CD83 localization in a recycling compartment of immature human monocyte-derived dendritic cells

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Abstract

Dendritic cells (DC) change their phenotype and functional properties during maturation. CD83 cell surface expression is induced on mature DC (mDC). In this study, we investigated intracellular CD83 localization and transport in human monocyte-derived DC. The enhanced level of CD83 cell surface expression in mDC resulted predominantly from increased protein synthesis, and in addition from regulated intracellular transport of CD83 protein. An internal pool of CD83 protein is present in immature DC (iDC). Although CD83 protein in iDC and in mDC was localized in the Golgi compartment and in recycling endosomes, only in mature cells did CD83 co-localize with MHC class II molecules in endocytic vesicles. CD83 cell surface expression on iDC was induced by inhibition of endocytosis. This result could be explained by CD83 cycling between endosomes and the cell surface in iDC. The mDC also rapidly internalized membrane-bound CD83 protein. Furthermore, a thiol protease inhibitor and specific cathepsin inhibitors impaired CD83 up-regulation in DC, indicating a role of endosomal proteases in the maturation-induced exposure of CD83 on the plasma membrane.

Introduction

Dendritic cells (DC) play a pivotal role as inducers and regulators of immune responses. After antigen uptake or as a consequence of inflammatory stimuli, DC undergo maturation events and acquire the ability to present antigens to T cells (1). In their immature state, DC internalize and process antigens. During maturation they acquire the ability to activate naive T cells but lose their antigen-processing activity. Pro-inflammatory stimuli like tumor necrosis factor-alpha (TNF-alpha) or mitogens such as LPS and double-stranded RNA like polyriboinosinic polyribocytidylic acid (poly I/C) induce maturation of DC. As a consequence of maturation, up-regulation of MHC class I and class II molecules (2), elevated levels of co-stimulatory molecules (CD80, CD86) and de novo exposure of CD83 on the cell surface are observed (1, 3).

In humans, plasma membrane expression of the CD83 protein is widely used as a differentiation marker of mature DC (mDC). CD83 is also expressed on activated lymphocytes (4) and was recently detected on cytokine-activated neutrophils (5, 6). The function of the CD83 polypeptide is not yet known. It has been speculated that CD83 influences DC–T cell interactions (7–10). In mice, CD83 in addition was detected on activated T cells and on thymic epithelial cells (11–13). CD83 gene-deficient mice display a specific block in developing CD4 single-positive thymocytes, which could be mediated by the absence of CD83 on thymic epithelial cells (13). A regulatory role of CD83 for positive selection of Tc, in the thymus was therefore suggested (13). In humans, in contrast to mice, CD83 is found predominantly on thymic DC rather than on epithelial cells (4). The biological role of the CD83 polypeptide expressed by human DC remains to be resolved.

CD83 was expressed after 1 or 2 days of stimulation on the cell surface of stimulated DC (8). The mechanism leading to CD83 plasma membrane exposure is unclear. Immature human monocyte-derived DC (MoDC) express only low amounts of CD83 on their cell membrane. By intracellular staining and flow cytometry, an intracellular pool of CD83
protein has been detected in immature MoDC, which discriminates them from monocytes and macrophages (14). In freshly isolated polymorphonuclear neutrophils, no intracellular pool of CD83 has been found (6). Recent work documents the intracellular expression of CD83 protein in EBV-transformed B cells (15).

In this study, we investigated intracellular localization and transport of CD83 protein in human MoDC. We show that immature MoDC contain an intracellular pool of CD83 protein in the Golgi complex and in endocytic vesicles. Membrane-exposed CD83 protein of mDC is rapidly internalized and directed to endosomes. Maturation-induced CD83 cell surface display is regulated on the level of protein synthesis and by intracellular protein transport and is in addition triggered by endosomal/lysosomal proteases.

**Methods**

**Reagents**

PE-labeled T6/RD1 (IgG1, Beckman-Coulter, Krefeld, Germany) reacts with CD1a, which is a marker for human DC. For detection of CD83, mAb HB15e (purified, IgG1, Caltag Laboratories, Burlingame, CA, USA) was used. For western blotting, CD83-specific mAb 1G11 (rat IgG1, Alexis, Lausen, Switzerland) and actin-specific mAb AC40 (Sigma, St Louis, MO, USA) were employed. HLA-DR was stained using mAb L243 hybridoma culture supernatant or mAb ISCR-3 culture supernatant, CD74, the invariant chain of MHC II, using Bu45 culture supernatant. CD80-specific mAb was from Pharmingen (IgG1, Mountain View, CA, USA). FITC-labeled F(ab)2 fragments of goat anti-mouse antibody, normal mouse serum and normal goat serum for blocking purposes were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Unlabeled IgG1, IgG2a and IgG2b and 7-aminoactinomycin D (7-AAD) were obtained from Sigma. PE-labeled IgG1 and PE-conjugated CD14-specific mAb were from Becton Dickinson (BD) (Mountain View, CA, USA).

