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Regulation of MHC Class I Transport in Human Dendritic Cells and the Dendritic-Like Cell Line KG-1

Anne L. Ackerman and Peter Cresswell

Dendritic cells (DCs), the “sentinels of the immune system,” progress through distinct maturational stages, each of which enhances aspects of the Ag presentation machinery (2–7). In peripheral tissues, immature DCs are optimized for Ag uptake, engulfing large amounts of extracellular material through macropinocytosis (8). Upon encountering a maturational stimulus, these cells down-regulate endocytosis and migrate to secondary lymphoid organs (9). Once there, mature DCs exhibit enhanced Ag presentation, up-regulating surface MHC class I and class II expression as well as costimulatory and adhesion molecules (10). These phenotypic changes allow DCs to stimulate strong T cell-mediated immunity against Ags acquired early in maturation (11, 12).

The temporal separation of Ag uptake and Ag presentation requires tight regulation of the trafficking and processing of Ag-presenting molecules. Both the processing and trafficking of MHC class II is altered with the DC maturational state (6, 13–15). In immature DCs, the majority of MHC class II is rapidly degraded after synthesis, preventing premature T cell activation to self-Ags (14). When DCs encounter a maturational signal, a transient intermediate state sequesters MHC class II in peripheral nonlysosomal vesicles, protecting newly loaded complexes from degradation (6, 13). Morphogenesis into a mature DC transports these complexes to the plasma membrane (6) where their increased stability (14), concomitant with the up-regulation of costimulatory molecules (16), allows the activation of CD4+ T cell-mediated immunity.

MHC class I Ag presentation stimulates a distinct branch of the adaptive immune system mediated by CTLs (17, 18). Peptide Ags destined for presentation by MHC class I are derived from cytosolic proteins (19), allowing surveying CTLs to identify virally infected cells (20). DCs, however, possess an alternate pathway of Ag acquisition, in which engulfed proteins are transported into the cytoplasm to allow presentation by MHC class I (21–24). This process, dubbed cross-presentation, accounts for cytotoxicity against tumors and tissue-specific viruses encoding Ags not normally synthesized by DCs (21).

Components of the MHC class I machinery are known to be developmentally regulated throughout DC maturation, with the up-regulation of IFN-γ-inducible immunoproteasome subunits, LMP2, LMP7, and MECL-1, in immature DCs and of TAP1, TAP2, tapasin, and the immunoproteasome regulator PA28 in mature DCs (4, 25). In CD34+ stem cell-derived Langerhans cells, up-regulation of surface MHC class I occurs rapidly after activation, reflecting a redistribution of internal complexes. MHC class I is found in endocytic structures in immature cells, but relocates to the cell surface upon maturation induced by LPS (26). Similar up-regulation of surface MHC class I is seen in monocyte-derived DCs, although they do not display an endosomal accumulation of MHC class I-peptide complexes (26).

Our initial investigation of DCs revealed that MHC class I trafficking and stability is significantly altered throughout the DC maturation process. Due to difficulties in the isolation of homogenous human DC populations (27, 28) and the sensitivity of DCs to manipulation (6, 29), we chose the KG-1 cell line to biochemically examine MHC class I regulation in DCs. KG-1, a highly endocytic, human erythromyelogenous leukemia line (30), has similar multipotent properties to PBMCs, possessing the capacity to mature into both macrophages (31) and dendritic-like cells (DLCs) (32). KG-1 DLCs can be produced in high numbers with a consistent purity.
providing an in vitro system in which to examine DC-specific processes. Upon stimulation with PMA and ionomycin, conditions which also induce the generation of DCs from hemopoietic progenitors (33), KG-1 differentiates into D LCS characterized by the extension of cellular processes and the up-regulation of MHC class I and II, costimulatory molecules, and DC-specific markers (32). KG-1 D LCS also up-regulate the DC-specific NF-kB isofrom, RelB (34, 35), which peaks late in stimulation (32). Although unstimulated KG-1 cells are poor activators of T cells, mature KG-1 D LCS stimulate allogeneic T cell proliferation at levels similar to PBMC-derived DCs (32), making KG-1 a good in vitro model to study human DC-specific processes.

Both PBMC-derived DCs and KG-1 cells regulate MHC class I assembly and trafficking throughout differentiation. Early in maturation, MHC class I is delayed in the Golgi apparatus, sequestering newly loaded complexes internally. Maturation into late stage cells results in the relocation of class I to the plasma membrane, accompanied by an increased half-life of cell surface complexes and an up-regulation of costimulatory molecules. Thus, in a manner similar to the regulation of MHC class II, monocyte-derived DCs tightly control the compartmentalization and transport of MHC class I molecules.

