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The main source for endogenous peptides presented by the MHC class I (MHC-I) pathway are de novo-synthesized proteins which are degraded via the ubiquitin proteasome pathway. Different MHC-I Ag pools can be distinguished: first, short-lived defective ribosomal products, which are degraded in concert with or shortly after their synthesis, and, second, functional proteins that enter the standard protein life cycle. To compare the contribution of these two Ag sources to the generation of MHC-I-presented peptides, we established murine cell lines which express as a model Ag the HIV-1 Gag polyprotein fused to ubiquitin (Ub) carrying the epitope SIINFEKL (SL). Gag was expressed either in its wild-type form (UbMGagSL) or as a variant UbRGagSL harboring an N-end rule degron signal. Although UbRGagSL displayed wild-type protein stability, its inherent defective ribosomal products rate observed after proteasome shutdown was increased concomitant with enhanced presentation of the SL epitope. In addition, UbRGagSL induces enhanced T cell stimulation of SL-specific B3Z hybridoma cells as measured in vitro and of adoptively transferred TCR-transgenic OT-I T cells in vivo. Furthermore, an elevated frequency of SL-specific T cells was detected by IFN-γ ELISPOT after immunization of naïve C57BL/6 mice with UbRGagSL/EL4 cells. These results further underline the role of the defective ribosomal product pathway in adaptive immunity.

Immune surveillance by CTLs represents a major mechanism for the detection and elimination of cells infected with intracellular pathogens, especially viruses. This task is accomplished by specific recognition of an antigenic peptide in complex with MHC class I (MHC-I) molecules on the cell surface by the TCR. Although MHC-I heterodimers may display peptides derived from exogenous Ags in a process called cross-presentation or cross-priming, the majority of MHC-I ligands are derived from the processing of virtually all endogenously and mostly de novo-synthesized proteins by the 26S proteasome (1, 2). The resulting peptides are translocated into the endoplasmic reticulum (ER) by TAP, with which they frequently undergo N-terminal trimming by luminal proteases, followed by loading onto nascent MHC-I molecules and transport to the cell surface (reviewed in Refs. 3 and 4).

The nature of the protein substrates that enter the MHC-I processing pathway has been an issue of intense investigation (5–9). It has now become widely accepted that newly synthesized and rapidly degraded polypeptides represent a significant source of MHC-I-restricted epitopes. These so-called defective ribosomal products (DRiPs) have been shown to constitute a large fraction of newly synthesized proteins (9). DRiPs do not attain their functional conformation due to errors in transcription, translation, or immediate posttranslational processes necessary for proper protein folding, subcellular targeting, or assembly into functional complexes (reviewed in Ref. 10). To prevent interference with cellular function, DRiPs are degraded irrespective of the half-life of their functional counterpart in a rapid process that occurs either immediately after translation or even cotranslationally by the ubiquitin proteasome system (UPS). The discovery of the DRiP pathway provides a reasonable explanation for the paradox of MHC-I determinants of stable viral structural proteins are presented on the cell surface for detection by CTLs instantaneously after infection of target cells (11). In addition, this concept gained support through the demonstration that protein de novo synthesis is essential for the efficient formation of MHC-I ligands as it was demonstrated by correlation of protein synthesis with TAP activity and export of MHC-I molecules from the ER (6, 7, 9). Furthermore, it has also been shown that the de novo synthesis of a stable viral Ag is essential for recognition of Ag-derived epitopes by specific CTLs (8).

Degradation of proteins via the UPS represents a tightly controlled process that is essential for the maintenance of cellular integrity and functionality (12). Specific amino acid sequence motives, so-called degron signals (13), have been shown to regulate degradation by the 26S proteasome. Some proteins with a metabolic half-life of 2 h or less contain regions rich in proline, glutamine, serine, and threonine,
which have therefore been named PEST sequences (14). Another way to attract proteins to the UPS includes the N-end rule which links the stability of a protein to the identity of its N-terminal amino acid (15, 16). Certain ubiquitin (Ub) ligases selectively target the so-called N-degron, consisting of a destabilizing N-terminal amino acid and internal lysine residues. Proteins exposing destabilizing N-terminal residues, like arginine, can be engineered by N-terminal in-frame fusions with Ub that is cotranslationally removed by Ub hydrolases allowing the generation of different N termini of otherwise identical proteins (17). When the C-terminal diglycine motif of Ub is mutated, cleavage of the isopeptide bond by Ub hydrolases is abolished and lysine residues within the Ub-moiety itself serve as attachment sites for poly-Ub chains, targeting the fusion protein for proteasomal degradation. This system is referred to as the Ub fusion degradation (UFD) pathway (18).

The correlation between Ag stability and generation of MHC-I antigenic determinants has been extensively studied (5, 6, 19–25). First, it has been demonstrated in a recombinant vaccinia virus (vVV) system that expression of an instable variant of the influenza A virus (IAV) nucleoprotein (NP) increased the efficiency in presentation of one particular epitope of NP (19), a finding that has subsequently been extended by others (5, 6). Recombinant Ub-X-galactosidase fusion proteins were differentially degraded according to the N-end rule in an Ub- and proteasome-dependent manner when introduced exogenously into cells and the instable counterparts of these fusion proteins evoked enhanced T cell activation (20). A correlation between protein half-life and generation of class I ligands has also been reported for the Listeria Ag p60 (21, 22). In contrast, no such correlation has been found for generation of the SIINFEKL (SL) epitope derived from instable OVA and MHC-I ligands has also been reported for the

**Materials and Methods**

**Plasmid constructs**

The generation of the expression plasmid psynag has been reported elsewhere (26). For the constructs pUbMsynag and pUbRsynag, ubiquitin was amplified from human genomic DNA using oligonucleotides Ub-1f (5'-ATG CAG ATC TTC TTG AAG ACC C-3') and Ub-1r (5'-ACC CCT CCA GGG CAG GAC C-3') and subcloned into pUC-Script. Using primers Ub_Af (5'-CGG TCA TTC TGC GCC ACC ATG CAG ATC TTC TTG AAG ACC C-3') and Ub_Br (5'-GCC CCT GCC CAT ACC CCC CCA GCG GAG CAC GAC-3') or Ub_Br (5'-GCC CCT GCC CAT ACC CCC CCA GCG GAG CAC GAC-3') and Ub_Dr (5'-gcc gtc ATC TAG ATC AGT TGC AGT GGG GCC CCG GC-3'); overlapping fragments of Ub and synag were generated, fused by PCR and cloned into pcDNA3.1 using EcoRI and XhoI sites. A sequence coding for the SL epitope, corresponding to aa 257–264 of OVA, was introduced by synag using a unique BsaEI site at position 1107 and oligonucleotides BsaEI-SL-fw (5'-GTT ACC TCG ATC ATC AAC TCT GAA AAG CTA-3') and BsaEI-SL-rc (5'-GTC ACT AGC TTT TTC AAG ATG ATC ATC GAG-3'). The SL epitope was also expressed from a minigene construct generated by inserting the SL coding sequence along with a start codon, a Kozak consensus sequence, and a double stop codon using oligonucleotides Hind-SL-Xho-fw (5'-AAG GCT CTT GCC CCC ACC ACC AGT ATG ATA ATC AAC TTC GAA AAA CTG TGA TAG C-3') and Hind-SL-Xho-rc (5'-CTC GAG CTA TCA CAG TTT TTC AAA GAT TAT ACT CAT GTG GGC GGC A-3') into the expression vector pcDNA3.1neo.

