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The Protease Inhibitor Cystatin C Is Differentially Expressed among Dendritic Cell Populations, but Does Not Control Antigen Presentation[1]

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Dendritic cells (DC) undergo complex developmental changes during maturation. The MHC class II (MHC II) molecules of immature DC accumulate in intracellular compartments, but are expressed at high levels on the plasma membrane upon DC maturation. It has been proposed that the cysteine protease inhibitor cystatin C (CyC) plays a pivotal role in the control of this process by regulating the activity of cathepsin S, a protease involved in removal of the MHC II chaperone Ii, and hence in the formation of MHC II-peptide complexes. We show that CyC is differentially expressed by mouse DC populations. $CD8^+$ DC, but not $CD4^+$ or $CD4^-CD8^-$ DC, synthesize CyC, which accumulates in MHC II^+Lamp^+ compartments. However, Ii processing and MHC II peptide loading proceeded similarly in all three DC populations. We then analyzed MHC II localization and Ag presentation in $CD8^+$ DC, bone marrow-derived DC, and spleen-derived DC lines, from CyC-deficient mice. The absence of CyC did not affect the expression, the subcellular distribution, or the formation of peptide-loaded MHC II complexes in any of these DC types, nor the efficiency of presentation of exogenous Ags. Therefore, CyC is neither necessary nor sufficient to control MHC II expression and Ag presentation in DC. Our results also show that CyC expression can differ markedly between closely related cell types, suggesting the existence of hitherto unrecognized mechanisms of control of CyC expression. ***The Journal of Immunology*****, 2003, 171: 5003–5011.**

Antigen presenting cells display on their surface MHC class II (MHC II) [4] molecules bound to peptides derived from proteins degraded in endosomal compartments (reviewed in Ref. 1). The MHC II molecules assemble in the endoplasmic reticulum with invariant chain (Ii), a type II transmembrane protein that acts as a chaperone, and carries the information required to deliver the MHC II molecules to the endocytic route. A region of Ii called class II-associated invariant chain peptide (CLIP) fills the peptide binding site of the MHC II $\alpha\beta$ dimer, stabilizing the site and protecting it from binding to other polypeptides of the endoplasmic reticulum or the Golgi. Ii also contains in its cytoplasmic tail a targeting motif that labels the MHC II-Ii complexes for delivery to the endocytic compartments. Once there, Ii is degraded, and its CLIP segment is substituted for antigenic peptides (see the scheme in Fig. 3*A*). Most of Ii is broken down in late endosomes/lysosomes in several steps (reviewed in Ref. 2). One or more noncysteine proteases eliminate the portion of Ii C terminally of CLIP, leaving the resulting Iip10 fragment still attached to the MHC II $\alpha\beta$ dimer (3). Then, Iip10 is cleaved N terminally of CLIP, yielding an MHC II-CLIP complex. The enzyme that cleaves Iip10 is a cysteine protease that varies among APC: B cells and dendritic cells (DC) use cathepsin (Cat) S, thymic epithelial cells use Cat L instead, and macrophages can use Cat L or S, and perhaps F (4–10). The conversion of Iip10 into CLIP is particularly important, because it releases the MHC II molecules of the cytosolic portion of Ii, which carries an endosomal retention signal that prevents the transport of MHC II-Ii and MHC II-Iip10 to the cell surface (11–13). Therefore, this step is quickly followed by the substitution of CLIP for antigenic peptides, a reaction catalyzed by the chaperone H-2DM (14). The resulting MHC II peptide complexes are then transported to the plasma membrane where they are recognized by $CD4^+$ T lymphocytes.

DC are "professional" APC that play a central role in the initiation and control of immune responses and probably in the maintenance of tolerance (15, 16). DC undergo complex developmental changes that make them highly efficient APC. In their so-called "immature" stage DC are very efficient at capturing Ags, but their MHC II molecules accumulate in endosomal compartments rather than on the cell surface (17). However, when the immature DC are stimulated by inflammatory stimuli they undergo a process of "maturation" (18). During maturation, DC acquire the capacity to activate naive T cells, and their MHC II molecules accumulate on the plasma membrane (19–22). What controls this developmental change in the subcellular distribution of MHC II? A study of bone marrow-derived dendritic cell (BMDC) suggested a pivotal role for the cysteine protease inhibitor cystatin C (CyC) (23). Immature BMDC contain CyC in endosomal compartments, so it was hypothesized that it might block the activity of Cat S. This would impair the cleavage of Iip10, thus preventing the generation of MHC II peptide complexes and provoking the retention of MHC II in the endocytic route. Upon maturation, CyC would be eliminated

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[4] Abbreviations used in this paper: Ii, invariant chain; CLIP, class II-associated invariant chain peptide; BMDC, bone marrow derived dendritic cell; Cat, cathepsin; CyC, cystatin C; DC, dendritic cell; D1DC, DC grown in culture from spleen precursors; HEL, hen egg lysozyme; ICM, immunofluorescence confocal microscopy; Hsp70, heat shock protein 70; DAPI, 4′,6′-diamidino-2-phenyindole.

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from the endosomal compartments, enabling Cat S to cleave Iip10, which in turn would allow the formation of MHC II peptide complexes and their accumulation on the cell surface.

Mouse spleens contain three distinct populations of DC that can be distinguished by their expression of the surface markers CD4 and CD8: $CD4^+$ DC, $CD8^+$ DC, and $CD4^-CD8^-$ DC (24, 25). These three DC types are phenotypically and functionally immature, but can be induced to mature in vitro or in vivo (26). We show in this study that the $CD8^+$ DC constitutively express high levels of CyC in $Lamp^+MHC\ II^+$ compartments as in immature BMDC, whereas the other two DC populations express little or no CyC. However, the distribution of MHC II before and after maturation was comparable in the $CD8^+$ and the $CD8^-$ DC populations. We then analyzed DC from CyC knockout mice. The developmental control of MHC II expression and the Ag presenting capacity were unaffected by the lack of CyC in splenic $CD8^+$ DC, BMDC, and DC generated in culture from spleen precursors (D1DC). We conclude that CyC expression is neither necessary nor sufficient to control MHC II Ag presentation in DC.

Materials and Methods

Mice

C57BL/6, Cat $S^{-/-}$ (6) and $CyC^{-/-}$ (27) mice were bred in the animal facilities of the Walter and Eliza Hall Institute (Melbourne, Australia) according to institutional guidelines and used at 6–8 wk of age. As described previously, neither of the knockout strains used in this study show major developmental or health defects.