Rabbit polyclonal antibodies for organelle labeling were directed against protein disulfide isomerase (PDI) in the endoplasmic reticulum (ER) (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada), giantin in the Golgi compartment (a kind gift of Y. Misumi and Y. Ikehara, Fukuoka University, Fukuoka, Japan) and cellubrevin in early, recycling endosomes (a kind gift of R. Jahn, Göttingen, Germany). Lamp-2 was stained using a rat mAb from the Developmental Studies Hybridoma Bank at the University of Iowa (http://www.uiowa.edu/~dshbwww/abi93.html) and a Cy3-coupled goat anti-rat IgG (Jackson). For CD83 and MHC class II co-localization studies, a rabbit antiserum specific for the HLA-DR beta chain was employed (16). Cy2- and Cy3-labeled F(ab)2 fragments of goat anti-mouse IgG as well as Cy2- and Cy3-labeled goat anti-rabbit IgG were purchased from Jackson.

**Isolation and culture of peripheral blood monocytes**

Monocytes were isolated from peripheral blood of healthy adults by density gradient centrifugation (Nycoprep, Nycomed, Oslo, Norway) as described previously (17). The purity and viability of the cell preparation was evaluated by flow cytometric analysis via staining with anti-CD14-PE and 7-AAD and was consistently greater than 70% of viable monocytes. A total of 10⁵ monocytes were cultured in 24-well plates with 1 ml very low endotoxin-RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 1% antibiotics/antimycotics in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) (500 U ml⁻¹; Novartis Pharma, Nürnberg, Germany) and IL-4 (500 U ml⁻¹; Strathmann Biotech GmbH, Hannover, Germany). On day 2 of culture, 250 U ml⁻¹ IL-4 and 250 U ml⁻¹ GM-CSF were added to the medium, and on day 4 half of the medium was replaced and fresh cytokines were added.

**Maturation of DC**

In vitro-generated MoDC were stimulated with 10 ng ml⁻¹ TNF-alpha (R&D Systems Inc., Minneapolis, MN, USA) or 25 µg ml⁻¹ poly I/C (Sigma) for 2–24 h. When indicated, cells were treated with 1 µg ml⁻¹ cycloheximide (CHX) (Sigma), which blocks protein synthesis (18), 1 µg ml⁻¹ brefeldin A (BFA, Golgi Plug, BD Pharmingen, San Diego, CA, USA) influencing cis- and trans-Golgi transport and endocytosis (19, 20), 3 µM Z-Val-Val-Nle-DMK (Bachem, Heidelberg, Germany), a specific cathepsin S (Cat S) inhibitor (21), 100 nM Z-Phe-Tyr(LBu)-DMK (Bachem), a specific cathepsin L (Cat L) inhibitor (21), 20 nM concanamycin B (Con B, kind gift of J. Villadangos, Melbourne, Australia), which is active in endosomes and lysosomes (22) or 100 µM E64 (Bachem), an inhibitor of thiol proteases (23) 1 h before stimulation. In some experiments, cells were incubated with 10 µg ml⁻¹ cytochalasin D (Sigma) for the indicated time points.

**Flow cytometric analysis**

A total of 10⁵ in vitro-generated cells were subjected to double-staining experiments for surface and intracellular staining with saponin and digitonin as described in detail elsewhere (24, 25). At least 3 × 10⁴ cells were analyzed with a FACSCalibur (BD).

For quantitative evaluation, 7-AAD staining excluded dead cells and the CD1a⁺ populations were gated out manually using the CellQuest software (BD). The results are expressed in percentage of positive cells and mean fluorescence intensity (MFI) ± SEM. Relative fluorescence indices (rFI) of the different structures were determined as follows:

\[
\text{rFI} = \frac{\text{MFI(} \text{specific antibody} \text{) \ - \ MFI(} \text{isotype control} \text{)}}{\text{MFI(} \text{isotype control} \text{)}}
\]

**Internalization of cell membrane molecules**

At 4°C, cell membrane-exposed CD83, MHC II molecules and MHC I-associated invariant chain (CD74) were stained with specific antibodies (HB15e, L243 and Bu45, respectively). Unbound antibodies were removed and cells were allowed to metabolize at 37°C. After the indicated time periods, aliquots of the cells were transferred to 4°C and the remaining cell membrane-bound antibody was detected by FITC-labeled goat anti-mouse Ig and FACS analysis. The rFI values were calculated as shown above.
Western blotting
A total of $1 \times 10^6$ in vitro-generated cells were lysed using buffer containing 0.5% Nonidet P40. The samples were separated with 12% SDS gel and subjected to western blot analysis with 1 µg per 2 ml 1G11 as described in detail elsewhere (26). Glycosidase treatment (PNGase F, New England Biolabs, Beverly, MA, USA) to remove N-linked carbohydrate structures was performed on lysates as prepared for western blotting. Proteins were de-glycosylated with PNGase F using the buffer recommended by the manufacturer. The reaction mixture was incubated for 16 h at 37°C. The samples were then separated on a 12% SDS gel and subjected to western blotting as described above.

