An Evaluation of Enforced Rapid Proteasomal Degradation as a Means of Enhancing Vaccine-Induced CTL Responses

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The HIV-1 Gag protein is an attractive target for CTL-based vaccine strategies because it shows less sequence variability than other HIV-1 proteins. In an attempt to increase the immunogenicity of HIV-1 Gag, we created Gag variants that were targeted to the proteasomal pathway for rapid degradation. This enhanced rate of degradation was associated with increased presentation of MHC class I-associated antigenic peptides on the cell surface. Despite this, immunizing mice with either plasmid DNA or recombinant vaccinia vectors expressing unstable Gag failed to produce significant increases in bulk CTL responses or Ag-specific production of IFN-γ by CD8+ T cells compared with mice immunized with stable forms of Gag. Production of IFN-γ by CD4+ T cells was also impaired, and we speculate that the abrogation of CD4+ T cell help was responsible for the impaired CTL response. These results suggest that vaccine strategies designed to increase the density of peptide-MHC class I complexes on the surfaces of APCs may not necessarily enhance immunogenicity with respect to CTL responses.

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It is unclear whether the enhancement of CTL responses observed in vitro assays of cytolytic function is of sufficient magnitude to warrant incorporation of this strategy into vaccines. It has been reported that targeting the hepatitis C virus core Ag for proteasomal degradation via the ubiquitin pathway does not enhance the Ag-specific CTL response (16). Recent studies suggest that while increasing epitope density generally primes a larger primary CTL response, excessive epitope density may not confer any additional benefit (17, 18). Careful studies by Bullock et al. (19) have uncovered a complex relationship between epitope density and the magnitude and avidity of primary, memory, and recall CTL responses. Importantly, they demonstrated that the induction of high avidity effectors was facilitated by priming with dendritic cells loaded with intermediate amounts of antigenic peptide. There is the additional concern that targeting an Ag into the class I pathway might reduce class II-restricted presentation and the provision of T cell help for the CTL response (20–22).

To evaluate the utility of degradation targeting strategies for enhancing vaccine-induced CTL responses, we have analyzed several different approaches for directing the HIV-1 Gag protein into the MHC class I Ag-processing pathway. Gag is an Ag of great interest because of its relatively high degree of conservation among HIV-1 isolates. Our results demonstrate that even when such strategies successfully increase the density of antigenic peptide-MHC complexes on the cell surface, the induction of enhanced CTL responses in vivo is not guaranteed and responses may in fact be blunted.

Materials and Methods

Plasmid construction

The ubiquitin fusion vector pUbMgag was constructed by amplifying ubiquitin from murine genomic DNA using the primers CCTGGTACCGC CACCATCGCAGTTCTCGTGAAAGACC and GACGCTGAGCCCAT ACCACGCAGGAAGCGAGACAGTCGAG. The resulting PCR product was digested with Acc651 and XhoI, then cloned into pGag (23) prepared by digestion with Acc65I and XhoI. The ubiquitin open reading frame (ORF) was then appended with a linker segment derived from the

Abbreviations used in this paper: ORF, open reading frame; DrhP, defective ribosomal initiation product; EGFP, enhanced GFP; UCH, ubiquitin C-terminal hydrolase.
The resulting PCR product was digested with AGCTCCCTGCTTGCCCATAC and ACCATGGGTGCTCGAGTCAG.–II and vector, pvMeK, was then appended with SIINFEKL epitope by PCR using CCTGGCTGTTGCCCGTCTCACTGGTG. The resulting Ub-M-eK–CTGTCGTGCC and GTCTCCGCGGTGGTATGCACGGCTCCGGCG/H11032 Escherichia coli strain XL1-blue (Stratagene, La Jolla, CA) using the primers encoding Gag aa 136. DNA amplification using the primers AGAA and ACGATAGCTAGCCTTGTCTAAAGCTTCCTTGGT. DNA amplification of the resulting vector was appended with the SIINFEKL epitope by PCR with pCI-PRE vector backbone (23) by transferring an NheI fragment of relevant vaccinia virus. Three days after vaccination, mice were challenged with 100 μl of HBSS (Invitrogen Life Technologies). Six days postvaccination, mice were homogenized by passage through a 70-µm mesh filter (Falcon). The resulting spleenocyte suspension was washed and resuspended in RBC lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.2), then washed twice and resuspended in RPMI-10. In DNA prime-vaccinia boost experiments, the mice were primed by i.m. immunization in each lower limb with 50 µg of plasmid DNA prepared using Endofree Plasmid Mega Kits (Qiagen, Valencia, CA) and diluted in a 1 l of HBSS (Invitrogen Life Technologies). Percent removal from each well and assayed on a Lumaplate using a TopCount solid-state scintillation counter (Packard Instrument, Meriden, CT). Percent removal from each well and assayed on a Lumaplate using a TopCount solid-state scintillation counter (Packard Instrument, Meriden, CT). Percent removal from each well and assayed on a Lumaplate using a TopCount solid-state scintillation counter (Packard Instrument, Meriden, CT). Percent removal from each well and assayed on a Lumaplate using a TopCount solid-state scintillation counter (Packard Instrument, Meriden, CT). Percent removal from each well and assayed on a Lumaplate using a TopCount solid-state scintillation counter (Packard Instrument, Meriden, CT).
Flow cytometry