**Cell culture, transfection procedure, and generation of cell lines**

EL4 is a thymoma cell line derived from the C57BL/6 mouse (H2-Kb). The EL4-derived cell line E.G7 synthesizes and secretes OVA (27). Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM l-glutamine, 100 mg/ml streptomycin, 0.01% sodium pyruvate, and 0.1% nonessential amino acids (all from Invitrogen Life Technologies). To generate stable gag-expressing cell lines, EL4 cells were transfected by electroporation using a GenePulserXcell facility (Bio-Rad,) and settings as follows: exponential decay, 140 V, 950 μF in a 0.2-cm cuvette. After at least 3 wk of maintenance in complete RPMI 1640 medium containing 1 mg/ml G418 (Invitrogen Life Technologies), single-cell cloning by limiting dilution was performed. At least 3 wk, cell clones were analyzed by an indirect ELISA. Fifty microliters of cell suspension was transferred to a MaxiSorp plate (Nunc) and mixed with 50 μl of 100 mM Na2CO3 (pH 9.0) and 0.1% (v/v) Nonidet P-40. After overnight coating at 4°C, plates were washed three times with 0.1% (v/v) Tween 20 in PBS (PBST) and blocked with 5% (w/v) BSA in PBST for 2 h at room temperature. Gag was detected using a pooled serum of 20 HIV-1-positive patients (National Institutes of Health AIDS Research Reference Reagent Program, catalog no. 3975) diluted 1/5000 in FACS buffer (5% (v/v) FCS and 0.02% (v/v) NaN3 in PBS), HRP-conjugated anti-human IgG Abs (GE Healthcare) and tetramethylbenzidine substrate (1-Step Turbo TMB ELISA; Pierce). Positive clones were further analyzed by p24 Ag ELISA, Western blotting, and FACS double staining (see below). Generally, two rounds of single-cell cloning were sufficient to achieve stable clones.

p24 Ag ELISA

To quantify Gag expression, 1.0 × 10⁶ cells were lysed in 100 μl of CHAPS/deoxycholate (Doc) buffer (100 mM NaCl, 50 mM Tris·HCl (pH 8.0), 0.5% (w/v) CHAPS, 0.3% (w/v) sodium deoxycholate, 1 mM PMSF, 5 mM N-ethylmaleimide, 2 μM carbonbenzoxyl-leucine-leucine-leucinal (zLLL; Sigma-Aldrich) and complete protease inhibitor mixture (Boehringer Mannheim), and whole protein content was measured using the bichinchoninic acid assay (Pierce). In parallel, the p24 content was determined using a modified sandwich ELISA consisting of a coating Ab, an alkaline phosphatase-conjugated detection Ab (Aalto Bio Reagents) and CDP star substrate (Tropix). Deviating from the standard protocol, to avoid unspecific binding, the recombinant p24 standard was diluted in CHAPS/Doc extract from parental EL4 cells.

**Immunofluorescence microscopy**

For immunofluorescence analysis, 10,000 cells were centrifuged onto a slide (Superfrost; Roth) and fixed with methanol. After two washing steps in PBS, the fixed cells were stained using a FITC-conjugated anti-capsid (CA) Ab (KC57; Beckman Coulter) and 1 μg/ml 4′,6-diamidino-2-phenylindole. Intra-cellular p24 was visualized using a fluorescence microscope (Axiovert 135; Zeiss) equipped with an Apochromat ×63/1.40 oil objective.

**Western blotting**

Stably transfected EL4 cells were lysed in CHAPS/Doc buffer as described above and 10 μg of whole protein was separated by SDS-PAGE on a gradient gel (6–15%). Proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare) and Gag was detected using anti-p62 (28) and anti-p24 sera (Seramun) followed by standard ECL detection.

**Metabolic labeling**

For detection of Gag-DRIps, short-term pulse-chase experiments were performed, whereas long-term pulse-chase experiments were used to analyze protein stability. Briefly, for short-term pulse-chase experiments, transgenic EL4 cells were washed in PBS and treated with 25 μM each of zLLL and lactacytin (LC; Sigma-Aldrich) or DMSO as solvent control during the last 10 min of a 30-min starvation period in methionine (Met)-free,
serum-free RPMI 1640 (Invitrogen Life Technologies). Cells were radio-labeled for 15 min with 3 mCi/ml [35S]Met (Amersham Life Sciences) and chased for up to 120 min while shaking at 37°C in DMEM supplemented with 10% FCS and 10 mM Met in the presence or absence of proteasome inhibitors. Cells were harvested and lysed in 200 μl of CHAPS/Doc buffer for 5 min on ice and separated from the insoluble fraction by centrifugation at 20,000 × g for 10 min. Gag was recovered by immunoprecipitation using a mixture of polyclonal rabbit anti-p6 and anti-p24 Abs prebound to G-Sepharose (GE Healthcare). Samples were separated by SDS-PAGE on a 10% (w/v) acrylamide ProSiege gel (Cambrex Bioscience) backed with Gel Bond film (FMC Bioproducts). Following fixation for 1 h in 40% methanol and 10% acetic acid, gels were rinsed with water, soaked in 1 M sodium salicylic acid solution for 5 h, and dried. Radioactivity in gels was analyzed using a phosphor imaging instrumentation (Fuji film BAS-2000). For surface staining using Bio-Rad MR Films (Kodak) and quantified by AIDA imaging software (Raytest). For long-term pulse-chase experiments, after a 30-min starvation period, cells were radiolabeled with 3 mCi/ml [35S]Met for 30 min and then chased for up to 48 h. Analyses of radioactivity in cell lysates were performed as described above.

Flow cytometry

For detection of intracellular p24 and H2-K1-bound SL epitope, double staining using KC57-FITC and mAb 25D1.16 (29) was performed. The latter was purified from hybridoma cell supernatant using fast protein liquid chromatography and a 5-ml G protein column (both GE Healthcare). One milligram of the purified Ab was labeled with Alexa Fluor 647 (Molecular Probes). After surface staining with 25D1.16, cells were fixed and intracellular staining using KC57-FITC was performed in FACS buffer containing 1% saponin. Cells were fixed in 1% paraformaldehyde and flow cytometry was performed on a FACSCalibur using CellQuest software (BD Biosciences).

