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Generation of Genome-Wide CD8 T Cell Responses in HLA-A*0201 Transgenic Mice by an HIV-1 Ubiquitin Expression Library Immunization Vaccine¹

Rana A. K. Singh,* Lei Wu,* and Michael A. Barry^{2*†‡§}

HIV-1 is a fundamentally difficult target for vaccines due to its high mutation rate and its repertoire of immunoevasive strategies. To address these difficulties, a multivalent, proteasome-targeted, live genetic vaccine was recently developed against HIV-1 using the expression library immunization approach. In this HIV-1 vaccine all open reading frames of HIV-1 are expressed from 32 plasmids as Ag fragments fused to the ubiquitin protein to increase Ag targeting to the proteasome to enhance CTL responses. In this work we demonstrate the ability of the HIV-1 library vaccine to simultaneously provoke robust HLA-A*0201-restricted T cell responses against all 32 HIV-1 library vaccine Ags after single immunization by gene gun. These CD8 T cell responses included HLA-A*0201-restricted CTL activity, CD8/IFN- γ T cell responses, and HLA tetramer binding against defined immunodominant epitopes in gag, pol, env, and nef as well as potent CD8/IFN- γ responses against undefined HLA-A*0201-restricted epitopes in all remaining Ags of the library. CD8 responses mediated by single gag, pol, env, and nef plasmids from the vaccine demonstrated little reduction in specific T cell responses when these plasmids were diluted into the context of the full 32-plasmid library, suggesting that Ag dominance or immune interference is not an overt problem to limit the efficacy of this complex vaccine. Therefore, this work demonstrates the ability of the HIV-1 library vaccine to generate robust multivalent genome-wide T cell responses as one approach to control the highly mutable and immunoevasive HIV-1 virus. *The Journal of Immunology*, 2002, 168: 379–391.

The development of an effective HIV-1 vaccine is essential for controlling this pandemic in humans. However, this goal has been difficult to accomplish due to the inherent biology of the virus, including its ability to infect immunologically relevant cells, actively block immune presentation (1), and undergo high rates of mutation to select immunoevasive virions (2, 3). There is mounting evidence demonstrating a role for CTLs in the control of HIV or SIV infections clinically and experimentally. Examples include individuals who have been exposed repeatedly to HIV without infection who have robust CTL responses in the absence of strong Ab responses (4, 5). When HIV-1-infected individuals have been followed over time, CTLs appear to play a role in controlling HIV following primary inoculation of the virus (6) and during ongoing infection (7) by destroying virus-infected cells. More recent experimental evidence for a role for CTLs in protection has been indicated in the macaque SIV model in which depletion of CD8⁺ T cells ablated the ability of their immune system to control SIV viremia (8, 9). Finally, a compelling role for CTL responses in vaccine protection has been demonstrated recently in work by Letvin and coworkers (10), who found a good correlation

between the control of SHIV-89.6P viremia and the ability of a given vaccine to elicit gag- and env-specific CTL responses.

Given the promise of these observations, a number of vaccine approaches are being developed to elicit CTL and other immune responses against HIV and SIV, including live/attenuated HIV or SIV (11, 12), and recombinant viral or bacterial vectors, including newer poxvirus, alphavirus, and adenovirus vectors (13–17). Genetic immunization represents one of these promising vaccine approaches in which plasmids encoding viral Ags are delivered into the host to elicit humoral and cellular immune responses against HIV-1 (18–24). This approach has recently demonstrated promise against HIV-1 and SIV in non-human primates when applied only as simple plasmids (10, 25, 26) and using DNA for priming and heterologous vaccines for boosting (24, 27).

Given these promising results, complementary efforts are needed to maximize the number of immune responses generated against dominant and subdominant viral epitopes as an approach to better control the diverse spectrum of HIV-1 variants present in the human population. Expression library immunization represents one approach to generate multivalent immune responses by delivering genetic vaccines expressing the whole genome of a pathogen (28). These genomic vaccines can expose the immune system to a diverse array of pathogen epitopes while being incapable of causing infection, since the genome of the pathogen has been fragmented and scattered across multiple plasmids.

