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Dendritic cells (DCs), macrophages (MPs), and monocytes are permissive to HIV. Whether they similarly process and present HIV epitopes to HIV-specific CD8 T cells is unknown despite the critical role of peptide processing and presentation for recognition and clearance of infected cells. Cytosolic peptides degrade endogenous proteins originating from self or pathogens, exogenous Ags preprocessed in endolysosomes, thus shaping the peptidome available for endoplasmic reticulum translocation, trimming, and MHC-I presentation. In this study, we compared the capacity of DCs, MPs, and monocyte cytosolic extracts to produce epitope precursors and epitopes. We showed differences in the proteolytic activities and expression levels of cytosolic proteases between monocyte-derived DCs and MPs and upon maturation with LPS, R848, and CL097, with mature MPs having the highest activities. Using cytosol as a source of proteases to degrade epitope-containing HIV peptides, we showed by mass spectrometry that the degradation patterns of long peptides and the kinetics and amount of antigenic peptides produced differed among DCs, MPs, and monocytes. Additionally, variable intracellular stability of HIV peptides prior to loading onto MHC may accentuate the differences in epitope availability for presentation by MHC-I between these subsets. Differences in peptide degradation led to 2- to 25-fold differences in the CTL responses elicited by the degradation peptides generated in DCs, MPs, and monocytes. Differences in Ag-processing activities between these subsets might lead to variations in the timing and efficiency of recognition of HIV-infected cells by CTLs and contribute to the unequal capacity of HIV-specific CTLs to control viral load. The Journal of Immunology, 2014, 193: 4322–4334.

Human immunodeficiency virus infects CD4-expressing cell subsets, CD4 T lymphocytes, monocytes, dendritic cells (DCs), and macrophages (MPs). Monocytes, MPs, and CD4 T cells can be productively infected, spread virus, and become viral reservoirs, whereas DCs do not sustain productive infection but transmit HIV to CD4 T cells and present HIV-derived Ags to prime HIV-specific CD4 and CD8 T cells (1, 2). All four cell types have the capacity to present MHC-I HIV epitopes to CD8 T cells (3–7). However, whether HIV epitope-specific CD8 T cells equally recognize all infected cell subsets is not known, despite their critical role in the clearance of HIV-infected cells.

HIV epotopes result from the intracellular degradation of proteins by the Ag-processing machinery (8). Cytosolic self and pathogen-derived proteins and pathogens are degraded into peptides of variable lengths by proteasomes (9), and to various extents by one or multiple cytosolic peptidases such as leucine aminopeptidase (LAP) (10, 11), thimet oligopeptidase (TOP) (12, 13), or tripeptidyl peptidase II (TPPII) (14, 15). After transfer into the endoplasmic reticulum, endoplasmic reticulum–resident aminopeptidases (ERAP)1 (16–18) and ERAP2 (19, 20) can further trim peptides before or after loading onto MHC-I complexes. Exogenous Ags, such as free or Ab-coated pathogens, can also be phagocytosed by target cells or by professional APCs and degraded into peptides by cathepsins in endo-lysosomes before transfer to the cytosol for further trimming and cross-presentation by MHC-I (8). Thus, the cytosol plays an important role in producing or destroying MHC-I epitopes in direct and cross-presentation pathways.

Differences in Ag-processing activities among HIV-infectable cell subsets may affect epitope production and presentation to CTL. We previously showed that CD4 T cells and monocytes present different levels of cytosolic peptidase activities that alter the kinetics and amount of antigenic peptides produced (21). Moreover, different virus-specific CTL responses were stimulated by mouse DC lines and fibroblasts infected with lymphocytic choriomeningitis virus (22), or by mouse DCs and lung MPs infected with Influenza (23), supporting the hypothesis that different cell types may present different epitopes. Higher lysosomal activities and subsequent higher degradation of Ags by MPs compared with DCs have been proposed to contribute to the inability of MPs to prime CTL responses due to insufficient peptide...
presentation (24). However, cytotoxic Ag-processing activities involved in degrading incoming HIV have not been systematically compared in these cell subsets.

Besides intrinsic differences in Ag-processing activities among cell subsets, external stimuli can alter the Ag-processing machinery. During pathogen infection, multiple components of the Ag-processing machinery, such as the immunoproteasome subunits (25), the proteasome activator PA28β complex, and aminopeptidases (26, 27), are induced by IFN-γ, which modifies the processing of various CTL epitopes (28–30). Bacteria, viruses, proinflammatory cytokines, CD40L, or TLR ligands such as LPS trigger maturation of DCs, alter proteasome composition (31–34), and, in a few cases, have been shown to alter the processing of a MHC-I peptide (28) and the cross-presentation of OVA (35). TLR7/8 agonists are encoded by HIV, and other synthetic TLR agonists used as adjuvants in HIV vaccination in animal models (36, 37) can affect T cell responses (38). Assessing the effect of TLR agonists on the production of HIV-derived epitopes is important to anticipate their impact on epitope production during vaccination.