Immunofluorescence staining and confocal microscopy
A total of $10^6$ immature or mature MoDC were allowed to adhere to poly (l.) lysine-coated slides for 1 h and fixed using 70% methanol for 5 min at –20°C. Slides were briefly air-dried and stored at –70°C until staining. After thawing of the slides, they were double stained with CD83-specific mAb (10 µg ml$^{-1}$), MHC class II-specific L243 culture supernatant and anti-giantin rabbit antiserum (1 : 300), anti-cellubrevin rabbit antiserum (1 : 300), Lamp-2-specific mAb (1 : 10) or the respective negative control for 1 h at room temperature in Tris-buffered saline (TBS) containing 2.5 mg ml$^{-1}$ intraglobin (Biotest, Dreieich, Germany). After two washes with TBS, the cells were incubated with Cy2- or Cy3-labeled goat anti-mouse antibody for 60 min at room temperature followed by two washes.

Additional staining steps as described above were performed with another set of non-cross-reactive primary and secondary antibodies to achieve labeling of a second epitope. After staining, the samples were washed with TBS. Cover slips were mounted with Gel/Mount (Biomed, Foster City, CA, USA) containing 50 mg ml$^{-1}$ 1,4 diazobicyclo[2.2.2]octane (Sigma) and analyzed on a Zeiss LSM510 microscope (Carl Zeiss Jena GmbH, Jena, Germany) using the Zeiss LSM 510 Image Browser/Examination software.

Statistical analysis
Statistical analysis was performed using the non-parametric two-tailed Wilcoxon/Mann–Whitney test, which does not require normal distribution of the data and is thus best suited for relatively small data sets. Values of $P < 0.05$ were considered statistically significant.

Results
MoDC contain intracellular deposits of CD83 polypeptides
We generated DC from peripheral blood monocytes by stimulation with GM-CSF and IL-4. After 6 days of culture, 80–95% of cells expressed the DC marker CD1a. At first, we investigated the kinetics of CD83 cell surface expression after the treatment of immature MoDC with TNF-alpha. As early as 2 h after stimulation, CD83 was detected on the cell membrane of MoDC. After 12–24 h virtually all cells exposed CD83 at the cell surface. Figure 1(A) summarizes results from six experiments where cells were stimulated with TNF-alpha for the indicated time periods and shows percentages of CD83-positive DC. Figure 1(B) depicts MFI of one representative experiment. MoDC remained CD83 positive for at least 2 to 3 days upon stimulation (Fig. 1A and data not shown).

Western blot analysis of immature MoDC (Fig. 2A, lane 3) and of MoDC treated for the indicated time periods with poly I/C (lanes 4 –7) or with TNF-alpha (lanes 8 –11) showed CD83 protein expression both in immature DC (iDC) and mDC. Upon induced maturation of DC with poly I/C or with TNF-alpha, CD83 expression was up-regulated. The molecular weight (m.w.) of CD83 protein increased gradually in 2 to 24 h from ~38–40 kDa to 50–60 kDa. Two hours after induction of maturation, a novel band with a m.w. of 32 kDa appeared. PNGase F digestion of N-linked carbohydrates (Fig. 2B) caused disappearance of the higher m.w. bands and resulted in a discrete 23-kDa band. This band corresponds to the CD83 polypeptide lacking N-linked glycosylation. The specificity of the bands was verified in lysates from COS-7 cells that had been transfected with CD83 cDNA (Fig. 2A, lane 2). No bands were detected by the CD83-specific antibody in lysates from non-transfected COS-7 cells (Fig. 2A, lane 1). Re-exposure of the western blot to an actin-specific antibody (Fig. 2A, bottom panel) demonstrated loading of the lanes with equal amounts of cell lysates. Poly I/C and TNF-alpha stimulated CD83 expression to the same extent and with comparable kinetics.
Intracellular protein transport mediates maturation-induced CD83 cell surface expression

Maturation-induced CD83 cell surface expression could be induced by de novo protein synthesis, since the CD83 gene is regulated by TNF-alpha (27), which was used as a maturation stimulus. In addition, a restricted transport of the detected intracellular pool of CD83 molecules could control exit to the cell surface. Protein biosynthesis or vesicular transport was blocked by using inhibitors of protein synthesis (CHX) or transport (BFA), and Con B. CHX inhibits protein synthesis by blocking translation of messenger RNA on the ribosome. BFA induces a disassembly of the Golgi complex thereby blocking the secretory pathway at the level of the ER–Golgi juncture and at the level of the trans-Golgi network (TGN). Con B, an inhibitor of vacuolar ATPases, inhibits the acidification and thus raises the pH in MHC loading compartments.

