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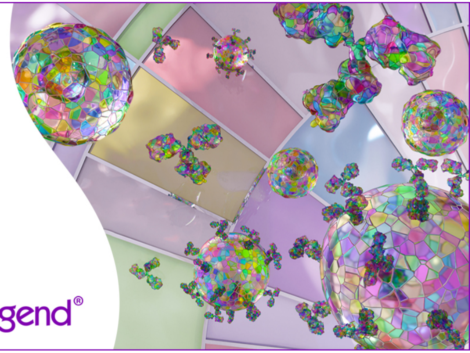

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Relative Expression Levels of the HLA Class-I Proteins in Normal and HIV-Infected Cells

Richard Apps,* Zhaojing Meng,† Gregory Q. Del Prete,‡ Jeffrey D. Lifson,‡ Ming Zhou,† and Mary Carrington*,§

The expression level of HLA class-I proteins is known to influence pathological outcomes: pathogens downregulate HLA to evade host immune responses, host inflammatory reactions upregulate HLA, and differences among people with regard to the steady-state expression levels of HLA associate with disease susceptibility. Yet precise quantification of relative expression levels of the various HLA loci is difficult because of the tremendous polymorphism of HLA. We report relative expression levels of HLA-A, HLA-B, HLA-C, and HLA-E proteins for the specific haplotype A*02:01, B*44:02, C*05:01, which were characterized using two independent methods based on flow cytometry and mass spectrometry. PBLs from normal donors showed that HLA-A and HLA-B proteins are expressed at similar levels, which are 13–18 times higher than HLA-C by flow cytometry and 4–5 times higher than HLA-C by mass spectrometry; these differences may reflect variation in the conformation or location of proteins detected. HLA-E was detected at a level 25 times lower than that of HLA-C by mass spectrometry. Primary CD4$^+$ T cells infected with HIV in vitro were also studied because HIV downregulates selective HLA types. HLA-A and HLA-B were reduced on HIV-infected cells by a magnitude that varied between cells in an infected culture. Averaging all infected cells from an individual showed HLA-A to be 1–3 times higher and HLA-B to be 2–5 times higher than HLA-C by flow cytometry. These results quantify substantial differences in expression levels of the proteins from different HLA loci, which are very likely physiologically significant on both uninfected and HIV-infected cells. *The Journal of Immunology*, **2015, 194: 3594–3600.**

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The HLA molecules are essential for immune function and have diverse clinical implications in infectious disease, autoimmunity, transplantation, cancer, and pregnancy (1–5). This study focuses on class-I HLA, comprising three classical loci (HLA-A, HLA-B, and HLA-C) and the additional nonclassical molecule HLA-E, all related by a common ancestral origin and retaining substantial sequence homology. The classical HLA class-I molecules are expressed by almost all human cells. They sample intracellular peptides and present them at the cell surface where they are recognized by CTLs, which can respond to foreign peptides. A defining feature of the classical HLA class-I is their tremendous polymorphism, concentrated in regions of the HLA molecule involved in peptide binding (6). Hundreds of distinct protein sequence allotypes are encoded by each of the three classical HLA class-I loci. HLA-E expression at the cell surface is also dependent on binding an intracellular peptide, but HLA-E specifically binds the leader peptide derived from classical HLA class-I molecules. HLA-E has very limited polymorphism, and it serves as the ligand for the inhibitory NKG2A receptor expressed by NK cells (7). Both classical and nonclassical HLA loci encode an ~45-kDa H chain that associates with a conserved β_2-microglobulin (β_2m) molecule of 12 kDa to form the complex that binds and presents small peptides of ~8 aa.

Numerous observations in vivo demonstrate that the expression level of HLA molecules has an important influence on their function. One of the most striking cellular changes in the inflammatory response is IFN-γ–mediated upregulation of HLA expression (8). In contrast, numerous pathogens downregulate HLA to evade T cell recognition (9–12). Cells of the innate-immune system carry multiple inhibitory receptors for classical HLA to detect this pathogen-mediated manipulation. Examples of these inhibitory receptors include LILR, which are expressed by cells of the myeloid lineage and bind all classical HLA class-I, and KIR, which are expressed by NK cells and bind specific HLA allotypes predominantly from the HLA-C locus. The level of expressed protein at some HLA loci varies among normal individuals. For example, allotypes of the HLA-C locus differ in expression level by up to 3-fold, and these differences correlate with clinical outcomes in some disease settings (13). Individuals with HLA-C allotypes that are expressed at higher levels show better control of viral load during HIV infection. Higher expression may result in more efficient initiation of T cell responses because both HIV-specific CTL responses and viral escape mutation associated more strongly with higher expressed HLA-C alleles (13). This effect in HIV infection is significant, because expression levels of HLA-C are marked by a single nucleotide polymorphism in the region 5′

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Abbreviations used in this article: B-LCL, B lymphoblastoid cell line; LC, liquid chromatography; β_2m, β-2-microglobulin; MFI, median fluorescence intensity; MS, mass spectrometry.