Surface staining of vaccinia-infected M57G cells for H-2Kb/SLNFEKL complexes was accomplished using mAb 25-D1.16, a kind gift from R. Germain (National Institutes of Health, Bethesda, MD) (28). The cells were infected with vaccinia vectors encoding EGFP, Gag-SLNFEKL Ags, or the control vaccinia vector vSC8 at a multiplicity of infection of 3 for the times shown in Fig. 3. The infected cells were then incubated with 50 µl of 25-D1.16 hybridoma supernatant for 1 h on ice, washed, and stained with FITC-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA) for 30 min. For intracellular staining assays, 5 million splenocytes were stimulated by incubation with 10 µg/ml peptide or 5 µg/ml vSC8. For Gag p24 capsid protein (Protein Sciences, Meriden, CT) in RPMI-10 supplemented with 55 µM 2-ME (Invitrogen Life Technologies) at 37°C in a humidified incubator with 5% CO2. Peptide stimulation was performed for 4 h in the presence of Golgistop reagent, after which the cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA), according to the manufacturer’s instructions. Permeabilized splenocytes were stained with FITC-conjugated rat anti-mouse IFN-γ (BD Pharmingen; clone XMG1.2), PE-conjugated rat anti-mouse CD8a (BD Pharmingen; clone 53-6.7), and PerCP-conjugated Armenian hamster anti-mouse CD3e (BD Pharmingen; clone 145-2C11). Stimulation with HIV-1 p24 was performed overnight, after which Golgistop was added for an additional 3 h. The splenocytes were then fixed, permeabilized, and stained with FITC-conjugated rat anti-mouse IFN-γ together with PE-conjugated rat anti-mouse CD4 (BD Pharmingen; clone GK1.5). Stained cells were analyzed using a FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA).

Cytokine ELISA

Five million splenocytes were stimulated by overnight incubation with 5 µg/ml control baculovirus protein or HIV-1 p24 (Protein Sciences) in RPMI-10 supplemented with 55 µM 2-ME. Supernatant samples were collected and assayed for secreted IFN-γ in triplicate using a mouse IFN-γ ELISA kit, according to the manufacturer’s instructions (Pierce, Rockford, IL).

Detection of H-2Kb/SLNFEKL complexes by SLNFEKL-specific T cells

OT-1 mice on a Rag1−/− background were kindly provided by T. Shin (Pardoll lab, Johns Hopkins School of Medicine). These mice were immunized i.v. with 1 × 107 PFU of either recombinant vaccinia vector expressing the SLNFEKL epitope as a minigene (provided by J. Yewdell, National Institutes of Health), or control vaccinia vector vSC8. Three days later, the mice were sacrificed, and effector splenocytes were obtained, as described above. MC57G target cells were infected for 1 h at a multiplicity of infection of 10 with recombinant vaccinia vectors expressing the various Gag-SLNFEKL fusion proteins or with the control vaccinia vSC8. For intracellular staining of IFN-γ, 3 × 10^5 target cells were mixed with 3 × 10^6 effectors in a flat-bottom 96-well microtiter plate for 4 h in the presence of Golgistop. The cells were fixed and permeabilized, then stained with FITC-conjugated rat anti-mouse IFN-γ (BD Pharmingen), PE-conjugated rat anti-mouse CD8a (BD Pharmingen), and PerCP-conjugated Armenian hamster anti-mouse CD3e (BD Pharmingen), and analyzed by flow cytometry.