Purification of splenic DC

Splenic mouse DC were purified as described elsewhere (24). The protocol used excluded $B220^+$ "plasmacytoid DC" and yielded 70–80% pure preparations of $CD11c^+$ DC, in which the contaminating cells were mostly macrophages. Segregation of the $CD4^+$ and $CD8^+$ DC populations was conducted by two different methods. For biochemical experiments in which relatively large numbers of cells were needed, the DC preparation was first incubated with the anti-CD4 rat mAb GK1.5, followed by anti-rat magnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway), to retrieve the $CD4^+$ cells. The $CD8^+$ cells were then purified sequentially using the rat anti-CD8 mAb 53-6.7 and anti-rat magnetic beads. The remaining cells consisted of autofluorescent macrophages and $CD4^-CD8^-$ DC. For the Ag presentation and confocal microscopy experiments in which the presence of the magnetic beads was undesirable, the splenic DC preparation was labeled with mAb against CD11c, CD4, and CD8, and the $CD4^+$ and $CD8^+$ DC populations were purified by preparative FACS. Where indicated, the splenic DC were cultured after preparative cell sorting in RPMI 1640 supplemented with 10% FCS and 200 U/ml recombinant mouse GM-CSF (PeproTech, Rocky Hill, NJ).

Culture of DC from BMDC and D1DC precursors

BMDC and D1DC were generated in culture as described elsewhere (22). The results shown in Fig. 5 were obtained with BMDC grown in the absence of IL-4; similar conclusions were obtained with cells grown in the presence of 1, 2, and 5 U/ml IL-4. Maturation of the BMDC and the D1DC was induced by addition of 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) to the culture medium 20 h before cell harvesting.

Analytical and preparative flow cytometry

The Abs used for preparative or analytical FACS were hamster mAb N418 (anti-CD11c), rat mAb YTS (anti-CD8), rat mAb GK1.5 (anti-CD4), rat mAb M5/114 (anti-MHC II), and rat mAb GL1 (anti-CD86). All the Abs were purified and conjugated to fluorochromes (FITC, PE, Texas Red, or Cy5) in our laboratory. Samples were analyzed using a FACS II, FACStar^{Plus}, FACScan, or LSR instrument (BD Biosciences, San Jose, CA). Preparative FACS was performed using a Mo-Flo (Cytomation, Fort Collins, CO), a DiVa, or a FACStar^{Plus} (BD Biosciences).

Pulse-chase, immunoprecipitation, and Western blot

Pulse-chase analysis, immunoprecipitations with the mAb N22, and Western blot of N22 immunoprecipitates were conducted as described elsewhere (5, 22). Normalization of [^{35}S]met/cys incorporation was conducted by pipetting 10 μl of cell lysate on filter paper, washing the paper five times in 5% TCA (Sigma-Aldrich), and counting the amount of radioactivity precipitated on the paper in a scintillation counter. The amount of cell lysate used for the immunoprecipitation was then adjusted accordingly. For Western blot of cell lysates, cells were subjected to 5–7 cycles of vortexing and heating at 95°C in 2× SDS-PAGE loading buffer. The anti-heat shock protein 70 (Hsp70) mouse mAb N6 was the kind gift of Dr. R. Anderson (Peter MacCallum Cancer Research Institute, Melbourne, Australia). CyC detection and immunoprecipitation was conducted with anti-human CyC rabbit sera from Upstate Biotechnology (Lake Placid, NY) or from DAKO (Glostrup, Denmark); the two antisera gave identical results.

Immunofluorescence confocal microscopy (ICM)

ICM analysis was conducted exactly as described elsewhere (22). The primary Abs used were: biotinylated anti-MHC II mAb N22, purified or biotinylated rat mAb 1D4B (anti-Lamp), a rabbit serum specific for the cytosolic portion of MHC II I-Aα, and the anti-CyC rabbit sera described above. The secondary Abs were: streptavidin conjugated to Cy5 (Amersham, Castle Hill, Australia), goat anti-rat serum conjugated to FITC (Caltag Laboratories, Burlingame, CA), and sheep anti-rabbit conjugated to FITC (Amrad, Boronia, Australia). Where indicated, 4′,6′-diamidino-2-phenyindole (DAPI) (Sigma-Aldrich) was also included in the second incubation to label the nuclei. The confocal microscope used was a Leica STS SP2 (Leica, Wetzlar, Germany).

Ag presentation assays

The T cell hybridomas used in this study have been described elsewhere (28, 29); their specificity is indicated in Fig. 5. For the assays, splenic DC were purified by preparative FACS and incubated for 20 h in RPMI 1640 supplemented with 10% FCS and 200 U/ml recombinant mouse GM-CSF (PeproTech) with 10^5 hybridoma T cells in the presence of 0.1 mM OVA or hen egg lysozyme (HEL) (Sigma-Aldrich). The amount of IL-2 released by the hybridomas was determined using a standard proliferation assay with the IL-2-dependent cell line CTLL-2, or by ELISA.

Results

CyC is differentially expressed among splenic DC populations

Mouse spleens contain in the steady state three distinct populations of DC: $CD4^+$ DC, $CD8^+$ DC, and $CD4^-CD8^-$ DC (Fig. 1*A*) (24). After purification, these DC are highly endocytic, cannot activate naive T cells in vitro unless they are induced to mature (26), express low levels of surface MHC II and CD86 (Fig. 1*B*) (30), and accumulate MHC II molecules in $Lamp^+$ compartments (Fig. 1*C*). These features define the splenic DC as immature (18). When put in culture, the three splenic DC undergo the phenotypic and functional changes that define DC maturation (18): they down-regulate endocytosis (data not shown), acquire the capacity to stimulate naive T cells (26), increase their surface expression of CD86 by 100-fold (Fig. 1*B*) (30), and their MHC II molecules accumulate on the cell surface whereas their $Lamp^+$ compartments cluster in a perinuclear region (Fig. 1, *B* and *C*).

It has been proposed that the cysteine protease inhibitor CyC may play a major role in the control of MHC II expression in BMDC (23). We were interested in testing whether CyC plays such a role in vivo, so first we determined whether the expression of CyC in splenic DC was similar to that in other APC such as B cells. Indeed, Western blot analysis showed that CyC was expressed at much higher levels in freshly isolated spleen DC than in total splenocytes (Fig. 2*A*). However, the expression of CyC varied drastically among the three splenic DC populations. The $CD4^+$ and the $CD4^-CD8^-$ DC expressed very little, whereas the $CD8^+$ DC contained virtually all the CyC detected in the unfractionated DC preparation (Fig. 2*A*). The $CD4^+$ and the $CD4^-CD8^-$ DC are located in different areas of the spleen compared to those where the $CD8^+$ DC are found (31), so it could be that the $CD8^+$ DC had simply captured CyC that had been secreted by other neighboring cells. Therefore we assessed, by metabolic labeling and immunoprecipitation, whether the $CD8^+$ DC synthesized CyC themselves. The $CD8^+$ DC synthesized 10-fold more CyC in 30 min than the $CD4^+$ or the $CD4^-CD8^-$ DC (Fig. 2*B*). We then assessed by Western blot whether the pattern of CyC expression was maintained