In this study, we compared activities and expression levels of the cytotoxic Ag-processing machinery in monocyte-derived DCs and MPs upon maturation with TLR4 and TLR7/8 ligands. Subset-specific differences in protease and peptidase activities led to variations in the degradation patterns of HIV peptides, the kinetics and amount of HIV epitopes produced, and the antigenicity of the degradation products. These results suggest that variable display of HIV peptides by infected cell subsets may lead to differences in the capacity of various CTLs to reduce viral load independently of their intrinsic immune functions.

Materials and Methods

Study participants

Buffy coats from anonymous blood donors were purchased from the Massachusetts General Hospital Blood Bank and approved for use by the Partners Human Research Committee under protocol 2005P001218 (Boston, MA).

Cell culture and HIV infection

Human PBMCs were freshly isolated from buffy coats by Ficoll-Hypaque (Sigma-Aldrich) density centrifugation. Monocytes were enriched from PBMCs using CD14+ immunomagnetic isolation kits, according to the manufacturer’s instructions (StemCell). DCs were differentiated from monocytes during a 6-d culture at 10^6 cells/ml in AIM-V media (Invitrogen), supplemented with 1% HEPES (Sigma-Aldrich), 1% human serum AB (Gemini Bio-Products), 20 nm IL-4 (CellGenix), and 10 ng/ml GM-CSF (CellGenix). On days 2 and 4, fresh IL-4 and GM-CSF were added. MPs were differentiated from monocytes during a 6-d culture at 10^6 cells/ml in low attachment flasks (Fisher) in AIM-V media supplemented with 1% HEPES and 10% human serum AB, as previously described (39). On day 6, maturation of DCs and MPs was induced by TLR ligand stimulation with 2 μg/ml LPS, 1 μg/ml CL097, or 1 μg/ml digitonin permeabilization in ice-cold lysis buffer (50 mM HEPES, 50 mM potassium acetate, 5 mM MgCl2, 1 mM DTT, 1 mM ATP, 0.5 mM MgCl2; Enzo Life Sciences), aminopeptidases (cell, 5 μM; extracts, 12.5 μM H-Leu-AMC; Bachem), TOP (cell, 20 μM; extracts, 5 μM Mca-PLGPK-DNP; Bachem), and TTPPI (cell and extracts, 100 μM H-AAF-AMC; Bachem) were measured by cleavage of peptide-specific fluorogenic substrates. The specificity of reactions was checked by preincubating extracts or cells for 30 min with the relevant inhibitor before measuring the rate of fluorescence emission. Aliquots were taken at various time points, and the reaction was stopped with 2.5 μL 100% trifluoroacetic acid. Peptide fragments present in the digestion mix were purified by 20% TCA precipitation and diluted in RPMP without serum. The pH was readjusted to 7.4 with NaOH. 51Cr-labeled HLA-matched B cells were pulsed with 30–90 ng/ml of a given peptides at 37°C in 50 μL degradation buffer (50 mM Tris-HCl, 137 mM potassium acetate, 1 mM MgCl2, and 1 mM ATP, and 0.5 mM EDTA [pH 7.4]). The rate of fluorescence emission, which is proportional to the proteolytic activity, was measured every 5 min at 37°C in a Victor-3 Plate Reader (Perkin Elmer), as described previously (21, 42).

In vitro epitope degradation and antigenicity assays

Highly purified HIV peptides (>98% pure) were purchased from the Massachusetts General Hospital peptide core facility or from BioSynthesis. Primers were designed with 30–90 ng/ml of peptides at 37°C in 50 μL degradation buffer (50 mM Tris-HCl, 137 mM potassium acetate, 1 mM MgCl2, and 1 mM ATP [pH 7.4]), as described previously (41). Aliquots were taken at various time points, and the reaction was stopped with 2.5 μL 100% trifluoroacetic acid. Peptide fragments present in the digestion mix were purified by 20% TCA precipitation and diluted in RPMP without serum. The pH was readjusted to 7.4 with NaOH. 51Cr-labeled HLA-matched B cells were pulsed with the purified digestion products (diluted to 0.4 μg/ml for 5-ATK9-2 and 5-RK9-3, and 0.8 μg/ml for 13-QY9-6) for 30 min at 37°C in the absence of serum and used as targets in a 4-h 51Cr release assay with HLA-matched, epitope-specific CTL clones at a 4:1 E:T ratio. The lysis percentages were compared with the lysis of B cells pulsed with undigestid long peptides and optimal epitope titrations.