Results

Construction of rapidly degraded forms of HIV-1 Pr55gag

Our initial attempts at destabilizing Gag showed that the protein is remarkably resistant to a variety of degradation signals, including a PEST sequence (30), the p53 degradation box (31), the yeast cup9 ORF (32), fusion to a signal sequence minus form of HIV-1 Env protein (33), and destabilizing mutations in the matrix domain and nucleocapsid domains (26, 34) (data not shown). We found that the matrix mutant with a deletion in residues 42–56 had a t1/2 of ~2 h (Table I), which we deemed too long to be suitable for our studies.

Cellular ubiquitin C-terminal hydrolases (UCHs) are proteases that cleave polyubiquitin precursor proteins into individual ubiquitin fragments. Some proteins have displayed dramatically reduced t1/2 when expressed as fusion proteins with ubiquitin mutants resistant to UCH activity (25). However, ubiquitin-Gag fusion proteins rendered noncleavable by either ubiquitin G76A or Δ74–76 mutations failed to demonstrate significantly reduced intracellular t1/2 (Table I).

We also investigated the possibility of targeting Pr55gag for rapid intracellular degradation using the N-end rule pathway, which was first described by Varshavsky and colleagues (35, 36). It was observed that proteins with certain destabilizing amino acids at the N termini were targeted for rapid proteasomal destruction. Exposure of destabilizing N-terminal residues can be accomplished by making use of UCHs, which cleave immediately following the C-terminal Arg-Gly-Gly motif of ubiquitin, regardless of the identity of the amino acid that follows the motif (with the exception of proline, which is cleaved slowly). This feature of the UCHs is useful for exposing destabilizing N-terminal amino acids, such as arginine. We found that ubiquitin-arginine wild-type Gag that is cleaved by UCHs to reveal a destabilizing arginine residue at the N terminus of Gag did not have a significantly reduced t1/2 (Table I). For some proteins, a short leader encoding exposed lysine residues (eK) is required for efficient degradation by the N-end

![FIGURE 1. Pulse-chase analysis of Gag stability in cells infected with vaccinia vectors expressing various forms of Gag. MC57G cells were infected with the indicated vaccinia vectors for 16 h. Infected cells were labeled with [35S]Cys-Met mixture for 20 min, then chased with an excess of unlabeled Cys and Met for 0, 30, 60, or 120 min. Labeled cells were subjected to immunoprecipitation using a polyclonal anti-HIV-1 Gag Ab. The t1/2 of each protein species (in minutes) was calculated by densitometric analysis (Molecular Devices).](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Degradation Signal</th>
<th>t1/2 (hours)</th>
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<tbody>
<tr>
<td>Wild-type Gag</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Noncleavable Ub-Gag</td>
<td>6</td>
<td>Ubiquitin fusion rendered noncleavable by ubiquitin G76A or Δ74–76 mutation</td>
</tr>
<tr>
<td>ΔUb (Ubiquitin)</td>
<td>2</td>
<td>Destabilizing matrix mutant discovered by random mutagenesis</td>
</tr>
<tr>
<td>Ub-R-eK-wt Gag</td>
<td>6</td>
<td>Ubiquitin-Arginine (no linker sequence from luc inhibitor) fused to wild-type Gag</td>
</tr>
<tr>
<td>Ub-R-eK-wt Gag</td>
<td>2</td>
<td>Ubiquitin-Arginine-eK fused to wild-type Gag</td>
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rule pathway, presumably by providing suitable substrates for ubiquitin isopeptide side-chain attachment (35, 36). Addition of such a Ub-R-eK leader reduced the $t_{1/2}$ of Pr55\textsuperscript{gag} to 2 h (Table I). A combined strategy of placing the Ub-R-eK leader onto the \Delta42–56 matrix mutant (see above) resulted in a protein with a $t_{1/2}$ of 10 min (Fig. 1).