Acid wash

SL-H2-K1/complex formation at the cell surface was followed by flow cytometry after an acid wash procedure as follows: cells were incubated for 2 min at pH 3 and 4°C in buffer containing 131 mM sodium citrate and 66 mM potassium phosphate (pH 3.5) in 0.1% saponin. After acid wash, cells were incubated in complete RPMI 1640 medium in the presence or absence of 20 μM ZLLL or protein synthesis inhibitors (6.3 μM etodolac, 8.3 μM cycloheximide, and 1.7 μM puromycin; all purchased from Sigma-Aldrich) for desired periods at 37°C. At least 500,000 cells were incubated with supernatants of hybridomas 25D1.16, B8-24-3 (30), or medium alone as a control for 1 h on ice, followed by two washing steps in PBS and staining with Cy2-conjugated anti-mouse IgG (Rockland) diluted 1:200 in FACS buffer for 30 min on ice. Cells were fixed and analyzed by flow cytometry as described above.

T cell activation assay

The SL-H2-K1-specific, murine CD8+ T cell hybridoma B3Z express the lacZ reporter gene under the control of the NFAT enhancer (31, 32). B3Z cells were provided by Prof. Nilabh Shastri (University of California, Berkeley, CA). In 96-well plates, 50,000 B3Z cells were cocultured with parental or transgenic EL4 cells in an E:T ratio ranging from 1:10 to 1:0.013 in a total volume of 200 μl/well at 37°C for 16 h. Cells were washed once with PBS and lysed by addition of 100 μl 0.15 mM chloroform and 100 μl of 10% (v/v) Nonidet P-40 in PBS. After incubation for 4–6 h at 37°C, the absorbance was determined at 595 nm.

Adoptive transfer of OT-1 T cells

OT-1 mice (33), which express a transgenic TCR (Vα2/Vβ5) specific for SL, were received with permission of Dr. F. Carbone (University of Melbourne, Australia). Transgene status was confirmed by flow cytometry using a mAb specific for Vβ5 (BD Biosciences). Congenic C57BL/6 mice (Charles River Laboratories) were used as recipients. Spleen and lymph nodes (LN; cervical, axillary, brachial, inguinal, and mesenteric) from OT-1 mice were harvested, homogenized, and washed in PBS. After lysis of erythrocytes using 0.8% (w/v) NH4Cl for 5 min at 37°C, cells were passed over a 70-μm cell strainer (BD Biosciences). Cells were labeled with CFSE (Vybrant CFDA SE Kit; Molecular Probes) and transferred i.v. into the tail vein of naive C57BL/6 recipient mice (10 × 106 cells/mouse). Parental or transgenic EL4 cells (0.2 × 106 cells/mouse) were transferred 5 min later into the contralateral tail vein. Animals were sacrificed 2 or 3 days later, spleen cells were harvested and analyzed by flow cytometry. To analyze the OT-1 cell proliferation, OT-1 cells were identified within the live lymphocyte population by staining for the transgenic TCR α-chain Vα2 and CFSE levels were quantified.

IFN-γ ELISpot

Naive C57BL/6 mice were injected into the tail vein with parental EL4 or UbGagSL/EL4 cells. As a positive control, 50 μg of synthetic SL peptide dissolved in 50 μl of IFA were s.c. injected near the tail root. After 9 days, the mice were sacrificed, the spleen was removed, and an ELISpot assay was conducted to monitor the SL-specific T cell response. Briefly, splenocytes were collected under sterile conditions and erythrocytes were lysed using 0.8% (w/v) NH4Cl. Cells were plated in 96-well ELISpot plates precoated with rat anti-mouse IFN-γ Ab (Mabtech). Following washing, splenocytes (10 × 106 cells/ml) were added and cells were pulsed with SL peptide (100 ng/ml) or PHA and schistosome egg Ag mitogen (5 μg/ml) each, in 10 μl of RPMI 1640 medium supplemented with 10% (v/v) FCS. To control for unspecific reaction, cells were treated in parallel without peptide. All experiments were performed in triplicates. After incubation at 37°C in a 5% CO2 atmosphere for 20 h, cytokine production was detected by using biotinylated Ab against IFN-γ (5 μg/ml; Mabtech) and alkaline phosphatase-streptavidin (0.2 U/ml). The IFN-γ spots were developed by addition of 100 μl of 5-bromo-4-chloro-3-indolyl phosphate/NBT solution. Spots in dried plates were counted using computer-assisted image analysis with a Zeiss Axioplan 2 and VisionKS ELISPOT version 4.9.15. For analysis, the number of spots without peptide (unspecific reaction) was subtracted from the number of spots with peptide (specific reaction). Statistical analysis was conducted using a t test, assuming unequal variance. To control for variations in the frequencies of CD8+ cells in the spleens of individual mice, FACS analyses using anti-CD8a-FITC (Beckman Coulter) were performed.

Statistical analysis

All statistical analyses were performed using the InstStat 2.01 software and Student’s t test.

Results

Expression of the HIV-1 Pr55 Gag polyprotein in the murine T cell line EL4

Assuming the importance of DRIps as the major source of antigenic peptides, we set out to enhance the generation of Gag-derived epitopes for MHC-I presentation by targeting the Gag polyprotein Pr55 originating from the isolate HIV-1HX10 for rapid degradation by the 26S proteasome. In the background of HIV-1, the coexpression of the regulatory HIV-1 Rev protein is strictly required for efficient nuclear export of Gag-specific mRNA (34). For analysis of Gag independently of other HIV-1 proteins, a codon-optimized, synthetic gag gene was expressed under the control of the CMV promoter, yielding high and Rev-independent expression levels of Gag in various cell lines (26). Unfortunately, there have been no Abs available which recognize MHC-I-bound Gag-derived epitopes. As an indicator for Ag processing, a sequence coding for the SL epitope of OVA was introduced into the p2 spacer region of the gag gene (Fig. 1A). In previous studies, it was shown that introduction of degron signals caused up to 3-fold reduction of the half-life of HIV-1 Gag (23). In an attempt to extrapolate these results to our Gag variant, a variety of degron signals, including a C-terminal PEST sequence, an N-end rule motif, as well as an UFD signal were inserted at various positions into Gag. Most astonishingly, among all of the degron signals tested, none affected the metabolic half-life of Gag significantly as measured by Western blotting and metabolic pulse-chase kinetics (data not shown).

However, when EL4 cell populations stably transfected with our Gag expression constructs were analyzed for presentation of the SL epitope using the mAb 25D1.16 (29), which recognizes specifically H2-K1/-SL complexes, an enhanced SL presentation was routinely detectable on the surface of cells expressing Gag variants harboring certain degron signals. This was especially observed for the N-end rule substrate UbGagSL, which was further compared with its counterpart UbMGagSL, expressing N-terminally Met instead of destabilizing residue arginine (Fig. 1A). In contrast to the
human cell lines HeLa and 293T, in which relatively high expression levels of Gag were obtained after transient transfection, much lower expression of the GagSL proteins was achieved after transient transfection of the GagSL expression constructs into the murine thymoma cell line EL4 (data not shown). In an attempt to select for high-level Gag-expressing cells, EL4-derived clonal cell lines were generated which stably express the UbMGagSL and UbRGagSL proteins, respectively. After single-cell cloning and several passaging steps, two stable clones of transgenic EL4 cells were selected and used for the following set of experiments.