The expression library immunization approach has recently been applied for HIV-1, where these genetic live vaccines were engineered to express all the open reading frames of the virus on 32 or more separate plasmids (29). Rather than expressing intact viral proteins, each plasmid was engineered to express a subgene fragment of ~400 bp fused to proteins such as ubiquitin (UB)³ to

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³ Abbreviations used in this paper: UB, ubiquitin; GFP, green fluorescent protein; EGFP, enhanced GFP.

target Ags to the proteasome to increase CTL responses (28). These fusion proteins inactivate the function and toxicity of problematic HIV-1 proteins, but at the same time retain all their T cell epitopes. These immune-targeting, fragmented Ags in the HIV-1 library mediate enhanced immune responses by increasing H-2^d-restricted CTL and Ab responses against dominant gag and env epitopes in BALB/c mice (29). Immunization with library vaccine Ags also generates CTL responses against an expanded set of subdominant H-2^d-restricted HIV-1 epitopes compared with immunization with wild-type HIV Ags such as gag (29).

Given the potential of this as a vaccine approach against HIV-1, this study was directed toward determining the diversity of multivalent CTL responses generated by the UB-HIV-1 library vaccine. This work was further directed at determining the ability of the HIV-1 library vaccine to elicit human-restricted CTL responses by testing the vaccine in humanized mice transgenic for the HLA-A*0201/K^b chimeric MHC I molecule (30). These HLA-A*0201/K^b transgenic mice express $\alpha 1$ and $\alpha 2$ domains of HLA-A*0201, and $\alpha 3$ transmembrane and cytoplasmic domains of H-2K^b; therefore, these mice generate CTLs and CD8 T cell responses essentially equivalent to those generated by human cells (30). Using four well-known immunodominant HLA-A*0201-restricted HIV-1 epitopes corresponding to the gag, pol, env, and nef regions of HIV-1, we describe a new assay system in which HLA-A*0201-restricted CTLs and CD8 T cell responses generated in transgenic mice can be accurately measured by using cell lines transiently or stably expressing individual UB-HIV-1 library vaccine members. Using this system we demonstrate that the UB-HIV-1 library genetic vaccine provokes simultaneous HLA-A*0201-restricted CD8 T cell responses against all 32 open reading frames encoded by the 32 plasmids of the library after a single round of gene gun immunization. Therefore, this work demonstrates the ability of this immune-targeting HIV-1 library vaccine to provoke multivalent human-specific CTL responses as an approach to control this highly mutable and immunoevasive pathogen.

Materials and Methods

Materials

Abs were purchased from BD PharMingen (San Diego, CA). Tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). Zeocin and pTracer-CMV2 was purchased from Invitrogen (Carlsbad, CA).

Endotoxin-free plasmid DNA purification columns were purchased from Qiagen (Chatsworth, CA). Peptides were synthesized by Research Genetics (Huntsville, AL). HLA-A*0201-HIV-1 gag and pol tetramers were supplied by the National Institutes of Health Tetramer Core (Bethesda, MD). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or VWR Scientific (Houston, TX).

Mice

Female C3H mice (H-2k) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice transgenic for HLA-A*0201/K^b (line 6) were provided by Dr. L. A. Sherman (The Scripps Research Institute, La Jolla, CA) (30). These mice express $\alpha 1$ and $\alpha 2$ domains of HLA-A*0201 fused to the $\alpha 3$ domain of H-2K^b, allowing the mice to generate HLA-A*0201-restricted T cell responses. The line 6 mice are backcrossed onto C57BL/6 mice such that HLA-A*0201 is expressed in an MHC background of H-2^b. These mice were further bred with C3H mice to generate the F₁ crosses used in the bulk of the experiments. All mice were maintained in the American Association for the Accreditation of Laboratory Animal Care-approved Center for Comparative Medicine at Baylor College of Medicine (Houston, TX).

Plasmids

The UB-HIV-1 library vaccine was supplied by K. Sykes and S. Johnston (University of Texas Southwestern Medical Center, Dallas, TX). Construction of the HIV-1 library vaccine or genetic live vaccine was described previously (29). Briefly, the entire HIV-1 genome was broken into 32 overlapping fragments by PCR using 32 sets of primers, and each fragment was cloned in pCMV_i plasmid. The relative position of each library member expressing a fragmented HIV-1 Ag is shown in a schematic format in Fig. 1. Each UB-HIV Ag from the library was subcloned from the original library into pTracer-CMV2 expressing the green fluorescent protein (GFP)-zeocin-resistance fusion protein to provide an antibiotic selection marker and fluorescent screening marker for the generation of stable cells for T cell stimulation and for use as CTL target cells. pEK was generated by deletion of enhanced GFP (EGFP) from pEGFP-N1. pEK-HLA-A*0201/K^b was generated by subcloning the HLA-A*0201/K^b chimera from pSV2-HLA-A*0201/K^b supplied by L. A. Sherman (30). pEK-HLA-A*0201/K^b expresses the $\alpha 1$ and $\alpha 2$ domains of HLA-A*0201 and the $\alpha 3$ transmembrane and cytoplasmic domains of H-2K^b to bind mouse β_2 -microglobulin (30). All plasmids were purified on Qiagen endotoxin-free columns.