The Ub-dependent pathway plays an important role in degrading damaged, misfolded proteins (37–39). We reasoned that if the normal secondary structure of Pr55\textsuperscript{gag} were disrupted, the resulting protein might be recognized as defective and targeted for rapid degradation. To accomplish this disruption, we rearranged the order of four fragments of the Pr55\textsuperscript{gag} ORF to construct a novel shuffled gag ORF predicted to encode a 51-kDa protein. The selected fragments of Pr55\textsuperscript{gag} preserved segments of the protein that are rich in known human MHC class I and II epitopes (40). In pulse-chase experiments, the protein encoded by the shuffled gag ORF had a $t_{1/2}$ of 20 min, whereas wild-type Pr55\textsuperscript{gag} has a $t_{1/2}$ of ~6 h (Fig. 1). Use of the proteasome inhibitor lactacystin prevented rapid degradation of the shuffled Gag protein, suggesting that the shuffled protein was degraded via the proteasomal pathway (data not shown).

**MHC class I processing of stable and unstable Gag proteins**

To test whether the rapid degradation of Gag protein increased the rate or amount of presentation of peptide epitopes by MHC class I, we appended each of the constructs shown in Fig. 1 with the H-2K\textsuperscript{b}-restricted SIINFEKL epitope derived from chicken OVA.

![FIGURE 2](http://www.jimmunol.org/) Flow cytometric analysis of H-2K\textsuperscript{b}/SIINFEKL staining of MC57G cells infected with vaccinia vectors expressing various forms of Gag. **Left panel**, Fluorescence of MC57G cells infected with EGFP-expressing vaccinia over the indicated time course. **Right panel**, MC57G cells were infected with the indicated recombinant vaccinia vectors expressing various forms of Gag appended with SIINFEKL. At various time points, infected cells were probed for surface SIINFEKL/H-2K\textsuperscript{b} complexes using the mAb 25-D1.16 and FITC-conjugated goat anti-mouse IgG. For both panels, net fluorescence intensity at each time point was calculated by subtracting the fluorescence of cells infected with the control vaccinia vector vSC8. Data from one of two independent experiments are shown.

![FIGURE 3](http://www.jimmunol.org/) The response of T cells to SIINFEKL/H-2K\textsuperscript{b} complexes on the cell surface. OT-I mice on a Rag1\textsuperscript{−/-} background were used to generate naive or activated splenocytes by immunization with control vaccinia vSC8 or recombinant vaccinia expressing the SIINFEKL epitope as a minigene, respectively. MC57G target cells were infected with vSC8 or the indicated recombinant vaccinia expressing either stable or unstable Gag-SIINFEKL fusion proteins for 8 h. Target cells were subsequently added to OT-I effectors for an additional 4 h at an E:T ratio of 10:1. The cells were then fixed, permeabilized, stained, and analyzed by flow cytometry. The analysis was conducted on cells gated for expression of both CD3\textsuperscript{+} and CD8\textsuperscript{+}. The percentages reflect CD3\textsuperscript{+}CD8\textsuperscript{+} cells, which are also IFN-\gamma\textsuperscript{+}. Similar results were obtained using unimmunized mice to generate naive splenocytes. Data represent one of two similar experiments.
Germain and colleagues (28) have generated a mAb with a high affinity for the complex of SIINFEKL with H-2K\(^d\). We used this Ab to track the appearance of H-2K\(^d\)/SIINFEKL complexes on the cell surface, as an indicator of the rate and degree of MHC class I-restricted processing of the degradation-targeted constructs. FACS analysis of murine MC57G cells infected for various times with vaccinia vectors encoding SIINFEKL-tagged Gag proteins showed that the appearance of H-2K\(^d\)/SIINFEKL complexes proceeded with faster kinetics and reached higher steady state levels in cells infected with the unstable Gag constructs (Fig. 2, right panel). Using GFP as a reporter, the kinetics of insert protein synthesis by the vaccinia vector is demonstrated in the left panel of Fig. 2. The results obtained are consistent with the concept that targeting Gag for rapid degradation increases the rate at which it is processed for MHC class I presentation.