Mass spectrometry analysis of the degradation peptides

The identity of the peptides in the digestion mix was determined by in-house mass spectrometry analyses. Equal amounts of peptide degradation samples at different time points were injected into a Nano-HPLC (Eksigent) in line with an Orbitrap mass spectrometer (LTQ Orbitrap Discovery; Thermo) with a flow rate of 400 nL/min. A Nano EchipLc trap column (200 μm × 0.5 mm ChromXP c18-C5 5 μm 300A; Eksigent) was used to remove salts from samples, and peptides were separated on a Nano EchipLC column (75 μm × 15 mm ChromXP c18-C5 5 μm 300A; Eksigent) over a gradient of 2–40% buffer B (buffer A, 0.1% formic acid in water; buffer B, 0.1% formic acid in acetonitrile) and electrosprayed in the mass spectrometer. Mass spectra were recorded in the range of 370–2000 Da. In tandem mass
spectrometry mode, the eight most intense peaks were selected with a window of 1 Da and fragmented. The collision gas was helium, and the collision voltage was 35 V. Tandem mass spectrometry spectra were searched against custom-made source peptide databases with Sequest and Proteome Discoverer (version 1.3; Thermo Scientific). The integrated area under a peak generated by a given peptide is proportional to the abundance of that peptide. Each degradation time point was run on the mass spectrometer at least twice.

Cytosolic stability of optimal epitopes

A quantity amounting to 1 nmol highly purified peptide was degraded in 15 μg cytosolic extracts at 37°C in degradation buffer, as described previously (43). Aliquots were taken at 0, 10, 30, and 60 min, and the reaction was stopped with 2.5 μL 100% trifluoroacetic acid. The remaining peptide in the digestion mix at each time point was quantified by reversed-phase HPLC (RF-HPLC). One hundred percent represents the amount of peptide detected at time 0 calculated as the area under the peptide peak. A stability rate of each peptide was calculated by a nonlinear regression (one-phase exponential decay) of the degradation profile obtained over a 60-min incubation.

Statistical analysis

Spearman’s rank correlation coefficient was used to examine bivariate associations. The paired t test and Wilcoxon signed rank test were used to compare measurements between groups. All p values are two sided, and p values <0.05 were considered significant. In figures, p value criteria are assigned as *p < 0.05, **p < 0.01, and ***p < 0.001. Statistical analyses were conducted using GraphPad Prism (GraphPad Prism Software, La Jolla, CA) and Microsoft Excel.

Results

TLR-mediated maturation differentially alters cytosolic Ag-processing activities in DCs and MPs

We first analyzed the cytosolic Ag-processing activities in live, intact iDCs, mature monocyte-derived DCs, iMPs, and mature monocyte-derived MPs (mMPs). Maturation was achieved by addition of LPS (TLR4 agonist), CL097, or R848 (TLR7/8 agonists) for 2 d. Proteasomal catalytic subunits (caspase-like, tryptic, and chymotryptic) and postproteasomal activities (aminopeptidases, TPPII, and TOP) in live DCs and MPs were measured using protease-specific fluorogenic peptidic substrates, and fluorescence emission upon peptide hydrolysis was monitored over time, as previously done (21, 42, 43).

iMPs displayed 1.8-fold higher proteasome chymotryptic activity than iDCs (p = 0.0318), whereas proteasomal caspase-like and tryptic activities were comparable in both cell types. After LPS- or CL097-induced maturation, proteasome caspase-like, tryptic, and chymotryptic hydrolytic activities of MPs increased, whereas those in DCs decreased or remained unchanged, demonstrating significant differences for all three proteasomal activities between MPs and DCs (1.8-, 2.1-, and 1.5-fold higher in LPS-matured MPs than DCs, and 1.2-, 2.0-, and 2.1-fold higher in CL097-matured MPs, respectively) (Fig. 1A). Similarly, significant higher proteasomal hydrolytic activities were observed in R848-matured MPs compared with R848-matured DCs (Supplemental Fig. 2A). Aminopeptidases and TPPII hydrolytic activities were comparable in both cell subsets regardless of the maturation stage (Fig. 1B, Supplemental Fig. 2B). In contrast, iMPs and LPS-, CL097-, and R848-matured MPs displayed ~2-fold higher TOP activities than their DC counterparts (Fig. 1B, Supplemental Fig. 2B, 2C).