Materials and Methods

Abs and peptides

Fluorescein-conjugated mAbs specific for HLA-ABC, HLA-DR, CD80 and CD83; PE-conjugated mAbs specific for CD1a, CD11c, and CD86; and the relevant isotype-matched controls were obtained from Beckman Coulter (Fullerton, CA). A fluorescein-conjugated anti-H-2Kb mAb was purchased from BD PharMingen (San Diego, CA). A Cy5-conjugated mAb specific for SIINFEKL-Kb complexes, 25-D1.16 (36), was kindly provided by Dr. J. Yewdell (National Institutes of Health, Bethesda, MD). The conformation-independent, MHC class I chain-specific rat mAb 3B10.7 was previously described (37) and the conformation-dependent, H chain-β2-microglobulin (β2-m)-peptide complex-specific mAb w6/32 (38) was obtained from the American Type Culture Collection (Manassas, VA). GAP.A3, an HLA-A3-specific mAb (39), was used as a negative control. A rabbit antisera against β2-m was obtained from Roche Applied Science (Indianapolis, IN). Anti-GM130 and anti-γ-adaptin Abs were both obtained from BD Transduction Laboratories (Lexington, KY). R.A3e7 and R.RING4C were affinity purified from rabbit antiserum and are specific for the HLA-A tyrosyl peptide signal (40) and TAP1 (41), respectively. HSC6, PaSta-1, and Ts219 are mouse mAbs specific for CD63 (42), tapasin (43), and CD58 (44), respectively. DR.DAB45 and L243 (46) are anti-HLA-DRαβ rabbit and mouse Abs, respectively. GILT is a rabbit antisera specific for IFN-γ-inducible thioeductase (GILT) (47). Rabbit anti-Grasp22 was a kind gift from Dr. G. Warren (Yale University, New Haven, CT). The SIINFEKL (OVA257-264) and RGYVYQGL (vesicular stomatitis virus nucleocapsid (VSV NC) 52–59) peptides were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Cells and cultures

KG-1 cells, obtained from American Type Culture Collection, were stimulated with PMA (10 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (100 ng/ml; Sigma-Aldrich) as described previously (32). Adherent cells were harvested by a 5-min incubation in 1 mM EDTA at 37°C. DC cultures were generated as described elsewhere (48). Briefly, immature DCs were harvested after 5 days of stimulation of PBMCs with GM-CSF (800 U/ml) and IL-4 (15,000 U/ml; R&D Systems, Minneapolis, MN) in RPMI 1640 medium supplemented with 20% FCS. The addition of 37.5 ng/ml LPS induced the maturation of these cells into mature DCs.

Immunofluorescence microscopy

Cells were fixed and prepared for indirect immunofluorescence as previously described (49) using Alexa Fluor 488-conjugated goat anti-rabbit Ig and Alexa Fluor 594-conjugated goat anti-mouse Ig secondary Abs (Molecular Probes, Eugene, OR). Cells were visualized using an Axioskop 2 fluorescence microscope (Zeiss, Oberkochen, Germany). Digital images were acquired with a charge-coupled device camera (Princeton Instruments, Trenton, NJ). Images were deconvoluted using OpenLab software (Improvision, Coventry, U.K.) and processed in (Adobe Photoshop Adobe Systems, Mountain View, CA).

Flow cytometry

Expression of cell surface Ags was measured by direct and indirect immunofluorescence flow cytometric analysis as previously described (50) using a FACS Calibur (BD Biosciences, Mountain View, CA) flow cytometer and CellQuest software. To compare the level of internal to total MHC class I, cells were first incubated at 4°C in the presence or absence of G46-2.6, a purified unlabeled anti-HLA-A, B, C Ab (BD PharMingen). Cells were then fixed in 3.7% formaldehyde in serum-free IMDM and permeabilized in a saponin solution (0.05% saponin in PBS supplemented with 5% bovine calf serum, 5 mM glucose, 0.05% sodium azide, and 10 mM HEPES). Cells were then labeled with fluorescein-conjugated G46-2.6 and analyzed by flow cytometry.

Macropinocytosis

Analysis of macropinocytosis by FITC-dextran uptake was performed as described elsewhere (8, 32) using fluorescein-conjugated M, 40,000 dextran (Molecular Probes). Dextran uptake was then quantitated by flow cytometry.

Radiolabeling

Cells were starved for 45 min at 37°C in DMEM (Life Technologies, Grand Island, NY) lacking methionine and cysteine and supplemented with 3% dialyzed FBS (Life Technologies). Cells were then incubated with [35S]methionine/cysteine (0.125 mCi/106 cells; ICN Pharmaceuticals, Costa Mesa, CA) for 15 min at 37°C. After the pulse period, cells were diluted in a 10-fold molar excess of unlabeled methionine and cysteine and cultured at 37°C for the specified chase period. PMA (10 ng/ml) and ionomycin (100 ng/ml) were included in culture conditions for all stimulated samples.

Immunoprecipitation

Following radiolabeling, cell pellets were extracted in 0.15 M NaCl/0.01 M TBS (pH 7.4) containing 1% Triton X-100 (Sigma-Aldrich) with protease inhibitors (5 mM iodoacetamide, 500 μM PMSF, and 75 μg/ml N-tosyl-l-lysine chloromethyl ketone; Sigma-Aldrich). Following pre-clearing with normal rabbit serum and protein G-Sepharose, postnuclear supernatants were subjected to primary immunoprecipitation with the designated primary Ab and protein G-Sepharose. Bound proteins were eluted in Laemmli SDS sample buffer and examined by 12% SDS-PAGE. Band intensities were quantified by image analysis using a GS-525 Molecular Image System and Molecular Analyst software (Bio-Rad, Hercules, CA).