We pre-treated immature MoDC for 1 h with inhibitors and subsequently stimulated the cells for 12 h with poly I/C and TNF-alpha. The lysates were separated on a 12% SDS gel, transferred to a Nylon membrane and subsequently detected with anti-CD83 mAb 1G11 (1 : 2000), which is directed against actin.

We examined in which intracellular organelles CD83 protein is detected in immature and mature MoDC. Double stainings using CD83 mAb (green) and antiserum specific for giantin as a Golgi marker (red) revealed co-localization (yellow color) of CD83 with giantin in both immature (Fig. 4A–C) and mature (Fig. 4D–F) MoDC. CD83 and PDI did not co-localize neither in immature nor in mature MoDC (data not shown), implying that CD83 is not retained in the ER. In immature (Fig. 4D, left panel) and reduced maturation-induced surface expression on MoDC stimulated with TNF-alpha instead of poly I/C with identical results compared with those presented in Fig. 3 (compare Table 1).

Incomplete inhibition of maturation-induced CD83 cell surface expression by CHX is consistent with an intracellular pool of preformed CD83 molecules in iDC. During maturation, CD83 molecules from this pool are transported to the cell membrane. Export of CD83 to the cell surface can be inhibited by transport inhibitors. We investigated the biosynthetic route of CD83 to the cell surface. Confocal microscopy analysis was applied to localize CD83-positive cellular compartments of iDC and mDC.

In immature MoDC, CD83 is localized in a Golgi compartment and in endosomes

We examined in which intracellular organelles CD83 protein is detected in immature and mature MoDC. Double stainings using CD83 mAb (green) and antiserum specific for giantin as a Golgi marker (red) revealed co-localization (yellow color) of CD83 with giantin in both immature (Fig. 4A–C) and mature (Fig. 4D–F) MoDC. CD83 and PDI did not co-localize neither in immature nor in mature MoDC (data not shown), implying that CD83 is not retained in the ER. In immature (Fig. 4D–F) as well as in mature MoDC (Fig. 4J–L), CD83 also co-localized with cellubrevin, a v-SNARE protein, which is a constituent of recycling endosomes. Cellubrevin (red) is present in an endosomal compact structure around the nucleus and in small peripheral puncta in the cytoplasm, as described by Wilcke et al. (28). Our results demonstrate the presence of CD83 in recycling compartments of iDC. DC maturation resulted in CD83 transport to the cellular membrane. In maturing cells, CD83 clearly co-localized with giantin and with cellubrevin. We observed the same staining pattern of CD83 protein at 30 min, 1, 2, 6, 12 and 24 h after stimulation (data not shown). This staining pattern is probably due to ongoing de novo biosynthesis during maturation, refilling CD83 deposits in the Golgi compartment and in endosomes.
to labeled intracellularly localized CD83, mDC display a pronounced staining of the plasma membrane (Fig. 4G, J and I, L), which was only marginally seen on iDC (Fig. 4A, C and D, F). The result in Fig. 4 shows that immature as well as mature MoDC contain CD83 deposits in the Golgi complex and in recycling endosomes, which upon maturation traffic to the cell membrane.

**Table 1.** Inhibition of maturation-induced cell surface expression of CD83 and HLA-DR

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<tr>
<th>Inhibitor</th>
<th>CD83</th>
<th>HLA-DR</th>
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<tr>
<td>CHX</td>
<td>56.3 ± 17.0</td>
<td>8.6 ± 22.0</td>
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<td>BFA</td>
<td>94.4 ± 5.1</td>
<td>46.7 ± 24.2</td>
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<td>Con B</td>
<td>69.7 ± 10.4</td>
<td>59.9 ± 12.8</td>
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Experiments were performed as outlined for Fig. 3. Mean percentages of inhibition ± SEM were calculated as follows: 100 – rFI (+inhibitor)/rFI (–inhibitor) × 100. Data from six independent experiments (CHX and BFA) and from three experiments (Con B) are shown.