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of HLA-C, and this polymorphism showed one of the two strongest effects in the human genome on the outcome of HIV infection (14–16). This variant is in strong linkage disequilibrium with a 3′ untranslated region insertion/deletion polymorphism in a microRNA binding site, which associates with HIV outcome and may account, in part, for differential HLA-C expression levels (17, 18).

Although expression level has important consequences for HLA function, relative levels of proteins expressed from the HLA-A, HLA-B, HLA-C, and HLA-E loci are not well established. Precise quantitation is made difficult by the extreme polymorphism of classical HLA loci. Most individuals express six classical HLA alleles (i.e., heterozygous at HLA-A, HLA-B, and HLA-C) in addition to HLA-E, so detecting a particular locus with specificity is challenging. Even if Abs specific to molecules from each locus can be identified, their binding cannot simply be compared because of differences in affinity for their respective Ags. In the 1970s, analysis of HLA from a B lymphoblastoid cell line (B-LCL) determined that HLA-A/B were expressed at a level that was >10-fold higher than HLA-C (19). HLA H chains were quantified indirectly via Ab to β_2m, after enrichment for surface membrane proteins and separation of the HLA allotypes by elution from a lectin column. But HLA allotypes were not resolved into fully distinct fractions, limiting the accuracy of the method. For example, HLA-C eluted in two fractions, of which only the first was used for HLA-C quantitation because the second contained both HLA-C and HLA-A. Further, HLA-C was detected at less than a tenth the level of HLA-A/B, but by how much less than this could not be defined. The experiment tested a single B cell line and did not consider HLA-E, which is thought to be expressed at even lower levels than HLA-C (14). The expression level of HLA-E is controversial, with differences in the conformations of HLA detected by Abs to HLA-E (20) and cross-reaction of HLA-E Abs with classical HLA allotypes (21) having complicated attempts to determine the level of HLA-E on primary cells.

Therefore, precise quantitation of the HLA-A, HLA-B, HLA-C, and HLA-E protein expression levels on normal primary cells would be valuable. Relative expression levels of the classical HLA class-I loci are of particular interest on HIV-infected cells, because HIV encodes the Nef protein, which downregulates HLA-A and HLA-B (9, 22). Nef has multiple functions but, specifically, downregulation of HLA/MHC was shown to be significant in vivo (23, 24). Because HLA-A and HLA-B are not reduced with equal efficiency by HIV, and Nef does not modulate HLA-C, it is not clear which HLA locus dominates on HIV-infected cells (25, 26). We describe two independent approaches, flow cytometry and mass spectrometry (MS), to determine the relative expression levels of HLA class-I proteins on normal and HIV-infected primary cells.

Materials and Methods

HLA genotyping and mAbs

This study was approved by the local Institutional Review Boards, and all donors gave informed consent. PBLs and archived B-LCL were genotyped for HLA-A/B/C to 4-digit resolution by PCR–sequence-specific oligonucleotide probing and PCR–sequence-based typing, as recommended by the 13th International Histocompatibility Workshop (27). Only samples homozygous for A*02:01, B*44:02, and C*05:01 were studied further. mAbs that bind HLA were characterized for reactivity against 97 common HLA class-I alleles using commercially available beads coated with individual HLA allotypes, as previously described (28). Of numerous Abs screened, the following were used for further analyses: purified mAbs W6/32 (29), BBM.1 (30), and L31 (31), purchased from Serotec, Santa Cruz Biotechnology, and MediaPharma, respectively; hybridoma supernatants of mAbs PA2.1 (32) and 22E-1 (33, 34), generously supplied by Drs. N. Holmes and I. Smith (both of University of Cambridge, Cambridge, U.K.); and mAb DT9 (14, 35), purified from supernatant of a hybridoma kindly provided by Dr. V. Braud (Centre National de la Recherche Scientifique, Valbonne, France).

Primary cell preparation and infection with HIV

Leukocytes were isolated by density gradient separation from peripheral blood freshly drawn from healthy donors and used immediately for cytometry, lysed and frozen for immunoprecipitation/MS, or selected for HIV infection using anti-CD4 mAb-based magnetic selection (EasySep) to attain a purity exceeding 95% CD4$^+$ with <1% CD8$^+$ cells, as assessed by cytometry staining with L120-PE and SK1-FITC (both from Becton Dickinson). CD4$^+$ cell preparations were expanded for 3–5 d in RPMI 1640 supplemented with 100 U/ml IL-2 (PeproTech), anti-CD3/28 beads (Invitrogen), FBS (Lonza), and PSG (Invitrogen) before infection with the HIV strain NL4-3 (36). NL4-3 was generated by infectious molecular clone transfection of HEK293T, with the infectious titer of culture supernatants determined using TZM-Bl (37). Infection was performed by incubation of cells with virus at a nominal multiplicity of infection of 0.1 for 4–6 h at 37°C. Cells were washed and cultured in RPMI 1640 with IL-2 for an additional 6 or 7 d before analysis by cytometry.