Endoglycosidase H (endo H) digestion was performed as described previously (51). When indicated, cell surface proteins were biotinylated with sulfo-NHS-biotin (Pierce, Rockford, IL) as described elsewhere (52) before lysis. MHC class I stability and rate of cell surface arrival were examined using samples derived from the same labeled cell extract subdivided into two equal samples. Thus, the total precipitated MHC class I shown in Fig. 5A serves as a control for the total amount of MHC class I present for the samples in Fig. 6A. All graphs quantitating the band intensities are the averages of three independent experiments.

MHC class I thermostability

To analyze newly synthesized proteins, cell proteins were extracted as described above following a 20-min radiolabel and 100-min chase. To analyze the steady-state population of MHC class I surface complexes, cell surface proteins were biotinylated with sulfo-NHS-biotin (Pierce, Rockford, IL) as described elsewhere (52) before lysis. MHC class I stability and rate of cell surface arrival were examined using samples derived from the same labeled cell extract subdivided into two equal samples. Thus, the total precipitated MHC class I shown in Fig. 5A serves as a control for the total amount of MHC class I present for the samples in Fig. 6A. All graphs quantitating the band intensities are the averages of three independent experiments.

Generation of KG1.Kb cells

KG1.Kb cells were generated by retroviral transduction with H2-Kb cDNA (kindly provided by Dr. R. Flavell, Yale University). The 1.6-kb EcoRI excisable DNA fragment containing the Kb coding sequence was subcloned into the LZRSpBMN-Z1 neo retroviral vector (kindly provided by

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Dr. A. Bothwell, Yale University). Retroviral infection and neomycin selection were performed as previously described (53). Stable cells were screened for Kb expression by flow cytometry.

**Cross-presentation studies**

KG1.Kb and C1R.Kb cells (54) were incubated for various times in 1 mg/ml OVA in IMDM supplemented with 1% FCS. To prepare PMA/ionomycin-stimulated cells, OVA was present 24 h before and throughout the stimulation unless otherwise specified. For peptide controls, cells were incubated with 100 μg/ml of either OVA257-264 (SIINFEKL) or the H-2Kb-binding VSV NC52-69 (RGYVYQGL) for 3 h at 37°C. Following extensive washing, cells were stained with Cy5-conjugated 25-D1.16 mAb and analyzed by flow cytometry. Cross-presentation was also detected using the T cell hybridoma B3Z, which produces IL-2 when presented with Kb-SIINFEKL complexes (55). KG1.Kb and C1R.Kb cells were prepared as described above with the indicated concentrations of either soluble OVA or the designated peptides before overnight coculture with equivalent numbers of B3Z T cells. The resulting IL-2 levels in these cultures were assayed in triplicate using an OptEIA Mouse IL-2 ELISA kit (BD Pharmingen) according to the manufacturer’s protocol. IL-2 secretion is shown as the average of three independent experiments.

**Results**

**MHC class I localization in primary DCs**

We generated PBMC-derived DCs and examined them for MHC class I distribution at distinct maturational stages. By immunofluorescence microscopy, immature DCs expressed low surface levels of MHC class I with an internal bolus that partially colocalized with GM130 (Fig. 1A, top left panel), γ-adaptin (Fig. 1A, top middle panel), and Grasp22 (data not shown) markers of the Golgi apparatus. This internal store of MHC class I did not colocalize with tapasin, a component of the MHC class I-loading complex found within the endoplasmic reticulum (ER; Fig. 1A, top right panel), ER-resident calnexin, trans-Golgi network-localized TGN38, or CD63, a lysosomal tetraspanin (data not shown). A contaminating macrophage demonstrated MHC class I-specific staining of the cell surface and ER (Fig. 1A, top right panel), typical of non-DC leukocytes and other nonhemopoietic cells. Following the induction of DC maturation by either LPS (derived from *Escherichia coli* 055:B5; Fig. 1A, lower panels) or TNF-α...
C of MHC class I in the two cell stages were similar (Fig. 2) dramatically with maturation (Fig. 2B). Although MHC class I levels at the cell surface increased dramatically with maturation (Fig. 2B), the immuno-fluorescence intensity (MFI) of 984 to 499, suggesting that only 23% of the total MHC class I resided internally at this stage (Fig. 2B). In contrast, preincubation of mature DCs with unlabeled Ab reduced MHC class I staining 77%, from a MFI of 562 to 106, suggesting that only 23% of the total MHC class I remained internal following maturation (Fig. 2B).

To determine the percentage of internal MHC class I at different stages in DC maturation, we preincubated DCs with unlabeled anti-MHC class I IgG before permeabilization and staining with directly conjugated anti-class I Abs. This analysis allows a direct comparison of the total and internal populations of MHC class I.

