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A Novel Trafficking Signal within the HLA-C Cytoplasmic Tail Allows Regulated Expression upon Differentiation of Macrophages

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MHC class I molecules (MHC-I) present peptides to CTLs. In addition, HLA-C allotypes are recognized by killer cell Ig-like receptors (KIR) found on NK cells and effector CTLs. Compared with other classical MHC-I allotypes, HLA-C has low cell surface expression and an altered intracellular trafficking pattern. We present evidence that this results from effects of both the extracellular domain and the cytoplasmic tail. Notably, we demonstrate that the cytoplasmic tail contains a dihydrophobic (LI) internalization and lysosomal targeting signal that is partially attenuated by an aspartic acid residue (DXXLI). In addition, we provide evidence that this signal is specifically inhibited by hypophosphorylation of the adjacent serine residue upon macrophage differentiation and that this allows high HLA-C expression in this cell type. We propose that tightly regulated HLA-C surface expression facilitates immune surveillance and allows HLA-C to serve a specialized role in macrophages. The Journal of Immunology, 2008, 180: 7804–7817.

Major histocompatibility complex class I (MHC-I) molecules are necessary for presentation of Ags to naive CD8+ CTLs through engagement with the TCR and CD8. There is evidence that professional APCs are required for this step, as they have unique costimulatory molecules needed for this process (1). APCs are thought to activate naive CD8+ T cells by internalizing exogenous Ags and presenting them in association with MHC-I in a process known as cross-presentation (2). Activated CTLs then mature into effector cells, which have the capacity to kill cells bearing foreign epitopes through the release of perforin and granzymes and via the activation of apoptotic pathways (3).

MHC-I can also be recognized by MHC-I-specific inhibitory receptors, such as killer cell Ig-like receptors (KIR) (4). These receptors are classically thought to be expressed on NK cells to facilitate the eradication of virally infected or tumorigenic cells that have down-modulated MHC-I. More recently, evidence has been accumulating that these receptors are also up-regulated with the acquisition of cytotoxic function in CD8+ T cells (5). In this case they may serve as an important negative feedback mechanism that aids in the prevention of autologous damage by raising the threshold for cell lysis (5).

There are three major subtypes of classical MHC-I molecules that serve these roles. HLA-A, B, and C all have the capacity to present viral Ags to CTLs. Additionally, almost all HLA-C molecules are recognized by KIRs. Perhaps because of the crucial role of HLA-C as an inhibitory molecule capable of sending a dominant-negative signal (6), HLA-C is normally expressed at low levels at the cell surface. HLA-C H chain mRNA is unstable (7), the HLA-C H chain protein is not stably expressed at the cell surface, and it does not associate efficiently with the MHC-I L chain (β2-microglobulin) (8–10). Additionally, HLA-C presents a more restricted repertoire of peptides causing it to be retained in the endoplasmic reticulum (ER) in complex with the TAP, which is responsible for transporting peptides into the ER for MHC-I loading. The retained HLA-C is then eventually degraded in the ER (8). The addition of HLA-C-specific peptides has been shown to release HLA-C from TAP in vitro (8) and to increase the cell surface expression of HLA-C (11).

We examined the expression of HLA-Cw*0401 relative to HLA-A*0201 in a variety of cell types, including T cell lines, primary T cells, and monocytic cell lines and confirmed that HLA-Cw*0401 was poorly expressed on the cell surface relative to HLA-A*0201. To better understand the amino acid sequences governing HLA-C surface expression, we examined the intracellular trafficking of chimeric molecules that contained the HLA-A*0201 extracellular domain and the HLA-C cytoplasmic tail (A2/C) or the HLA-Cw*0401 extracellular domain and the HLA-A cytoplasmic tail (Cw4/A). Not surprisingly, the extracellular domain of HLA-C was responsible for promoting retention in the ER. Remarkably, however, the cytoplasmic tail also had an effect on cell surface expression by increasing internalization at the cell surface and targeting the molecules for degradation in acidic organelles. Mutagenesis studies revealed that aspartic acid at position 333, serine at position 335, and isoleucine at position 337 were key amino acids that affected the activity of this motif. Finally, we found that the complex regulation of HLA-C surface expression allowed the specific up-regulation of HLA-C upon differentiation.
of primary monocytes and monocyctic cell lines into macrophage-like cells. The specific induction of HLA-C expression with differentiation strongly suggests that there is a unique role for HLA-C in APCs. We propose that inhibitory signals sent via HLA-C play a role in down-modulating the normal CD8+ cellular immune response and/or that it functions to specifically limit the lysis of APCs that are cross-presenting Ag.

Materials and Methods

DNA constructs

MSCV 2.1 hemagglutinin (HA)-HLA-A*0201 was constructed as previously described (12). For MSCV 2.1 HA-HLA-Cw*0401 (11), the HLA-Cw*0401 open reading frame (Peter Parham, Stanford University) was amplified with the following primers, 5'-CAACTCTCCACGCGCCGA GATGCG-3' and 5'-CCGGCTGAGTTAGCTTACAAAGCGATGAGA GAGA-3'. The PCR product was digested with NaeI and XhoI and the 3' fragment was gel purified. This fragment was then ligated to the 5' leader sequence plus the HA tag from HA-HLA-A*0201 (isolated by digesting MSCV 2.1 HA-HLA-A*0201 with EcoRI and NotI) and MSCV 2.1 digested with EcoRI and XhoI. MSCV 2.1 HA-Cw*0401 was constructed by digesting MSCV 2.1 HA-HLA-Cw*0401 with EcoRI and XhoI, subcloning this fragment into the same sites in Litmus 29 to generate Litmus 29 HLA-Cw*0401. A three-way ligation was then performed with a EcoRI to SapI fragment from Litmus 29 HA-HLA-A*0201 that encodes the intracellular domain of HLA-Cw*0401, a DNA fragment encoding the HA tail of HA-HLA-A*0201 cyttoplasmic tail digested with SapI and XhoI (generated by PCR amplification of MSCV 2.1 HA-HLA-A*0201 with the following primers 5'-GTGATCAGGCTCAGGTTAGCTTACAA AGCGATGAGACATCAGAGCCCTGGGC-3' and 5'-CCGGCTGAGTAGCTTACAAAGCGATGAGA GAGA-3'). This fragment was then ligated to pcDNA3.1 (+EcoRI and XhoI). MSCV 2.1 A2/Cw4 was constructed by first PCR amplifying the Cw*0401 cytoplasmic tail using the following primers 5'-CACGCTGAGCTTACAAAGCGATGAGACATCAGAGCCCTGGGC and 5'-CCGGCTGAGTAGCTTACAAAGCGATGAGA GAGA-3'. The PCR product was then ligated into pcDNA3.1 (EcoRI and XhoI). This fragment was then digested with NaeI and SnaI, ligated into PCMV IRES GFP digested with the same enzymes. The C320Y point mutation was generated using a two-round PCR. The resulting PCR product was digested with EcoRI and XhoI and ligated into MSCV 2.1 digested with the same enzymes.