Flow cytometry

B-LCL from culture, PBLs freshly isolated from normal donors, or CD4-selected cells isolated from normal donors and infected in vitro with HIV were incubated with unlabeled primary Ab to HLA or isotype controls, followed by PE-conjugated anti-mouse IgG (Sigma-Aldrich). Free secondary Ab binding sites were blocked with murine Ig before further staining of primary cell samples with directly conjugated mAb to identify specific leukocyte populations on which HLA staining is reported. CD3$^+$ cells were identified in freshly isolated PBL preparations using UCHT1-FITC (Beckman Coulter). Cultures infected in vitro with HIV were stained with CD4-PB (BioLegend), CD8-allophycocyanin, CD3–allophycocyanin–Cy7 (both from Becton Dickinson), and yellow fluorescent reactive viability dye (Invitrogen). Expression of CD4 was used to discriminate HIV-infected cells after gating on CD3$^+$ CD8$^-$ cells, as previously described (13, 38). Briefly, uninfected cells remained CD4$^+$, whereas infected cells lost CD4 expression, and HLA staining could be compared between these two populations (Fig. 1A). To verify that CD4 downregulation identified infected cells, a parallel stain was performed in which HIV was detected directly. A sample of cells from each culture well in which HLA

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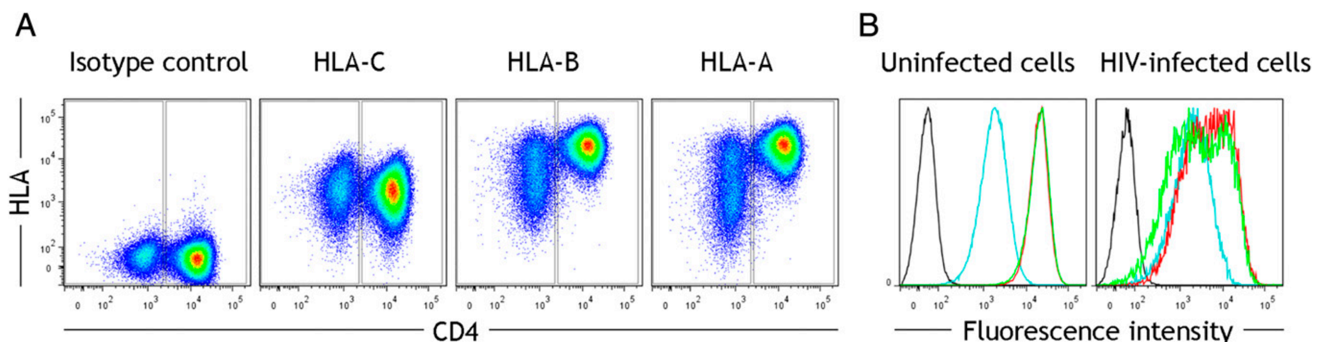

FIGURE 1. Representative flow cytometry staining of each HLA locus on CD4 cells purified from normal donor 2 and infected in vitro with HIV. (**A**) After gating the CD3$^+$ CD8$^-$ population, CD4 downregulation was used to discriminate infected (CD4$^-$) from uninfected (CD4$^+$) cells in a culture (*x*-axis). Staining for each classical HLA class-I locus was then measured (*y*-axis). (**B**) Relative staining of HLA-A (green), HLA-B (red), HLA-C (blue), and isotype control (black) is shown in separate graphs for uninfected (*left panel*) and infected (*right panel*) cells from the culture.

Table I. Abs PA2.1, 22E-1, and DT9 bind with similar strength to their respective alleles of the haplotype studied in flow cytometry, when correcting for variation in Ag levels in the screening panel, as measured by staining β_2m with mAb BBM.1

	MFI			Binding Relative to BBM.1		
Ab	A*0201	B*4402	C*0501	A*0201	B*4402	C*0501
PA2.1	9927	29	37	1.3	0.0	0.0
22E-1	29	5563	148	0.0	1.3	0.0
DT9	27	55	7107	0.0	0.0	1.3
BBM.1	7367	4413	5959	—	—	—

was assayed was stained with the same panel of conjugated mAbs, but instead of the indirect HLA stain, cells were fixed and permeabilized by incubation with paraformaldehyde and saponin (Becton Dickinson) before staining of intracellular gag with KC57-PE (Beckman Coulter). Within the $CD3^+$ $CD8^-$ population, >97% of $CD4^-$ cells were $KC57^+$ and >97% of $CD4^+$ cells were $KC57^-$ for every culture from which HLA expression was reported, confirming that CD4 downregulation could be used to discriminate HIV-infected cells from uninfected cells (data not shown). Staining results were acquired using a FACScan or LSR II flow cytometer (both from Becton Dickinson) with statistical analysis performed using FlowJo software (Tree Star).