To determine and compare the intracellular steady-state level of Gag, a p24 ELISA was performed on cell extracts standardized for protein content. The level of Gag in the UbMGagSL/EL4 clone determined in the UbRGagSL/EL4 clone (1.59 ± 1.14 ng of p24/Mio cells) was almost twice the amount of Gag determined in the UbRGagSL/EL4 clone (1.14 ng of p24/Mio cells; Fig. 1A). This slight difference in Gag expression was routinely observed when transgenic EL4 cell lines expressing UbMGagSL and UbRGagSL were compared. Thus, it was conceivable to assume that the lower steady-state level of Gag in the UbRGagSL/EL4 clone represents an inherent characteristic of the N-end-ruled Gag substrate. Similar results were obtained by Western blot analysis using both, anti-p24 and anti-p6 Abs to visualize Gag proteins (Fig. 1B). Gag and GagSL proteins migrated at the appropriate molecular mass of ~55 kDa, confirming the complete cleavage of the Ub moiety. A slight difference in the molecular mass of Gag proteins reflects the presence or absence of the SL epitope. Consistent with the ELISA data, less Gag was observed for the R-Gag proteins irrespective of the presence of the SL epitope. In addition to the 55-kDa Gag signal, a second band migrating at ~40 kDa was observed that presumably originates from an internal translation event as was described for another Gag expression system (35).

To control whether functional Gag proteins were expressed and not targeted to the centrosome, as it was observed for similar Ub fusion variants of the HIV-1 accessory protein Nef (36), the intracellular distribution of Gag was analyzed by immunofluorescence. In both, the UbMGagSL/EL4 and the UbRGagSL/EL4 clones, Gag proteins were equally distributed throughout the cytoplasm (Fig. 1C). Transport of Gag to the cell membrane and budding of virus-like particles further support the notion that, after cleavage of the Ub moiety, a functional Gag protein was expressed similar to its viral counterpart (data not shown).

**Half-life and DR iP rate of UbRGagSL and UbMGagSL proteins**

Protein turnover can be characterized by two different parameters: first, the overall metabolic half-life, which describes the degradation rate of a functional protein in its standard life cycle following folding and assembly into operating complexes, and, second, the DR iP rate, which describes the fraction of de novo synthesized proteins that do not follow the quality criteria and are degraded instantaneously in concert with or shortly after translation (6).

By performing distinctly designed short- and long-term pulse-chase experiments in the presence or absence of proteasome inhibitors, we addressed the question in which of these characteristics the UbRGagSL and UbMGagSL proteins differ. The overall protein half-life was determined by pulse radiolabeling of EL4 cells with [35S]Met for 20 min followed by a 48-h long chase. [35S]Met Gag proteins that were recovered by precipitation using p24- and p6-specific Abs, subjected to SDS-PAGE, and visualized by fluorography displayed in both cases a half-life of ~6 h (Fig. 2A). In parallel, total cell lysates were resolved by SDS-PAGE and the major band corresponding to β-actin served as an internal control (Fig. 2A, lower panel). Nevertheless, the decay of Gag expressed in the UbRGagSL/EL4 clone was somewhat faster during the first 24 h of chase when compared with the UbMGagSL/EL4 clone. This was further supported by phosphor imager analysis of three independently conducted pulse-chase experiments (Fig. 2B), demonstrating that the overall half-life of Gag is not governed by the N-end rule, although in the case of UbRGagSL, the decay was to some extent enhanced in the first hours of chase, indicating that the slightly shorter half-life cycle of UbRGagSL might be affected by increased turnover occurring in concert with de novo synthesis.

Reasoning that DR iP s are extremely short-lived products which are at least partially ubiquitinated and degraded by the 26S proteasome, they can only be detected biochemically after shutdown of proteasome activity and blocking deubiquitinating enzyme activities (9). To determine the DR iP rate of UbM- and UbMGagSL proteins, short-term pulse-chase experiments were conducted according to previously elaborated DR iP pulse-chase protocols (9, 37). Gag-transgenic EL4 cells were treated during the final 10 min of a 30-min starvation period with a combination of distinctly acting proteasome inhibitors, the peptide aldehyde zLLL (1) and the irreversibly and highly specifically acting inhibitor LC (38). Cells were pulsed with [35S]Met for 15 min and chased for up to 120 min in the presence or absence of zLLL/LC. Soluble Gag proteins in the detergent cell extracts that bound to Gag-specific Abs were subjected to SDS-PAGE and analyzed by fluorography (Fig. 2C). The quantities of radioactivity in dried gels corresponding either to Pr55 or the total proteins migrating in the molecular mass range of ~60–250 kDa were measured using a phosphor imager (Fig. 2D).

Similar to the long-term pulse-chase (Fig. 2A), the decay of Gag

**FIGURE 1.** Characterization of EL4 cell lines expressing UbGag fusion proteins. A, Schematic representation of Gag proteins and quantification of Gag protein content of transgenic EL4 cell lines. HIV-1 Gag N-terminally fused in frame to Ub and harboring a SL-encoding sequence within the p2 linker region was expressed from a Rev-independent synthetic gag gene under the control of a CMV promoter. Gag protein content was measured using a p24 ELISA. Values are given as mean ± SD (n = 8). MA, Matrix; NC, nucleocapsid; amino acids are indicated in single-letter codes. B, Analysis of Gag expression by Western blotting. To examine Gag expression in UbM- and UbRGagSL/EL4 cells, similar amounts of whole cellular protein were subjected to SDS-PAGE and Western blotting using anti-p6 or anti-p24 antiserum. C, Intracellular localization of UbGagSL fusion proteins. The subcellular distribution of UbMGagSL and UbRGagSL was examined by immunocytochemistry using an anti-p24-FITC Ab.
was slightly faster in UbRGagSL/EL4 cells. Although data obtained in both cell lines with active proteasomes were comparable, the situation after proteasome shutdown was quite different: although addition of zLLL/LC rescued Gag proteins in both cultures from degradation, the amount of Pr55 recovered from UbRGagSL/EL4 cells increased by 50% immediately after the pulse, reaching maximum values of up to 80% increase within 15 min (Fig. 2D, upper right panel) compared with only a 30% increase of Pr55 recovered from UbMGagSL/EL4 cell extracts (Fig. 2D, upper left panel). The same effect was observed for the Gag fragment migrating at 40 kDa: the rate of recovery of which was again more pronounced in UbRGagSL/EL4 cells when compared with UbMGagSL/EL4 cells. In addition, an increase in the smear of proteins migrating in the molecular mass range of 60–250 kDa was detected in cells treated with zLLL/LC. This smear recovered by anti-Gag Abs was not recovered under the same conditions from nontransfected EL4 cells (data not shown), indicating that the smear represents polyubiquitinated Gag-DRiPs and not cellular proteins that bind nonspecifically to Gag-anti-Gag immune complexes. Similar to previous calculations in HIV-1-expressing cells (9), the radioactivity in this molecular mass range was taken into account for the estimation of the DRiP rates of both UbGagSL fusion proteins. Although treatment of UbMGagSL/EL4 cells with zLLL/LC resulted in modestly enhanced recovery of proteins in the molecular mass range of 60–250 kDa, the magnitude of increase was clearly higher in UbRGagSL/EL4 cells (Fig. 2D, lower panels), confirming results observed for Pr55. Similar to the kinetics of DRiP formation reported previously (9), the accumulation of labeled Gag proteins during the pulse and the first 15 min of chase is of transient nature (Fig. 2D). After reaching a certain plateau, processes like deubiquitination, proteolysis, or aggregation, as well as association with membranes (39), altogether continuously remove DRiPs from the pool of total Gag proteins accessible for immunoprecipitation. Thus, the results of the pulse-chase analyses demonstrate that the DRiP rate of the Gag