Cell lines

THP-1 and U937 macrophage cell lines were obtained from the American Type Culture Collection. THP-1 cells were cultured in RPMI 1640 supplemented with 2 mM t-glutamine, 10 mM HEPEs, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS, 0.05 mM 2-ME, and 2 mM penicillin, streptomycin, and glutamine. U937 and CEM cells were cultured with RPMI 1640 supplemented with 10% FBS, 10 mM HEPEs, and 2 mM penicillin, streptomycin, and glutamine (R10). Cell lines expressing various MHC-I molecules were generated using murine retroviral vectors as previously described (14, 15) except that they were pseudotyped with pCMV VSV-G (Dr. Nancy Hopkins, Massachusetts Institute of Technology). Cells (1 × 10^6) were spin infected with the retroviral supernatants by centrifuging at 2500 rpm in a table top centrifuge for 2 h with 8 μg/ml polybrene. The cells were then selected with neomycin (1 mg/ml).

PBMC isolation and electroporation

PBMCs were isolated from buffy coats provided by the Lansing Red Cross by Ficoll-Hypaque centrifugation. Following isolation they were stimulated with 10 μg/ml PHA (Sigma-Aldrich); 24 h after IL-2 was added at 50 U/ml and fresh IL-2 was added after 3 days. Five days after stimulation, 5 × 10^7 stimulated PBMCs were electroporated using the Amaxa Nucleofector system. Electroporations were performed according to the manufacturer's protocol except following electroporation, the cells were placed in 500 μl of medium in 1.5 ml Eppendorf tubes and incubated for 10 min at 37°C before being placed in a 12-well dish.

Macrophage differentiation

Buffyc coated by the New York Blood Center were purified by Ficoll-Hypaque centrifugation, and CD14+ mononuclear cells were isolated using the EasySep human CD14-positive selection kit (StemCell Technologies). Purity of the sorted CD14-positive cells was assessed by flow cytometry using FITC-conjugated mouse anti-human CD14 Ab (clone M5E2; BD Pharmingen). To assess MHC-I expression levels, freshly purified, undifferentiated cells were preincubated with 10% Fc block (Accu- rate Chemical & Scientific) in FACS buffer (10 mM HEPEs, 2% FBS, 1% human serum, 0.02% azide) for 20 min on ice and then stained with Abs directed against HLA-A2 (BB7.2), HLA-C (L31; gift of Patrizio Giacomin, Regina Elena Cancer Institute, Italy), BW4 (One Lambda), and BW6 (One Lambda), depending on the donors MHC-I phenotype. The cells were also stained with matched, isotype control Abs (protein A purified IgG2b for BB7.2, IgG1 ascites for L31, and IgM for anti-Bw4 and anti-Bw6). For staining with L31, a citrate-phosphate buffer (pH 3.0) was used to release β2-microglobulin and expose the epitope as described previously (16, 17). To induce maturation, the CD14+ cells were plated at 1 × 10^6/ml in R10 plus CM-CSF for 5 days. The cells were then harvested and stained again with anti-MHC-I Abs as described above.

For differentiation of monocyctic cell lines, one million THP-1 or U937 cells were treated with LPS (100 ng/ml for THP-1 and 10 ng/ml for U937) solubilized in DMSO in 1 ml of medium in a 24-well plate. Twenty four hours later, an additional 1 ml of medium was added containing PMA (200 ng/ml for THP-1 and 10 ng/ml for U937) and LPS. After 72 h at 37°C, cells were harvested by treatment with cell dissociation solution (Sigma-Aldrich) for 20 min at 37°C.

Western blot analysis

Cells were lysed in PBS, 0.3% CHAPS, 0.1% SDS (pH 8), and 1 mM PMSF. They were then normalized for total protein and separated by SDSPAGE. Western blot analysis was performed with the following Abs: HA (HA.11, 1:5,000; Covance Research Products), and anti-mouse-HRP (1:2,500; Zymed Laboratories).