Immunoprecipitation

B-LCL from culture or PBLs freshly isolated from normal donors were lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor (Roche), and HLA class-I molecules were purified by immunoprecipitation. mAb W6/32 or isotype control covalently conjugated to protein G–Sepharose (GE Healthcare), as previously described (39), were incubated with lysate from 12.5×10^6 B-LCL cells or 25×10^6 PBLs for 90 min rotating at 4˚C, after preclearing of the lysate against protein G–Sepharose beads alone. Bound complexes were washed with RIPA and frozen for subsequent analysis by MS. To verify immunoprecipitation efficiency, parallel immunoprecipitations were eluted with LDS sample buffer, resolved by PAGE, and transferred to a polyvinylidene difluoride membrane (all from Invitrogen Novex). Blots were probed with mAb L31 (Media-Pharma), reactive with HLA-C*05:01 in the haplotype studied (40, 41), which was detected by peroxidase-conjugated anti-mouse Ab visualized using ECL (both from GE Healthcare).

Mass spectrometry

Target peptides unique to each HLA protein to be used for quantitation were determined empirically after immunoprecipitation of the four HLA proteins from B-LCL. Both "heavy" isotope–labeled (single amino acid at the C terminus of each peptide labeled with ^{13}C and ^{15}N) and "light" nonlabeled synthetic sets of the target peptide sequences were obtained (New England Peptide). HLA proteins were eluted from immunoprecipitation beads using 2% SDS in 50 mM Tris-HCl (pH 8.3) and subsequently reduced, alkylated, and digested using Trypsin-LyC (Promega) overnight, following a filter-aided procedure using FASP digestion kits (Expedeon). Tryptic peptides generated were combined with synthetic heavy isotope–labeled peptides, desalted using C18 ZipTip (Millipore), lyophilized, and reconstituted in 0.1% TFA. Nanobore reversed-phase liquid chromatography (LC) tandem MS was performed using an Agilent 1200 nanoflow LC system coupled online with a LTQ Orbitrap XL mass spectrometer. Reversed-phase LC columns (75 μm i.d. × 10 cm) were slurry packed in-house with 5-μm, 300-Å pore size C-18 stationary phase into fused silica capillaries with a flame-pulled tip. After sample injection, the column was washed for 20 min with 98% mobile phase A (0.1% formic acid in water) at 0.5 μl/min. Peptides were eluted using a linear gradient of 2% mobile phase B (0.1% formic acid in ACN) to 35% B in 100 min, then to 80% B over an additional 20 min. The column flow rate was maintained at 0.25 μl/min throughout the separation gradient. The mass spectrometer was operated in a data-dependent mode; each full MS scan was followed by three tandem MS scans wherein the three most abundant molecular ions were dynamically selected for collision-induced dissociation using a normalized collision energy of 35%. To quantify precipitated proteins, a calibration curve was generated for each peptide monitored by analyzing increasing amounts of synthetic light peptide mixed with the fixed amount of heavy peptide added to biological samples, with data processing performed using Xcalibur software (Thermo Scientific).

Results

Sequenced-based HLA typing of 300 healthy donors identified two unrelated individuals who were homozygous for one of the most frequent White haplotypes: A*02:01, B*44:02, C*05:01. These individuals were studied to simplify the task of comparing relative expression levels from each HLA class-I locus. A B-LCL from a third donor homozygous for this haplotype was also identified. Three mAbs appropriate for measurement of HLA-A, HLA-B, and HLA-C expression levels by cytometry were identified by demonstrating specificity to each of the three allotypes of this haplotype and comparable binding strengths (Supplemental Fig. 1). mAb PA2.1 is specific to HLA-A*02:01, mAb 22E.1 is specific to B*44:02, and mAb DT9 is specific to C*05:01 in subjects homozygous for the A*02:01, B*44:02, C*05:01 haplotype. mAb DT9 is known to recognize HLA-E in addition to HLA-C (35), but we and other investigators determined previously that binding of DT9 to PBLs is dominated by reactivity with HLA-C (14, 42), likely because expression levels of HLA-E are very much lower than HLA-C. mAb BBM.1 recognizes the

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Table II. Relative expression levels of HLA class-I proteins measured by flow cytometry