FIGURE 2. Pulse-chase analyses of UbMGagSL/EL4 and UbRGagSL/EL4 cells. A and B, Half-life of UbGagSL fusion proteins. For long-term pulse-chase experiments, UbMGagSL/EL4 and UbRGagSL/EL4 cells were radiolabeled for 20 min with [35S]Met and chased for up to 48 h. A, Fluorograph of Gag proteins recovered by immunoprecipitation using anti-p6 and anti-p24 Abs and separated by SDS-PAGE. The band corresponding to β-actin was identified based on its molecular mass in fluorographs of total cell lysates resolved by SDS-PAGE. B, Densitometric quantification of 35S-labeled Pr55. The radioactivity of the Pr55 band was quantitated using a phosphor imager and plotted as percentage of the initial signal. Values represent the mean and SD from three independent pulse-chase experiments. C and D, DRiP rate of UbGagSL fusion proteins. For short-term pulse-chase experiments, UbMGagSL/EL4 and UbRGagSL/EL4 cells treated with 20 μM zLLL/LC each during the final 10 min of a 30-min starvation were radiolabeled for 15 min and chased for up to 60 min the presence or absence of zLLL/LC. C, Fluorograph of Gag proteins recovered by immunoprecipitation and resolved by SDS-PAGE as described above. D, Densitometric quantification of 35S-labeled Pr55 (upper panels) and the high molecular mass smear recovered with anti-Gag Abs (lower panel). The radioactivity recovered at each time point is plotted as percentage of the radioactivity recovered at the time point 0 in the absence of proteasome inhibitors. Mean values and SD based on data from three independent pulse-chase experiments are depicted.
proteins clearly followed the N-end rule, although the N-terminal fusion of UbR to Gag does not result in a significant reduction of the overall metabolic half-life.

Correlation of DRiP rate with the efficiency of MHC-I presentation of Gag proteins

Although the stability of both Gag proteins was not governed by the N-end rule, an observation which is consistent with previous reports by others using different Gag expression systems (23), we observed an elevated DRiP rate of the UbRGagSL variant compared with UbMGagSL. To analyze whether UbRGagSL enters the MHC-I processing pathway more efficiently, the presentation of SL in complex with MHC-I molecules by flow cytometry was assessed using the fluorescently labeled mAb 25D1.16, which is specific for H2-Kb-bound SL (29). In parallel, the expression of Gag was monitored by intracellular staining with a CA-specific Ab. Parental EL4 cells and a cell clone expressing relatively high levels of the SL epitope from a minigene construct, called SL/EL4, served as negative and positive controls, respectively. Similar to previous studies using rVV expression systems (5, 40, 41), a CMV promoter construct that directs high-level expression of the sequence MSINFEKL was used to generate the stably transfected SL/EL4 clone. FACS analyses revealed that the cell surface presentation of the SL epitope generated from UbR-GagSL clearly surpasses the level of presentation observed for the UbMGagSL counterpart (Fig. 3A). However, consistent with the ELISA and Western blot data, the level of Gag in UbR-GagSL-expressing cells is about half of that observed in the UbMGagSL-transgenic cells as shown by double staining with mAb 25D1.16 and a CA-specific Ab (Fig. 3B). To further support the notion that the increased presentation of H2-Kb-SL complexes on the surface of UbR-GagSL/EL4 cells is not simply dependent on variations in expression levels of Gag, the presentation of H2-Kb-SL complexes was correlated with the intracellular amount of Gag in five independently performed double-staining experiments (Fig. 3C). The ratio of the mean fluorescence intensity (MFI) at 647 nm (staining with 25D1.16-Alexa Fluor 647) and the MFI at 488 nm (staining with anti-CA-FITC) was calculated demonstrating ~2-fold increase in H2-Kb-SL presentation (p = 0.019; Student’s t test).

If the main source of SL epitopes is generated from short-lived Gag-DRiPs, the processing of UbR-GagSL should lead to a higher intracellular steady-state level of the SL epitope compared with UbMGagSL. This should result in more efficient loading of empty H2-Kb molecules with SL epitopes. To challenge this assumption, cells were subjected to a standard acid wash procedure causing the dissociation of MHC-peptide complexes and subsequent decay of MHC class-I heterodimers on the cell surface (6, 42) (Fig. 4). The fate of the total H2-Kb molecules in both UbMGagSL/EL4 and UbR-GagSL/EL4 cell clones after the acid wash (time point 0) was comparable and revealed a reduction below 50% of the original value (time point ~10 min; Fig. 4A). After the acid wash, a slow, but continuous recovery of cell surface H2-Kb molecules was observed that followed comparable kinetics in both cell clones. Although both the steady-state levels and the kinetics of reloading of H2-Kb were almost identical in both cell clones, specific staining of SL-H2-Kb complexes revealed a different picture: first, and as observed already above (Fig. 3), the presentation of SL epitopes was ~2-fold higher in UbR-GagSL-processing cells (Fig. 4B, time point 0). Second, after acid wash that causes a drop to almost identical baseline levels (Fig. 4B, time point 0), the kinetics of recovery of SL-H2-Kb complexes on the cell surface was clearly faster in the UbR-GagSL/EL4 clone. According to previous studies that demonstrated the generation of epitopes from de novo-synthesized Ag of different origin in a proteasome-dependent manner (1, 5, 7–9, 43–45), control experiments using protein synthesis inhibitors or proteasome inhibitors (Fig. 4, C and D) revealed that both protein biosynthesis and the activity of the proteasome are necessary in both cell clones for the efficient presentation of H2-Kb molecules on the cell surface. This was shown for total H2-Kb (Fig. 4C) and SL-loaded H2-Kb molecules (Fig. 4D).