Immunofluorescence microscopy

CEM T cells were prepared for immunofluorescence microscopy as previously described (12) except that they were permeabilized with 0.1% digitonin (Wako Chemicals) diluted in Dulbecco’s PBS with calcium and magnesium and blocked with equal parts wash buffer and Fc receptor blocker (Accurate Chemical & Scientific). To identify cell surface staining (Fig. 1G), ConA conjugated to AlexaFluor 488 (Molecular Probes) was diluted to 40 μg/ml and incubated with cells for 5 min on ice. To identify acidic compartments (Fig. 4C), CEM cells were pretreated with 100 nm bafloycin A or DMSO for 4 h at 37°C. Following treatment, CEM cells were adhered to glass slides, fixed, permeabilized, and stained for indirect
FIGURE 1. The extracellular and cytoplasmic tail domains of HLA-C influence surface expression. A, The cytoplasmic tail sequences of HLA-A, HLA-B, and HLA-C molecules. Amino acid differences unique to HLA-C are boxed. B, Schematic diagram of HA-tagged MHC-I molecules. Open boxes represent HLA-A*0201 sequences, black boxes represent HLA-Cw*0401 sequences, the gray box represents the HA tag, and L indicates the position of the leader sequence. C, Expression of HA-tagged chimeric MHC-I molecules. CEM T cells (control) or CEM T cells expressing the indicated MHC-I were pulse-labeled with $^{[35S]}$methionine and cysteine for 15 min. Lysates from these samples were immunoprecipitated with an Ab against HA, and separated by SDS-PAGE. D, Quantitation of pulse-labeling. A phosphor imager and ImageQuant software were used to quantify samples. Values were corrected for differences in methionine and cysteine content. The counts from the HLA-A*0201 sample were set to 100% and the values from other samples were expressed relative to it. The results are displayed as the means ± SD from three experiments. E, Cell surface expression of HA-A2, HA-Cw4, HA-A2/C, and HA-Cw4/A. CEM T cell lines were generated expressing the indicated HLA-C molecules. The cells were stained with an Ab directed against the HA tag and analyzed by flow cytometry. The filled gray curve represents the expression of the indicated molecules while the filled black curve represents control cells stained with the HA Ab. The MFI ± SD from six experiments is shown in the upper right hand corner. F, Cell surface expression of untagged HLA-A2 and A2/C in CEM T cells. The CEM T cell line expressing un-tagged HLA-A2 has been previously described (12, 39, 40). Untagged MSCV A2/C was generated from pcDNA3.1 A2/Cw4 (see Materials and Methods) and was expressed in CEM cell lines as described in Materials and Methods. The cell surface expression of the indicated untagged MHC-I was assessed by flow cytometry using the HLA-A2-specific Ab, BB7.2 as described in Materials and Methods. The lightly shaded curve represents parental CEM cell lines. The darkly shaded curve represents CEM cells expressing untagged HLA-A2 and the unshaded curve represents CEM cell expressing untagged A2/C. G, Subcellular distribution of MHC-I molecules. The indicated CEM T cell lines were incubated with ConA to label the cell surface (green). They were then fixed, permeabilized, and stained with HA to examine MHC-I localization (red). Merge panels represent the product of these two overlapping images in which regions of overlap are highlighted in yellow. All images were collected on a Zeiss LSM 510 confocal microscope and single z-sections are displayed. H, Quantification of ConA and HA colocalization. Quantification is shown for four cells expressing HA-A2 and 4 cells expressing HA-A2/C. I, Cell surface expression of HA-A2 and HA-Cw4 primary T cells. PBMCs were electroporated with a bicistronic GFP-expressing construct as described in Materials and Methods. Twenty four hours post electroporation PBMCs were stained using an Ab directed against HA and analyzed by flow cytometry. Cells with equal GFP expression were selected and their GFP expression is shown in the top panels. The corresponding HA stain is shown in the panels below. In the bottom panels, the filled curve is the background staining on mock-transfected cells and the black line represents HA staining for cells transfected with the indicated construct. As a control, stable CEM T cells expressing the same molecules were also stained in parallel and are shown in the panels on the right.
immunofluorescence as previously described (12). MHC-I was visualized using BB7.2 (1:100), and AlexaFluor 488 goat anti-mouse IgG2b (1:250; Invitrogen). LAMP-1 was visualized using a anti-LAMP-1 Ab (clone H4A3, 1:500; BD Pharmingen) and AlexaFluor 546 goat anti-mouse IgG1 (1:250; Invitrogen). For identification of the ER (Fig. 2C), HA-tagged molecules and KDEL were stained using mouse mAbs (HA.11, 1:50 and anti-KDEL, 1:200 (Stressgen)) followed by staining with appropriate secondary Abs (goat anti-mouse IgG1 AlexaFluor 488, and goat anti-mouse IgG2a AlexaFluor 546, 1:250; Molecular Probes). Images were collected using a Zeiss LSM 510 confocal microscope and processed with Adobe Photoshop software.

**Microscopy quantification**

Microscopy images were quantified independently by two individuals who assessed the relative amount of colocalization and assigned a colocalization score between 0 and 3, where 3 was the maximal possible colocalization. For each condition, the samples were averaged and the percentage of maximum colocalization was determined by dividing each score by the highest score achieved.

**Flow cytometry**

Stains were performed as previously described (18) using an anti-HA Ab (HA.11, 1:150) or an anti-HLA-A2 Ab (BB7.2) (19) and goat-anti-mouse-PE (1:250; Biosource or Invitrogen). FACS analysis of the THP-1 and U937 cells was the same except the cells were incubated with Fc receptor blocker (Accurate Chemical & Scientific) for 20 min at 4°C before the anti-HA Ab incubation.

**Transport, internalization, and metabolic labeling assays**

The transport and endocytosis assays were performed essentially as previously described (20) except that an Ab directed against the HA tag (HA.11) was used. For metabolic labeling of total protein, fifteen million CEM T cells were pulse-labeled for 15 min with [35S]methionine and cysteine. For inhibitor studies, one third of the sample was harvested after the pulse while the remaining cells were then chased for 12 h in either RPMI 1640 with DMSO or 100 nM bafilomycin A (Sigma-Aldrich). Lysates were generated in PBS, 0.3% CHAPS, 0.1% SDS (pH 8), 1 mM PMSF, and precleared overnight. They were immunoprecipitated for 2 h with an Ab against HA (HA.11) and washed three times in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). The immunoprecipitates were then eluted by boiling in 10% SDS, re-precipitated with an Ab against HA, and washed three times in RIPA buffer. The final immunoprecipitates were then separated by SDS-PAGE.

For metabolic labeling of phosphorylated protein, five million CEM T cells were labeled for 4 h with 0.5 mCi/ml [32P]orthophosphate in phosphate-free medium (RPMI 1640; Specialty Media) supplemented with 10% diazylzed FBS (Invitrogen). The cells were lysed with Nonidet P-40 lysis buffer, immunoprecipitated at 4°C, and analyzed by SDS-PAGE and autoradiography.

**FIGURE 2.** The HLA-C tail does not affect transport from the ER into the Golgi apparatus. A and B, CEM T cells expressing the indicated MHC-I were pulse-labeled with [35S]methionine and cysteine for 15 min, chased for the indicated time period and harvested. Lysates were immunoprecipitated with an Ab against HA, treated with endo H, and separated by SDS-PAGE. C indicates control CEM cells that do not express the HA tag. B, A phosphor imager and ImageQuant software were used to quantify the gel shown in part A. The percentage of molecules that were resistant to endo H digestion over time was calculated as follows: \[ \frac{(\text{endo H resistant}) - (\text{endo H sensitive})}{\text{endo H sensitive}} \times 100 \]. C, The HLA-C extracellular domain promotes colocalization with markers of the ER. CEM T cells expressing the indicated MHC-I were fixed, permeabilized, and stained for HA to examine MHC-I localization (green), or KDEL (red) to mark the ER. The merge panels depict regions of overlap in yellow. All images were collected on a Zeiss LSM 510 confocal microscope and single z-sections are displayed. D, Quantification of KDEL and MHC-I colocalization. The relative amount of colocalization between KDEL and BB7.2 staining was determined as described in Materials and Methods. Quantification is shown for 6 cells expressing HA-A2, five cells expressing HA-A2/C, four cells expressing HA-Cw4, and five cells expressing HA-Cw4/A.
buffer (1% Nonidet P-40, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 1 mM PMPSF) and precleared overnight. They were immunoprecipitated with BB7.2 Ab for 2 h and washed three times in RIPA buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). The immunoprecipitates were then separated by SDS-PAGE and the dried gel was exposed to a phosphor imager screen and analyzed on a Typhoon Trio PhosphorImager (GE Healthcare). Where indicated, cells were treated with 100 nM bafilomycin A or DMSO solvent control for 18−24 h.