We wanted to demonstrate that this increase in the presentation of H-2K\(^d\)/SIINFEKL complexes on the cell surface could be distinguished by T cells. T cells from OT-I mice are transgenic for a TCR specific for H-2K\(^d\)/SIINFEKL complexes. We assayed the response of OT-I splenocytes to MC57G targets infected with recombinant vaccinia vectors expressing wild-type or rapidly degraded Gag tagged with the SIINFEKL epitope. By 10 h after infection with vaccinia vectors, levels of H-2K\(^d\)/SIINFEKL complexes on the surface of MC57G cells have reached a plateau (Fig. 2). We found that the IFN-\(\gamma\) response of naive OT-I splenocytes correlated directly with the amount of cell surface H-2K\(^d\)/SIINFEKL as assayed by staining with Ab (compare Figs. 2 and 3, upper panel). The increased amount of H-2K\(^d\)/SIINFEKL complexes generated on the cell surface by rapidly degraded Gag constructs tagged with SIINFEKL was associated with an increase in the proportion of IFN-\(\gamma\)-producing naive OT-I CD3\(^+\) CD8\(^+\) splenocytes in this assay. No such correlation was observed when the assay was repeated using activated H-2K\(^d\)/SIINFEKL-specific effectors derived from OT-I mice that had been previously immunized with recombinant vaccinia expressing the SIINFEKL epitope as a minigene (Fig. 3, lower panel). Taken together, these results demonstrate that the increased level of epitope expression resulting from enforced proteasomal degradation improves responses of naive CD8\(^+\) T cells, while activated T cells, which have a lower triggering threshold, do not exhibit increased responses in vitro.

**Primary CTL responses to stable and unstable Gag proteins**

The faster kinetics and higher overall levels of MHC class I processing of rapidly degraded Gag Ags might lead to an enhanced induction of CTL responses in mice vaccinated with vShuf or vUbRgag. To address this question, we examined the primary CTL responses of mice vaccinated i.v. with these vaccinia vectors. BALB/c mice were vaccinated with 3 \(\times\) 10\(^8\) PFUs of vGag, vShuf, vUbMgag, vUbRgag, or the control vaccinia vector vSC8. Six days after this vaccination, splenocytes were prepared for use in \(^{51}\)Cr release assays. By using relatively high E:T ratios and long (10- to 12-h) incubation periods, the induction of CTL in a primary immune response can be directly measured without an in vitro stimulation (6). Fig. 4 shows the net specific lysis of \(^{51}\)Cr-labeled P815 target cells pulsed with the P7G Gag peptide is shown.
FIGURE 5. Continues
We also used a plasmid DNA prime/vaccinia challenge strategy to examine the memory responses of C57BL/6 mice to the H-2K\(^b\)-binding SIINFEKL peptide appended to the C terminus of each of the Gag species. As shown in Fig. 2, this peptide is processed for MHC class I presentation with faster kinetics when appended to the unstable Gag species encoded by vShuf and vUbRgag. Despite the faster processing kinetics, CTL responses to the SIINFEKL epitope were blunted in mice challenged with the vaccinia viruses vShuf or vUbRgag (Fig. 6A). The same result was obtained when splenocytes were stimulated with SIINFEKL peptide and analyzed for IFN-\(\gamma\) production by CD3\(^+\)CD8\(^+\) cells (Fig. 6B). Thus, the failure of enforced rapid degradation to enhance Gag-specific CTL responses was observed with different epitopes, including one for which enhanced processing was directly demonstrated in vitro. It is interesting to note that the Gag construct with the shortest \(t_{1/2}\), UbRgag, also exhibited the most drastic reduction in SIINFEKL-specific CTL activity and IFN-\(\gamma\) production (compare Figs. 1 and 6, A or B). This was associated with a decrease in the CD4\(^+\) response to rGag p24 (Fig. 6C).