**Fig. 3.** Upon maturation of MoDC, CD83 protein is transported to the cell surface. After 6 days of culture, MoDC were pre-treated for 1 h with inhibitors of protein synthesis or intracellular transport and subsequently stimulated for 12 h in the presence of inhibitors to achieve maturation. (A) No stimulator, no inhibitor, B–E: poly I/C, (B) no inhibitor, (C) CHX, (D) BFA, (E) Con B. Cells were analyzed by double labeling of CD1a and CD83 (left panel of histograms) and HLA-DR (right panel of histograms) and flow cytometry as described in Methods. Numbers in histograms represent MFI of analyzed cells. Histograms representative for three to seven experiments (see Table 1) are shown (gray line: isotype control, black line: CD83 or HLA-DR, respectively).

**Fig. 4.** In immature MoDC, CD83 is localized in the Golgi compartment and in recycling endosomes. Immature (A–F) and mature (G–L) MoDC were allowed to adhere to slides, fixed with methanol and first stained with CD83-specific mAb and goat anti-mouse IgG Cy2-labeled secondary reagent (green). Subsequently, giantin-specific antiserum (A–C and G–I) or cellubrevin-specific antiserum (D–F and J–L) and goat anti-rabbit IgG Cy3-labeled secondary antibody (red) were used as described in Methods. Scale bars represent 10 μm.

To examine endosomal localization of CD83, co-localization with MHC class II molecules was investigated. In iDC, MHC class II molecules are localized in endosomal/lysosomal compartments containing the lysosomal markers Lamp,
CD63 and β-hexosaminidase, which are negative for the early endosomal marker transferrin receptor. This compartment has been termed MHC class II-rich compartment (MIIC) (29). Staining of iDC with mAb L243 specific for mature, peptide-loaded MHC class II molecules (green) and with a cellubrevin-specific antiserum (Fig. 5A) revealed partial co-localization of MHC class II molecules and cellubrevin (yellow) in only a small minority of cells (marked by asterisks). The absence of cellubrevin from most MIIC structures suggests that the CD83-positive endosomal structures are different from MIIC. Staining of iDC with CD83-specific antibody (green) and a rabbit serum specific for the beta chain of HLA-DR (red) (Fig. 5B) showed no co-localization and confirmed the result shown in Fig. 5(A). Moreover, CD83 staining in iDC did not merge with Lamp-2-specific antibody labeling (Fig. 5E). Thus, the intracellular pool of CD83 molecules in iDC is localized in an endosomal compartment positive for the v-SNARE protein cellubrevin and distinct from MIIC structures.

In mDC, MHC class II molecules stained by L243 mAb (green) were found on the cell surface. No co-localization with cellubrevin (red) was observed (Fig. 5C). In these cells (Fig. 4J–L), CD83 (green) was expressed on the cell surface and was co-localizing with cellubrevin (red) intracellularly. In contrast to iDC, in mDC we observed co-localization (yellow) of CD83 (green) with MHC class II molecules (red), both on the cell surface and intracellularly (Fig. 5D). CD83 co-stained with Lamp-2 (Fig. 5F), as expected from Lamp-positive MIIC structures. Neither in iDC nor in mDC were MHC II molecules detected in Golgi compartments, which was examined by staining of giantin (Fig. 5, G and H). In iDC, we found CD83 protein in the Golgi complex and in recycling endosomes. CD83 does not co-localize with MHC class II molecules in iDC. In contrast, in mDC, CD83 and MHC class II molecules co-localize in an endosomal/lysosomal compartment defined by Lamp-2 expression, which is distinct from recycling endosomes.

Immature and mature MoDC internalize CD83 from the cell membrane

Rapid endocytosis and re-expression of low levels of CD83 molecules on the cell surface co-exist with a deposit of CD83 protein in recycling endosomes of iDC. If endocytosis of cell surface-expressed CD83 protein occurred on iDC, blockage of endocytosis should enhance the number of cell surface CD83 molecules and allow cell surface detection. Therefore, we treated immature MoDC with cytochalasin D, an inhibitor of endocytosis. Cytochalasin D depolymerizes and sequesters F-actin filaments and thus blocks endocytosis without affecting the exocytotic pathway (30). After cytochalasin D treatment for 3 h, we observed CD83 cell surface expression in immature MoDC. As a control, the diluent dimethyl sulfoxide had no effect (Fig. 6A and B). Twenty-four hours after cytochalasin D treatment, up to 30% of immature MoDC expressed CD83 on the cell surface. Elevation of CD83 expression upon cytochalasin D treatment was not due to DC maturation. Other markers of maturation like CD80 (Fig. 6C) and MHC I and MHC II antigens (data not shown) were not enhanced after cytochalasin D treatment of iDC. Inhibition of endocytosis promotes CD83 cell surface expression on iDC.