Recycling assays
A measurement of the rate of recycling of internalized molecules was performed as described previously (21), except that washes were performed at room temperature to avoid inhibiting recycling with cold temperature (22). Briefly, cells were incubated with 150 μg/ml cycloheximide (Sigma-Aldrich) for 2−3 h in RPMI 1640 plus 10% serum. Then cells were harvested and an aliquot was removed and placed on ice. The remainder of the samples was stripped of stainable HLA-A2 by washing in 50 mM glycine, 100 mM NaCl (pH 3.4), twice, at room temperature for 1 min. The stripped cells were then washed in PBS and incubated at 37°C, 5% CO2 in medium without serum for the indicated period of time, in the presence of 150 μg/ml cycloheximide. (Samples were incubated without serum to avoid substitution of bovine β2-microglobulin present in serum for the human β2-microglobulin removed with the stripping protocol.) Cells were then placed on ice and stained for HLA-A2 with BB7.2, using wash buffers that included BSA instead of serum.

Results
HLA-C allotypes are classical MHC-I molecules that play a dual role. They are able to activate CD8+ CTLs via their ability to present Ags. Additionally, HLA-C allotypes have the capacity to send inhibitory signals to CD8+ T cells bearing inhibitory receptors. Perhaps as a result of this unique role, HLA-C molecules are known to be expressed at much lower levels on the cell surface than other classical MHC-I molecules (HLA-A and HLA-B allootypes). However, is unclear what elements determine these differences in expression. The extracellular domains of MHC-I proteins are known to be highly polymorphic, whereas the cytoplasmic tail domains are generally highly conserved within allotypes (Fig. 1A). A comparison among the three types of classical MHC-I molecules revealed that there are four amino acids unique to the HLA-C cytoplasmic tail domain (Fig. 1A).

To determine which amino acid differences played a role in reducing HLA-C surface expression, we first developed a way to clearly compare expression of these molecules. This was accomplished by attaching an HA tag to the N terminus of HLA-A*0201 and HLA-Cw4*0401 (HA-A2 and HA-C4; Fig. 1B). The HA tag, which was inserted just after the leader cleavage site, allowed us to compare the expression of heterologous proteins using the same Ab so that differences in Ab affinity did not confound our results. In prior publications, we have demonstrated that the presence of this tag does not affect the maturation and expression of HLA-A2 (12). Additionally, we have demonstrated that this tag does not affect recognition by the conformationally sensitive anti-HLA-A2 Ab, BB7.2 (12, 19).

We also made chimeric molecules in which the cytoplasmic tail domains of HLA-A or HLA-C were fused to the transmembrane domain of HA-Cw4 and HA-A2 to create HA-Cw4/A and HA-A2/C, respectively (Fig. 1B). DNAs encoding each of these proteins were cloned into murine retroviral vectors, and viral supernatants were used to transduce CEM T cells at a low multiplicity of infection to limit the number of transductants with multiple integrated copies. Bulk cell lines were then grown in selective medium to obtain a uniform population. To ensure that our results were not influenced by arbitrary variations introduced by individual

![FIGURE 3. The HLA-C tail disrupts protein transport and accelerates internalization from the cell surface.](http://www.jimmunol.org/)

A. CEM T cells expressing the indicated MHC-I were pulse-labeled with [35S]methionine and cysteine. They were then chased for 1 or 4 h in bion. Lysates from these samples were immunoprecipitated with an Ab against HA and one of the immunoprecipitate was analyzed by SD±PAGE (total). The remaining two thirds was reciprocated with avidin-agarose and then analyzed by SD±PAGE (surface). NC indicates an immunoprecipitation of control CEM cells that do not express the HA tag. B. Quantitation of cell surface transport. A phosphor imager and ImageQuant software were used to quantify recovered protein. The percentage of MHC-I molecules that had reached the cell surface at the indicated time point was calculated as follows: [(surface MHC-I/(total MHC-I)×100). The mean from two experiments ± SD is shown. C, A2/C is internalized at an accelerated rate. CEM T cells stably expressing the indicated protein were incubated on ice with the anti-HLA-A2 Ab, BB7.2. The cells were shifted to 37°C for the indicated time and then stained with a secondary Ab to determine the percentage of HA-A2 (black square) or HA-A2/C (gray square) remaining on the cell surface over time. The mean ± SD for an experiment performed in quadruplicate is shown. D, Internalization of CD4 by the HIV Nef protein. CEM T cells were transduced with adenoviral vectors as previously described (40, 41) and the flow cytometric internalization assay was used to measure endocytosis as described for HLA-A2 and A2/C. Briefly, CEM T cells were incubated on ice with the anti-CD4 Ab, OKT4. The cells were shifted to 37°C for the indicated time and then stained with a secondary Ab goat α-mouse-PE (1:250; Invitrogen). The cells were analyzed by flow cytometry and the percentage of CD4 remaining on the surface of control cells (black square) or HIV-1 Nef expressing cells (gray square) was calculated. The mean ± SD for an experiment performed in duplicate is shown. E, The cytoplasmic tail of HLA-C does not inhibit recycling. The rates of HA-A2/C, HA-A2/C I337T, and HA-A2 recycling in CEM T cells were measured as described previously (20) and as outlined in Materials and Methods.)
transfections or transductions, the cell lines used in our investigations were re-made with new transfections and transductions on three separate occasions and in each case yielded similar relative expression levels.

Because, the translation initiation and leader sequences were the same for each molecule, we were able to measure initial protein synthesis as an estimate of the amount of translatable RNA in the cell. As shown in Fig. 1C and quantified in Fig. 1D, the expression level of HA-A2 and HA-A2/C was not significantly different (p/H11005/0.12). Despite this, we found that there was 3-fold less HA-A2/C on the cell surface (mean fluorescence intensity (MFI) = 50 ± 4 for HA-A2/C, compared with 149 ± 18 for HA-A2) (Fig. 1E). This was not an artifact of the presence of the HA tag, as independently constructed and expressed HLA-A2 and A2/C that lacked the tag behaved similarly when stained with the HLA-A2-specific mAb, BB7.2 (Fig. 1F). In addition, these data were corroborated by confocal microscopy (Fig. 1, G and H), which confirmed that HA-A2 was largely expressed on the cell surface, where it colocalized significantly with ConA. In comparison, HA-A2/C had a staining pattern that was distinctly different relative to that of ConA (Fig. 1G, compare panels 3 and 7, and quantification shown in Fig. 1H).

