DM-DO failed to inhibit the peptide-loading activity of pure DM (*16*), indicating that DM-DO complexes are not competitive inhibitors of DM function.

To ensure that the DO inhibitory activity was not restricted to DR3, we also assayed the activity of DM-DO on ^{35}S-methionine–labeled DR1- and DR4-CLIP complexes purified from T2.DR1 or T2.DR4 cells, respectively. In this case, the peptide used in the assay (HAp) (*17*) was derived from influenza virus hemagglutinin derivatized at the COOH-terminus with biotin. This peptide functions as both a DR1- and DR4-restricted epitope (*18*). Formation of DR-HAp complexes was quantitated by capture with streptavidin-agarose beads, SDS-PAGE, and image analysis (Fig. 3). For both DR1 and DR4, DM-DO was less effective than free DM in mediating the exchange of HAp for bound CLIP. For DR4, the difference between DM and DM-DO was less apparent because DR4 has a lower affinity for CLIP (*19*) and shows a relatively high rate of spontaneous exchange (Fig. 3B).

The relative inability of DM-DO complexes to mediate the exchange of CLIP for antigenic peptides suggested that DO might serve to depress class II–restricted antigen processing in vivo. To test this prediction, we transfected cDNA expression constructs encoding DOα (also called DNα) (*8*, *12*) and DOβ into a human T cell line that had been transfected with the class II transactivator gene, CIITA (*20*, *21*). CIITA induces the expression of class II, invariant chain, and DM genes but not DO (*22*, *23*). As shown by protein immunoblotting, this cell line, CEM.CIITA,

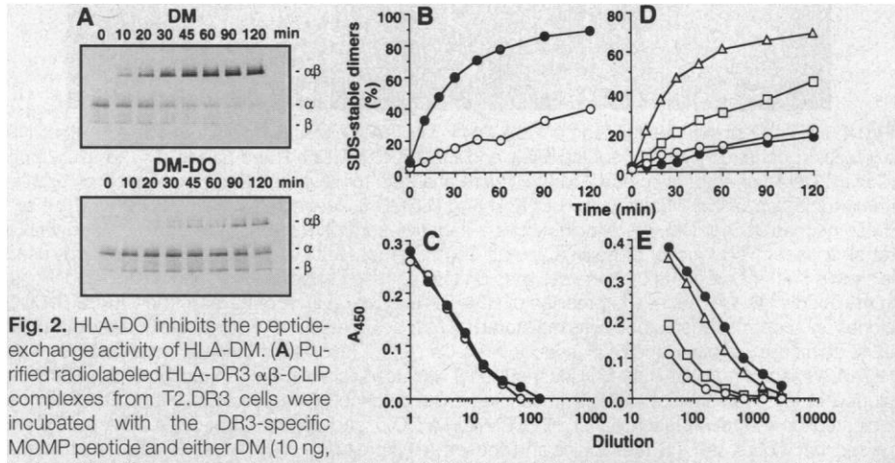

Fig. 2. HLA-DO inhibits the peptide-exchange activity of HLA-DM. (**A**) Purified radiolabeled HLA-DR3 αβ-CLIP complexes from T2.DR3 cells were incubated with the DR3-specific MOMP peptide and either DM (10 ng, top) or DM-DO complexes (containing 10 ng of DM, bottom) for the indicated times at pH 5.0. After neutralization, samples were analyzed by SDS-PAGE (11%). (**B**) The percentage of SDS-stable dimers formed at each time point by DM (●) and DM-DO (○) complexes in (A) as quantitated by image analysis. (**C**) Quantitation by ELISA of the amount of DM added in (A), showing that the amount of DM in the DM (●) and DM-DO (○) preparations was equal. (**D**) DR3 αβ-CLIP complexes were incubated with peptide and DM-DO (●) or DM [undiluted (▲) or diluted 1:4 (□) or 1:8 (○)] for the indicated times at pH 5.0. After neutralization and analysis of the samples by SDS-PAGE, the percentage of SDS-stable dimers formed at each time point was quantitated by image analysis. (**E**) Quantitation by ELISA of the amounts of DM added in (D). Symbols are as in (D).