Preincubation with unlabeled Ab reduced MHC class I staining of immature DCs by 44%, from a mean fluorescence intensity (MFI) of 984 to 499, suggesting that only 23% of the total MHC class I resided internally at this stage (Fig. 2A). In contrast, preincubation of mature DCs with unlabeled Ab reduced MHC class I staining 77%, from a MFI of 562 to 106, suggesting that only 23% of the total MHC class I remained internal following maturation (Fig. 2B). Although MHC class I levels at the cell surface increased dramatically with maturation (Fig. 2D), the total steady-state levels of MHC class I in the two cell stages were similar (Fig. 2C). These results suggest that the up-regulation of MHC class I at the cell surface upon maturation results at least partially from a redistribution of previously synthesized molecules from an internal site, and the immunofluorescence data in Fig. 1 indicate that the site may be the Golgi apparatus. Further analysis showed that class I expression and distribution are similarly regulated in the KG-1 DLC line (Figs. 1B and 2, E–H, described below).

**KG-1 DLCs model human DC maturation**

To follow-up on the above findings, we wanted to analyze MHC class I trafficking biochemically. Because it is difficult to isolate pure DC populations and primary human DCs are highly sensitive to manipulation (6), we explored the KG-1 DLC line as a model system. Our first goal was to extend the analysis of St. Louis et al. (32) by kinetic analysis of the maturational process in KG-1 cells.

Primary DC maturation is characterized by the physical extension of dendrites, the up-regulation of MHC and costimulatory molecules, the down-regulation of macropinocytosis, and altered MHC class II trafficking (1, 56). During a 72-h stimulation with PMA and ionomycin, KG-1 cells underwent similar morphological changes to those seen in primary DCs throughout maturation (Fig. 3A). The initial 12- to 24-h culture in PMA/ionomycin allowed KG-1 cells to mature into early DLCs, growing in size, but remaining in suspension. Since unstimulated KG-1 cells retained monocyte-like characteristics, this early period of DLC maturation may be similar to the commitment of monocytes to the DC lineage following incubation in GM-CSF and IL-4. An additional 48-h culture of KG-1 cells in PMA/ionomycin (72 h after initial stimulation) produced semiadherent late DLCs possessing dendritic processes (Fig. 3A, far right panel) and expressing DC maturation markers. Throughout the differentiation of KG-1, surface expression of the Ag presentation molecules MHC class II and CD1a, the costimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD58 (LFA-3), and the DC-specific markers CD83 and CD11c (Fig. 3, B–F and data not shown) gradually increased. The macrophage-specific marker CD14 was absent from all DLC stages (data not shown).

We also examined the ability of KG-1 cells to internalize fluid-phase Ags by quantitating fluorescent dextran uptake by flow cytometry (Fig. 3G). Unstimulated KG-1 cells exhibited potent endocytosis; the MFI of cells incubated with 40 kDa of FITC-dextran at 37°C was 20-fold over background levels observed at 4°C. This uptake was reduced in early DLCs (9-fold over 4°C controls) and decreased to near background levels in late DLCs (2-fold over 4°C).

**FIGURE 2.** Immature DCs and early DLCs contain a significant internal store of MHC class I molecules. A–D, Monocyte-derived DCs; E and F, KG-1 DLCs. Immature (A) and mature (B) DCs and early (E) and late (F) DLCs were incubated in either the absence (small dashes for immature DCs and early DLCs, long dashes for mature DCs and late DLCs) or presence (solid line) of unlabeled purified anti-HLA-ABC Ab and then were fixed and permeabilized. Cells were then directly stained for intracellular, unblocked MHC class I with a fluorescein-conjugated anti-HLA ABC Ab and were analyzed by flow cytometry. The reduction in the MHC class I signal following preincubation with unlabeled Ab reflects the proportion of MHC class I molecules on the cell surface. C and D, MHC class I staining of permeabilized (C, total) and unpermeabilized (D, surface) immature DCs (small dashes) and mature DCs (long dashes). E and F, MHC class I staining of permeabilized (G, total) and unpermeabilized (H, surface) unstimulated KG-1 cells (solid line), early KG-1 DLCs (small dashes), and late KG-1 DLCs (long dashes).
controls). This internalization was not saturable, indicating independence from membrane binding (5).

KG-1 DLCs also altered MHC class II localization in a manner similar to primary DCs (6). In unstimulated KG-1 cells and early DLCs, MHC class II was found in cytoplasmic vesicles partially colocalizing with CD63-positive lysosomes (Fig. 3A, far and near left panels) and the MHC class II chaperone HLA-DM (our unpublished data). During further maturation, however, MHC class II was transiently observed in peripheral vesicles lacking CD63 (Fig. 3A, near right panel). Although some of the phenotypic changes discussed above were observable as early as 30 min after stimulation, this altered MHC class II localization did not occur until the end of the early maturation period at ~24 h after stimulation, a maturation phase we have defined as intermediate. In late DLCs, surface and vesicular staining replaced this peripheral MHC class II staining (Fig. 3A, far right panel). Although some vesicular staining remained, class II surface levels increased dramatically, creating a staining pattern unlike that seen in the earlier stages. The remaining vesicular staining likely reflects the normal trafficking of newly synthesized MHC class II. These data indicate that KG-1 DLCs regulate the compartmentalization of MHC class II trafficking during maturation in a manner similar to primary DCs.