Recycling of CD83 in immature MoDC

Fig. 5. Co-localization of CD83 and MHC class II molecules in endosomes. Immature (A, B, E, G) and mature (C, D, F, H) MoDC were allowed to adhere to slides and were fixed with methanol. Cells were stained with MHC class II-specific L243 mAb and with goat anti-mouse Ig Cy2-labeled secondary reagent (A, C, green) followed by cellubrevin-specific antiserum and goat anti-rabbit Ig Cy3-labeled secondary antibody (red). In (B and D), staining was performed with CD83-specific mAb and goat anti-mouse Ig Cy2-labeled secondary reagent (B, D, green), and MHC class II-specific rabbit antiserum and goat anti-rabbit Ig Cy3-labeled secondary antibody (red). In (E and F) staining was performed with CD83-specific mAb and goat anti-mouse Ig Cy2-labeled secondary reagent (green), and a Lamp-2-specific rat mAb followed by a Cy3-coupled goat anti-rat IgG reagent (red). (G and H) show stainings with culture supernatant from HLA-DR-specific mAb ISCR-3 and goat anti-mouse Ig Cy2-labeled secondary reagent (green) and giantin-specific antiserum and goat anti-rabbit IgG Cy3-labeled secondary antibody (red). Scale bars represent 10 μM.
Recycling of CD83 in immature MoDC
Membrane-bound CD83 protein was also rapidly internalized from mature MoDC. This is shown in Fig. 6(D, solid line). Within 30 min at 37°C, CD83 membrane exposure on mDC was reduced by >50%. Figure 6(E, left panel) shows corresponding FACS histograms. Results from five internalization experiments show a cell surface half-life of \( t_{1/2} = 20 \text{ min} \) for CD83 on mature MoDC. In comparison, MHC II surface expression was stable within 90 min (Fig. 6D, dashed line and Fig. 6E, middle panel), whereas 80% of membrane-exposed MHC II-associated invariant chain (II, CD74) was endocytosed in 10 min (Fig. 6D, dotted line and Fig. 6E, right panel). Within 30 min, CD83-bound antibodies were internalized and detected in endosomes by laser scanning microscopy (data not shown).

Inhibition of endocytosis of mature MoDC with cytochalasin D increased the cell surface half-life from \( t_{1/2} = 20 \text{ min} \) to \( t_{1/2} = 90 \text{ min} \) (Fig. 6F and G). Delivery of CD83 to the cell membrane followed by internalization is important to maintain CD83 steady state levels on the cell surface of DC.

Proteases impact on CD83 cell surface expression

We showed that in mDC, CD83 protein co-localized in endocytic vesicles with MHC II heterodimers. Processing of MHC II molecules associated to II requires the activity of endosomal proteases, e.g. Cat S and Cat L (31). Since endocytic vesicles of mDC containing CD83 protein display proteolytic activity, we investigated whether lysosomal proteases impact on the maturation-induced up-regulation of CD83.

We employed a specific inhibitor of Cat S (Z-Val-Val-Nle-DMK), a Cat L inhibitor [Z-Phe-Tyr(Bu)]-DMK (25) and E64d, an inhibitor of thiol proteases, and examined maturation-induced up-regulation of CD83 surface expression. Immature MoDC were incubated with the inhibitors for 1 h and subsequently activated with TNF-alpha or poly I/C for 12 h.

Figure 7(A and B) shows that E64d almost completely inhibited CD83 surface expression (\( P < 0.001 \)), while Cat S and Cat L inhibitors only slightly impaired (\( P < 0.05 \)) maturation-induced display of CD83 surface cell molecules (Fig. 7A and B). Cat S and E64d thiol protease inhibitors significantly reduced maturation-induced HLA-DR up-regulation, whereas Cat L inhibitor had no remarkable effect (Fig. 7C). In contrast to mDC, CD83 expression on iDC is not affected by protease inhibitors (data not shown). This result suggests a role of thiol proteases in the maturation-induced CD83 surface expression of DC.

Discussion

Maturation of human DC is a prerequisite for efficient antigen presentation to CD4+ T cells. De novo CD83 cell surface expression of CD83 is highly characteristic for mDC, albeit a function for CD83 has not been described. CD83 has been implicated in binding to ligands present on monocytes (7, 32) and on DC (9). Recently, killing of human T lymphoblasts and activated DC in an allogeneic mixed lymphocyte reaction was shown to be enhanced by anti-CD83 antibody-dependent cytotoxicity of NK cells (33). Cell surface expression appears to be implicated in the biological role of CD83.

We studied CD83 transport and intracellular localization during maturation of human MoDC. Intracellular staining of CD83 from in vitro-generated, detergent-permeabilized immature, CD83 cell surface-negative DC displayed CD83 intracellularly (14 and our data, not shown).