**FIGURE 3.** The UbR-GagSL/EL4 cell line presents more SL epitope on the cell surface compared with the UbMGagSL/EL4 cell line. Flow cytometry of UbMGagSL/EL4 and UbR-GagSL/EL4 cells double stained for intracellular Gag using a CA-specific mAb (KC57-FITC) and extracellular H2-Kb-bound SL (29). In parallel, the expression of Gag was monitored by intracellular staining with a CA-specific Ab. Parental EL4 cells and a cell clone expressing relatively high levels of the SL epitope from a minigene construct, called SL/EL4, served as negative and positive controls, respectively. Similar to previous studies using rVV expression systems (5, 40, 41), a CMV promoter construct that directs high-level expression of the sequence MSINFEKL was used to generate the stably transfected SL/EL4 clone. Flow cytometry revealed that the cell surface presentation of the SL epitope generated from UbR-GagSL clearly surpasses the level of presentation observed for the UbMGagSL counterpart. FACS analyses revealed that the cell surface presentation of the SL epitope generated from UbR-GagSL clearly surpasses the level of presentation observed for the UbMGagSL counterpart. If the main source of SL epitopes is generated from short-lived Gag-DRiPs, the processing of UbR-GagSL should lead to a higher intracellular steady-state level of the SL epitope compared with UbMGagSL. This should result in more efficient loading of empty H2-Kb molecules with SL epitopes. To challenge this assumption, cells were subjected to a standard acid wash procedure causing the dissociation of MHC-peptide complexes and subsequent decay of MHC class-I heterodimers on the cell surface (6, 42). The fate of the total H2-Kb molecules in both UbMGagSL/EL4 and UbR-GagSL/EL4 cell clones after the acid wash (time point 0) was comparable and revealed a reduction below 50% of the original value (time point ~10 min; Fig. 4A). After the acid wash, a slow, but continuous recovery of cell surface H2-Kb molecules was observed that followed comparable kinetics in both cell clones. Although both the steady-state levels and the kinetics of reloading of H2-Kb were almost identical in both cell clones, specific staining of SL-H2-Kb complexes revealed a different picture: first, and as observed already above (Fig. 3), the presentation of SL epitopes was ~2-fold higher in UbR-GagSL-processing cells (Fig. 4B, time point 0). Second, after acid wash that causes a drop to almost identical baseline levels (Fig. 4B, time point 0), the kinetics of recovery of SL-H2-Kb complexes on the cell surface was clearly faster in the UbR-GagSL/EL4 clone. According to previous studies that demonstrated the generation of epitopes from de novo-synthesized Ag of different origin in a proteasome-dependent manner (1, 5, 7–9, 43–45), control experiments using protein synthesis inhibitors or proteasome inhibitors (Fig. 4, C and D) revealed that both protein biosynthesis and the activity of the proteasome are necessary in both cell clones for the efficient presentation of H2-Kb molecules on the cell surface. This was shown for total H2-Kb (Fig. 4C) and SL-loaded H2-Kb molecules (Fig. 4D).

**In vitro activation of the SL-H2-Kb-specific T cell hybridoma B3Z**

So far, we have demonstrated more efficient SL presentation in UbR-GagSL-processing cells when compared with UbMGagSL-processing cells, although both clones differ only in the DRiP rate.
but not in the overall protein stability of Gag. To analyze whether the augmented SL presentation led to an enhanced T cell activation, we used the SL-H2-K\textsuperscript{b}-specific T cell line B3Z, which had been generated by fusion of the NFAT-lacZ T cell clone with a SL-specific V\textalpha\textsubscript{2}V\beta\textbeta T cell clone (46). Over

night cocultivation of B3Z cells with corresponding EL4 target cells, the specific T cell activation was detected by a colorimetric assay detecting TCR signaling. First, we established that the experimental conditions were adequate to detect specifically the antigen presentation of H2-K\textsuperscript{b}-SL complexes. Incubation of B3Z cells with increasing concentrations of the synthetic peptide SIINFEKL or a control peptide SIIKFEKL demonstrated that exogenously added epitopes are bound to cell surface H2-K\textsuperscript{b} molecules and presented to B3Z cells (Fig. 5A).

A linear correlation between peptide concentration and activation of B3Z cells was observed in the range of 0.3–30 nM of available peptides. Complete absence of T cell activation in the case of the control peptide confirms the high specificity of the B3Z line. In a further experiment, parental EL4 cells were incubated for 1 h with increasing concentrations of exogenously added peptides. After intensive washing, the cells were mixed with B3Z cells in an E:T ratio of 1:1 (Fig. 5B), revealing that even a short peptide pulse can induce T cell activation that started at 10 ng/ml added SL.

Thus, the B3Z cell line represents a very sensitive and specific indicator for SL presentation which is able to detect subtle differences in the presentation of H2-K\textsuperscript{b}-SL complexes. Incubation of B3Z cells with increasing concentrations of the synthetic peptide SIINFEKL or a control peptide SIIKFEKL demonstrated that exogenously added epitopes are bound to cell surface H2-K\textsuperscript{b} molecules and presented to B3Z cells (Fig. 5A).

In vivo activation of SL-H2-K\textsuperscript{b}-specific OT-1 cells and naive SL-specific T cells

To test whether the enhanced MHC-I presentation of SL epitopes generated from the UbR-GagSL variant also augments T cell stimulation in vivo, UbMGagSL/EL4 and UbR-GagSL/EL4 cells were adoptively transferred together with OT-1 splenocytes into naive C57BL/6 mice. The OT-1 mouse is transgenic for the SL-specific TCR (V\textalpha\textsubscript{2}/V\beta\textbeta), with approximately one-third of all splenocytes and LN cells representing naïve, SL-specific CD8\textsuperscript{+} T cells (47). As positive controls both, the OVA-expressing EL4-derived cell line E.G7 (27) as well as the minigene-expressing SL/EL4 cell clone (Fig. 3) were used, while the parental EL4 cells served as negative control. To quantify proliferation of OT-1 T cells, splenocytes and LN cells derived from OT-1 mice were labeled with CFSE before injection. This method is meant to track the number of cell divisions in vivo (48). To prevent target and effector cell encounter before injection and, in addition, to enhance the chance of interaction of both cell types in the recipient mouse, OT-1 cells were first injected into one tail vein and, after a delay of 5 min, the SL-presenting EL4 cells were injected into the contralateral vein. Two days after injection, the spleens of the recipient C57BL/6 mice were removed and the splenocytes were analyzed by flow cytometry. Among splenocytes the OT-1 cells were identified as V\textalpha\textsubscript{2}- and CFSE-positive cells (Fig. 6A, gate R3). In contrast to V\textalpha\textsubscript{2}-negative cells (Fig. 6A, gate R4), a certain fraction of the V\textalpha\textsubscript{2}-positive cell population showed clear evidence for proliferation, which was evident from a decaying CFSE fluorescence intensity. Analyzing V\textalpha\textsubscript{2}- and CFSE-positive cells derived from the UbR-GagSL/EL4 recipient revealed a relatively higher number of OT-1 cells that were primed and had started to proliferate compared with OT-1 cells derived from the UbMGagSL/EL4 recipient mice (Fig. 6A, lower panels). This notion was further supported by the downregulation of CD62L expression on the surface of V\textalpha\textsubscript{2}- and CFSE-positive cells, which is indicative of an activated T cell phenotype (data not shown). The statistical analysis of 10 independent experiments revealed that SL-expressing cells stimulate the V\textalpha\textsubscript{2}- and CFSE-positive population of the transferred spleen and LN cells to proliferate at different levels (Fig. 6B).