KG-1 cells cross-present exogenous Ags

Normal primary DCs cross-present exogenous Ags to CD8-positive T cells in the context of MHC class I. To further the comparison between KG-1 and primary DCs, we wanted to determine whether KG-1 cells could also perform this function. We first created KG-1 cells (KG-1.Kb) expressing the mouse MHC class I molecule H-2 Kb. The 25-D.1 mAb and the B3Z T cell hybridoma were used to detect the OVA-derived SIINFEKL (OVA257-264) peptide complexed with Kb. Following incubation with soluble OVA, unstimulated KG-1.Kb cells displayed low levels of SIINFEKL-Kb complexes, detectable by both 25-D.1 staining (Fig. 4A) and B3Z IL-2 secretion (Fig. 4H). After stimulation, the magnitude of cross-presentation increased (Fig. 4, B and H), despite similar surface Kb levels (Fig. 4E). Late KG1.Kb DLCs induced B3Z-mediated IL-2 secretion at protein concentrations three orders of magnitude lower than unstimulated cells (Fig. 4H).

C1R.Kb, a control B cell line exhibiting 5-fold higher levels of Kb than KG1.Kb (Fig. 4E), displayed high levels of SIINFEKL-Kb complexes when incubated with OVA257-264 peptide (Fig. 4G). No SIINFEKL-Kb complexes, however, could be detected upon incubation with whole protein, even after stimulation with PMA and ionomycin (Fig. 4, D and H).
FIGURE 4. KG-1 cross-presents exogenous Ags in the context of MHC class I. Unstimulated KG1.Kb cells (A), late KG1.Kb DLCs (B), late Kb-negative KG1 control DLCs (C), and PMA/ionomycin-treated C1R. Kb cells (D) were incubated with OVA throughout the stimulation period and stained with the 25-D1.16 mAb conjugated with Cy5. This mAb is specific for Kb molecules bound to the OVA 257–264 (SIINFEKL) peptide. Only late DLCs efficiently generate the epitope. E, The expression level of Kb present on stimulated KG1.Kb cells (solid line, MFI = 15) does not increase substantially over that observed on unstimulated cells (dashed line, MFI = 12). C1R.Kb cells (small dashes, MFI = 36) express a 5-fold higher level of Kb than KG1.Kb cells. In all panels, dotted lines indicate staining with isotype mAb controls. F, Preincubation with OVA for 24 h followed by its removal during PMA/ionomycin treatment (small dashes, MFI = 28) generates a higher number of Kb-SIINFEKL complexes than when Ag is present throughout the 72-h stimulation only (dashed line, MFI = 13). Maximal expression is observed when OVA is present both before and during stimulation (solid line, MFI = 56). G and H, IL-2 production by the B3Z hybridoma, specific for Kb-SIINFEKL complexes, following coculture with KG1.Kb cells preincubated with either OVA, SIINFEKL, or the control peptide VSV NC32–49. G, Incubation of both CIR.Kb (○) and KG1. Kb (●) cells with the SIINFEKL peptide induces significant IL-2 secretion, whereas incubation of KG1.Kb with the control VSV NC32–49 peptide (□) does not. H, Following incubation with OVA, late (△), early (□), and unstimulated (●) KG1.Kb cells all induce dose-dependent IL-2 secretion whereas PMA/ionomycin-treated C1R.Kb cells (●) do not.

Flow cytometry provides a semiquantitative readout of the number of cell surface SIINFEKL-Kb complexes, allowing a comparison of cross-presentation efficiency under different conditions of Ag administration. KG-1.Kb cells were subjected to a 24-h preincubation followed by a 72-h maturation period, each in either the presence or absence of OVA. 25-D1.1 staining in the absence of Ag was minimal (Fig. 4F, dotted line). When given Ag during both the preincubation and stimulation phases, late DLCs exhibited intense 25-D1.1 staining (Fig. 4F, solid line). Late DLCs also displayed high 25-D1.1 reactivity when Ag was provided only during the 24-h preincubation (Fig. 4F, small dashes). Administration of Ag during the 72-h stimulation period alone, however, reduced the number of SIINFEKL-Kb complexes present from that seen when Ag was administered during the 24-h preincubation alone (Fig. 4F, long dashes). Thus, late KG-1 DLCs present Ags acquired early in maturation better than those acquired later, mimicking DC regulation of cross-presentation throughout maturation (57, 58).

MHC class I expression during KG-1 maturation

Having further established the credentials of KG-1 as a model for human DCs, we investigated the cellular distribution of class I molecules in KG-1 cells during maturation. By immunofluorescence microscopy (Fig. 1B), KG-1 DLCs exhibited alterations in MHC class I localization throughout maturation similar to those in primary DCs. In early DLCs, MHC class I colocalized with the Golgi markers GM130, γ-adaptin, and Grasp22 (Fig. 1B, top panels). This pattern persisted until differentiation into late DLCs, when the majority of MHC class I resided on the cell surface (Fig. 1B, bottom panels). This distribution pattern was specific for MHC class I, as neither HLA-DR, HLA-DM, tapasin, TAP1, nor the costimulatory molecule CD58 exhibited colocalization with Golgi markers (data not shown). A proportion of the Kb molecules in early KG-1.Kb DLCs were, like the endogenous HLA class I molecules, detectable in the Golgi apparatus (Fig. 1 and data not shown).