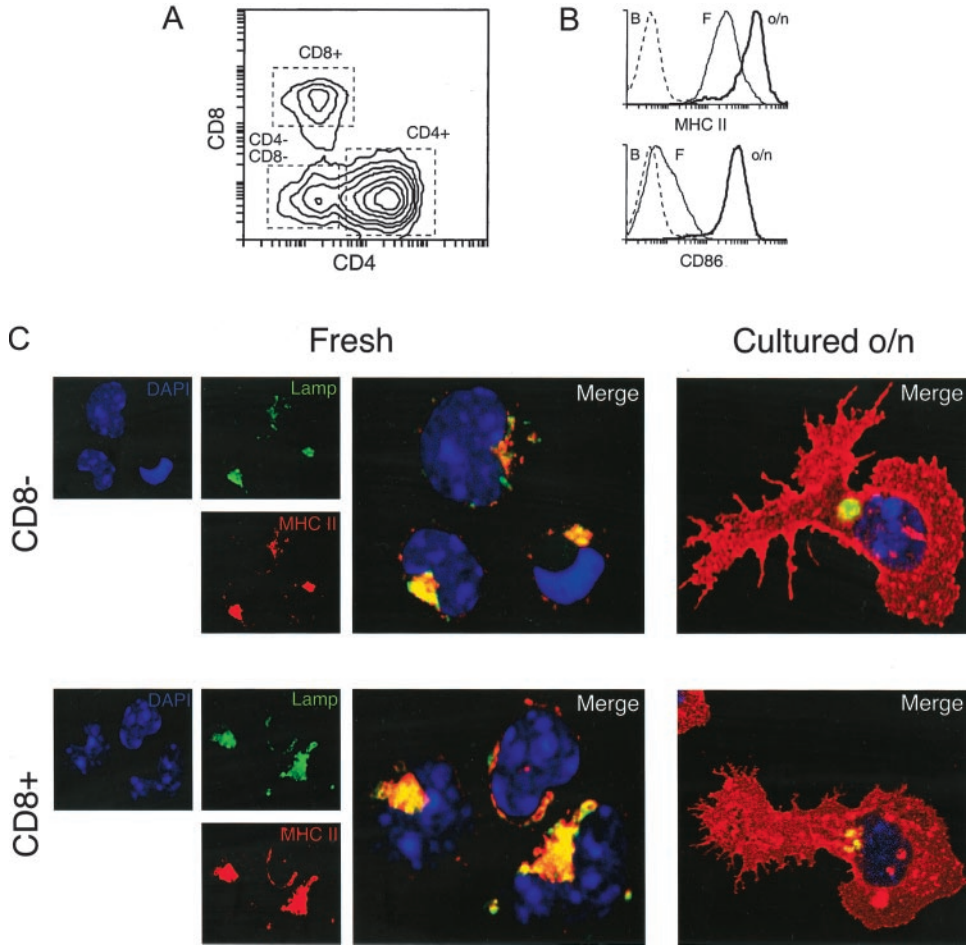

FIGURE 1. Splenic DC populations and maturation in culture. *A*, A DC preparation from mouse spleens was stained with anti-CD11c, anti-CD4, and anti-CD8 mAb, and analyzed by FACS. The plot shows the expression pattern of CD4 and CD8 in the $CD11c^+$ cells (70–80% of the DC preparation, the rest being mostly macrophages). $CD45RA^+$ "plasmacytoid DC" were excluded during the purification protocol. Mouse spleens contain three DC populations distinguished by their expression of CD4 and CD8: $CD4^+$ DC, $CD8^+$ DC, and $CD4^-CD8^-$ DC. The result is representative of multiple experiments. *B*, Splenic DC mature in culture, increasing the surface expression of MHC II and CD86 by 4- to 5- and 100-fold, respectively. The expression of MHC II and CD86 was analyzed by FACS in the splenic DC immediately after purification (thin continuous line, F) or after culture overnight (thick line, o/n). The dashed line corresponds to nonlabeled cells (background, B). Profiles for the total DC preparation are shown; the results for each of the three splenic DC populations were comparable. The result is representative of multiple experiments. *C*, MHC II molecules accumulate in the $Lamp^+$ compartments of both $CD8^-$ and $CD8^+$ freshly isolated (immature) splenic DC, but on the plasma membrane in their cultured (mature) counterparts. DC were analyzed by ICM immediately after purification (Fresh) or after overnight culture using Abs for Lamp (green), MHC II (red) and the nuclear dye DAPI (blue). The result is representative of multiple experiments.

after DC maturation. The relative difference in CyC content between the $CD8^-$ and the $CD8^+$ DC did not vary after overnight culture (Fig. 2*C*). Therefore, differences in the expression levels of CyC among the three DC types were constitutive rather than linked to their maturation status.

In immature BMDC, CyC is located in $MHC\ II^+Lamp^+$ compartments (22, 23) where it might interfere with MHC II peptide loading by inhibiting the cysteine protease Cat S (23). Similarly, in $CD8^+$ spleen DC, CyC colocalized strongly with MHC II and Lamp (Fig. 2*C*). Consistent with the Western blot and the immunoprecipitation experiments shown above, CyC was not detected by microscopy in the $CD8^-$ DC (data not shown).

Because the subcellular distribution of MHC II, before and after maturation, was comparable between $CD8^+$ and $CD8^-$ DC, regardless of their expression of CyC, these results did not support a role for CyC in controlling MHC II expression. However, it could be that the $CD8^+$ DC and the $CD8^-$ DC used two different mechanisms of control, one dependent and the other independent of CyC (32). If so, Ii degradation and MHC II peptide loading might follow different kinetics in the $CD8^+$ and the $CD8^-$ DC. This possibility was assessed using biochemical methods.

Normal processing of Ii in $CD8^+$ DC

If CyC was inactivating Cat S in the $CD8^+$ DC, it would be expected that these DC would not break down Iip10, or would do it more slowly than the CyC-negative $CD4^+$ DC (Fig. 3*A*). To test this hypothesis, we compared Ii processing and MHC II peptide loading in both DC types by pulse-chase and immunoprecipitation. Freshly purified $CD4^+$ and $CD8^+$ DC were metabolically labeled with [^{35}S]Met/Cys for 30 min, and then chased for 60 and 240 min. The cells were lysed, and their MHC II molecules immunoprecipitated with the mAb N22. The immunoprecipitates were divided into two, incubated at room temperature (nonboiled) or at 98°C (boiled), respectively, and then analyzed by 12.5% SDS-PAGE (Fig. 3*B*). This protocol allows following the conversion of $\alpha\beta$-Ii into $\alpha\beta$-Iip10, and of this into $\alpha\beta$-peptide (Fig. 3*A*). The lanes