To compare differences in OT-1 T cell stimulation, we calculated the proliferation index which takes into account the number of proliferating cells relative to the number of cell divisions. The comparison of the proliferation index induced by UbR-GagSL/EL4 cells (0.85 ± 0.31) and UbMGagSL/EL4 cells (0.53 ± 0.25) revealed that UbR-GagSL induces a significantly stronger proliferation of adoptively transferred OT-1 T cells. Thus, the efficiency of SL presentation in UbR-GagSL/EL4 cells correlates with a better T cell activation in vivo as quantified on the level of T cell proliferation.
To further substantiate the notion that the increased DRiP rate and MHC-I Ag presentation correlate with a more efficient induction of a T cell response in vivo, naive C57BL/6 mice were injected i.v. with UbMGagSL/EL4 or UbRGagSL/EL4 cells, respectively, and activation of SL-specific T cells was quantified by IFN-γ ELISPOT. Parental EL4 cells served as negative control, whereas for positive control the SL peptide was injected s.c. Results shown in Fig. 7 reveal that higher frequencies of SL-specific T cells were induced by immunization with UbRGagSL/EL4 cells when compared to immunization with UbMGagSL/EL4 cells, indicating that, in naive mice, UbRGagSL/EL4 cells are more potent in activating not only transgenic OT-1 T cells, but also naive SL-specific T cells.

FIGURE 5. In vitro activation of the SL-H2-Kb-specific T cell line B3Z. A, B3Z cells were incubated with the synthetic peptides SL or SIIFKEKL in rising concentrations and activation of B3Z cells was assessed. B, Parental EL4 cells were incubated for 1 h with externally added peptides in various concentrations. After intensive washing, the cells were cocultured with B3Z cells in an E:T ratio of 1:1. C, Transgenic EL4 cells were cocultured with B3Z cells in an E:T ratio ranging from 1:10 to 1:0.0013 in 96-well plates for 16 h. In all assays, the T cell activation was analyzed by addition of 0.15 mM chlorophenyl red β-galactopyranosid/0.5% Nonidet P-40 in PBS and measuring the absorbance at 595 nm. AUFS, Arbitrary units full scale.

FIGURE 6. In vivo activation of OT-1 cells. A, Two days after adoptive cotransfer of UbM- and UbRGag/EL4 cells and CFSE-labeled, SL-H2-Kb-specific OT-1 cells into wt C57BL/6 mice, splenocytes were analyzed by flow cytometry. The CFSE signal of and Vα2-positive OT-1 cells in the live lymphocytes gate were analyzed. B, To calculate the proliferation stimulus of the transgenic EL4 cells, the number of cell divisions undergone by primed OT-1 cells was calculated. Therefore, the cell numbers of primed relative to naive Vα2-positive cells (nprimed/nnaive) was multiplied by the number of cell divisions (log₂(MFIprimed/MFINaive)). Values are given as mean ± SD (n = 10).

FIGURE 7. Quantification of SL-specific T cell response in mice after immunization with UbMGagSL/EL4 or UbRGagSL/EL4 cells by IFN-γ ELISPOT. Naive C57BL/6 mice were i.v. injected with UbMGagSL/EL4 or UbRGagSL/EL4, respectively. Parental EL4 cells served as negative, synthetic SL peptide injected s.c. as positive control. Splenocytes were isolated 9 days after immunization and incubated with or without synthetic SL peptide. Each circle represents the frequency of IFN-γ-secreting T cells from one individual mouse (mean of triplets). Mean values are depicted as bars (n = 14).
Discussion

Data presented in this study further support the general assumption that DRiPs constitute a major source for delivering endogenous peptides into the MHC-I Ag presentation pathway. In this work, we established two murine EL4-derived cell lines which express HIV-1 Gag exposing distinct N-terminal amino acids after cotranslational cleavage of the Ub fusion part. Although the N-end rule did not apply to Gag in our system inasmuch as both Gag proteins displayed a comparable metabolic half-life of ~6 h, the formation of DRiPs derived from the R-Gag variant was about twice the DRiP rate of wild-type (wt) M-Gag. Using the OVA-derived SL as an indicator epitope, it was shown that the intracellular steady-state level of Gag and the efficiency of SL presentation were inversely proportional: although UbRGGagSL/EL4 cells expressed less Gag, the number of H2-Kb-SL complexes on the cell surface and the kinetics of loading of empty H2-Kb molecules were elevated when compared with UbMGagSL/EL4 cells. In consistency with the enhanced Ag presentation, the UbRGGagSL/EL4 cell line also induced a better CD8+ T cell activation in vitro and in vivo.

Regardless of all of the efforts spent, there is a certain need to improve current strategies for protective or therapeutic vaccinations against HIV-1. Although high-frequency T cell responses can be observed in HIV-1-infected individuals, even in those with progressing disease (49), there is evidence that virus-specific CD8+ T cells display a key function in the immune control of virus spread (50). Even more, there has been cumulating evidence reported that indicate differences in the capability of virus-specific T cell clones to interfere with HIV-1 infection. Therefore, vaccine strategies aimed at the generation of potent virus-specific CTL response are now generally considered as a therapeutic option to interfere with disease progression subsequent to HIV infection (51), particularly as stimulation of sterilizing immunity by induction of neutralizing Abs has not been achieved so far (52). Most interestingly, it has been shown recently that an effective CTL response acting specifically against HIV-1 Gag, but not against other viral Ags, can be correlated with a significant reduction in viral load in HIV-1-infected patients (53). In addition, HIV-1 Gag can be considered as an interesting model Ag because it is exclusively present in the cytosolic compartment and thus fully accessible to the UPS and subsequent entry into the Ag processing pathway. Furthermore, principal biochemical procedures to measure the DRiP rate of Gag have been established (9). However, although a number of Gag-derived MHC-I epitopes are characterized, there is no specific Ab available to analyze the quantity of Gag-derived epitopes in complex with MHC-I molecules at the cell surface. To circumvent this problem we found that insertion of the standard model epitope SL into the polymorphic p2 spacer region of Gag (54) resulted in efficient processing and presentation of SL on the cell surface that did not interfere with virus budding and release of the respective Gag proteins (data not shown).