We also found that molecules with an HLA-C extracellular domain were expressed poorly on the cell surface relative to those containing HLA-A extracellular domains (MFI = 12 ± 3 for HA-Cw4 and 20 ± 2 for HA-Cw4/A2) (Fig. 1E). Given that we noted a slightly lower level of initial protein synthesis for these molecules relative to HLA-A2 (Fig. 1C and D), we also verified these results in a different system that could better account for potential differences in gene copy number. In this case, HA-A2 and HA-Cw4...
FIGURE 5. Isoleucine at position 337 (I337) is required for accelerated internalization and lysosomal targeting. A and B, Expression of A2/C cytoplasmic tail point mutations. CEM T cells (negative control) or CEM T cells expressing the indicated MHC-I were pulse-labeled as described in Fig. 1C, and analyzed by SDS-PAGE. Bands were quantified using a phosphor imager and ImageQuant software. The results are displayed in B as the means ± SD from three experiments. C and D, I337 is necessary for reduced steady-state cell surface expression of HA-A2/C. CEM T cell lines were generated expressing the indicated MHC-I. CEM T cells expressing the indicated mutant were stained with an Ab directed against the HA tag and analyzed by flow cytometry. The filled gray curve represents the expression of the indicated molecule while the filled black curve represents untagged cells stained with the HA Ab. D, Quantitation of the HA-A2/C cytoplasmic tail mutant surface expression. The results are depicted as MFI ± SD from three experiments. E, I337 is not required for slow export of HA-A2/C. A transport assay was performed as in Fig. 3A, except cells were chased in biotin for only 1 h. The transport assay was quantitated as in Fig. 3B, except that percentage of HA-A2 transported to the cell surface was set to 100%. The mean ± SD is shown for two experiments. F, I337 is necessary for accelerated internalization of HA-A2/C. A flow cytometric internalization assay was used as in Fig. 3C to determine the percentage of each molecule remaining on the cell surface over time. The mean ± SD for an experiment performed in triplicate is shown. G and H, I337 is necessary for lysosomal targeting of HA-A2/C. A pulse chase was performed as in Fig. 4E and was quantitated as in Fig. 4F. The mean ± SD is shown for two experiments.
We also examined whether HLA-A2 and A2/C differed with respect to how rapidly they were recycled to the cell surface following internalization. As shown in Fig. 3E, we did not detect any significant differences in recycling of HA-A2/C relative to HLA-A2 that would explain its lower expression levels. In fact, a slightly higher percentage of HA-A2/C was recycled to the cell surface from internal compartments (p = 0.036, n = 3).

We then examined the ultimate fate of HA-A2/C with longer pulse-chase experiments to determine whether it was targeted to lysosomal compartments after internalization. As shown in Fig. 4A and quantified in Fig. 4B, we found that mature, endo H resistant HA-A2/C was degraded approximately twice as fast as HA-A2. (HA-A2 had a half-life of 10.5 h, compared with 6 h for HA-A2/C). These data were confirmed by confocal microscopy in which we noted that HA-A2/C displayed extensive colocalization with LAMP-1, a marker of lysosomal organelles, when degradation was inhibited by bafilomycin, an inhibitor of the vacuolar ATPase that is required for efficient acidification and degradation in lysosomal compartments (Fig. 4C and quantified in Fig. 4D).

Finally, to provide further evidence that the degradation of HA-A2/C occurred in acidic compartments, such as lysosomes, we treated CEM T cells with bafilomycin to determine whether it reversed the degradation observed by pulse-chase analysis. As shown in Fig. 4E and quantified in Fig. 4F, bafilomycin treatment resulted in a 6-fold increase of HA-A2/C compared with a 2.2-fold increase for HA-A2 (p = 0.015, n = 3). In sum, these data suggest that the HLA-C cytoplasmic tail contains an internalization and lysosomal targeting signal.

Identification of a trafficking signal in the HLA-C cytoplasmic tail that promotes intracellular localization and lysosomal targeting

To determine which amino acids were responsible for the effects of the HLA-C tail, we focused on four amino acid differences between HLA-C and HLA-A/B molecules (Fig. 1A). Each of these amino acids was mutated in HA-A2/C, and stable CEM T cell lines were made as described above for HA-A2/C. Initial protein synthesis measurements indicated that the expression of each of these molecules was not significantly different from that of HA-A2/C, except for HA-A2/C D333A, which was expressed slightly less (p = 0.04, Fig. 5, A and B).

We then examined surface expression via flow cytometric analysis on cells stained with an anti-HA Ab. As shown in Fig. 5, C and D, only one of the amino acids substitutions reversed the effect of the HLA-C tail. Specifically, changing isoleucine at position 337 in the HLA-C tail to the threonine found in HLA-A and B tails (I337T) increased surface expression by 3-fold compared with HA-A2/C (p < 0.001, n = 3). Conversely, we found that the reciprocal mutation in the HLA-A cytoplasmic tail (A2 T337I), reduced surface expression of HA-A2 × 3-fold (p < 0.0001, n = 3, Fig. 5, C and D).

An analysis of intracellular transport, using the assay described above, revealed that HA-A2/C I337T transport was reduced compared with wild type HA-A2 (p < 0.001, n = 3), but was not significantly different from A2/C (Fig. 5E). Whereas, the flow cytometric internalization assay (described above) revealed that substitution of I337 reduced the internalization rate 15-fold (from 3.73% per minute to 0.25% per minute p < 0.01, Fig. 5F).

Finally, we used pulse-chase analysis plus or minus bafilomycin to measure the degree to which wild type and mutant molecules were degraded in acidic compartments. As shown in Fig. 5G and quantified in Fig. 5H, substitution of I337 in HA-A2/C increased the amount of recovered protein 4-fold in the control, DMSO treated, sample (compare lanes 5 and 8 in Fig. 5G, and quantification in Fig. 5H, p < 0.01), resulting in expression that was similar to that of HA-A2 (compare lanes 3 and 8 in Fig. 5E). Thus, I337 is a determinant required for accelerated internalization and degradation of molecules containing an HLA-C cytoplasmic tail domain.
To further define the internalization and lysosomal targeting motif in HLA-C, the highly conserved aspartic acid (D) at position 333 was changed to an alanine. Based on initial protein synthesis, this mutant was expressed slightly less than HA-A2/C (Fig. 5, A and B, \( p /H11005 \leq 0.04 \)), but its expression was not significantly different from HLA-A2 and most A2/C mutants (C320Y, N327D, I337T, T337I; Fig. 5, A and B). Substitution of D333 resulted in a substantial loss of cell surface expression compared with HA-A2/C, HA-A2 and the other HA-2/C mutants (Fig. 5, C and D, \( p /H11021 \leq 10^{-4} \)). The intracellular transport rate was not the explanation for the reduction in surface expression as this was similar to HA-A2/C (Fig. 6A). However, we found that substitution of D333 resulted in a 2-fold increase in internalization rate compared with HA-A2/C (\( p < 1 \times 10^{-4} \)) and a 5-fold increase relative to wild type HA-A2 (\( p < 1 \times 10^{-4} \), Fig. 6B). Additionally, pulse-chase analysis revealed that mutation of D333 also caused an increase in turnover of mature, endo H-resistant molecules (Fig. 6C, C and D). In sum, these data indicate that D333 functions to attenuate the downstream dihydrophobic internalization and lysosomal targeting signal.