We also observed reductions in the quantity of internal MHC class I throughout maturation (Fig. 2, E and F). Although the total steady-state levels of MHC class I remained similar throughout maturation, the MHC class I detectable at the cell surface increased on late DLCs from a MFI of 23 to 89 (Fig. 2G). Preincubcation of early DLCs with unlabeled anti-MHC class I IgG reduced the total MHC class I-specific staining from a MFI of 67 to 51, or 27% (Fig. 2E), in contrast to a 42% reduction, from a MFI of 86 to 42, in late DLCs (Fig. 2F). These data indicate that ~72% and 58% of the total MHC class I remained internal at these respective cell stages. Although the magnitude of the change in distribution is not as great, the overall pattern correlates well with that seen for primary DCs. Surface and total class I expression levels in unstimulated KG-1 cells were similar to those in early DLCs, arguing that the distribution of class I was unchanged between those stages of differentiation (Fig. 2, G and H).

MHC class I stability and trafficking in KG-1 cells

Having shown that MHC class I was regulated in KG-1 DLCs similarly to primary DCs, we examined the kinetics of transport
and degradation of newly synthesized MHC class I-β2m complexes in these cells by pulse-chase analysis (Fig. 5A). In unstimulated KG-1 cells, MHC class I was rapidly degraded, with a half-life of <2 h. MHC class I was more stable in early DLCs; a higher percentage of labeled MHC class I molecules escaped degradation within the first 8 h (Fig. 5B). The proportion remaining 12 h after synthesis, however, did not differ from unstimulated cells; both cell types degraded >90% of the total MHC class I molecules by this time. In late DLCs, the half-life increased further to >5 h (Fig. 5B). Degradation of these complexes was not complete; after 8 h, the amount of MHC class I-β2m complexes plateaued, with ~25% remaining stable 24 h after synthesis (Fig. 5B).

MHC class I stability at late time points in DC maturation likely results from lower levels of endocytosis (Fig. 3F), leading to reduced MHC class I internalization and degradation. Because early DLCs remain capable of endocytosis, increases in stability at this stage could result from the internal retention of complexes, which delays trafficking of MHC class I molecules to the cell surface and protects them against internalization and degradation. To determine whether ER retention of MHC class I was responsible, we subjected the pulse-chase samples obtained above (Fig. 5, A and C) to digestion with the enzyme endo H, which cleaves N-linked oligosaccharides before modification in the medial Golgi (59). In all KG-1 stages, MHC class I molecules acquired resistance to endo H cleavage at similar rates (Fig. 5, A and C). More than 80% of all molecules became resistant within 2 h (Fig. 5C), indicating rapid transit out of the ER in all DLC stages and demonstrating that the low surface expression on early DLCs is not due to ER retention.

By combining metabolic labeling with surface biotinylation, we sought to compare the ER to plasma membrane transport rates for MHC class I in the three stages of KG-1 differentiation. Following a short metabolic label and variable chase times, cell surface molecules were biotinylated. Class I molecules were immunoprecipitated and biotinylated H chains were isolated on streptavidin beads and separated by SDS-PAGE (Fig. 6A). In unstimulated KG-1 cells, MHC class I transport to the plasma membrane was rapid, with surface levels peaking within the first hour. These complexes, however, rapidly disappeared, with few remaining after 4 h. In early DLCs, MHC class I appearance at the cell surface was delayed, peaking between 4 and 6 h, a time when more than half of the molecules had already been degraded (Fig. 5, A and B). This suggests that, although a portion of MHC class I may traffic rapidly to the cell surface in a manner similar to unstimulated cells, a

FIGURE 5. The stability of MHC class I molecules increases during KG-1 DLC maturation but their rate of transport out of the ER is unaltered. A, Pulse-chase analysis of MHC class I expression. KG-1 cells in different maturation states were pulse labeled with [35S]methionine for 20 min and chased for the indicated times. Following lysis in 1% Triton X-100, MHC class I molecules were immunoprecipitated using the conformation-dependent mAb w6/32. Samples were then incubated for 18 h in the absence (−) or presence (+) of endo H and subjected to 12% SDS-PAGE. The upper band is the glycosylated protein, while the lower band is the deglycosylated protein. B, Quantitation of MHC class I levels throughout the chase. Total levels of radiolabeled MHC class I H chain were measured by image analysis. C, Quantitation of MHC class I trafficking out of the ER, measured by the acquisition of endo H resistance calculated as the percentage of the total MHC class I H chain (resistant plus sensitive). Graphs shown in B and C represent the averages of three independent experiments. Unstim, Unstimulated.