	MFI				Level Relative to HLA-C	
Sample Type	IgG	HLA-A	HLA-B	HLA-C	HLA-A	HLA-B
B cell line	5	1,134	1,357	76	15	18
Normal PBL (donor 1)	4	922	1,208	70	13	17
Normal PBL (donor 2)	4	806	947	51	16	18
Cultured uninfected PBL (donor 1)	63	20,839	20,627	1,551	13	13
Cultured uninfected PBL (donor 2)	33	19,844	19,629	1,678	12	12
Cultured HIV-infected PBL (donor 1)	58	4,363	7,043	1,387	3	5
Cultured HIV-infected PBL (donor 2)	47	2,508	4,445	1,795	1	2

Staining is reported for total $CD3^+$ cells from normal PBLs and from $CD4^+$ (uninfected) or $CD4^-$ (infected) cells within the $CD3^+$ $CD8^-$ population for cultured PBLs. Expression levels are shown for a single representative replicate of two or three from independently obtained biological samples. The ranges of expression relative to HLA-C in these replicates were B cell line, $n = 3$, HLA-A (14–16), HLA-B (17–19); normal PBL donor 1, $n = 3$, HLA-A (13–15), HLA-B (17–19); normal PBL donor 2, $n = 3$, HLA-A (13–16), HLA-B (18–18); cultured uninfected PBL donor 1, $n = 2$, HLA-A (13–14), HLA-B (13–13); cultured uninfected PBL donor 2, $n = 3$, HLA-A (12–13), HLA-B (11–13); HIV-infected PBL donor 1, $n = 2$, HLA-A (3–4), HLA-B (5–6); and HIV-infected PBL donor 2, $n = 3$, HLA-A (1–3), HLA-B (2–6). Absolute MFIs are higher for all cultured PBLs due only to use of a different cytometer.

```
HLA-A*0201   GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDG   62
HLA-B*4402   GSHSMRYFYTAMSRPGRGEPRFITVGYVDDTLFVRFDSDATSPRKEPRAPWIEQEGPEYWDR   62
HLA-C*0501   CSHSMRYFYTAVSRPGRGEPRFIAVGYVDDTQFVQFDSDAASPRGEPRAPWVEQEGPEYWDR   62
HLA-E        GSHSLKYFHTSVSRPGRGEPRFISVGYVDDTQFVRFDNDAASPRMVPRAPWMEQEGSEYWDR   62
              ***::**.*::***********:******* **:**.**:* *  *****:****.****

HLA-A*0201   ETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGK    121
HLA-B*4402   ETQISKTNTQTYRENLRTALRYYNQSEAGSHIIQRMYGCDVGPDGRLLRGYDQDAYDGK    121
HLA-C*0501   ETQKYKRQAQTDRVNLRKLRGYYNQSEAGSHTLQRMYGCDLGPDGRLLRGYNQFAYDGK    121
HLA-E        ETRSARDTAQIFRVNLRTLRGYYNQSEAGSHTLQWMHGCELGPDXRFLRGYEQFAYDGK    121
             **:  :  :*  * :* .   ********** :* *:**::*.* *:****.* *****

HLA-A*0201   DYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRT  182
HLA-B*4402   DYIALNEDLSSWTAADTAAQITQRKWEAARVAEQDRAYLEGLCVESLRRYLENGKETLQRA  182
HLA-C*0501   DYIALNEDLRSWTAADKAAQITQRKWEAAREAEQRRAYLEGTCVEWLRRYLENGKKTLQRA  182
HLA-E        DYLTLNEDLRSWTAVDTAAQISEQKSNDASEAEHQRAYLEDTCVEWLHKYLEKGKETLLHL  182
             **::*:*** ****.* *** :::* : *  **: *****. *** *::***:**:** :

HLA-A*0201   DAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQK  243
HLA-B*4402   DPPKTHVTHHPISDHEVTLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQK  243
HLA-C*0501   EHPKTHVTHHPVSDHEATLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQK  243
HLA-E        EPPKTHVTHHPISDHEATLRCWALGFYPAEITLTWQQDGEGHTQDTELVETRPAGDGTFQK  243
             : ****:***.:****.*******.***********:***.:************* ****

HLA-A*0201   WAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEPSSQPTIPIVGIIAGLVLFGAV-ITGAVV  303
HLA-B*4402   WAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEPSSQSTVPIVGIVAGLAVLAVV-VIGAVV  303
HLA-C*0501   WAAVVVPSGEEQRYTCHVQHEGLPEPLTLRWGPSSQPTIPIVGIVAGLAVLAVLAVLGAVM  304
HLA-E        WAAVVVPSGEEQRYTCHVQHEGLPEPVTLRWKPASQPTIPIVGIIAGLVLLGSV-VSGAVV  303
             *********:*************:*:**** *:**.*:*****:***.::. : : ***:

HLA-A*0201   AAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV                        341
HLA-B*4402   AAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSLTA---                        338
HLA-C*0501   AVVMCRRKSSGGKGGSCSQAASSNSAQGSDESLIACKA                        342
HLA-E        AAVIWRKKSSGGKGGSYSKAEWSDSAQGSESHSL----                        337
             *.*: *:***. **** *:*  *:*****:
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FIGURE 2. Peptides unique to each HLA used for quantitation in an MS-based assay. Mature protein sequence of each HLA class-I protein present in the individuals studied is shown. Peptides unique to an HLA locus within this individual that were used for quantitation are highlighted: three peptides for HLA-A (yellow), four peptides for each of HLA-B (green) and HLA-C (blue), and two peptides for HLA-E (red). In the HLA-E sequence, X represents either R or G.