To compare how each TLR agonist alters cytosolic Ag-processing activities in DCs or MPs, we calculated a ratio of activities in mature cells over their immature counterpart (Fig. 1C). Both TLR4 and TLR7/8 stimulations increased proteasomal caspase-like, tryptic, and chymotryptic hydrolytic activities in MPs by 20–30%. In contrast, mature DCs showed decreased proteasomal activities upon maturation with a 30% lower caspase-like activity, and a 40–50% lower tryptic activity in LPS- and R848-matured DCs. In DCs, caspase-like, tryptic, and chymotryptic hydrolytic activities inversely correlated with the expression of maturation markers CD83 and CD86 after LPS- or R848-induced maturation [r_spearman (rs) = −0.4191, p = 0.042; rs = −0.4200, p = 0.029; and rs = −0.6018, p = 0.002, respectively], which supports decreased proteasomal activities upon maturation of DCs (Fig. 1D), as demonstrated by others in IFN-treated human primary DCs (44). To assess how TLR stimulation changes protease activities in maturing DCs, we measured proteasomal and aminopeptidase activities at 5, 24, and 48 h poststimulation with LPS or CL097 and calculated a ratio of activities in maturing cells over their immature counterpart at each time point (Fig. 1E, Supplemental Fig. 2D). In line with previous results, LPS- and CL097-maturing DCs showed 30% decreased proteasomal caspase-like and tryptic activities as early as 24 h poststimulation. Further maturation with both TLR ligands decreased proteasomal activities by 40% and tryptic activities up to 50% compared with immature cells. Chymotryptic activities were increased as early as 5 h upon maturation with LPS, but not affected by CL097 at any time point. Aminopeptidase hydrolytic activities were unchanged upon maturation with LPS or CL097 (Supplemental Fig. 2D). We next analyzed whether HIV infection of DCs and MPs affected Ag-processing activities. Single-round infection with vesicular stomatitis virus glycoprotein-pseudotyped lentivirus expressing HIV-1 NL4.3 without Env resulted in infection rates of 23.7 and 14.4% 6 d postinfection in MPs and DCs, respectively (data not shown). In line with results from TLR-matured cells, we detected 10–20% increased proteasomal caspase-like, tryptic, and chymotryptic hydrolytic activities in infected MPs. In contrast, infected DCs showed decreased proteasomal activities with 20% lower caspase-like activity and 30% lower tryptic activities. Aminopeptidase activities did not change upon infection of both cell subsets (Supplemental Fig. 2E, 2F). These results indicate that TLR-induced maturation triggered significant changes in proteasome and TOP activities of DCs and MPs in divergent ways, which corresponded to changes seen upon HIV infection.

Expression of cytosolic proteases involved in Ag processing is higher in MPs than in DCs

To examine whether the elevated hydrolytic activities observed in mMPs compared with mature monocyte-derived DCs were due to different levels of peptide expression, we analyzed cytosolic extracts by dual infrared fluorophore Western blotting, which allows for multiplex detection of signals over a wider quantifiable linear range than chemiluminescence (45) (Fig. 2A). The constitutive proteasome subunits responsible for caspase-like (β1), tryptic (β2), and chymotryptic activities (β5) in MPs were 326, 315, and 269% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A). The expression of immunoproteasome catalytic subunits β1i and β5i in MPs was 162 and 142% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A). The expression of immunoproteasome catalytic subunits β1i and β5i in MPs was 162 and 142% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A). The expression of immunoproteasome catalytic subunits β1i and β5i in MPs was 162 and 142% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A). The expression of immunoproteasome catalytic subunits β1i and β5i in MPs was 162 and 142% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A). The expression of immunoproteasome catalytic subunits β1i and β5i in MPs was 162 and 142% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A). The expression of immunoproteasome catalytic subunits β1i and β5i in MPs was 162 and 142% that of DCs, and showed similar differences upon LPS maturation (285, 266, and 242%, respectively) (Fig. 2B, Supplemental Fig. 3A).
FIGURE 1. Ag-processing activities in live intact human monocyte-derived DCs and MPs change differentially upon TLR-induced maturation. (A) Proteasomal caspase-like, tryptic, and chymotryptic activities were measured with peptidase-specific fluorogenic substrates in live iDC (□), iMP (■), mature monocyte-derived DC (mDC) LPS (○), mMP LPS (●), mDC CL097 (△), and mMP CL097 (▲). Results are from n > 10 healthy donors. (B) Postproteasomal aminopeptidase, TPPII, and TOP activities were measured in the same cells. Results are from n > 7 healthy donors. (A and B) Paired t tests (chymotryptic, aminopeptidases) and Wilcoxon signed rank test (caspase-like, tryptic, TPPII, TOP) were performed (*p < 0.05, **p < 0.01, ***p < 0.001), and error bars show SD. (C) Ratios of protease activities in TLR-matured over immature cells of the same donor were calculated and represented as mean ± SD for DCs (open bars) and MPs (solid bars). Results are from n > 10 healthy donors. (D) Proteasomal activities in iDC and mDCs were plotted versus the percentage of CD86⁺CD83⁺ DCs for each experiment. Surface expression was analyzed by flow cytometry. Comparison by Spearman test is indicated. n > 23 measurements. (E) Ratios of protease activities in TLR-maturing DCs over immature cells of the same donor were calculated and represented as mean ± SD at the indicated time points poststimulation. Results are from n = 2 healthy donors.
LPS-matured cells (148 and 194% in iMPs; 206 and 231% in LPS-matured mMPs for ERAP1 and ERAP2, respectively; Fig. 2B, Supplemental Fig. 3E). This analysis demonstrates that, regardless of cell maturation states and variability among donors, the higher expression of catalytic subunits of proteasomes, TOP, and aminopeptidases most likely contributes to elevated hydrolytic activities observed in MPs compared with DCs.