transfected (*24*) with the vector alone or expressing DOα only, was positive for DMβ but not DOβ (Fig. 4A). Two examples are shown of cells expressing DOβ. Both these lines expressed DOα mRNA by reverse transcriptase–polymerase chain reaction (RT-PCR) (*25*). To determine whether DO expression reduced class II peptide loading, we examined the same cell lines using the mAb Cer.CLIP.1, which reacts with surface class II–CLIP complexes (*13*). All the cell lines reacted readily with the DR-reactive mAb L243, but only the DO-expressing CEM.CIITA cells reacted with the CLIP-specific antibody (Fig. 4B). To confirm that the surface CLIP was expressed in association with class II molecules, we labeled the cell line expressing DOα only and one of the DOα- and DOβ-expressing lines with ^{35}S-methionine, then extracted the cells with detergent, and the class II molecules were purified with CerCLIP.1 or L243 mAb affinity columns to generate CerCLIP$^+$, CerCLIP$^-$, and total class II complexes (*4*). Analysis by SDS-PAGE (Fig. 4C) showed that DRα and β subunits were purified from both cell lines by L243, but CerCLIP.1$^+$ DR complexes were isolated only from the DO-positive cells. CLIP can be seen running in its characteristic position (*19*) in both the CerCLIP.1$^+$ and total class II complexes from the DO-positive cells.