FIGURE 6. Low surface expression of MHC class I on early KG-1 DLCs results from a delay in transport to the cell surface. KG-1 cells at different maturation states were pulse labeled with [35S]methionine for 20 min and chased for the indicated times. Samples were incubated at 4°C for 30 min with sulfo-NHS-SS-biotin to biotinylate cell surface proteins. After extracting the cells in 1% Triton X-100, total MHC class I was first isolated by immunoprecipitation with w6/32. Biotinylated class I H chains were released by SDS, isolated by streptavidin-agarose, and separated by SDS-PAGE (A). Appearance of radiolabeled MHC class I on the cell surface was quantitated by image analysis (B). Unstim, Unstimulated.
substantial portion does not reach the cell surface until much later. After reaching the surface, however, much of the class I was quickly degraded. In late DLCs, surface MHC class I levels peaked after reaching the cell surface. This transient delay in trafficking may preclude internalization and degradation, accounting for the increased steady-state levels of MHC class I observed in early DLCs.

DLC maturational stages possess altered peptide repertoires

Human MHC class I-β2m dimers are stable when associated with a specific peptide and bind the conformation-specific Ab w6/32. However, empty MHC class I-β2m dimers dissociate and the released free H chains do not bind w6/32 (60). We capitalized on this to examine the efficiency of peptide loading at different stages of KG-1 differentiation. Following pulse-chase analysis, we subjected cell lysates to either a 16- or 1-h pre-clear. Dissociation of unstable, empty MHC class I-β2m dimers during the extended pre-clear causes a reduction in the w6/32-precipitable MHC class I signal compared with the 1-h pre-clear. Although the intensity of the MHC class I signal decreased following the long pre-clear, all KG-1 stages exhibited similar decreases by image analysis, indicating that they possessed similar proportions of loaded MHC class I molecules (Fig. 7A).

Despite similar loading efficiencies, the quality of peptides present in the MHC class I binding groove may differ between maturation stages. The affinity of the peptide bound to the MHC class I-β2m dimer affects the complex to thermal denaturation (43, 61, 62). MHC class I-peptide complexes possessing lower affinity peptides dissociate at lower temperatures than those bound to high-affinity peptides. We examined MHC class I thermostability after a short radiolabel and 2-h chase. At this time, the majority of labeled complexes have left the ER in all stages of maturation (Fig. 5C), although in early DLCs the majority have yet to reach the cell surface (Fig. 6) and presumably reside in the Golgi (Fig. 1). We quantitated the H chain coprecipitating with β2m after exposure to a range of temperatures (Fig. 7B and C). Although MHC class I complexes assembled in both the unstimulated cells and late DLCs display similar thermostability curves, those formed in early DLCs were more thermostable, indicating a higher affinity repertoire of peptides (Fig. 7B). The patterns of thermal stability changed when we examined surface MHC class I molecules following surface biotinylation. Immunoblotting with streptavidin after an anti-β2m immunoprecipitation revealed that, at steady state, the surface complexes present on early and late DLCs exhibit similar stabilities (Fig. 7C). Therefore, complexes newly formed in late DLCs are relatively unstable, while those at the cell surface are far more stable. This suggests that nascent MHC class I complexes in late DLCs are not loaded with high-affinity peptides but complexes containing high-affinity peptides are enriched at the cell surface.

Discussion

Examination of both primary monocyte-derived DCs and KG-1 DLCs demonstrates that maturation affects the trafficking of MHC class I molecules, with Golgi localization observed in the immature state. The KG-1 cell line can be induced to differentiate in vitro, exhibiting similar morphological and phenotypic changes to those seen during normal DC differentiation (Fig. 3 and Ref. 32). We have also demonstrated in KG-1 cells similar regulation of MHC class II trafficking necessary for proper control of CD4+ T cell-mediated immunity (Fig. 3A). In addition, KG-1 cells can cross-present exogenous Ags to CD8 T cells (Fig. 4). The efficiency of cross-presentation decreases throughout KG-1 maturation, probably due to reduction in Ag acquisition because of down-regulation of endocytosis (Fig. 3G). Although Ag uptake in the absence of a maturation signal generates few specific MHC class
I complexes, the same degree of uptake results in their efficient generation when combined with a maturation signal. In contrast to nonhemopoietic MHC class I-bearing cells, in which Ags are presented, displayed, and degraded, primary monocyte-derived DCs and KG-1 DLCs retain Ags acquired early in maturation for stable presentation at later stages (63). It seems unlikely that the increased presentation efficiency of late stage cells results from the up-regulation of surface MHC class I alone, suggesting that altered regulation of MHC class I processing throughout maturation facilitates these changes. All of these data argue that the KG-1 cell line is an excellent model for molecular analysis of DC functions.