Cytosolic extracts of MPs produce more ATK9-containing antigenic peptides with faster kinetics than DC extracts

We aimed to determine whether higher expression and activities of the Ag-processing machinery in MPs altered the processing of HIV-derived epitopes. We previously developed an in vitro degradation assay to analyze epitope processing in cytosolic extracts (21, 41). A prerequisite to using this assay was to confirm that the differences in peptidase activities in live DCs and MPs were replicable in cytosolic extracts used for the epitope-processing assay. Using equal amounts of extracts normalized to actin levels, we observed that LPS-matured MPs had 5.4-, 6.5-, and 2.8-fold higher caspase-like, tryptic, and chymotryptic activities, respectively, compared with LPS-matured DCs ($p < 0.0001$). Similar trends were observed in extracts prepared from cells matured by CL097 and R848 (Fig. 3A, 3B). All three proteasomal activities measured in cells correlated significantly with their activities measured in extracts ($rs = 0.32$, $p = 0.002$, caspase-like; $rs = 0.30$, $p = 0.010$, tryptic; $rs = 0.23$, $p = 0.025$, chymotryptic; Fig. 3C), thus validating the use of cytosolic extracts to compare epitope processing in DCs and MPs.

We used cytosolic extracts from DCs and MPs to degrade a synthetic 16-mer peptide representing HIV-1 reverse transcriptase, 5-ATK9-2 (WKGSPAIFQSSMTKIL, aa 153–168), which contains the HLA-A03/A11–restricted ATK9 epitope (AIFQSSMTK, aa 158–166) (46). Degradation products were identified by mass spectrometry (21, 41, 42). The degradation of 5-ATK9-2 led to production of optimal ATK9 and N-extended ATK9 that could be further trimmed into ATK9, N- and C-extended ATK9, and also fragments lacking intact ATK9, called antitopes (Fig. 4A, Supplemental Fig. 4). MP extracts produced a large variety of N-extended ATK9 at each degradation time point, and optimal epitope ATK9 was detectable within 3 min in MP extracts, whereas it appeared after 10 min in DC extracts (Supplemental Fig. 4A, 4B). To assess and compare the production of all peptides over time in each cell subset, we calculated for each time

![FIGURE 2](http://www.jimmunol.org/)

The expression of the Ag-processing machinery is higher in MPs than in DCs. (A) Cytosolic extracts from immature, LPS-matured, or CL097-matured DCs and MPs were probed in Western blots for the expression of chaperone Hsp90; constitutive proteasome catalytic subunits $\beta_1$, $\beta_2$, and $\beta_5$; proteasomal core $\alpha_7$; lid S1; regulator PA28$\alpha$; and ERAP1, using actin as a loading control. (B) Signal intensities were normalized to actin and quantified for each cell type. The difference in protein expression levels in MPs over DCs from the same donor was calculated and represented as mean ± SEM. Proteasomal subunits (black bars), postproteasomal peptidases (gray bars), ERAP1/2, and cytosolic Hsp90 (white bars) are shown.
FIGURE 3. Changes in proteasomal activities in cell extracts reflect those observed in live intact DCs and MPs. (A) Proteasomal caspase-like, tryptic, and chymotryptic activities were measured in cytosolic extracts from iDC (□), iP (●), LPS-matured DC (○) and MP (●), CL097-matured DC (△), and MP (▲) using protease-specific fluorogenic substrates. Results are from n > 16 healthy donors. (B) Proteasomal caspase-like, tryptic, and chymotryptic activities were measured, as described in (A), including cytosolic extracts from R848-matured monocyte-derived DC (mDC) (○) and MP (●), CL097-matured DC (△), and MP (▲) using protease-specific fluorogenic substrates. Results are from n > 13 healthy donors and show mean ± SD. Paired t tests [caspase-like, tryptic (B), chymotryptic] and Wilcoxon signed rank test [tryptic (A)] were performed (*p < 0.05, **p < 0.01, ***p < 0.001). (C) The proteasomal hydrolytic activities measured in live intact immature or mature DCs (□) and MPs (○) were plotted against their activities in corresponding extracts. A partial correlation on Spearman ranked data was performed to control for cell type-dependent effects. n > 73 measurements.

The kinetics and amount of HIV optimal epitopes produced in cytosolic extracts from monocytes, DCs, and MPs vary among epitopes

To investigate whether differential peptidase activities in DCs and MPs impacted the processing of other HIV-1 epitopes, we next analyzed the processing of the following: 1) a 28-mer Nef-derived precursor peptide 13-QY9-6 (QVEEEVGFVPVPQRPMTYKAAVVDL, aa 60–87 in Nef), which contains HLA-B35–restricted QY9 and 16 other epitopes, and also 2) a 17-mer p17 fragment 5-RK9-3 (RWEKIRLPBGKKKYKL, aa 15–31) containing HLA-A03–restricted RK9 and 6 other epitopes (Fig. 5) (47).