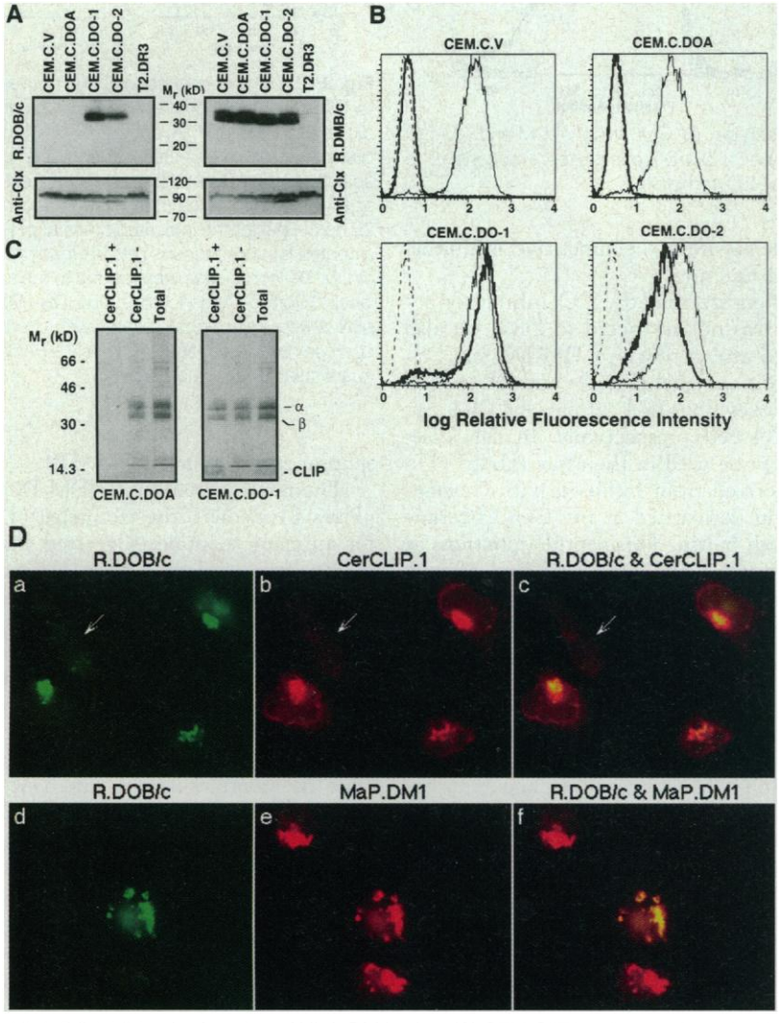

Fig. 4. HLA-DO inhibits the function of HLA-DM in vivo. (**A**) CEM.CIITA cells transfected with cDNA expression constructs (*24*) encoding DOα and DOβ (CEM.C.DO-1 and CEM.C.DO-2), DOα only (CEM.C.DOA), or empty vector (CEM.C.V) were analyzed for DOβ and DMβ expression by protein immunoblotting of cell lysates with R.DOB/c and R.DMB/c, respectively. T2.DR3 cells, which express neither DO nor DM, were included as a negative control. Each blot was also probed with a rabbit antiserum specific for calnexin (*32*) (Anti-Clx) to demonstrate equal loading. The antibody used is shown on the side of the blot, the cell lines on the top, and molecular size markers (in kilodaltons) in the middle. (**B**) Cell surface expression of HLA-DR and αβ-CLIP complexes in the CEM.CIITA.DO clones and control transfectants was measured by flow cytometric analysis with the DR-specific mAb L243 (thin solid lines) and the CLIP-specific mAb CerCLIP.1 (thick solid lines) as described (*13*). The HLA-A3–specific mAb GAP.A3 (*33*) (dashed lines) was used as a negative antibody control. Data are plotted as log fluorescence intensity (mean fluorescence channel) versus cell number. Cell lines are indicated on the top of each panel. (**C**) CEM.CIITA.DO-1 and control CEM.CIITA.DOA cells were pulsed for 6 hours with ^{35}S-methionine and chased in the presence of a 15-fold excess of methionine and cysteine overnight. Class II molecules were affinity-purified from each cell line (*34*) with the DR-specific mAb L243 (Total), the CLIP-specific mAb CerCLIP.1 (CerCLIP.1$^+$), or L243 after depletion by CerCLIP.1 (CerCLIP.1$^-$) and analyzed by SDS-PAGE (11%). DRα, βDR, and CLIP are indicated on the right, molecular size markers (in kilodaltons) are on the left, the purified complex is on the top, and the cell line is on the bottom. (**D**) Subcellular localization of αβ-CLIP complexes, HLA-DO, and HLA-DM. CEM.CIITA.DO-1 cells were mixed with untransfected CEM.CIITA cells (3:1 ratio) and costained with R.DOB/c serum (anti-DO) and CerCLIP.1 (anti-CLIP) (a to c), or costained with R.DOB/c serum and MaP.DM1 (mAb specific for HLA-DM) (d to f). The antibody used in each panel is indicated at the top.

The DM-mediated exchange of CLIP for endocytically generated peptides probably occurs in the MIIC. Thus, replacement of DM by DM-DO should result in the transient accumulation of class II–CLIP complexes in the MIIC before their expression on the cell surface. We examined the DO-expressing CEM.CIITA cells by immunofluorescence for the presence of intracellular DO, DM, and CLIP (*26*) (Fig. 4D). The DO-expressing cells were mixed with DO-negative CEM.CIITA cells before fixation to provide an internal control. Only cells expressing DO (panel a) accumulated CLIP (panel b), presumably as class II–CLIP complexes, and the intracellular CLIP colocalized with the DO-containing vesicles (panel c). Cell surface CLIP was also apparent. The arrow indicates a DO-negative and therefore CLIP-negative cell present in the field. To demonstrate that both the DO-positive and the DO-negative cells express DM, we also stained cells for DO (panel d) and DM (panel e). Colocalization of DO and DM (panel f) showed that the intracellular DO and DM reside in the same compartment, as expected (*8*).

In class II–negative cells, DM and class II expression is markedly up-regulated by the interferon-γ (IFN-γ)–induced expression of CIITA (*23*). Whereas DOα-subunit mRNA expression is IFN-γ responsive, the level of β-subunit mRNA does not increase, either because of RNA instability or a lack of transcriptional up-regulation (*9–12, 27*). Thus, stimulation by IFN-γ results in DM expression without functional DO. Nonprofessional antigen-presenting cells that lack DO would be expected to acquire maximal antigen-pro-

cessing capacity when exposed to an inflammatory cytokine environment in vivo. B cells, however, constitutively express CIITA and therefore DM. Dampening their antigen-processing ability by DO may reduce the level of endogenous self peptides presented and thus reduce the possibility of activating autoreactive CD4-positive T cells. In support of this idea, high levels of class II–CLIP complexes can be detected on the cell surface of human peripheral B cells (*16*). Activated B cells present antigens to class II–restricted CD4-positive T cells more efficiently than resting B cells (*28*). A part of this enhanced activity may result from increased DM expression relative to DO.