Finally, we examined the efficiency of CTL priming in mice vaccinated with plasmid constructs encoding stable or unstable forms of Gag. For these experiments, BALB/c mice were first vaccinated with plasmids pUbMgag, pUbRgag, or a plasmid encoding irrelevant Ag (pCAT). The mice were then challenged i.v. with \(3 \times 10^6\) PFUs of a vaccinia vector expressing a stable form of Gag (vGag). Mice primed with plasmid DNA expressing an unstable form of Gag (pUbRgag) generated much less Gag-specific CTL activity than mice primed with a stable form of Gag (Fig. 7A). Similar results were obtained when P7G peptide was used to stimulate splenocytes that were then assayed for IFN-\(\gamma\) production by flow cytometry (Fig. 7B). We also analyzed the production of IFN-\(\gamma\) by CD4\(^+\) splenocytes stimulated with HIV-1 p24 and obtained much weaker Ag-specific responses in mice primed with plasmid expressing unstable Gag (data not shown). Because the frequency of CD4\(^+\) IFN-\(\gamma\)\(^+\) cells was low, particularly in mice primed with pUbRgag, we decided to assay the culture supernatant of p24-stimulated splenocytes for IFN-\(\gamma\) by ELISA. Splenocytes from mice primed with unstable Gag secreted less IFN-\(\gamma\) than cells from mice primed with stable Gag (Fig. 7C). Thus, the decreased response to unstable Gag was observed both at the initial priming stage as well as the subsequent memory phase of the response.

**Discussion**

Successful attempts to increase the induction of Ag-specific CTL by forcing rapid degradation of the vaccine Ag have been reported by several groups (4–12). We show in this study that even when enhanced processing and presentation of a vaccine Ag can be directly demonstrated, this does not necessarily result in increased immunogenicity. Two different rapidly degraded forms of the HIV-1 Gag protein failed to show significantly increased immunogenicity in mice following delivery by DNA vaccination or by vaccinia vectors. The degradation targeting strategies used in this study clearly increased the rate of appearance and steady state levels of antigenic peptide/MHC class I complexes on the surfaces of APCs. The failure of these strategies to enhance CTL induction in vivo provides an important caveat to this approach for enhancing immunity.

We have considered several potential explanations for the failure of enforced rapid degradation to enhance Gag-specific CTL responses in vivo. Recent studies have suggested that defective ribosomal initiation products (DRiPs) may be a major source of antigenic peptides presented in association with MHC class I (3, 42, 43). One prediction of the proposition that DRiPs are a principal source of antigenic MHC class I peptides is that the targeting of fully translated proteins to the MHC class I processing pathway should result in only modest increases in the levels of antigenic peptide. However, when mature Gag-SIINFEKL fusion protein was targeted for rapid intracellular destruction, we observed substantial increases in the levels of MHC class I/SIINFEKL complexes on the surface of cells expressing the Ag (Fig. 2). Although details concerning the biogenesis of DRiPs remain unclear, it is assumed that they arise when translation fails to produce a full-length, correctly folded protein, producing instead a defective protein product that is subject to rapid destruction by the proteasome. It is generally thought that the majority of ribosomes scan from the 5’ cap of a given mRNA, then translate a single ORF. It is thus conceivable that DRiP activity is focused around reading frames nearer the 5’ end of an mRNA. Because the SIINFEKL epitope we have studied in this work was appended to the extreme C terminus of Gag, it is possible that the epitope resides in a portion of the mRNA that is relatively free from DRiP activity. It would thus be interesting to examine the MHC class I processing of epitopes located closer to the 5’ end of rapidly degraded Ags.

During the primary response, the priming of CTLs has been demonstrated to proceed in a graded fashion with increasing levels of epitope density (17, 18, 44). However, excessive levels of epitope may not prime larger numbers of functional Ag-specific CTLs as measured by bulk CTL activity or by intracellular staining for IFN-\(\gamma\) at the peak of the primary response ~1 wk after immunization (17, 18). In agreement with these previous findings, the immunization of mice with recombinant vaccinia expressing unstable Gag constructs that gave rise to higher levels of peptide-MHC class I complexes on the cell surface failed to generate larger primary CTL responses than control vaccinia expressing stable Gag (compare Figs. 2 and 4). The priming of naive T cells with supraoptimal epitope levels also appears to have consequences for the resultant memory cells. Bullock et al. (19) reported that priming with excessive levels of epitope generated memory CTLs of lower avidity. Furthermore, Wherry et al. (45) demonstrated that priming T cells in the presence of excessive levels of epitope subsequently led to a gradual reduction in numbers of functional antigenic-specific memory cells. This result was obtained using a model of vaccinia immunization that controls for the dose of total Ag available to other components of the immune system. We did not evaluate the long-term effects of immunizing mice with vaccinia expressing unstable Gag. However, it is interesting to note that in mice previously primed with plasmid DNA encoding stable Gag, eliciting a memory response by boosting with vaccinia expressing unstable Gag giving higher than normal levels of surface peptide.