Western blot analysis of CD83 protein expressed by iDC revealed the presence of preformed CD83 protein with a m.w. of ~38–40 kDa. During maturation of the cells in the presence of TNF-alpha or poly I/C, the m.w. gradually increased to ~50 kDa. This reflects further glycosylation of CD83 protein during transport through the Golgi compartments. In addition, in TNF-alpha and in poly I/C-treated cells, a novel band with a m.w. of ~32 kDa appeared, which is also the prominent band observed in CD83 cDNA-transfected COS cells. Removal of N-linked carbohydrates by PNGase F treatment of lysates from mature cells reduced CD83 forms with higher m.w. to 23 kDa. Therefore, the 32 kDa band presumably corresponds to a newly synthesized precursor form containing three N-linked glycans. The presence of the 32 kDa band in all lysates from TNF-alpha and from poly I/C-treated cells reflects ongoing de novo biosynthesis of this CD83 precursor molecule.

Hock et al. had shown that constitutive CD83 cell surface expression on human leukemia cell lines could be partially reduced by CHX (34). Similar results were obtained in activated polymorphonuclear neutrophils with almost complete inhibition of CD83 surface expression upon treatment with CHX. However, in contrast to MoDC, freshly isolated polymorphonuclear neutrophils did not contain CD83 protein (6). In immature PBMC-derived DC, Kruse et al. (8) found evidence for a CD83 RNA pool while these cells lack detectable CD83 protein by western blot analysis or immunofluorescence staining. In the absence of a CD83 protein depot in iDC, CD83 cell surface expression would depend on de novo synthesis of CD83 protein.

Fig. 6. CD83 is internalized from the cell surface of immature and mature MoDC. After 6 days of culture, MoDC were treated with 10 μg ml−1 cytochalasin D or the diluent dimethyl sulfoxide (DMSO) for up to 24 h. At the indicated time points, cells were analyzed by CD83 labeling and flow cytometry as described in Methods. A gate was set on CD1a+ viable cells. (A) Inhibition of endocytosis results in CD83 cell surface expression on immature MoDC. FACS histograms from one representative experiment of four are shown. (B) Shown are percentages of CD83-positive cells at the indicated time points. Mean data ± SEM from four independent experiments are shown. (C) Treatment of MoDC with cytochalasin D does not induce maturation. MoDC were treated with cytochalasin D or with TNF-alpha for 6 h or left untreated. CD80 or CD83 expression was analyzed by flow cytometry, as indicated. (D) Mature MoDC internalize CD83. Mature MoDC were incubated with CD83-specific mAb (solid line), HLA-DR-specific antibody L243 (dashed line) and II-specific antibody Bu45 (CD74, dotted line). Unbound antibody was removed and cells were incubated at 37°C for the indicated time periods. Cell surface-exposed antibody was detected using FITC-labeled goat anti-mouse Ig. The rFI values from time \( t = 0 \) are taken as 100% and values for later times are calculated accordingly. Mean data ± SEM from five independent experiments are summarized. (E) Representative FACS histograms from one of the experiments summarized in (D) are shown. (F) CD83 internalization by mature MoDC can be inhibited by cytochalasin D. CD83-stained mature MoDC were pre-treated with cytochalasin D or with DMSO, the solvent of cytochalasin D, for 1 h and were subsequently incubated at 37°C. At the indicated time points, cell surface-exposed antibody was detected. Representative FACS histograms from one of five experiments summarized in (G) are shown. (G) CD83 internalization by mature MoDC can be inhibited by cytochalasin D. CD83-stained mature MoDC were pre-treated with cytochalasin D or with DMSO, the solvent of cytochalasin D, for 1 h and were subsequently incubated at 37°C. At the indicated time points, cell surface-exposed antibody was detected. Mean values of rFI ± SEM from five independent experiments are summarized. The rFI = [MFI(specific antibody) – MFI(isotype control)]/MFI(isotype control).
Protease activity is required for maturation-induced CD83 cell surface expression in MoDC. After 6 days of culture, MoDC were pre-treated with 3 μM of the specific Cat S inhibitor Z-Val-Val-Nle-DMK, 100 nM Z-Phe-Tyr(+Bu)-DMK, a specific Cat L inhibitor, or 100 μM E64d for 1 h before inducing maturation of the cells for 12 h. Representative histograms for CD83 (solid lines) and a non-specific isotype-matched control antibody (dashed lines) are shown. (A) CD83 expression upon maturation of MoDC in the presence of protease inhibitors was analyzed by flow cytometry. Results (mean ± SEM) obtained in 12 experiments (Cat S inhibitor) and in 6 experiments (Cat L inhibitor and E64d) are summarized. The rFI were determined as follows: rFI = [MFI(specific antibody) – MFI(isotype control)]/ MFI(isotype control). * = P < 0.05, ** = P < 0.001. (C) After 6 days of culture, MoDC were pre-treated with 3 μM of the specific Cat S inhibitor Z-Val-Val-Nle-DMK, 100 nM Z-Phe-Tyr(1Bu)-DMK, a specific Cat L inhibitor, or 100 μM E64d for 1 h before inducing maturation of the cells for 12 h. HLA-DR expression upon maturation of MoDC in the presence of protease inhibitors was analyzed by flow cytometry. Results obtained in 10 experiments (Cat S inhibitor), in 6 experiments (Cat L inhibitor) and in 4 experiments (E64d) are summarized. We calculated the relative increase of HLA-DR molecules on the cell membrane as rFI (+stimulator)/rFI (–stimulator). * = P < 0.05.