Genetic immunization

Four- to 6-wk-old mice were genetically immunized by gene gun transfection of the epidermis using the Helios biolistic device (Bio-Rad, Hercules, CA) using 250 psi of helium. Plasmid DNA (2.5 μ g/shot of 1.6- μ m gold particles) was delivered according to the instructions provided by the manufacturer of the device. In most cases two shots were delivered into the back of the ears on each mouse at each immunization except where noted in the text. In all cases mice were vaccinated in a single round of immunization without further boosts.

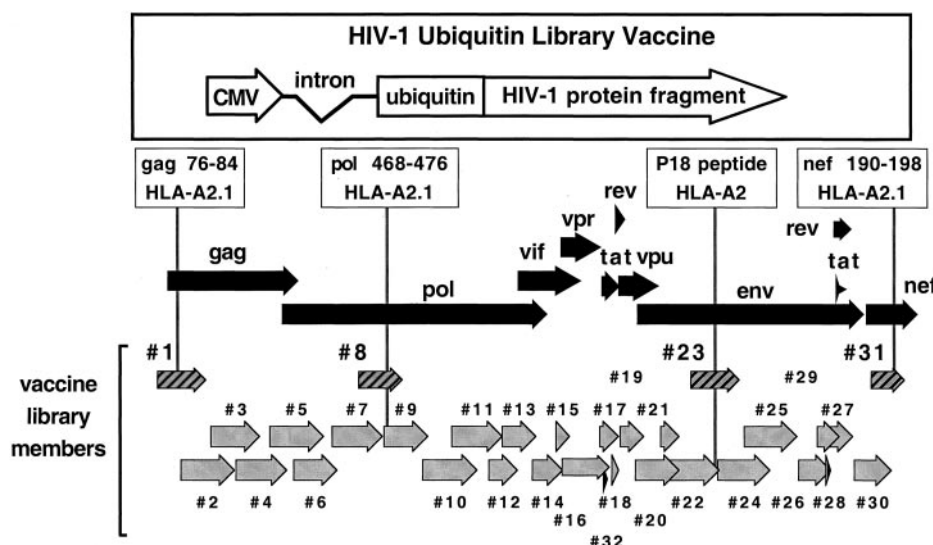


FIGURE 1. Schematic of UB-HIV-1 library and locations of known HLA-A*0201-restricted CTL epitopes.

Generation of mouse cell lines expressing HLA-A*0201 and UB-HIV-1 library members

A panel of murine cell lines was screened for the absence of MHC I molecule by flow cytometry and for low cytotoxicity toward T cells. The pluripotent 10T1/2 cell line derived from a C3H/J mouse (H-2^k) (31) was used for generating stable Ag-expressing cell lines, because these cells were negative for H-2K, -D, and -L molecules of MHC I and were nontoxic to T cells (data not shown). The 10T1/2 cells were cotransfected with plasmid encoding UB-tagged individual members of the HIV-1 library vaccine and plasmid-encoding chimeric HLA-A*0201/K^b using GenePorter (Gene Therapy, San Diego, CA). UB-HIV-1 library vaccine is expressed from the pTracer-CMV2 backbone, and HLA-A*0201/K^b was expressed from pEK backbone, which also expresses kanamycin resistance gene to provide selection of cell lines with G418. Stable transfectants were generated by selection of transfected cells in complete DMEM (DMEM supplemented with 10% FBS and antibiotic/antimycotic) containing both zeocin (200 µg/ml) and G418 (1 mg/ml). Each stable cell population was cloned in a 24-well plate. Each clone was screened for combined GFP and HLA-A*0201 expression by flow cytometry using the BB7.2 mAb against HLA-A*0201 (Ref. 30 and data not shown). Cell lines expressing the highest amounts of GFP and HLA-A*0201 were used in all experiments. For transient transfection, cells were analyzed 72 h after transfection for GFP and HLA-A*0201 expression by flow cytometry. In most cases, >70% of the cells were positive for both GFP and HLA-A*0201.