**Evidence for regulated cell surface expression of HLA-C in APCs**

The data we have acquired indicates that there are multiple mechanisms by which cells precisely regulate HLA-C expression. The extracellular domain promotes retention and degradation in the ER.

**FIGURE 7.** Macrophage differentiation up-regulates HLA-C and HA-A2/C. A, Up-regulation of HLA-C upon differentiation of primary CD14+ cells into macrophages. CD14+ mononuclear cells were isolated from the blood of a normal donor. A subset of the cells was stained immediately (untreated) with anti-HLA-C (L31), an Ab against a subset of HLA-B allotypes (HLA-Bw6) or isotype control Abs. The remainder of the cells were cultured for 5 days in GM-CSF, harvested and stained with the same Abs. B, Up-regulation of HA-Cw4 and HA-A2/C with differentiation of monocytic cells lines into macrophage-like cells. U937 (left panels) and THP-1 (right panels) cell lines were derived as described in Materials and Methods. Cell surface expression was determined by flow cytometry using an Ab to HA for undifferentiated (DMSO) and differentiated (LPS/PMA) cells. C, Macrophage differentiation stabilizes the endo H-resistant form of HA-A2/C and HA-Cw4. U937 and THP-1 cell lines were treated with LPS and PMA as in B. Lysates were generated, digested with endo H, and analyzed by Western blot using an Ab directed at HA. R, Endo H-resistant band; S, endo H-sensitive band. D, Macrophage differentiation inhibits internalization of HA-A2/C. The flow cytometric internalization assay described in Fig. 3C was used to determine the percentage of HA-A2 or HA-A2/C remaining on the cell surface over time in undifferentiated (DMSO) or differentiated (LPS/PMA) U937 cell lines. For these experiments an HLA-A2-specific mAb (BB7.2) was used. The mean ± SD for an experiment performed in quadruplicate is shown.
and the cytoplasmic domain promotes a limited amount of internalization and degradation. Thus, it seems that complex mechanisms exist to maintain significant intracellular levels of HLA-C while limiting (but not eliminating) surface expression. It makes sense that a molecule capable of sending a dominant inhibitory signal to killer T cells should be tightly regulated, as high expression could result in higher activation thresholds for the detection of virally infected or tumorigenic cells. However, the fact that intracellular pools of HLA-C are maintained suggests that there may be conditions in which it is advantageous to rapidly up-regulate HLA-C cell surface expression. We were unable to specifically induce HLA-C surface expression in T cells with a variety of stimuli, such as IFNs α and γ or with compounds that stimulate T cells, such as IL-2 and PHA. Additionally, we were unable to up-regulate HLA-C expression by treatment of T cells with specific pathogens, such as HIV and adenovirus (data not shown).

It remained possible, however, that HLA-C had evolved to present peptides from certain types of pathogens, or that it functioned to inhibit killing under some conditions. For example, when APCs activate naive CTLs by cross-presenting exogenous Ag, it would not be advantageous to lyse the uninfected, cross-presenting APC. Thus it would make sense for these cells to up-regulate molecules that would send inhibitory signals to effector T cells that might otherwise mistake the APC for an infected target cell and lyse it. Indeed, it has recently been reported that coincident with acquisition of killer T cell effector functions, CTLs up-regulate the KIR family of inhibitory receptors (5).

To examine this possibility further, we isolated primary human CD14+ mononuclear cells from a normal, healthy donor’s peripheral blood. Some of the cells were stained immediately for HLA-C expression, and the remainder was incubated in GM-CSF for 5 days to induce macrophage differentiation. To measure HLA-C surface expression, we obtained an Ab that specifically recognizes most HLA-C allotypes, (17, 25). As shown in Fig. 7A, left panel, HLA-C staining, as measured with the L31 Ab, was low in freshly isolated, undifferentiated CD14+ (~2.8-fold above background and more than 40-fold less than Bw6). After 5 days of culture in GM-CSF, HLA-C surface expression was dramatically up-regulated more than 20-fold relative to Bw6.

The data described above confirms that HLA-C expression is normally much lower than that of HLA-B allotypes in undifferentiated primary monocytes using Abs that recognize natural epitopes. However, this approach does not allow the determination of which domains of HLA-C are responsible for regulated expression in differentiated macrophages. To further investigate this observation in a more well defined system, we expressed HA-A2, HA-Cw4 and HA-A2/C in cell lines (THP-1 and U937) that were capable of differentiating into macrophage-like cells by the addition of LPS and PMA. For comparison, we also expressed HA-tagged B*4405 and B*4402 in these cells. These two HLA-B molecules differ by only a single amino acid, but are known to vary substantially in terms of peptide loading and rates of ER egress (26).

In undifferentiated nonadherent solvent (DMSO)-treated cells, HA-A2, HA-A2/C and HA-Cw4 were expressed in a manner that was very similar to what we observed in primary T cells and stable CEM T cell lines (Fig. 7B, panels 1 and 3). HA-A2 was expressed at comparably high levels and was largely endo H resistant (Fig. 7C, lanes 2 and 6), whereas HA-Cw4 was expressed at very low levels at the cell surface (Fig. 7B, panels 1 and 3) and was largely endo H sensitive (Fig. 7C, lanes 18 and 22). HA-A2/C was expressed at intermediate levels (Fig. 7B, panels 1 and 3) and had reduced amounts of endo H-resistant material relative to HLA-A2 (Fig. 7C, lane 10 and 14), presumably due to increased internalization and degradation in acidic compartments as was observed in other cell types (Figs. 3 and 4). B*4405 was expressed well on the cell surface (Fig. 7B, panels 5 and 7) and was largely endo H resistant (Fig. 7C, lanes 34 and 38), whereas B*4402 was expressed comparatively less well on the cell surface (Fig. 7B, panels 5 and 7) and was largely endo H sensitive due to ER retention (Fig. 7C, lanes 26 and 30; see also Ref. 26).

When U937 and THP-1 cell lines were treated with LPS and PMA to induce differentiation into macrophage-like cells, we observed no change in the surface expression of HA-A2 or HA-B*4402, and we noted a small decrease in the surface expression of HA-B*4405 (Fig. 7B). In contrast, we observed that A2/C cell surface expression was increased to achieve levels that were similar to wild-type HA-A2 (Fig. 7B, compare panels 1 and 2 or 3 and 4). Additionally, as shown in Fig. 7A, we also observed an increase in full-length HA-Cw4 cell surface expression.