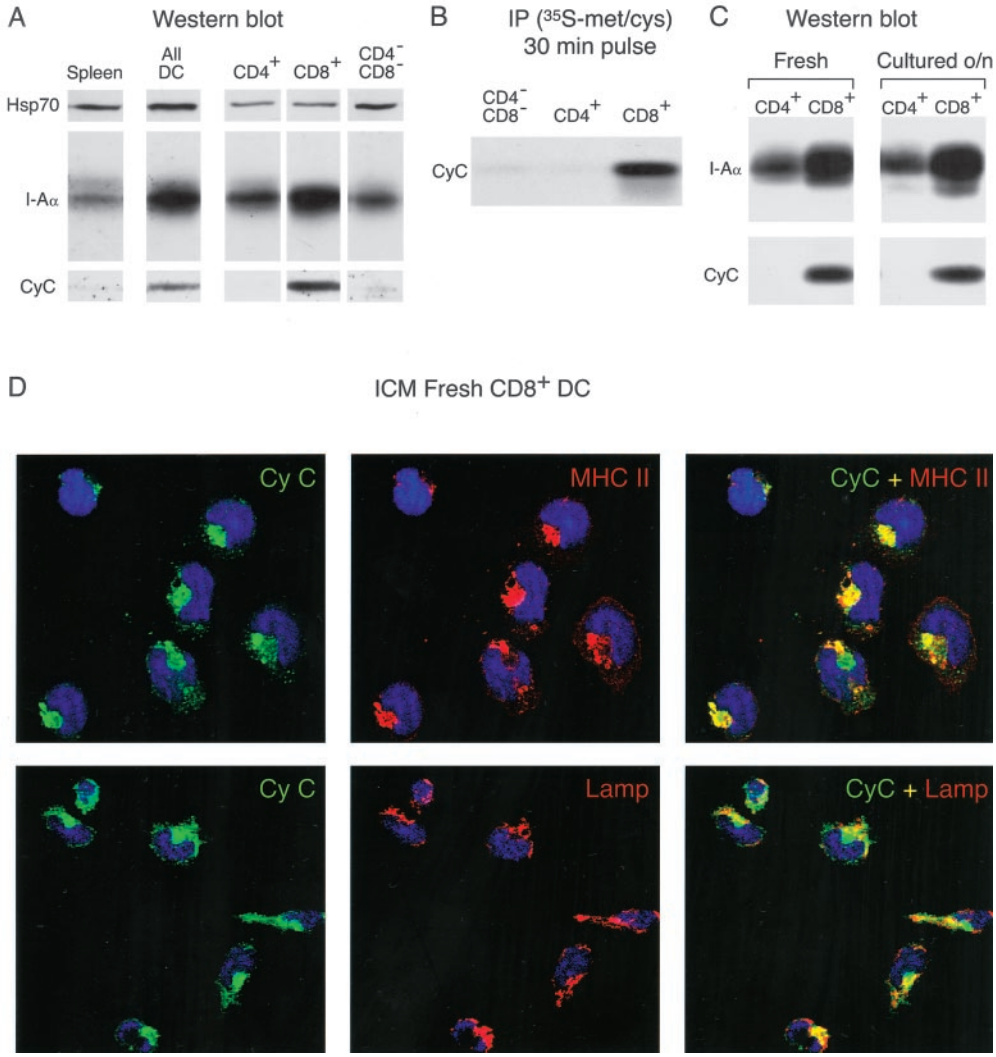

FIGURE 2. Expression pattern and subcellular localization of CyC in splenic DC. *A*, Cell lysates from total splenocytes, freshly purified splenic DC, and each of the three splenic DC populations, were loaded in a 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was cut transversally into three pieces, and each piece was analyzed by Western blot with Abs for Hsp70 (molecular mass 70 kDa, *top*), MHC II α-chain (molecular mass 35 kDa, *middle*), and CyC (molecular mass 15 kDa, *bottom*). For normalization purposes, three different amounts of cell lysate from each sample were loaded in adjacent lanes; the results shown correspond to the lanes in which the signals for Hsp70 were comparable, and are representative of multiple experiments. *B*, Freshly isolated $CD4^-CD8^-$ DC, $CD4^+$ DC, and $CD8^+$ DC were metabolically labeled with [^{35}S]met/cys for 30 min. The cells were lysed, and CyC was immunoprecipitated from equal amounts or radiolabeled cell lysate, quantitated by TCA precipitation. The immunoprecipitate was run in a 12.5% SDS-PAGE and visualyzed by autoradiography. Sequential immunoprecipitation of MHC II showed that the three DC populations had synthesized comparable amounts of this molecule (data not shown). The result is representative of multiple experiments. *C*, $CD4^+$ and $CD8^+$ splenic DC were purified by FACS and frozen immediately (Fresh) or after overnight culture (o/n). Cell lysates were prepared and analyzed by Western blot as in *A*. *D*, Freshly purified $CD8^+$ DC were analyzed by ICM using Abs for CyC (green), and either MHC II (*top*, red), or Lamp (*bottom*, red). DAPI was included to label the nuclei (blue).

loaded with the boiled samples show the composition of the MHC II molecules immunoprecipitated at each time point; those loaded with the nonboiled samples also allow to visualize the formation of peptide-loaded MHC II complexes (SDS-stable dimers), which are resistant to dissociation at room temperature (33).

The newly synthesized MHC II molecules were associated with full-length Ii, forming complexes that were mostly labile at room temperature (Fig. 3*B*, pulse) (32). At the 240 min timepoint, most of Ii had been degraded (note that the carbohydrates contained in the MHC II α- and β-chains are modified in the Golgi, causing the β-chain precipitated at this timepoint to run at the same position as full-length Ii (32)), and most of the MHC II molecules were forming SDS-stable $\alpha\beta$-peptide complexes (Fig. 3*B*, 240 min). The amount of $\alpha\beta$-peptide complexes generated in the $CD4^+$ and the $CD8^+$ DC were comparable. The Iip10 fragment, the substrate of Cat S, could be seen at the 60 min timepoint as a free polypeptide in the boiled lanes and as part of a relatively stable $\alpha\beta$-Iip10 complex in the nonboiled ones (Fig. 3*B*, 60 min) (5, 12, 32). Both DC types generated similar amounts of $\alpha\beta$-Iip10 at the 60 min timepoint, and both converted this complex into $\alpha\beta$-peptide. Thus, the presence of CyC in MHC II^+Lamp^+ compartments did not impair Ii processing or MHC II peptide loading in the $CD8^+$ DC.