CTL assay

Four weeks after immunization, splenocytes from two or more nonimmunized or identically immunized mice were pooled and stimulated *in vitro* for 6 days either with 1 µM of the appropriate peptide or with irradiated (6000 rad) 10T1/2 stimulator cells expressing the indicated gene as shown in the figures. The responder:stimulator ratio was 10:1. The splenocytes were cultured in RPMI 1640 medium supplemented with 10% FBS, 10% T-stim (New England Biolabs, Beverly, MA), 50 µM 2-ME, 10 mM HEPES, and antibiotic/antimycotic at 37°C in a humidified CO₂ incubator. On day 7 the CTL activity of the cultured splenocytes was assayed by a standard 4-h ⁵¹Cr release assay. Target cells used in the CTL assay were either stably or transiently transfected 10T1/2 cells expressing the indicated genes. For HIV-1 HLA-A*0201-restricted peptide loading, 10T1/2 cells stably transfected with HLA-A*0201 were labeled with 1 µM of the indicated peptide for 1 h at 37°C, and excess peptide was removed by washing. All target cells were labeled with 100 µCi ⁵¹Cr (as sodium chromate) at 37°C for 1 h in a 1-ml volume. After washing, 1 × 10⁴ labeled target cells were added to effector cells at the indicated E:T cell ratios. The percent specific lysis was calculated as: ((counts per minute of sample – counts per minute of spontaneous release)/(counts per minute of maximum release – counts per minute of spontaneous release)) × 100. Spontaneous release was defined as the mean counts per minute released from six replicates of 1 × 10⁴ labeled cells incubated in medium alone. Maximum release was defined as the mean counts per minute released from six replicates of 1 × 10⁴ labeled cells incubated in medium containing 1% SDS. Each experiment was performed in triplicate and was repeated at least three times. The SD was consistently <10% of the mean value.

Intracellular IFN-γ staining

Splenocytes were recovered from nonimmunized and immunized mice, and RBCs were removed by hypotonic treatment using water and 10× PBS. Fresh lymphocytes or lymphocytes cultured for 6 days with stimulator cells as described above were washed and stimulated *in vitro* (1 × 10⁶ cells/sample) for 4–6 h at 37°C in a CO₂ incubator with the indicated 10T1/2 cells or 10T1/2 HLA-A*0201 cells labeled with 5 µM of the indicated peptide at a responder:stimulator ratio of 10:1. During the second hour of stimulation 5 µg/ml brefeldin A was added to enhance intracellular accumulation of IFN-γ. These stimulated cells were then washed with FACS buffer (PBS containing 2% FBS and 0.02% sodium azide) and stained with 100 ng each of anti-CD3-PerCP-conjugated hamster anti-mouse CD3ε (ε-chain) mAb and anti-CD8-FITC-conjugated rat anti-mouse CD8α (Ly-2) mAb or with the isotype control of anti-CD8-FITC conjugate for 30 min at 4°C in the dark in 100 µl of FACS buffer. Cells were then washed twice with FACS buffer, and intracellular IFN-γ staining was performed using the Cytofix/Cytoperm Plus kit from BD Pharmingen according to the manufacturer's instruction. Briefly, CD3- and CD8-labeled cells were fixed and permeabilized for 20 min at 4°C in 250 µl of Cytofix/Cytoperm solution and then washed twice with Perm/Wash solution. The permeabilized cells were incubated with 100 ng of PE-conjugated anti-IFN-γ Ab or isotype control Ab for 30 min at 4°C. The cells were then washed twice with Perm/Wash solution and resuspended in FACS buffer for further analysis.

Sample data were acquired and analyzed on a FACScan instrument and analyzed using CellQuest software (both from BD Biosciences, Mountain View, CA).

Tetramer staining

Four weeks after immunization splenocytes from control or immunized mice were harvested. RBCs were removed by hypotonic treatment using water and 10× PBS. Fresh lymphocytes or lymphocytes cultured for 6 days as described above were used for tetramer staining. Lymphocytes (1 × 10⁶/sample) were stained with 100 ng each of PE-conjugated MHC-I-SI-INFELK or MHC-I-SIINFEDL tetramers and anti-CD3-PerCP-conjugated hamster anti-mouse mAb and anti-CD8-FITC-conjugated rat anti-mouse mAb or with the isotype control of anti-CD8-FITC conjugate for 30 min at room temperature in the dark in 100 µl of FACS buffer (PBS containing 2% FBS and 0.02% sodium azide). Cells were then washed twice with FACS buffer, and sample data were acquired and analyzed on a BD Biosciences FACScan instrument and analyzed using CellQuest software.