The increase in surface expression of A2/C was reflected by an increase in the amount of endo H-resistant protein detected by Western blot analysis (Fig. 7C, compare lanes 10 and 12 for U937 cells or lanes 14 and 16 for THP-1 cells). We also noted an increase in the ratio of endo H resistant: sensitive forms of full length HLA-Cw*0401 (Fig. 7C, compare lanes 18 and 20 for U937 or lanes 22 and 24 for THP-1). Albeit, most of the full-length HLA-C molecules remained endo H sensitive.

The relative amount of endo H-resistant material for HA-A2 and HA-B*4405 remained unchanged. However, we did note an increase in the fraction of HA-B*4402 that became resistant to endo H (Fig. 7C, compare lanes 26 and 28 or 30 and 32). Thus, macrophage differentiation resulted in a complex set of effects that enhanced ER exit of some MHC-I molecules, like B*4402, that are normally retained in the ER because of problems with protein
loading (26). In addition, macrophage differentiation increased the amount of endo H-resistant HA-A2/C, which is normally low because of lysosomal targeting of mature molecules.

To better understand the striking up-regulation of surface HA-A2/C and the stabilization of mature, endo H-resistant forms of A2/C in differentiated macrophage-like cells, we first examined internalization rate. As shown in Fig. 7D, we found that HA-A2/C was internalized 3.6 times more rapidly than HA-A2 in DMSO treated monocytic cells. However, after treatment with LPS and PMA, HA-A2 and HA-A2/C were internalized at very similar rates, suggesting that the activity of the cytoplasmic tail signal was modified with differentiation.

Evidence that HLA-C cell surface expression in macrophages is regulated by phosphorylation

The HLA-C internalization and lysosomal targeting signal is surrounded by serine residues (SDXSLI) and thus could be regulated by phosphorylation (27, 28). To examine this, we mutated these serine residues to alanines to prevent phosphorylation (A2/C SSAA). These constructs were expressed in both CEM cells and in U937 cells as described above. Then, phosphorylation of HA-A2, HA-A2/C and the mutant A2/C SSAA was directly assessed by labeling the cells with 32P orthophosphate and immunoprecipitating each molecule with the HLA-A2-specific Ab, BB7.2. As shown in Fig. 8A and B, we readily detected phosphorylation of HLA-A2/C through hypophosphorylation of Ser335. An internalization assay performed as described in Fig. 3C was used to determine the percentage of the indicated MHC-I molecules remaining on the cell surface over time in DMSO vs LPS/PMA-treated U937 cells. The anti-HLA-A2 mAb BB7.2 was used to detect surface expression in these experiments. The mean ± SD for an experiment performed in triplicate is shown.

To better understand the striking up-regulation of surface HA-A2/C and the stabilization of mature, endo H-resistant forms of A2/C in differentiated macrophage-like cells, we first examined internalization rate. As shown in Fig. 7D, we found that HA-A2/C was internalized 3.6 times more rapidly than HA-A2 in DMSO treated monocytic cells. However, after treatment with LPS and PMA, HA-A2 and HA-A2/C were internalized at very similar rates, suggesting that the activity of the cytoplasmic tail signal was modified with differentiation.
To determine whether phosphorylation affected surface expression, we examined these molecules by flow cytometry in undifferentiated U937 and CEM cells. As shown in Fig. 9A, we found that mutation of both serines to alanines increased HA-A2/C expression to that of wild type HA-A2. Mutation of serine 332 alone had no effect (data not shown), indicating that serine 335 was sufficient for this phenotype. Mutation of this serine residue to glutamic acid (S335E), to mimic phosphorylation, did not affect expression of HA-A2/C in undifferentiated cells. Remarkably, however, when macrophage differentiation was induced in U937 cells with LPS and PMA, we found that mimicking phosphorylation prevented up-regulation, whereas unmodified HA-A2/C was up-regulated 4.7-fold (p < 1 x 10^-6, Fig. 9, A and B). Thus, these data provide confirmation that the phosphorylated form of A2/C is degraded and that inhibition of A2/C phosphorylation results in a stabilization of A2/C protein.

To further examine the mechanism by which S335 affected HA-A2/C expression, we examined degradation by pulse-chase analysis. As shown in Fig. 9C, HA-A2/C expression was reduced by 97% by 12 h (compare lanes 15 and 16 with lanes 17 and 18 in Fig. 9C). Inhibition of phosphorylation by alanine substitutions (SSAA) resulted in stabilization of the molecule nearly 4-fold (compare lanes 17 and 18 with lanes 29 and 30 in Fig. 9C). In contrast, mimicking phosphorylation of S335 by glutamic acid substitution resulted in degradation similar to HA-A2/C (compare lane 17 and 18 with lanes 41 and 42 in Fig. 9C). In all cases, the observed degradation was rescued with bafilomycin, indicating that it was secondary to lysosomal targeting.

Following treatment with LPS and PMA to induce macrophage differentiation, we observed a 4-fold increase in HA-A2/C protein at the 12 h chase point compared with DMSO-treated cells (compare lanes 17 and 18 with lanes 23 and 24 in Fig. 9C). Mimicking phosphorylation at position 335 (S335E) prevented differentiation-induced stabilization of HA-A2/C (compare lanes 23 and 24 with lanes 47 and 48 in Fig. 9C). Conversely, inhibiting phosphorylation by substituting alanine at the same position maintained protein stability under all conditions (Fig. 9C, lanes 35-38). In sum, these data strongly indicate that phosphorylation of S335 in the HLA-C tail is necessary for lysosomal targeting and that macrophage differentiation inhibits phosphorylation of this residue.

To examine the effect of mimicking or inhibiting phosphorylation on internalization rate, we used the flow cytometric internalization assay described above. As shown in Fig. 9E (left graph), mimicking phosphorylation (S335E) accelerated internalization 1.7-fold (p < 0.01), whereas preventing phosphorylation at this position (SSAA) inhibited it 2.0-fold (p < 0.01). Following treatment with LPS and PMA, we again observed a decrease in the internalization rate of HA-A2/C (Fig. 9E, right graph). However, the phosphorylation mimic (S335E) did not respond, and continued to be internalized at a rapid rate. These data further support the model that phosphorylation is necessary for the internalization and degradation of HLA-C, and that macrophage differentiation resulted in hypophosphorylation, and increased expression of HLA-C.