The changes in MHC class I trafficking in DCs and KG-1 DLCs may reflect a system optimized to present the subset of Ags internalized during inflammation. In precursors and early stage cells, the fluid-phase uptake of extracellular components requires the rapid turnover of the plasma membrane, internalizing MHC class I molecules for subsequent degradation in lysosomes. This regulation of the MHC class I-peptide half-life would prevent the surface accumulation of self-peptide-containing complexes generated before an inflammatory stimulus. In early cells, however, sequestration of MHC class I molecules in the Golgi may be responsible for both increased in vivo stability (Fig. 5B) and productive peptide loading reflected in increased thermostability (Fig. 7B). MHC class I complexes prevented from reaching the surface would escape internalization and degradation. Also, removal of loaded complexes from the ER would allow them to escape the degradative countdown of mannose trimming, which serves as a signal for retrotranslocation and cytoplasmonic destruction by the proteasome (64, 65). Transporting these complexes to the Golgi would also separate them from the MHC class I-loading machinery, preventing further peptide exchange. The complexes presented by late cells would therefore display peptides derived from Ags acquired earlier in differentiation. Although the mechanism whereby these Ags are retained remains unclear, the delay of complexes in the Golgi could provide temporary internal storage for epitopes bound to MHC class I.

A similar pattern of transient MHC class I accumulation in the Golgi has been observed in cells expressing an antisense construct inhibiting expression of CD99 (66). These cells exhibit a similar staining pattern to that seen in early DCs. Signaling through CD99 appeared to be necessary to induce the relocation of MHC class I complexes to the cell surface, since anti-CD99 Abs could induce class I retention in the Golgi. Interestingly, although unstimulated KG-1 cells express significant levels of CD99, we have observed that early DLCs down-regulate CD99 shortly after stimulation (data not shown). CD99 ligation is involved in the diapedesis of leukocytes across endothelial cell junctions (67) and may serve as a signal triggering the relocation of MHC class I complexes to the cell surface during the migration of maturing DCs to secondary lymphoid organs. How CD99 controls MHC class I localization remains unknown, but this pathway may function in early DCs to retain MHC class I complexes in the Golgi.

The increase in total MHC class I stability of late DLCs (Fig. 5B) correlates with the down-regulation of endocytosis (Fig. 3F), which may allow MHC class I-peptide complexes relocalized from the Golgi to persist at the cell surface for longer periods of time. In addition, the up-regulation of TAP and other molecules associated with MHC class I loading in later DC stages (data not shown and Ref. 4) may partially account for the prolonged half-life of class I molecules observed upon transition from resting KG-1 cells to early DLCs: increased loading efficiency results in reduced degradation in the ER by quality control mechanisms (68).

MHC class I molecules have been localized to lysosomes in DCs (26, 69), and it has been suggested that this may play a role in cross-priming. We examined phagolysosomes generated in KG-1 DLCs by the internalization of latex beads and confirmed that these structures contained MHC class I derived from the cell surface (data not shown). In addition, continuous culture of DLCs following binding of anti-MHC class I Abs demonstrated that surface MHC class I-peptide complexes were internalized into GILT- and transferrin receptor-positive vesicular structures (data not shown). These complexes were rapidly degraded after internalization. Comparison of the internalized class I with total MHC class I staining, however, suggested that in early DLCs, the proportion of MHC class I found within endocytic compartments is significantly less than that observed within the Golgi. These findings support the hypothesis that the internal retention of loaded complexes within the Golgi is a major mechanism controlling MHC class I presentation in DCs.

Although MHC class I complexes synthesized at all stages of differentiation are occupied by peptides, the quality of peptide loading varies throughout maturation. Complexes formed early in maturation are more thermostable, suggesting that higher affinity peptides are available. The IFN-γ-inducible proteasomal subunits, LMP-2, LMP-7, and MECL-1, are up-regulated in early DCs (4, 70), perhaps accounting for these qualitative differences. Although the complexes generated in late cells are less stable than those formed early, the complexes residing on the surface of late cells have a similar stability to those seen in the early cells. This selective enrichment of stable complexes at the cell surface may ensure a productive interaction with cognate T cells. The enrichment may result from reduced degradation of stable complexes and/or their retention at the cell surface, and possibly explaining the increased cross-presentation of Ags acquired early in maturation (Fig. 4B). Such changes in peptide availability and MHC class I stability may also influence the quality of the immune response, allowing sustained interactions with T cells (63).

Our data suggest that maturing DCs stably load nascent MHC class I-β2m dimers while protecting successfully loaded complexes from both degradation and peptide exchange by sequestering them in the Golgi. The presentation of these Ags by MHC class I also occurs in the context of costimulatory molecule up-regulation (Fig. 3). Only fully mature DCs reach secondary lymphoid organs having both accumulated stable MHC class I complexes loaded with antigenic peptides and up-regulated the costimulatory factors necessary to generate a T cell response. Thus, the DC-specific mechanisms regulating MHC class I-mediated Ag presentation may be coordinated to maximize the capacity of the immune system to respond to Ags acquired during a finite period surrounding an inflammatory stimulus. The internal sequestration and degradation of MHC class I in immature DCs may serve to prevent premature T cell stimulation. Self-Ags, encountered in the absence of inflammation, will be presented by DCs lacking costimulatory factors, possibly leading to peripheral tolerance (71). In addition, as the generation of long-lived complexes may be critical to the induction of immunological memory, the regulation of MHC class I complex half-life may ensure that only those epitopes generated at sites of inflammation (in the presence of a maturation signal) will persist in secondary lymphoid organs. Thus, the regulation of compartmentalization and loading of Ag presentation molecules facilitates the complex role of DCs in immune surveillance.

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References