MP and DC extracts similarly degraded 13-QY9-6 at a slow rate with 85% of the total peak intensity contributed by peptides longer than 26 aa after 10 min, whereas 10 min monocyte extracts yielded 60% of 8–25 aa that were later trimmed to 8–25-aa-long peptides (Fig. 5A). HLA-B35–restricted QY9 epitope (QVPLRPMTY, aa 73–81) was efficiently generated by all three of the cell types, with monocyte extracts producing significantly higher amounts (2.7-fold after 210 min) than DC and MP extracts (Fig. 5B). These differences in QY9 production were independently verified in a killing assay with QY9-specific CTL, in which the antigenicity of the degradation products measured by CTL-killing assay correlated strongly with the production of optimal ATK9 epitope detected by mass spectrometry (rs = 0.6068, p < 0.0001), providing additional validation of the quantification of epitope production by mass spectrometry (Fig. 4E). These results show that the processing of ATK9 epitope from HIV reverse transcriptase–derived precursor peptide, 5-ATK9-2, was faster and yielded more antigenic peptides in MP than in DC cytosolic extracts.

The proteasomal activities in cell extracts reflect those observed in live intact DCs and MPs.
maturation states induced by TLR4, 7, and 8 stimulation (data not shown). The antigenicity of the degradation products correlated strongly with the detection of optimal QY9 epitope by mass spectrometry (rs = 0.8329, p < 0.0001; results from two independent experiments) (Fig. 5D). In contrast to A11-ATK9, A03-QY9 was similarly produced in DCs and MPs. Differences in QY9 epitope production in monocytes, as compared with DCs or MPs, may be due to differences in activities of peptidases involved in QY9 production or destruction, such as aminopeptidases, which are significantly higher in monocytes than in DC and MP (data not shown).

We had previously shown that peptide degradation patterns and kinetics of epitope production contribute to immunodominance patterns in HIV infection (41). In PBMC or monocyte extracts, immunodominant HLA-A03 RK9 (RLRPGGKKK, aa 20–28) and RY10 (RLRPGGKKKY, aa 20–29 in p17) (48) are processed faster and accumulate, whereas subdominant overlapping HLA-A03 KK9 (KIRLRPGGK, aa 18–26) tends to be destroyed (41). Whether this hierarchy of epitope production exists in all cell subsets is not known. We used cytosolic extracts from monocytes, DCs, and MPs to degrade Gag p17 peptide fragment 5-RK9-3 containing the three overlapping CTL epitopes (KK9,
KIRLRGGK, aa 18–26; RK9, RLRPGGGKKK, aa 20–28; and RY10, RLRPGGGKKKY, aa 20–29) (Fig. 5E). Mass spectrometry detection of the fragments produced after 120 min of degradation was in line with our previous study (21), as more RK9-containing fragments were generated, compared with fragments containing KK9. In contrast, extracts from CL097-matured DCs and MPs produced more KK9-containing fragments than RK9-containing fragments (Fig. 5E). The higher aminopeptidase activities of monocytes may lead to frequent cleavages between KI or IR residues, destroying KK9 and preserving RK9, as we previously showed in cytosolic extracts from PBMCs, monocytes, and CD4 T cells (21). The differences in RK9 and KK9 production were independently verified in a killing assay using RK9-specific and KK9-specific CTLs. The 5-RK9-3

FIGURE 5. Extracts from monocytes, DCs, and MPs generate variable amounts of Nef- and p17-derived antigenic peptides at different rates. (A) A total of 2 nmol 13-QY9-6 (aa 60–87 in Nef) was degraded with 45 μg cytosolic extracts from monocytes, mature monocyte-derived DC (mDC) CL097, and mMP CL097 for 10, 120, and 210 min. Degradation products identified by mass spectrometry were grouped according to their lengths of fragments, as follows: longer than 26 aa (light gray), 19–25 aa (black), 13–18 aa (medium gray), 8–12 aa (dark gray), and fragments equal or shorter than 7 aa (white). The relative amount of fragments was defined as the intensity of all degradation products. (B) QY9 production in extracts from monocytes (○), mDC CL097 (△), and mMP CL097 (▲) detected by mass spectrometry. (C) The 13-QY9-6 degradation products were purified and pulsed onto HLA-B35 B cell targets. QY9-specific CTL responses were assessed by a 51Cr release assay. (A–C) Data are representative of three independent experiments with different healthy donors. (D) Mass spectrometry peak areas of peptide QY9 produced in extracts from monocytes (○), mDC CL097 (△), and mMP CL097 (▲) from two independent experiments were plotted against their respective target cell lysis values from the 51Cr release assay. Spearman correlation test was performed; n = 27 data points. (E) A total of 2 nmol 5-RK9-3 (aa 15–31 in Gag p17) was degraded in 90 μg cytosolic extracts from monocytes, CL097-matured DC, and MP for 10, 120, 210, and 360 min. Peptides encompassing both RK9 and KK9 epitopes (black bars), RK9 only (dark gray bars), KK9 only (light gray bars), or no epitopes (white bars) were identified by mass spectrometry. Optimal epitopes A03-RK9 (★), A03-KK9 (●), A03-RY10 (☆), and B27-IK9 (○) are indicated. The degradation profiles of 5-RK9-3 in extracts from monocytes (○), mDC CL097, and mMP CL097 are shown at 120 min. (F) The 5-RK9-3 degradation products in extracts from monocytes (○), CL097-matured DC (△), and MP (▲) were purified and pulsed onto HLA-A03 B cells, and specific CTL responses against A03-RK9 (top) and A03-KK9 (bottom) were measured by 51Cr release assay. (E and F) Data are representative of three independent experiments with extracts from different healthy donors.
degradation peptides in monocyte extracts resulted in significant higher target cell lysis by RK9-specific CTL than KK9-specific CTL (53 and 0% lysis by RK9- and KK9-specific CTL, respectively, at 120 min), whereas the opposite trend was observed for the degradation products of CL097-matured DC and MP extracts (17 and 37% lysis by KK9 CTL for DC and MP extracts; 0 and 9% lysis by RK9 CTL, respectively, at 120 min) (Fig. 5F). No significant differences were detected within DC or MP subsets upon LPS, CL097, or R848 maturation (data not shown). This suggests that, even for overlapping epitopes restricted by the same HLA, different infectable myeloid subsets may present variable ratios of epitopes, and therefore epitope-specific CTLs will be better suited to clear some subsets than others.