The pulse-chase experiment was conducted in vitro immediately after DC purification. Because culturing the DC triggered their maturation (26), and mature DC may down-regulate the inhibition of Cat S by CyC (23), it could be argued that the newly synthesized MHC II molecules that we analyzed by pulse-chase encountered endocytic compartments with normal Cat S activity. To address this

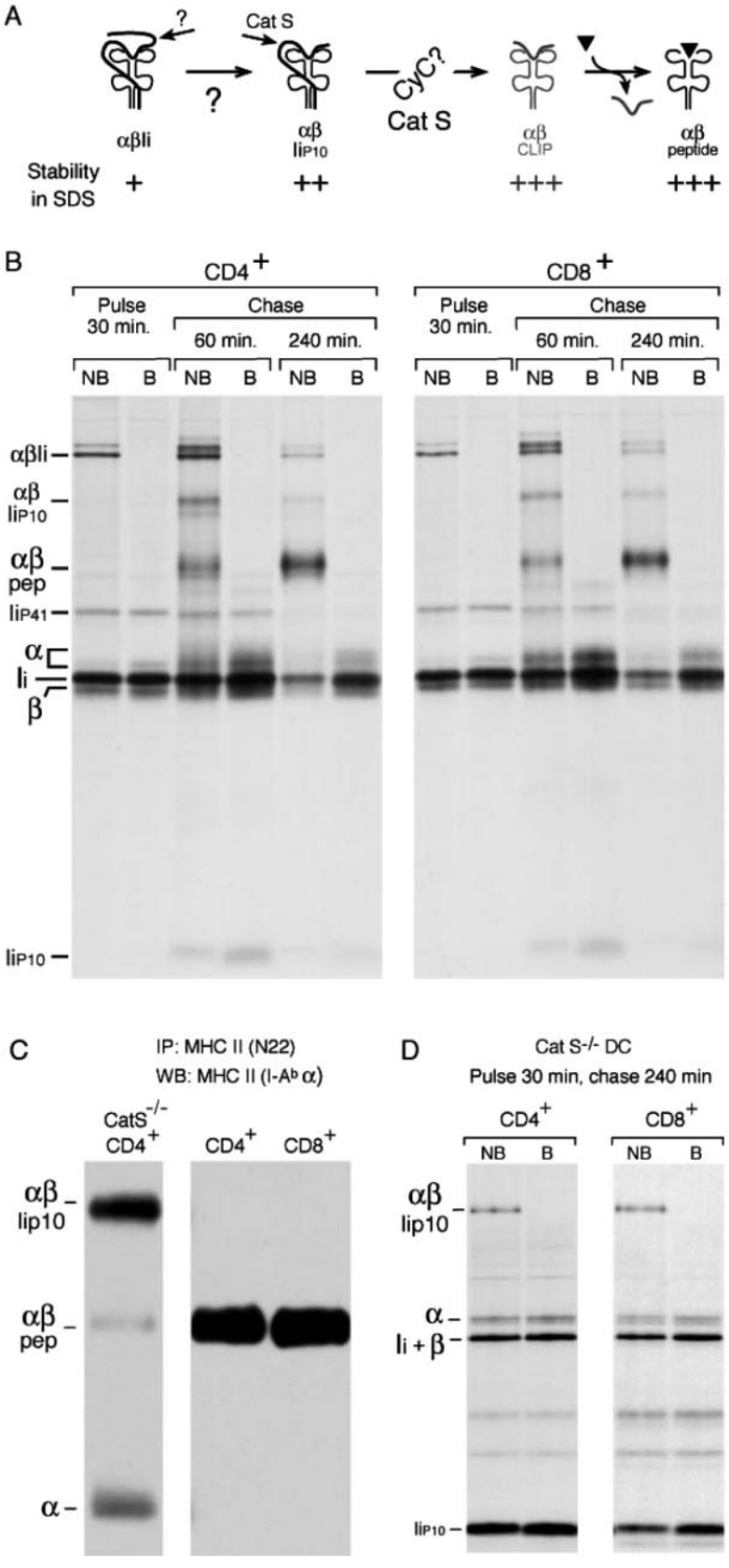

FIGURE 3. Both $CD4^+$ and $CD8^+$ DC process Ii and load their MHC II molecules with antigenic peptides in a Cat S-dependent manner. *A*, Schematic representation of the stepwise conversion of newly syntyhesized MHC II $\alpha\beta$-Ii complexes into $\alpha\beta$-peptide (1). The chaperone Ii (thick line) is first cleaved by one or more noncysteine proteases to generate Iip10, which is then processed by Cat S to yield CLIP. The CLIP is then exchanged for antigenic peptides, generating $\alpha\beta$-peptide complexes. CyC might act as an inhibitor of Cat S. The depicted complexes have a variable resistance to denaturation in SDS-PAGE at room temperature (32), which has been indicated by one to three plus signs (+) under each complex. The $\alpha\beta$-CLIP complex has been drawn in a lighter color to represent that unless the chaperone H-2DM is absent, it is quickly converted into $\alpha\beta$-peptide and therefore not detectable by pulse-chase analysis and immunoprecipitation (48, 49). *B*, Analysis of Ii degradation and MHC II peptide loading in splenic $CD4^+$ and $CD8^+$ DC. Freshly isolated $CD4^+$ and $CD8^+$ DC were metabolically labeled for 30 min, washed, and then cultured in normal medium for 60 and 240 min. The MHC II molecules were immunoprecipitated from each sample with mAb N22, loaded in a 12.5% SDS-PAGE without boiling (NB) or after boiling (B), and visualized by autoradiography. The positions occupied by the free MHC II α and β polypeptides, full length Ii, the Ii spliced variant Iip41, the degradation intermediate Iip10, and the SDS-stable complexes $\alpha\beta$Ii, $\alpha\beta$Iip10, and $\alpha\beta$-peptide, are indicated on the *left*. *C*, Western blot analysis of MHC II immunoprecipitates obtained from freshly isolated splenic DC. Cat $S^{-/-}$ $CD4^+$ DC and wild-type $CD4^+$ and $CD8^+$ DC were lysed immediately after purification. Their MHC II molecules were immunoprecipitated with mAb N22, loaded in a 12.5% SDS-PAGE without boiling, and detected by Western blot using a rabbit serum that recognizes the cytoplasmic portion of the MHC II α-chain. Most of the MHC II molecules contained in the Cat $S^{-/-}$ DC were associated with Iip10, whereas in both the $CD4^+$ and the $CD8^+$ wild-type DC, most of the MHC II molecules were forming $\alpha\beta$-peptide complexes. *D*, Both $CD4^+$ and $CD8^+$ splenic DC require Cat S to fully degrade Ii. The two DC populations were purified from the spleens of Cat $S^{-/-}$ mice and analyzed by pulse-chase and immunoprecipitation as in *B*. Degradation of Iip10 was impaired in both DC types, causing the accumulation of $\alpha\beta$-Iip10 complexes, instead of $\alpha\beta$-peptide, after a 240-min chase. Two additional intermediates of Ii degradation can be observed above the position occupied by Iip10 (9, 13).

possibility, we analyzed the structure of the MHC II moleculescontained in the $CD4^+$ and $CD8^+$ DC immediately after purification. Because the purification protocol did not include incubations at 37°C, if the $CD8^+$ DC were accumulating $\alpha\beta$-Iip10 complexes in vivo, such complexes would still be present in the fresh DC preparations. MHC II was immunoprecipitated with mAb N22 from the lysates of purified $CD4^+$ and $CD8^+$ DC and loaded in a 12.5% SDS-PAGE without boiling. An immunoprecipitate from $CD4^+$ DC obtained from a Cat $S^{-/-}$ mouse was also included as a control. The MHC II complexes were then detected by Western blot