β_2m molecule with which all HLA 45-kDa H chains associate (30). Binding of mAbs PA2.1, 22E.1, and DT9 was normalized to that of BBM.1 in our screening panel to quantify the relative strength of Ab binding, corrected for variation in the amount of each HLA allele present (Supplemental Fig. 1). This approach revealed that PA2.1, 22E.1, and DT9 bind at very similar levels to their respective Ags in the target haplotype (Table I). Therefore, binding of these mAbs to primary cells in cytometry can be directly compared to estimate relative expression levels of the HLA loci present.

Staining of mAbs PA2.1 (HLA-A), 22E.1 (HLA-B), and DT9 (HLA-C) was compared for the samples homozygous for the A*02:01, B*44:02, C*05:01 haplotype. HLA-A was detected at 15 times the level of HLA-C and HLA-B was detected at 18 times the level of HLA-C on B-LCL (Table II). On freshly isolated PBLs from two normal donors, HLA-A was detected at 13–16 times the level of HLA-C, and HLA-B was detected at 17–18 times the level of HLA-C (Table II). Staining of normal PBLs is reported specifically for CD3^{+} cells, but relative differences between HLA loci were similar for all major leukocyte pop-

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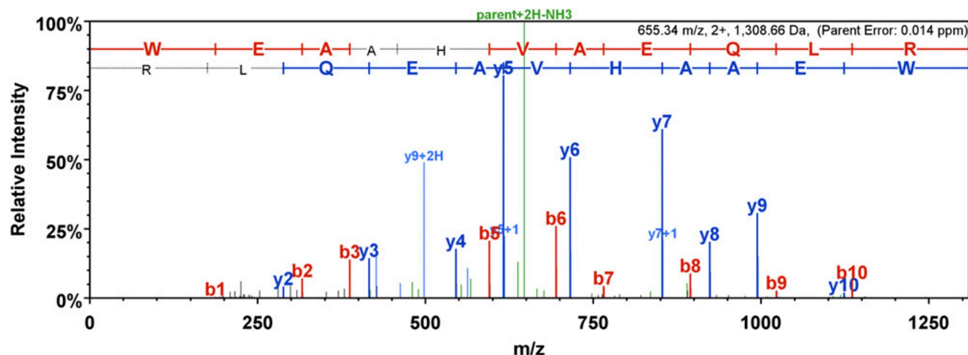

FIGURE 3. Representative MS fragmentation spectrum. The doubly charged HLA-A–specific peptide WEAAHVAEQLR, detected for normal PBL donor 1, is shown visualized using Scaffold software. N-terminal b fragment ions are labeled in red, C-terminal y fragment ions are labeled in blue, and other fragment ions are labeled in green.

Table III. Representative results for the peptides monitored by MS at each HLA locus, determined for an immunoprecipitation sample from normal PBL donor 1

Target Allele	Quantitating Peptide	Amount Detected (fmol)	Median for Locus (fmol)
HLA-A*0201	WAAVVVPSGQEQR	3185	2905
	WEAAHVAEQLR	2905	
	VDLGTLR	2504	
HLA-B*4402	THVTHHPISDHEVTLR	2117	2503
	FITVGYVDDTLFVR	2298	
	APWIEQEGPEYWDR	2977	
	AYLEGLCVESLR	2708	
HLA-C*0501	THVTHHPVSDHEATLR	333	582
	DYIALNEDLR	836	
	APWVEQEGPEYWDR	756	
	GYNQFAYDGK	408	
HLA-E	AEWSDSAQGSESHSL	18	23
	SWTAVDTAAQISEQK	29	

ulations. CD4$^+$ cells from these donors were activated and then infected with HIV-1 in vitro before staining each HLA allotype on both infected and uninfected cells from the same cultures. Uninfected cells show that in vitro culture marginally reduced HLA-A and HLA-B expression relative to HLA-C to 12–13 times higher for both donors (Table II). In contrast, HLA-A and HLA-B were reduced substantially on HIV-infected cells; the median fluorescence intensity (MFI) of HLA-A staining was reduced to 1–3 times higher than HLA-C, and HLA-B staining was reduced to 2–5 times higher than HLA-C (Table II). Specifically for HIV-infected cells, HLA-A/B expression levels were not distributed normally across cells from a given individual. Many infected cells have HLA-A/B expression levels that are only marginally higher than HLA-C, and for some cells, HLA-A is expressed at a level lower than HLA-C in the representative example shown (Fig. 1).

The cytometry method relied on comparisons between Abs that were not identical in affinity and that could not assess HLA-E. Therefore, we applied an MS-based method to differentiate and quantify the classical HLA class I molecules, as well as HLA-E. Cells from the single B-LCL or two normal PBL donors homozygous for the A*02:01, B*44:02, C*05:01 haplotype were analyzed. mAb W6/32, which binds all HLA class-I alleles equally (28), was used to immunoprecipitate total HLA from cell samples (Supplemental Fig. 2). Precipitated material was digested with trypsin and analyzed by LC-MS. Multiple peptides unique to each HLA H chain among the known HLA alleles present were monitored and quantified (Fig. 2). These peptides were selected from those detected in initial analyses of the B-LCL sample on the basis of high signal intensity, distance from miscleavage sites, low modification potential, and high LC performance. A tandem mass spectrum showing representative identification of one of the peptides specific to HLA-A in a PBL sample is shown (Fig. 3). Peptides were quantified relative to a heavy isotope–labeled synthetic peptide with the same sequence that was added to the biological samples (Supplemental Fig. 3) using a calibration plot generated for each peptide (Supplemental Fig. 4). Amounts of all peptides monitored for each HLA locus are shown for a representative analysis of one PBL donor (Table III). The median expression level indicated by all peptides monitored for a locus was taken as the overall measure for expression level at that locus. All peptides were measured in the single B-LCL and two normal PBL donors analyzed; the results are summarized in Table IV. In B-LCL, HLA-A was detected at 6 times the level of HLA-C, HLA-B was detected at 5 times the level of HLA-C, and HLA-C was observed at a level 17 times higher than HLA-E. The results were the same for the two normal PBL donors. HLA-A was detected at 5 times the level of HLA-C, HLA-B was detected at 4 times the level of HLA-C, and HLA-C was observed at a level 25 times higher than HLA-E.