Fig. 6 summarizes peptide production for the 29 epitopes studied. Five epitopes (17%) were produced as optimal epitopes and 11 (38%) as N-extended epitopes in all three subsets, but only 5 epitopes were detected at the same time point in all subsets. Thus, due to heterogeneity in peptidase activities among subsets,

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**FIGURE 6.** Variable production of 29 HIV-1 epitopes in cytosolic extracts of monocytes, DCs, and MPs. The maps show the location of epitopes (arrows) within sequences 5-ATK9-2 (aa 153–168 in RT), 5-RK9-3 (aa 15–31 in Gag p17), and 13-QY9-6 (aa 60–87 in Nef). The tables show a summary of the relative amount of optimal epitopes and corresponding N-terminal extensions detected by mass spectrometry in the degradation products of 5-ATK9-2, 5-RK9-3, and 13-QY9-6 in monocytes, and mature monocyte-derived DC (mDC CL097 and mMP CL097 extracts after 10, 120, and 210 min of digestion. Numbers represent the contribution of optimal epitopes and N-extended epitopes to the total intensity of all degradation products at each time point. The presence of optimal epitopes is indicated (*). For each epitope, the data are representative of three mass spectrometry analyses from independent experiments.
peptides presented by HIV-infectable subsets may differ in their timing of presentation, amount, and length (optimal or extended epitopes), all of which could affect recognition by T cells.

**Intracellular stabilities of different HIV epitopes are highly variable but display similar hierarchies in DCs and MPs**

We previously showed that epitope stability in PBMC cytosol is highly variable among epitopes and contributes to defining the amount of epitopes present at the cell surface (43). To analyze the cytosolic epitope stability in extracts of DCs and MPs, we followed the degradation of three HLA-B57–restricted optimal epitopes located in p24 Gag (KF11, KAFSPEVIPMF, aa 30–40; ISW9, ISPRTLNAW, aa 15–23; TW10, TSTLQEQIGW, aa 108–117) (47, 49) in extracts of iDCs and iMPs. Using RP-HPLC analysis to follow the disappearance of each peptide (43), we showed that peptide ISW9 was rapidly degraded in extracts of both cell subsets with <50% epitope remaining after 10 min ($t_{1/2}$ 7 min in iDCs, 9.4 min in iMPs). In contrast, TW10 and KF11 were more stable with 87 and 60% epitope remaining after 10-min incubation in cytosolic extracts, which corresponds to cytosolic $t_{1/2}$ of 51.7 and 22.1 min in iDCs, and 49.8 and 20.4 min in iMPs, respectively (Fig. 7A). To rank epitopes, we calculated a stability rate as a nonlinear regression (one-phase exponential decay) of the degradation profile obtained over a 60-min reaction, as described in our previous study (43) (Fig. 7B). TW10, RK9, and ATK9 were more stable than KF11 and ISW9 in accordance with our previously published results in PBMC cytosolic extracts (43). Maturation of DCs and MPs with LPS or R848 did not affect the cytosolic $t_{1/2}$ of the epitopes tested (Fig. 7C, 7D). These results demonstrate that the respective intracellular stability of different optimal HIV epitopes is highly variable in DCs and MPs, but follows a specific hierarchy that is comparable in both subsets. The combined effects of differential epitope production and stability among various subsets are likely to be a major factor in the relative presentation of different epitopes by different cell types and to modulate epitope-specific CTL recognition of infected cell subsets.

**Discussion**

The capacity of cell subsets to degrade proteins into epitopes is likely to have an impact on the capacity of CD8 T cells to clear infected cells, but is less well defined than intrinsic differences in T cell functions. In this study, we uncovered differences in the capacity of DCs, MPs, and monocytes to degrade HIV proteins into epitopes.