Finally, to directly demonstrate that phosphorylation of A2/C is reduced upon macrophage differentiation, phosphorylation of HA-A2 and HA-A2/C was directly assessed by labeling the cells with 32P orthophosphate and each molecule was immunoprecipitated with the HLA-A2-specific Ab, BB7.2. As shown in Fig. 8C, induction of differentiation dramatically increased the recovery of total A2/C relative to that of HA-A2, as measured by Western blot analysis (compare Fig. 8C, lanes 5 and 6 with Fig. 8B, lanes 8 and 9) without demonstrating a corresponding increase in phosphorylated forms (Fig. 8C, lanes 2 and 3). Thus, these data support our model, that differentiation reduced A2/C phosphorylation, which in turn resulted in stabilization of A2/C protein.

Discussion

In this study, we demonstrated that HLA-C is a highly regulated MHC-I molecule and in most cell types its expression is limited at almost every step in the biosynthetic pathway. Maintenance of a low level of HLA-C surface expression may allow a balance between the signals needed for Ag presentation and appropriate inhibition of NK cells, without sending strong dominant signals that might overly increase activation thresholds. The extracellular domain of HLA-C reduced expression by promoting ER retention whereas the cytoplasmic tail affected expression via the activity of an internalization and lysosomal targeting signal.

The influence of the extracellular domain of MHC-I on its surface expression was not surprising. This region of the molecule dictates the peptides MHC-I will bind and ultimately regulates release from the ER and transport to the cell surface. Indeed, the addition of HLA-C-specific peptides has been reported to promote the release of HLA-C from TAP in vitro (29), and thus would be expected to decrease ER retention. Based on these data, it is tempting to speculate that HLA-C expression would increase with a broadening of intracellular peptides such as would occur with infection by viruses or intracellular bacteria. To this end we did examine several viruses (HIV and adenoavirus) without detecting any significant change in HLA-C expression. Obviously, however, we cannot rule out the possibility that specific peptides found in other kinds of pathogens might stimulate the release of HLA-C from the ER.

The bigger surprise was the discovery of an internalization and lysosomal targeting signal within the HLA-C cytoplasmic tail. This motif was identified by the demonstration that mutating isoleucine at position 337 to a threonine reversed the phenotype conferred by swapping the HLA-C cytoplasmic tail for the HLA-A2 cytoplasmic tail. Interestingly, the sequence surrounding this residue resembles a Golgi localized, γ-ear containing, ARF-binding (GGA) consensus binding motif (DXXLL; reviewed in (30)). GGAs are localized to the TGN and endosomal compartments, and are thought to play a role in trafficking between the TGN and endosomes. Thus, it was possible that GGAs played a role in targeting HLA-C into the endolysosomal pathway from the cell surface or the TGN. However, while we observed some reduction in surface expression and some alteration in intracellular localization with knockdown of GGA-2 and -3, we observed no significant change in the surface transport rate, internalization, recycling or degradation rates. Also, arguing against a role for the GGAs, we found that mutation of the required aspartic acid residue at position 333 (DXSLI) to an alanine, actually increased the activity of the signal. Based on these data, the role of this amino acid was not to provide a GGA binding site, but rather to attenuate the dihydrophobic signal so as to allow some HLA-C to remain on the cell surface. Thus, we have defined a set of amino acids in the HLA-C cytoplasmic tail, which comprise a novel signal that serves to maintain a precise, low level of HLA-C surface expression. Further work will be needed to identify the corresponding trafficking protein that binds it.

Interestingly, the activity of the HLA-C internalization and lysosomal targeting signal also depended on an adjacent serine (DXSLI), which we directly demonstrated was phosphorylated in vivo. Changes in this position increased or decreased internalization and degradation, depending on the substitution that was made. When serine 335 was changed to a glutamic acid residue, which
mimicked the negative charge provided by phosphorylation, internalization and degradation occurred rapidly. When phosphorylation was prevented by changing the serine to an alanine, internalization and degradation were inhibited and cell surface expression was increased. The complex regulation of HLA-C trafficking was puzzling in that HLA-C cell surface expression was kept low while HLA-C intracellular levels remained fairly high. These observations suggested that under most conditions it is beneficial to keep HLA-C expression low to reduce inhibitory signals that might limit immune surveillance, but that there might be some circumstances in which HLA-C might be rapidly up-regulated, either to increase the capacity of the cell to present certain types of Ags, or to turn down an immune response by increasing signaling to KIRs.

To examine whether HLA-C might be specifically up-regulated under some conditions of immune activation, we treated CEM cells with IFNs (α and γ) or with chemicals known to activate T cells (PHA and IL-2), without success. Additionally, we infected the cells with viral pathogens such as adenovirus and HIV, again without significant affect. We then turned to APCs, because these cells have unique roles in Ag presentation (e.g., the capacity to present exogenous Ags in association with MHC-I).

We found that undifferentiated primary monocytes and monocytic cell lines expressed low levels of HLA-C, similar to the other cell types we examined. Upon differentiation, however, we observed a reduction in phosphorylation of A2/C, which correlated with a reduction in internalization and degradation and a corresponding up-regulation of HLA-C and molecules bearing the HLA-C cytoplasmic tail. Under the same conditions, the surface expression of HLA-A and HLA-B molecules remained essentially unchanged or was even reduced somewhat. The dependence of this effect on the cytoplasmic tail, which we demonstrated governs the capacity to present exogenous Ags in association with MHC-I.

These observations, together with the strong evidence that HLA-C plays a crucial role as an inhibitor of NK cell lysis by virtue of its specific binding of KIRs, suggests that HLA-C is up-regulated on macrophages to down-regulate and/or specifically inhibit lysis of cells bearing these receptors. Interestingly, it has recently been demonstrated that CTLs acquire KIRs coincident with acquisition of effector functions (5). Thus, HLA-C may be up-regulated to provide feedback inhibition of CTLs, once they have fully matured. Alternatively, however, perhaps more intriguing possibility is that HLA-C is specifically up-regulated on APCs to protect them from lysis by mature CTLs while they are cross-presenting exogenous Ags to naive CTLs. The capacity to specifically prevent the lysis of cross-presenting APCs would be advantageous in the setting of a chronic infection in which it was necessary to continuously present Ags over an extended period of time. In preserving these cells by such a mechanism, the resulting increased threshold to lysis may inadvertently create a protected reservoir that aids in the persistence of certain organisms. Indeed, there is a long list of persistent pathogens that can be found in macrophages, including HIV, leishmania, brucella, salmonella, herpes viruses, tuberculosis, legionella, plus others (31–38). Thus, HLA-C may be precisely regulated to balance the need for continued immune activation by APCs presenting Ags against the cost of allowing some pathogens to persist.

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Disclosures

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