Fig. 7. Protease activity is required for maturation-induced CD83 cell surface expression in MoDC. After 6 days of culture, MoDC were pre-treated with 3 μM of the specific Cat S inhibitor Z-Val-Val-Nle-DMK, 100 nM Z-Phe-Tyr(+Bu)-DMK, a specific Cat L inhibitor, or 100 μM E64d for 1 h before inducing maturation of the cells for 12 h. (A) Representative histograms for CD83 (solid lines) and a non-specific isotype-matched control antibody (dashed lines) are shown. (B) CD83 expression upon maturation of MoDC in the presence of protease inhibitors was analyzed by flow cytometry. Results (mean ± SEM) obtained in 12 experiments (Cat S inhibitor) and in 6 experiments (Cat L inhibitor and E64d) are summarized. The rFI were determined as follows: rFI = [MFI(specific antibody) – MFI(isotype control)]/ MFI(isotype control). * = P < 0.05, ** = P < 0.001. (C) After 6 days of culture, MoDC were pre-treated with 3 μM of the specific Cat S inhibitor Z-Val-Val-Nle-DMK, 100 nM Z-Phe-Tyr(1Bu)-DMK, a specific Cat L inhibitor, or 100 μM E64d for 1 h before inducing maturation of the cells for 12 h. HLA-DR expression upon maturation of MoDC in the presence of protease inhibitors was analyzed by flow cytometry. Results obtained in 10 experiments (Cat S inhibitor), in 6 experiments (Cat L inhibitor) and in 4 experiments (E64d) are summarized. We calculated the relative increase of HLA-DR molecules on the cell membrane as rFI (+stimulator)/rFI (–stimulator). * = P < 0.05.
could be T cell membrane components, which could facilitate DC–T cell interaction during antigen presentation. Our finding that cell surface-bound CD83 molecules from mDC are endocytosed with a half-life of 20 min supports this hypothesis. Endosomes in DC are rich in proteases, e.g. Cat S and Cat L, which during antigen processing degrade the class II-associated invariant chain and facilitate transport of peptide-loaded class II molecules to the cell surface. Therefore, cell surface transport of MHC class II molecules can be blocked by inhibition of thiol proteases and by specific inhibitors of Cat S and Cat L. We investigated whether protease inhibitors would also influence CD83 cell surface expression. To limit the cytotoxicity of the inhibitors, CD83 surface expression was assessed after 12 h instead of 24 h in other experiments. DC matured for 12 h show moderate expression of CD83 on the cell surface. Despite the reduced maturation time, we observed an inhibitory effect of the protease inhibitors, which proved to be statistically significant in the Wilcoxon/Mann–Whitney rank sum test. Our finding that cathepsin inhibitors and E64d inhibit maturation-induced cell surface expression of CD83 suggests a role of thiol proteases for processing of CD83 transport. CD83 has also been described in other cell types than DC. Investigation of intracellular CD83 traffic and its recycling from the membrane of neutrophils, B cells and T cells may reveal additional insights in its potential target structures and function.

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Abbreviations

7-AAD 7-amino-actinomycin D
BFA brefeldin A
Cat L cathepsin L
Cat S cathepsin S
CHX cycloheximide
Con B concanamycin B
DC dendritic cells
ER endoplasmic reticulum
GM-CSF granulocyte macrophage colony-stimulating factor
iDC immature DC
mDC mature DC
MFI mean fluorescence intensity
MIIC MHC class II-rich compartment
MoDC monocyte-derived dendritic cell
m.w. molecular weight
PDI protein disulfide isomerase
poly I/C polyribosinic polyribocytidylic acid
rFI relative fluorescence indices
TBS Tris-buffered saline
TGN trans-Golgi network
TNF tumor necrosis factor

References


7 Scholler, N., Hayden-Ledbetter, M., Hellstrom, K. E., Hellstrom, I. and Ledbetter, J. A. 2001. CD83 is a sialic acid-binding Ig-like lectin (Siglec) adhesion receptor that binds monocytes and a subset of activated CD8+ T cells. J. Immunol. 166:3965.