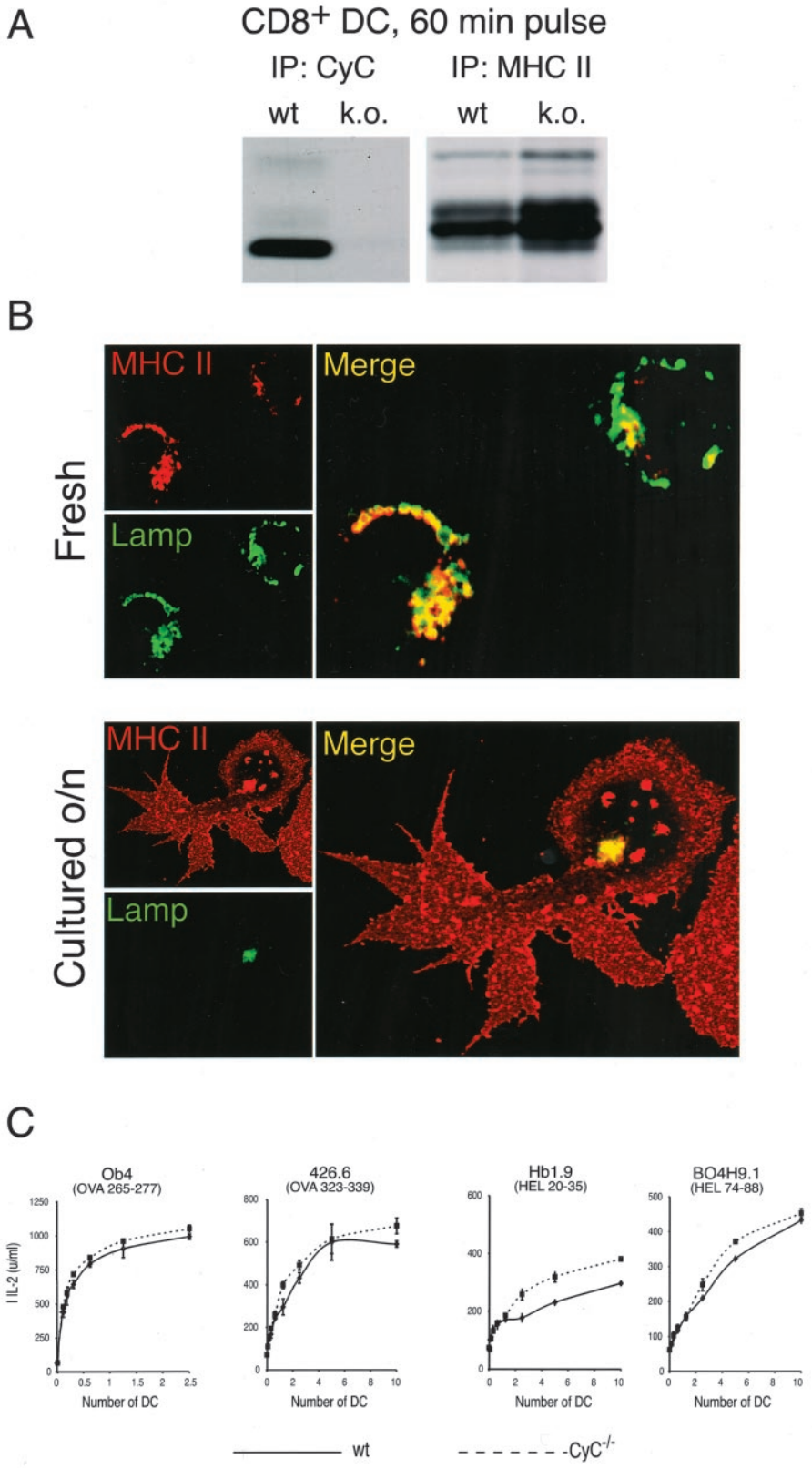

FIGURE 4. Analysis of CyC-deficient $CD8^+$ DC. *A*, Freshly isolated $CD8^+$ splenic DC from wild-type or $CyC^{-/-}$ mice were metabolically labeled for 60 min. The cells were lysed, and CyC was immunoprecipitated from equal amounts or radiolabeled cell lysate, quantitated by TCA precipitation. MHC II was immunoprecipitated sequentially from the same lysates, showing that both cell samples had synthesized comparable amounts of this molecule. The immunoprecipitates were analyzed by 12.5% SDS-PAGE and autoradiography. *B*, CyC-deficient $CD8^+$ DC were analyzed by ICM immediately after purification (Fresh, *top*) or after overnight culture (o/n, *bottom*) as in Fig. 1 (except that DAPI was not included). *C*, The indicated number of purified $CD8^+$ DC from normal or $CyC^{-/-}$ mice were used in assays of Ag presentation to the hybridomas shown. The specificity of each hybridoma is indicated. Note that the Ags used were OVA or HEL protein, not the antigenic peptides.

using a rabbit serum against the MHC II α-chain (Fig. 3*C*). Most of the MHC II molecules immunoprecipitated from the Cat $S^{-/-}$ DC were forming $\alpha\beta$-Iip10 complexes (7, 9, 13, 22), but the MHC II from both the $CD4^+$ and the $CD8^+$ DC of normal mice were forming $\alpha\beta$-peptide complexes. The same immunoprecipitates were also loaded in SDS-PAGE after boiling and analyzed by Western blot using Abs against the cytoplasmic portion of Ii, showing the presence of Iip10 in the immunoprecipitate obtained from the Cat $S^{-/-}$ cells, but not in those obtained from normal $CD4^+$ or $CD8^+$ DC (data not shown). We conclude that degradation of Ii and MHC II peptide loading proceeds normally in $CD8^+$ DC.