Discussion

We report the relative expression levels of HLA-A, HLA-B, HLA-C, and HLA-E proteins measured for normal and HIV-infected primary cells. Two independent methods were used: flow cytometry and MS. On freshly isolated PBLs from normal donors, the HLA-A/B proteins were expressed at similar levels to each other, but relative to HLA-C, they were 13–18 times higher when measured by flow cytometry and 4–5 times higher when measured by MS. HLA-E was expressed at levels 25 times lower than HLA-C, as measured by MS. On HIV-infected cells, HLA-A and HLA-B were reduced by a magnitude that varied between cells in an infected culture. Some infected cells expressed lower HLA-A than HLA-C, but when averaging across all infected cells from an individual, HLA-A/B were between 1 and 5 times higher than HLA-C in measurements made using cytometry. Although we studied only one haplotype common in Whites, the differences between certain loci (e.g., HLA-B versus HLA-C) are large compared with the variation in the expression level of alleles that is seen at a single locus (13). Therefore, we expect that the observed magnitudes

Table IV. Relative expression levels of HLA class-I loci measured by MS

	Amount Detected (fmol)				Level Relative to HLA-C		
Sample Type	HLA-A	HLA-B	HLA-C	HLA-E	HLA-A	HLA-B	HLA-E
B cell line	2888	2759	521	32	6	5	0.06
Normal PBL (donor 1)	2905	2503	582	23	5	4	0.04
Normal PBL (donor 2)	3424	3137	726	31	5	4	0.04

Amounts detected are medians from the multiple peptides used to quantitate each HLA locus. For each donor and the cell line, expression levels shown are from a single representative experimental replicate. The ranges of expression relative to HLA-C in these replicates were B cell line, n = 8, HLA-A (5.5–6.2), HLA-B (4.8–5.6), HLA-E (0.06–0.07); PBL donor 1, n = 4, HLA-A (4.3–5.0), HLA-B (3.7–4.3), HLA-E (0.03–0.06); and PBL donor 2, n = 4, HLA-A (4.3–5.0), HLA-B (3.6–4.3), HLA-E (0.03–0.05).

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of differences in expression levels between HLA loci are broadly applicable across individuals.

Two PBL donors were analyzed and yielded almost identical results within each assay on normal cells. For example, HLA-B was detected at 4 times the level of HLA-C for both individuals by MS, and it was detected at 17–18 times the level of HLA-C for both individuals by flow cytometry. HLA-A/B expression levels on HIV-infected cells differed to some extent between the donors, likely as a consequence of variable in vitro infection efficiency between individuals. The cytometry and MS assays gave corresponding results, but the former estimated a greater difference between HLA-A/B versus HLA-C expression levels. Multiple differences between the two assays might account for this variation. Cytometry specifically detects surface HLA expression level, whereas the MS-based approach measures total cellular HLA. Characterization of the mAbs used in cytometry detected HLA molecules expressed in vitro, which may not wholly reflect the reactivity on primary cells. MS analyzed HLA purified by immunoprecipitation with mAb W6/32. This mAb binds the predominant functional conformation of HLA, H chain associated with β_2m and peptide, whereas the cytometry mAbs may also recognize unfolded conformations. Despite these caveats, the overall concordance observed with such different assays as cytometry and MS strengthens our confidence in the relative HLA expression levels reported.