It is unclear whether DO has a function in addition to the DM inhibition described here. Its expression in thymic epithelium (*11*, *29*) could argue that DO has a role in antigen presentation. However, cell surface expression of DO has not been demonstrated (*8*, *11*). In addition, the interaction of DM with DO is very stable compared to that with conventional class II molecules, arguing that DM is unlikely to be involved in loading DO with peptides. Thymic expression of DO may instead be required for the maintenance of self-tolerance. DM expression reduces T cell recognition of certain allogeneic class II–restricted epitopes (*30*). Therefore, down-regulating DM function by DO might increase the expression of such epitopes. In this case, matching thymic class II–peptide levels to those in the periphery would also serve to avoid autoimmunity mediated by CD4-positive T cells.

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21. We cotransfected class II–negative CEM cells with CIITA-pcDNAI/Amp and pMCFR-Neo (*13*) at a ratio of 10:1 using previously described conditions [B. K. Sadasivan, A. Cariappa, G. L. Waneck, P. Cresswell, *Cold Spring Harbor Symp. Quant. Biol.* **55**, 267 (1995)]. Clones resistant to geneticin were screened for expression of cell surface class II by flow cytometric analysis with the DR-specific mAb L243 [L. A. Lampson and R. Levy, *J. Immunol.* **125**, 293 (1980)]. Positive clones were subcloned by limiting dilution, and the clone with the highest class II expression was used for subsequent DO transfection.
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24. DOA was amplified by PCR from a Raji cDNA library with primers specific for the 5′ end (5′-TCCCCC-GGGTACCGCCACCATGGCCCTCAGAGCAGG-GCTG-3′) and 3′ end (5′-TCCAGTGTCCCCAGG-TAATGATCGATGG-3′). The resulting PCR product was cloned into pCR2.1 (Invitrogen), sequenced, and subcloned into the eukaryotic expression vector pMCFR-PAC (*13*) to generate pMCFR-PAC/DOA. CEM.CIITA cells were transfected with pMCFR-PAC/DOA and pDOB#163 (*10*) at a ratio of 1:10, PMCFR-PAC/DOA alone, or empty vector alone as described (*13*). Clones resistant to puromycin were screened for DO expression by immunofluorescence with R.DOB/c. Two of the positive cell lines (CEM.CIITA.DO-1 and CEM.CIITA.DO-2) were chosen and used for subsequent experiments. CEM.CIITA-DOA cells were screened for DOA expression by RT-PCR analysis and CEM.CIITA. Vector-only cells were isolated by resistance to puromycin.
25. D. B. Sant'Angelo, unpublished data.
26. CEM.CIITA.DO-1 cells were mixed at a ratio of 3:1 with untransfected CEM.CIITA cells and processed for immunofluorescence as described [N. F. Robbins, C. Hammond, L. Denzin, M. Pan, P. Cresswell, *Hum. Immunol.* **45**, 13 (1996)] with the following modifications. After fixation of the cells on coverslips, they were treated with permeabilization solution [PBS containing 5% fetal bovine serum, 1% BSA, and saponin (15 μg/ml; Sigma)], 10 mM glycine, 10 mM Hepes (pH 7.4) for 15 min at room temperature, and the coverslips were placed in a 20-μl drop of a solution of R.DOB/c (50 μg/ml) in permeabilization solution for 30 min. The coverslips were then washed three times in permeabilization solution and treated with sequential rounds of CerCLIP.1 or MaP.DM1 (C. Hammond, in preparation) and secondary antibodies as described above, except that the saponin concentration was increased to 500 μg/ml. After extensive washing, the coverslips were mounted onto slides with Mowiol 4-88 solution (Calbiochem).
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34. Metabolic labeling of CEM.CIITA transfectants and affinity purifications were done as described (*4*, *13*).

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