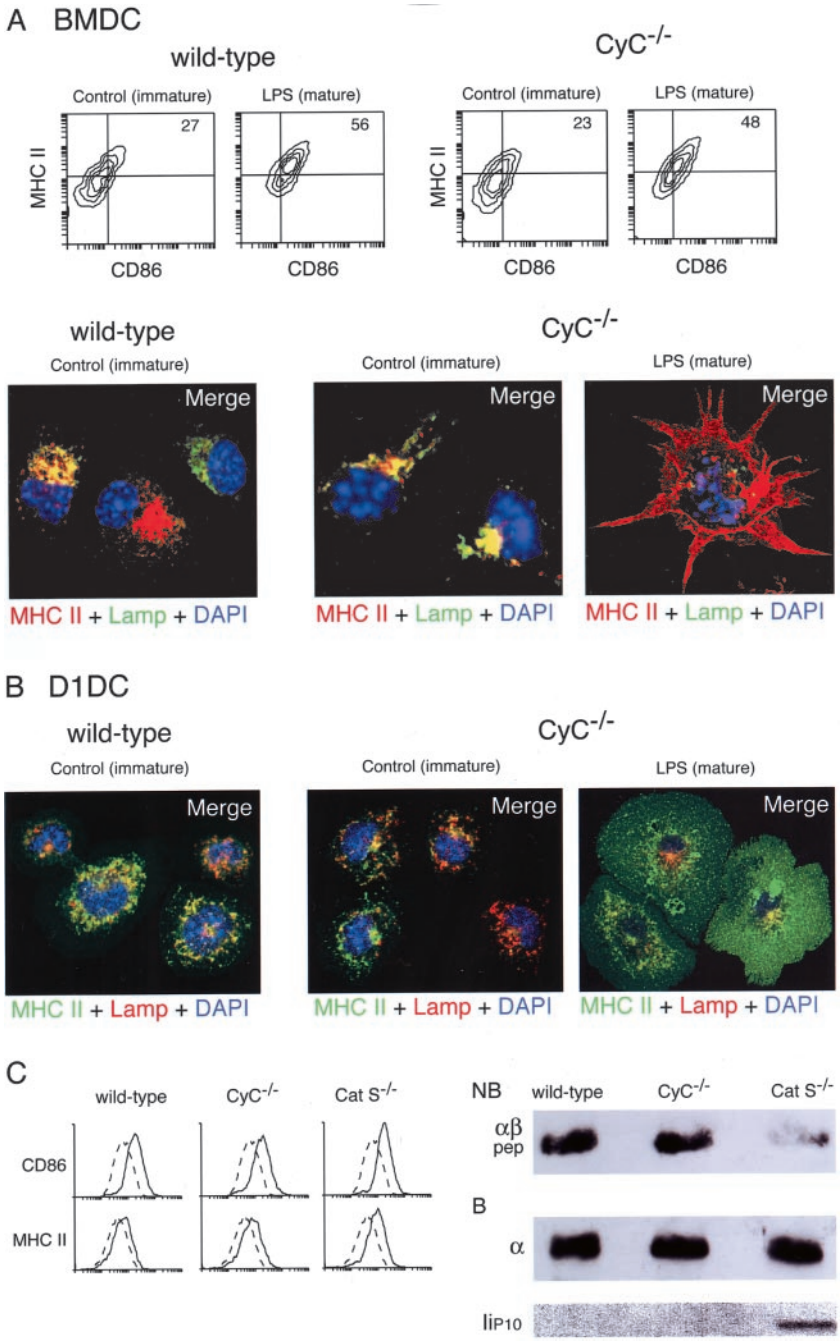

FIGURE 5. CyC does not control MHC II expression or peptide loading in BMDC or D1DC. *A*, BMDC were generated from wild-type or CyC-deficient animals and analyzed by FACS (*top*) or ICM (*bottom*) 5 days after initiation of the cultures. The samples labeled LPS (mature) received LPS at a final concentration of 1 μg/ml 20 h before cell harvesting. For the FACS analysis, cells were labeled with Abs for CD11c, CD86, and MHC II; the results shown were gated on live (propidium iodide$^-$) CD11c$^+$ cells. The numbers indicate the percentage of MHC II^{high}CD86^{high} cells. For the ICM analysis, CD11c$^+$ cells were sorted by preparative FACS, plated on microscopy coverlips, and labeled with anti-MHC II mAb N22 (red), anti-Lamp1 mAb 1D4B (green) and the nuclear dye DAPI (blue). Only the merged images are shown. *B*, D1DC were generated from the spleens of wild-type or CyC-deficient animals and analyzed by FACS (data not shown) or ICM. The cells labeled LPS (mature) were treated with 1 μg/ml LPS for 20 h before harvesting. Cells were plated directly on microscopy coverslips (preparative cell sorting was not necessary because the D1DC cultures were over 90% CD11c$^+$ (data not shown)). ICM analysis was performed with an anti-MHC II α rabbit serum (green), anti-Lamp 1 mAb 1D4B (red), and the nuclear dye DAPI (blue). Only the merged images are shown. The micrograph of the mature cells was taken at lower magnification than the control. *C*, Wild-type, CyC-deficient, and Cat S-deficient CD11c$^+$ BMDC were purified by preparative FACS. Analysis of the surface expression of CD86 and MHC II confirmed that virtually 100% of all the DC were immature (*right histograms*, the dashed line corresponds to the background level in the wild-type BMDC). The MHC II molecules contained in each cell preparation were immunoprecipitated and run in 12.5% SDS-PAGE before (*top*) or after boiling (*middle* and *bottom*). The immunoprecipitates were then analyzed by Western blot with Abs specific for I-Aα (*top* and *middle*), and the N terminus of the Ii (*bottom*). The regions containing the SDS-stable $\alpha\beta$-peptide complexes (around 50 kDa, *top*), the free I-Aα-chain (35 kDa, *middle*), and Iip10 (10 kDa, *bottom*) are shown.

Cat S is necessary to degrade Iip10 in both CD4$^+$ and CD8$^+$ splenic DC

The results shown above suggested that the constitutive expression of CyC in the endosomal compartments of CD8$^+$ DC did not inhibit Cat S activity, but it could be that CD8$^+$ DC degraded Iip10 by a mechanism insensitive to CyC. Ii can be eliminated in early endosomal compartments independently of Cat S or other cysteine proteases (32), so such mechanism could be enhanced in the CD8$^+$ DC. Alternatively, other proteases capable of cleaving Iip10, and not inhibited by CyC, could be selectively expressed in CD8$^+$ DC. To address these possibilities, we analyzed Ii processing and MHC II peptide loading in splenic CD8$^-$ and CD8$^+$ DC from Cat S-deficient mice.

The spleens of Cat S$^{-/-}$ mice contained similar amounts of CD4$^+$ and CD8$^+$ DC compared with normal mice (data not shown). Both DC types were analyzed by pulse-chase and immunoprecipitation of MHC II, as described above for normal DC. As shown in Fig. 3*D*, maturation of MHC II was blocked at the $\alpha\beta$-Iip10 stage in both the CD4$^+$ and CD8$^+$ Cat S$^{-/-}$ DC so that no MHC II-peptide complexes were generated after 240 min of chase. Therefore, the CD8$^+$ DC depend on Cat S to degrade Iip10, just as CD4$^+$ DC and splenocytes do.

MHC II expression and subcellular localization are not affected by the lack of CyC

The results above showed no correlation between CyC expression and control of MHC II Ag presentation in CD8$^+$ and CD8$^-$ splenic DC. However, this did not formally exclude that the complete absence of CyC might have an impact on MHC II expression or trafficking. Therefore, we analyzed DC from CyC-deficient mice (Fig. 4*A*) (27). The subcellular distribution of MHC II in

freshly isolated (immature) and cultured (mature) $CD8^+$ DC was unaffected by the lack of CyC (Fig. 4*B*).

The role of CyC in the $CD8^+$ DC could be to interfere with processing of exogenous Ags rather than Ii degradation. To examine this possibility, we compared the capacity of normal and CyC-deficient $CD8^+$ DC to present exogenous OVA and hen egg lysozyme (HEL) to T cell hybridomas. The lack of CyC did not affect the Ag presentation efficiency of the $CD8^+$ DC (Fig. 4*C*). These results exclude a role for CyC in controlling MHC II Ag presentation in splenic DC.