We find that HIV-infectable cell subsets have heterogeneous epitope-processing activities, with MPs harboring the highest proteasome activities (specifically after maturation with TLR4 or 7/8 ligands), monocytes the highest aminopeptidase activities, and CD4 T cells the lowest cytosolic peptide activities (21, 42) (Fig. 1 and data not shown). Higher peptide activities were often linked to higher expression of peptides but could also be due to the expression of additional subset-specific peptides such as the monocyte-specific serine protease or other cell-specific peptides yet to be identified (50).

Differences in values of peptide activities between intact live cells and cell extracts may be due to variations in uptake of substrates between DCs and MPs, differences in ratio between peptidase and substrate between extracts and intracellular volumes of live cells, and the fact that proteasome activities measured in live cells include both cytosolic and nuclear proteasomes. However, despite differences in values, the alterations of peptide activities induced by TLR ligands and infection occurred similarly in live cells and extracts used for in vitro degradation.

The degradation of a protein into epitopes is a multistep process involving one or several proteasomes and peptidases. Some HIV epitopes are produced solely by the proteasome, whereas others require proteasome and ERAP, or proteasome, aminopeptidase and TPPII, TOP, or Nardylsin processing (12, 41, 42, 51, 52). We showed that the cleavability of epitope-flanking and intraepitopic motifs by various peptidases defines how efficiently an epitope is produced and destroyed, and is variable among epitopes located in the same protein (41–43). The substrate preference will define the requirement for specific peptides to make or degrade peptides.
and eventually determine whether epitopes may be equally or differently produced and presented among HIV-infectable subsets. The faster and higher production of the proteasome- and aminopeptidase-dependent epitope A11-ATK9 in MPs corresponds to higher proteasome and aminopeptidase activities measured in MPs compared with DCs. Because proteasomes and aminopeptidases are involved in the processing of most epitopes, the degradation patterns of peptides between DCs, MPs, and monocytes are likely to differ, as evidenced by the variable kinetics or amount of peptide produced for the 29 epitopes studied to date.

If all infected subsets display sufficient amount of peptides, they may be recognized and cleared by CD8 T cells, and this broader recognition of infected targets may contribute to the superior antiviral function of these T cells—indeed, of their polyfunctionality, avidity, and TCR (53–59). However, if peptide presentation by one subset is slower or below threshold of detection for a specific TCR, these cells may produce and propagate virus before immune recognition occurs. Due to low infectivity of DCs and insufficient amount of cells to sort infected cells, we were not able to directly compare the endogenous processing and presentation of HIV epitopes by DCs and MPs to CLT clones. However, the similar modulations of Ag-processing activities observed in HIV-infected and in TLR-stimulated DCs and MPs suggest that our observations are relevant to epitope presentation in the context of HIV infection.

The impact of differences in epitope production among infectable subsets will be in part determined by TCR avidity for a specific peptide, and also by the amount of peptides displayed by each infected cell. The expression of specific HLA alleles or allomorphs in HIV-infected persons has been correlated with spontaneous control or progression of HIV (60–63). Pereya et al. (64) have recently found that the course of infection is better predicted by a combination of the specificities of the epitopes recognized and the HLA type compared with HLA type alone. One possible explanation for this association—in addition to variability in T cell functions or peptide avidity—may be a better presentation of epitopes linked to control by all infectable subsets.

The cytosol is the place in which HIV enters or traffics before assembly and budding, and is critical for the degradation of incoming virions or newly produced proteins (4, 65). However, HIV may also be endocytosed and subjected to degradation by cathepsins (6, 65–69). MPs have higher cathepsin activities than DCs (J. Dinter and S. Le Gall, unpublished data). These differences in peptidase activities of the cross-presentation pathway may further contribute to differences in epitopes available for presentation and cross-presentation. Because many experimental vaccines deliver protein/peptide immunogens to endocytic compartments via viral vectors or nanoparticles, assessing how these vaccine peptides are degraded in the context of vaccination is necessary to ensure that the peptides produced and presented by HIV-infected cells are also presented by DCs after vaccination. DC subsets, such as Langherans cells, BDCAs1* DCs, or even monocyte-derived DCs receiving a vaccine against a given pathogen, prime T cell responses that may differ in specificity, magnitude, and phenotype (70–72). Further variations come from a combination of factors, including DC subsets, adjuvants (73), immunomodulating cytokines or TLR ligands included in the vaccine (36, 37, 74), variations in endolysosomal peptide activities among DC subsets (75, 76), and receptor-mediated targeting (72, 77–79). Approaches to HIV peptide immunogens include focus on conserved areas to induce immune pressure crippling HIV replication or covering the diversity of HIV through chimeras of variants (80, 81). In addition to sequence coverage of HIV and ways to induce sustainable polyfunctional responses, identifying HIV protein areas efficiently presented by all infected cells may help to improve vaccine design by expanding the recognition of target cells.

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Disclosures

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References