**FIGURE 5.** Memory responses of BALB/c mice primed with plasmid pGag, then challenged with various vaccinia constructs. Mice were primed with plasmid DNA expressing stable Gag and allowed to rest for 2-5 wk, and the memory response was assessed 3 days following challenge with vSC8 or recombinant vaccinia expressing stable or unstable forms of Gag using ex vivo assays without further effector cell restimulation. A, Standard 4-h \(^{3}Cr\) release assay using P7G-loaded P815 cells as targets. The background lysis of P815 targets has been subtracted. B, Intracellular staining for IFN-\(\gamma\) production in splenocytes stained with P7G peptide. Cells were gated for expression of both CD3e and CD8a. The percentages refer to CD3e\(^+\)CD8a\(^-\) cells, which are also IFN-\(\gamma\)\(^-\). C, Intracellular staining for IFN-\(\gamma\) production in splenocytes stimulated overnight in the presence of rHIV-1 p24. The percentages refer to CD4\(^+\) cells, which are also IFN-\(\gamma\)\(^-\). Data from one of two independent experiments are shown.
FIGURE 6. Memory responses of C57BL/6 mice primed with pGag, then challenged with various vaccinia constructs. Mice were primed with plasmid DNA expressing stable Gag and allowed to rest for 2–5 wk, and the memory response was assessed 3 days following challenge with vSC8 or recombinant vaccinia expressing stable or unstable forms of Gag-SIINFEKL fusion proteins using ex vivo assays without further restimulation of effectors. A, Standard 4-h 51Cr release assay using SIINFEKL-loaded MC57G cells as targets. The background lysis of MC57G targets has been subtracted. B, Intracellular staining for IFN-γ production in splenocytes stimulated for 4 h in the presence of SIINFEKL peptide. Cells were gated for expression of both CD3e and CD8a. The percentages refer to CD3+CD8+ cells, which are also IFN-γ+. C, Intracellular staining for IFN-γ production in splenocytes stimulated overnight in the presence of rHIV-1 p24. The percentages refer to CD4+ cells, which are also IFN-γ+. Data from one of two independent experiments are depicted.
FIGURE 7. Memory responses of BALB/c mice primed with plasmid DNA expressing stable or unstable Gag. Mice were primed with pUbMgag, pUbRgag, or plasmid DNA encoding irrelevant Ag (pCAT) and allowed to rest for 2–5 wk, and the memory response was assessed 3 days following challenge with recombinant vaccinia expressing stable Gag (vGag) using ex vivo assays without further restimulation of effectors. A, Standard 4-h 51Cr release assay using P815 cells stably expressing Gag-EGFP fusion protein as targets. The background lysis of P815 targets has been subtracted. B, Intracellular staining for IFN-γ production in splenocytes stimulated for 4 h with P7G peptide. Cells were gated for expression of both CD3ε and CD8α. The percentages refer to CD3ε−CD8α+ cells, which are also IFN-γ+. C, ELISA for IFN-γ in the supernatant of splenocytes cultured overnight in the presence of rHIV-1 p24 or control baculovirus protein. The error bars indicate values for ±1 SD. Data representative of three independent experiments are depicted.
peptide-MHC class I complexes also led to a reduction in functional CTL activity (Figs. 5 and 6).

The poor CD8$^{+}$T responses to rapidly degraded forms of Gag may be due to their failure to elicit strong CD4$^{+}$ T cell responses. It is possible that targeting Ags to the MHC class I pathway may reduce the availability of the Ag for endosomal MHC class II processing and presentation to CD4$^{+}$ T cells. CD4$^{+}$ T cells provide help for CTLs by secreting cytokines such as IL-2 (46) and by delivering maturational signals in the form of CD40L-CD40 interactions to APCs that enable them to prime CTLs autonomously (47–49). It has also been shown that activated CD8$^{+}$T cells express CD40, permitting direct interactions with CD4$^{+}$ T cells (50).