Our results support the overall findings of Snary et al. (19) and address several of the limitations of that approach from 1977. A major limitation of their approach was that HLA H chains were quantified indirectly based on the β_2m signal from an elution fraction, but serial elutions from a lectin column did not completely isolate all proteins from different HLA loci into distinct fractions. In contrast, our quantification by cytometry and spectrometry was based on direct detection of the HLA class-I H chain proteins themselves. This was achieved by Ab specificity for each H chain in the case of cytometry and by quantitation of peptides unique to each HLA H chain by MS. Further, Snary et al. (19) imprecisely defined the magnitude by which HLA-A and HLA-B expression was estimated to exceed that of HLA-C, which could only be determined as “at least 10 times” because HLA-C was observed below the threshold for quantitation by β_2m detection. Our new measurements were able to quantify the low amount of HLA-C and, thus, define the absolute levels by which HLA-A/B are more highly expressed. HLA-A and HLA-B were detected at levels 13–18 times higher than HLA-C by flow cytometry and 4–5 times higher than HLA-C by MS using PBLs, consistent with the lower extreme of the estimate provided by Snary et al. (19).

Our findings extend the previous results in three significant ways: quantifying HLA-E expression level, measuring expression level on primary PBL for each of the HLA types studied, and measuring the levels on HIV-infected cells for classical HLA class-I specifically. mAbs can recognize HLA-E on transfectants, but precisely which Ags these mAbs bind on primary cells is not clear (20, 21); therefore, we avoided quantifying HLA-E by cytometry. HLA-E was identified and quantified by the MS-based assay and found to be nearly 20 times lower than HLA-C in B-LCL and 25 times lower than HLA-C in PBLs. Relative levels of the HLA loci for PBLs freshly isolated from normal donors were broadly similar to those for B-LCL. Primary CD4 T cells infected in vitro with HIV showed the expected reduction in specifically HLA-A and HLA-B (22, 25). The magnitude of this HLA-A/B reduction varied between infected cells in any given culture, which may correlate with the time since infection and accumulation of Nef in each cell. On average, HLA-A/B expression was still higher than HLA-C, but only by 1–5-fold on infected cells compared with 12–13-fold on uninfected cells from the same culture. Our results also confirm a previous observation made using HLA transfectants: Nef downregulates HLA-A by a greater amount compared with HLA-B (26), showing that this is also the case for infection of primary cells. The reduction in HLA-A/B that we observed was not as dramatic as that reported in the seminal studies defining Nef modulation of HLA (22, 25). These previous studies used HLA transfectants rather than primary cells and were designed to maximize the effects of Nef by using strains of HIV engineered to overexpress Nef. Consequently, the smaller magnitude of the HLA reduction that we see using primary cells infected with the HIV strain NL4-3 more likely represents the magnitude of HLA reduction by HIV in vivo. This is supported by the similarly modest magnitude of HLA-B*57 downregulation that was observed by infection of primary cells in vitro with HIV (43). The locus specificity and modest magnitude of HLA downregulation by HIV may be precisely selected for an optimal balance of reducing CTL response while minimizing the induction of responses by innate cells with inhibitory receptors for HLA.

The large differences in expression levels observed between HLA class-I loci are likely to be functionally significant. Higher HLA expression levels are known to more efficiently initiate CTL responses (13, 44) and modulate the cytokines that CTLs secrete (45). Differential expression levels of MHC class I loci have also been well characterized in chickens, and they were shown to associate strongly with the risk for Marek’s disease (46, 47), indicating that this phenomenon may be common across species. Further, some inhibitory receptors (such as LILRB1/2 and KIR3DL1) recognize Ags from multiple HLA loci, so allotype-specific expression levels may affect the innate-immune response, as well as the acquired-immune response. Given the accumulating data pointing to a significant impact of differential allotype-specific and locus-specific expression levels on the immune response, it is necessary to define this property for each HLA locus to determine its potential effect across human diseases.

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Disclosures

The authors have no financial conflicts of interest.

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