The validity of the N-end rule has been successfully demonstrated for a number of Ags derived from viruses and bacteria, like the IAV NP, the E. coli β-Gal, the Sindbis virus RNA polymerase, the Listeria p60 protein, or the HIV-1 accessory protein Nef (5, 19–21, 25, 55, 56). However, the N-end rule does not seem to apply ubiquitously for all proteins tested so far (23, 57). In agreement with others, we could not detect significant changes in protein half-life for the UbRGGagSL fusion protein (23). Even addition of a short lysine-rich leader sequence to a similar UbRGGag fusion protein resulted only in moderate destabilization of Gag, as shown by others (23). A possible explanation for the resistance of Gag to the N-end rule might be its inherent capacity to self-assemble after transport to virus-budding sites at the cell membrane. This might obscure the recognition of the N-end degron in the context of completely folded Gag during the formation of multi-Gag protein complexes of budding virus particles. In contrast, the destabilizing R-residue at the N terminus of the nascent protein should be better accessible to cognate Ub ligases during translation. As part of the N-end degron, accessible internal lysine residues are essential for Ub attachment and, thus, for targeting of the substrate for degragation by the 26S proteasome. Moreover, inconsistent reports about the validity of the N-end rule might be consequent to differences of proteasome subunit composition of the cell type used for expression of the model Ag driven by various expression systems. Although we can merely speculate about the underlying reason for the variability of the N-end rule, it seems reasonable that damaged proteins or polypeptides tagged for destruction by degron signals are removed as quickly as possible, preferentially during synthesis and thus affected by destabilizing N-end residues preferentially during the accessibility to the DRiP pathway.

Accumulation of polyubiquitinated Gag-DRiPs derived from UbRGGagSL occurred after proteasome inhibition, as it was also observed for HIV-1-expressing cells (9). Nevertheless, Ub-independent degradation by the proteasome has been described (58) and might also be regulated by destabilizing N-terminal residues, for instance by affecting the folding state of the nascent peptide chain.

Distinct to these previous observations of inaccessibility of HIV-1 Gag to the N-end rule in terms of overall protein half-life (23), we now demonstrate that the N-end rule regulates the DRiP rate and thus the Ag-processing rate of Gag. Wong et al. (23) already reported that rVV-mediated expression of subgenomic, altered, and highly turned over fragments of Gag resulted in increased numbers of SL-H2-Kb complexes presented at the cell surface. However, when both primary and memory CTL responses were compared in vaccination studies in mice using Gag-encoding plasmid DNA or rVVs, the stimulation of T cell responses in vivo did not correlate with the processing rate of those Gag variants. Mechanisms that regulate the entry into the DRiP pathway have been intensively investigated by others, and it is becoming increasingly clear that the efficiency in translation and protein folding strongly affect the DRiP rate of a given protein inasmuch the efficiency of protein folding is also critical for entry of a given Ag into the MHC-I processing pathway (45, 59, 60). Variations between full-length wt Gag studied here and fragments of Gag studied by others (23) might contribute to those differences. Impaired protein folding might be directly related to enhanced DRiP rate of a given Ag, and induction of mistaggregation by introduction of mutations into structured regions of the protein (59, 60) or chaperone inhibition (45) should support entry into the MHC-I pathway.

Rapid cotranslational degradation of a certain proportion of total Gag should slightly reduce the steady-state level of R-Gag that otherwise exhibits the same turnover rate during the posttranslational “second” half-life as wt M-Gag. This might explain why less Gag was observed in R-Gag-expressing cells on the steady-state level when compared with the M-Gag-expressing cells. If aged proteins that enter the MHC-1 pathway during their second half-life were the main source of antigenic peptides, this reduced availability in the second half-life should lead to less MHC-I presentation of Gag-derived epitopes. Nonetheless, we detected clearly more H2-Kb-SL complexes presented at the cell surface of the UbRGGagSL/EL4 cell line.

Importantly, the increase in MHC-I Ag presentation was accompanied by enhanced T cell activation and clearly less UbRGGagSL/EL4 cells compared with UbMGagSL/EL4 cells were necessary to achieve the same level of T cell activation, as demonstrated in vitro using the B3Z T cell hybridoma. Similarly, in the in vivo model of adoptive transfer, 2 × 10⁶ of both target cell lines UbRGGagSL/EL4...
and UbMGagSL/EL4 were sufficient to induce maximal stimulation of $1 \times 10^7$ cotransferred SL-specific OT-1 T cells. However, when the number of target cells was limited to $2 \times 10^5$, a clear difference in OT-1 T cell proliferation was observed. This indicates that for efficient stimulation of CD$^8^+$ T cells in vivo, limited availability of target cells can be compensated by a higher presentation rate of specific MHC-I complexes per target cell. A correlation between the amount of TCR ligands at the surface of professional APCs and the induction of a CTL response has been shown in other models (61–64).

The MHC-I pathway is considered to be a complex and multistep process. We analyzed the initial step, the synthesis and degradation of an Ag, and the final step, the amount of peptide-MHC-I complexes on the cell surface. Because the amount of total H2-K$^b$-molecules and the rate of reappearance after the acid wash procedure were similar in both EL4 cell lines, we assume that the H2-K$^b$ expression levels as well as the intermediate steps in the MHC-I pathway do not markedly differ between the two cell lines, whereas the differences in the proportion of H2-K$^b$ molecules loaded with SL epitope were consequent to disparities in the DRiP rate. Similar to previous studies, the generation of the SL epitope was dependent on proteasome activity and ongoing protein biosynthesis (1, 5, 7–9, 43–45), further supporting the notion that this phenomenon studied in our system is related to the DRiP pathway.

During the peptide loading of MHC-I molecules in the ER, a great number of peptides, derived from turnover of an estimated number of $2–3 \times 10^6$ cellular proteins, compete for the available number of binding grooves (65). Thus, an enhanced DRiP rate should result in an increased generation and steady-state level of antigenic peptides. DRiPs as a source for antigenic peptides may become especially crucial for efficient MHC-I Ag presentation in early stages of viral infection during the onset of synthesis of viral proteins, especially of stable, structural proteins like the retroviral Gag polyproteins (65, 66).

Several DNA vaccination studies, using rVV vectors or plasmids expressing an instable variant of HIV-I Nef, UbRNef, and an HIV-I envelope (Env) protein mistargeted for expression in the cytoplasm (55) as well as lymphocytic choriomeningitis virus NP targeted for degradation using an UFD signal (67) or N-end-ruled variants of (55) as well as lymphocytic choriomeningitis virus NP targeted for degradation using an UFD signal (67) or N-end-ruled variants of, were shown to increase the number of peptide/MHC complexes at the cell surface. Although CD$^8^+$ T cells generally exhibit an exceptionally high sensitivity for specific epitope/MHC-I complexes (76), enhancing Ag processing and thereby MHC-I Ag presentation might be advantageous in situations where low-avidity T cells encounter target cells with insufficient MHC-I presentation, e.g., virus-infected cells at an early stage of virus replication.

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References