Three recent papers have now clarified the role played by CD4$^{+}$ T cell help in the generation of an effective memory CTL response (20–22). Cytokine secretion and cytolytic activity of CD8$^{+}$ T cells during the primary response appear to be comparable irrespective of the presence or absence of CD4$^{+}$ T cell help. During the memory response, however, the reactivation and proliferation of CD8$^{+}$ CTLs as well as their ability to secrete effector cytokines such as IFN-γ are critically dependent upon CD4$^{+}$ T cell help previously delivered during the primary response. Thus, deficiencies in the MHC class II processing of rapidly degraded forms of Gag might explain their poor ability to subsequently generate robust memory CTL responses, particularly when unstable Gag is delivered in the form of a plasmid DNA prime before T cell help directed at vaccinia-specific Ags (Fig. 7). Furthermore, this lack of help may compound the functional defect of memory CD8$^{+}$ T cells primed in the presence of excessive levels of epitope described earlier, and explain why mice initially primed with plasmid DNA encoding unstable Gag exhibited such profound unresponsiveness when we attempted to elicit memory responses.

The susceptibility of APC to CTL killing during the development of an immune response is controversial, but might explain why challenging mice with vaccinia expressing rapidly degraded Gag led to poor memory responses. Some studies have suggested that the survival of APCs can be enhanced by the interaction of CD40 and CD40L (51), perhaps due to the acquisition of resistance to CTL-mediated killing (52). Other studies have demonstrated rapid in vivo clearance of APCs by CTLs, and postulate a role for this in the feedback regulation of an immune response (53, 54). In this second scenario, the faster appearance of Gag-derived peptides on surface class I molecules could have led to a more rapid clearance of cells infected with vaccinia expressing rapidly degraded forms of Gag. In other words, the rapid destruction of APCs infected with vShuf or vUbRgag in mice already primed by DNA vaccination against Gag (Figs. 5 and 6) might have resulted in diminished viral spread or reduced numbers of APCs for eliciting memory responses.

An additional consideration in assessing the utility of the degradation targeting strategy is the nature of the APC. A substantial body of evidence suggests that bone marrow-derived APCs are critical for the induction of CTL responses in vivo following DNA vaccination (55, 56) and viral infection (57). Ags that are initially expressed in cells lacking appropriate T cell costimulatory function can induce a CTL response through a cross-priming mechanism in which some form of the Ag is transferred to professional APCs (57). The mechanism of transfer is unclear, but in the case of virally infected cells it might involve uptake of Ag residing in apoptotic debris following the death of the infected cell (58). The degradation targeting strategies described in this work would be expected to be most effective in situations in which the cell that initially expressed the Ag is the same cell that presents Ag to CTL. If intact Ag has to be transferred, then forcing rapid degradation may actually be counterproductive. We and others have recently used a novel experimental approach for distinguishing direct priming from cross-priming in vivo (59, 60). This approach uses the human CMV proteins US2 and US11 to inhibit MHC class I-restricted Ag presentation in cells initially infected with recombinant vaccinia viruses carrying these genes, thereby functionally blocking direct priming, but not cross-priming. With this approach, we have shown that both direct and cross-priming are involved in the induction of vaccinia virus-specific CTL responses in vivo. Interestingly, the relative contribution of these two pathways depends on the site of infection. In i.p. infections, direct presentation predominated over cross-priming mechanisms. In light of these results, we have used i.p. infections in some of the experiments tested in this study. However, even with i.p. infections, we did not see a dramatic enhancement of CTL induction with rapidly degraded constructs.

Finally, it is possible that some difference in the overall kinetics of the response to rapidly degraded Ag prevented us from observing an enhancement in immunogenicity. However, within a period of 2–5 wk following initial priming with plasmid DNA, no differences in the relative memory responses to stable or unstable Gag were observed after challenge with recombinant vaccinia vectors in any of the experiments depicted in Figs. 5–7.

In summary, we have explored the utility of enforced rapid degradation strategies for inducing CTL responses to HIV-1 Gag. Although the strategies tested did increase the presentation of antigenic epitopes on the cell surface, and this increase was discernible by naïve CD8$^{+}$ T cells in vitro, immunization with unstable Gag constructs was not associated with a significant increase in the induction of CTL responses in vivo. These results highlight the complexities involved in the use of Ag-targeting strategies in vaccine design.

References