The study that suggested a role for CyC in controlling MHC II Ag presentation was performed on BMDC generated in vitro (23), so it could be that CyC only plays a role in these DC or other DC types obtained by in vitro culture. Therefore, we analyzed BMDC and D1DC (21) from $CyC^{-/-}$ and wild-type mice. The surface expression of MHC II and CD86, before and after maturation, were not affected by the lack of CyC in either BMDC or D1DC (Fig. 5, *A* and *C*, and data not shown). The MHC II molecules of immature $CyC^{-/-}$ BMDC and D1DC accumulated in $Lamp^+$ compartments, as they did in wild-type cells, and they redistributed to the cell surface after maturation (Fig. 5, *A* and *B*).

Finally, we determined whether the lack of expression of CyC had an impact on the generation of peptide-loaded MHC II complexes in BMDC. We immunoprecipitated MHC II from immature BMDC grown from normal, CyC-deficient, or Cat S-deficient mice (Fig. 5*C*). The immunoprecipitates were incubated at room temperature or 95°C, and analyzed by SDS-PAGE followed by Western blot (Fig. 5*C*). As expected, the MHC II molecules of the Cat $S^{-/-}$ BMDC coprecipitated with Iip10 and were forming few $\alpha\beta$-peptide SDS-stable complexes. In contrast, most of the MHC II molecules contained in the normal and the $CyC^{-/-}$ BMDC were forming $\alpha\beta$-peptide complexes and were not associated with Iip10. These results demonstrate that as in immature D1DC (22) and lymphoid organ DC (Fig. 3*C*), Cat S is active in immature BMDC, enabling the constitutive generation of MHC II molecules loaded with antigenic peptides (22, 34, 35). Therefore, it is not surprising that the CyC deficiency did not have a deleterious effect on Iip10 degradation or MHC II-peptide complex formation. The BMDC shown in these experiments were generated in culture medium supplemented with GM-CSF, but without IL-4; similar results were obtained with BMDC generated in culture medium containing GM-CSF plus 1, 2, or 5 U/ml IL-4 (data not shown).

Discussion

The developmental control of MHC II Ag presentation is one of the most distinctive features of DC. Several studies aimed at characterizing the mechanisms responsible for this control have led to propose two major models. According to the first model, immature DC are inefficient at loading their MHC II molecules with peptides. This would be caused, at least in part, by CyC inhibiting Cat S, thus blocking Ii processing at the Iip10 stage (23). The blockade of Cat S activity would be lifted during maturation, enabling the generation of MHC II-peptide complexes, which would then accumulate on the plasma membrane. The second model suggests that immature DC do generate MHC II-peptide complexes, which are then expressed on the cell surface, but the complexes are quickly endocytosed and ultimately degraded in lysosomal compartments (20, 22). Endocytosis of the MHC II-peptide complexes would be slowed down during maturation, thus causing their accumulation on the surface of the mature DC.

In support of the first model, two studies showed by ICM that a complex between I-A^k and a HEL-derived peptide was not generated until the DC were induced to mature (36, 37). However, FACS analysis revealed that the fraction of surface MHC II molecules that were forming the I-A^k-HELpep complex in immature and mature DC was comparable (36). This suggested that the I-A^k-HELpep complex was in fact generated and exported to the plasma membrane in immature DC, although perhaps it could not be detected by ICM because of low sensitivity of the Ab used. This possibility was supported by the observation that the I-A^k-HELpep complex was presented to T cells by the immature DC (36). Indeed, a recent study that used a more sensitive Ab has demonstrated that the I-A^k-HELpep complex was generated and displayed on the surface of both immature and mature DC, where it was recognized by T cells (35). Similarly, another study showed that immature DC generated intracellularly a complex between I-E^k and a pigeon cytochrome C (PCC)-derived peptide within five minutes of exposure to the Ag, and exported the I-E^k-PCCpep complex to the plasma membrane within an hour (34). Therefore, these studies support a model of control of MHC II expression in DC based on events occurring after peptide loading and cell surface expression.

The hypothesis that DC control MHC II expression by regulating Cat S activity has been challenged by other studies: 1) Cat S-deficient DC express normal levels of MHC II on their plasma membrane (6, 9, 13); 2) DC can control MHC II surface expression in the absence of Ii (38); 3) Cat S-independent MHC II allotypes, which do not remain associated with Iip10 when Cat S is inactive, also accumulate in the endosomes of immature DC (22); 4) most importantly, biochemical experiments performed on BMDC (Fig. 5*C*) (19, 22, 35), D1DC (22, 39), lymphoid organ DC (Fig. 3*C*), and human monocyte-derived DC (20), have demonstrated that immature DC degrade Iip10 normally, and constitutively load the bulk of their MHC II molecules with peptides.

Nevertheless, it could be argued that CyC only required to subtly slow down the Cat S-mediated cleavage of Iip10, to induce transferring the MHC II-Iip10 complexes to lysosomal compartments, from where the MHC II molecules would not be able to escape even if they were later converted into MHC II-peptide complexes. In this study we have taken advantage of the differences in CyC expression among DC types, and the availability of CyC-deficient mice, to directly assess the role of CyC on DC Ag presentation. Despite the high level of CyC in the MHC II^+, $Lamp^+$ compartments of the $CD8^+$ DC, Ii degradation and MHC II peptide loading was as efficient in this DC population as in the CyC-negative $CD8^-$ DC. Furthermore, MHC II localization was controlled in CyC-deficient spleen DC, BMDC, and D1DC as in their normal counterparts. Our study thus demonstrates that CyC is neither necessary nor sufficient to control MHC II Ag presentation in DC, and supports that such control is based on the regulation of the rate of endocytosis of the MHC II-peptide complexes (20, 22).

Our finding that CyC is differentially expressed among closely related DC subsets is very striking, because CyC has generally been considered a ubiquitously expressed protein (40, 41). Indeed, neither the mouse nor the human *CyC* gene contain obvious regulatory sequences that might explain this expression pattern (40, 41). We are currently studying in more detail the basis for these differences in CyC expression. Such studies may reveal novel mechanisms of control that could be potential targets for the treatment of pathologies associated with abnormal CyC secretion, which include atherosclerosis, aortic aneurism, hereditary CyC amyloid angiopathy, chronic inflammation, and tumor metastasis (42–45).

Does CyC play a specific role in $CD8^+$ DC? At this stage we can only speculate. Most of the studies on CyC function have focused on the putative role of this inhibitor in controlling extracellular proteolysis. Secretion of CyC might be required to maintain an appropriate proteolytic environment in the areas of the lymphoid organs enriched in $CD8^+$ DC (31). There are also indications

that CyC may play immunomodulatory roles (46) and act as a growth factor (47). In addition, the presence of CyC in the endosomal compartments of the $CD8^+$ DC could affect their proteolytic activity. However, if this is the case, those alterations did not impair the processing and presentation of the antigenic epitopes that we have analyzed in this study. Additional work will be required to determine whether CyC plays a specific role in $CD8^+$ DC.

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