Results

Use of HLA-A*0201-transgenic mice and transfected cell lines to detect human (HLA-A*0201)-restricted CTL responses against UB-HIV-1 library vaccine Ags

This work was directed toward screening the functionality of the UB-HIV-1 library vaccine plasmids for their ability to provoke CD8/CTL responses in mice transgenic for the human MHC molecule HLA-A*0201, because this system has previously been used to generate human-restricted CTL responses in mice (30). Because it is impractical to synthesize every potential HLA-A*0201-restricted peptide from HIV-1 to measure genome-wide CTL activity, APC were needed for this study to not only act as targets for CTL activity, but also to be used to expand memory T cells *in vitro*. These APC needed not only to express HLA-A*0201 and the HIV-1 Ags, but at the same time had to avoid allogeneic and xenogeneic responses in the transgenic mice. Given these constraints, we constructed mouse HIV-1 APC that coexpressed the HLA-A*0201/K^b chimeric MHC I molecule. A number of potential mouse cell lines were screened for ease of transfection and low H-2 MHC I expression. Of the screened cells, 10T1/2 cells derived from C3H mice could be easily transiently transfected to 75% with liposomes and readily generated stable transfectants. In addition, these cells expressed undetectable levels of mouse MHC I molecules H-2K^k, -D^k, or -L^k by flow cytometry (data not shown), making them less likely to present mouse-restricted epitopes from HIV-1. Therefore, these cells, when engineered to express HLA-A*0201, could be used as both stimulator and target cells to measure HLA-restricted responses from the mice rather than mixed HLA- and H-2-restricted responses. In contrast, cells derived from the transgenic mice would display both H-2 and HLA. The ability to use transient transfection to generate the target cells was particularly attractive, because several HIV-1 proteins are known to be toxic to transfected cells, making stable Ag-expressing cells difficult to generate and maintain. Therefore, in this study both transiently and stably transfected 10T1/2 cells expressing HLA-A*0201/K^b and HIV-1 Ags were tested as both Ag-expressing cell lines and target cell lines. These cells were used in conjunction with the HLA-A*0201 transgenic mice crossed with C3H mice to minimize possible allogenic responses against the 10T1/2 cells due to the low level of MHC I or minor histocompatibility complex recognition.

The functionality of the system was determined by immunizing mice with UB-tagged HIV-1 library vaccine library plasmids 1, 8, 23, and 31 expressing dominant HLA-A*0201-restricted HIV-1 epitopes from gag, pol, env, and nef (gag_{76–84} and pol_{468–476} (32), env P18 (33), and nef_{190–198} (34); Fig. 1). The gag, env, and nef epitopes are displayed once each in the library, and the pol epitope is displayed twice in overlapping sequences (Fig. 1). The gag and

pol HLA-restricted peptides were of particular interest, because they are relatively conserved among different strains of HIV-1 (32). Mice were immunized in the ear with 5 μ g of each plasmid by gene gun in two different shots in a single round of immunization with no boosting. Four weeks later splenocytes were harvested from the mice and stimulated for 6 days in vitro with 1) 10T/2 cells, 2) 10T/2 cells transfected with HLA-A*0201/K^b (HLA), 3) 10 T/2 cells transiently transfected with HLA and appropriate UB-HIV-1 library vaccine library members, 4) 10T/2 cells stably transfected with HLA and appropriate UB-HIV-1 library vaccine library members, or 5) 10T/2-HLA stable cell lines loaded with the HLA-A*0201-restricted peptides from gag, pol, env, and nef. On day 7 CTL activity from the cultured splenocytes was measured by standard 4-h ⁵¹Cr release assay using the same panel of HLA- and Ag-expressing target cells (Fig. 2). Splenocytes derived from nonimmunized mice and variously stimulated in vitro for 6 days demonstrated only background CTL activity against all the target cells (Fig. 2 and data not shown).

In contrast to these controls, splenocytes from mice immunized with the UB-HIV-1 library vaccine library members 1, 8, 23, and 31 demonstrated robust CTL responses against cells presenting the appropriate epitopes (Fig. 2). For example, splenocytes from mice immunized with plasmid 1 from the library and stimulated in vitro with 10T/2 cells transiently transfected with UB-HIV plasmid 1 showed significantly high CTL activity against transiently transfected target cells, stably transfected target cells, and peptide-loaded target cells (Fig. 2) compared with their CTL activity against control target 10T/2 and 10T/2-HLA cells (~7% specific lysis; E:T cell ratio, 40:1). A similar pattern of CTL activity against all five targets was observed when the same splenocytes were stimulated in vitro with 10T/2 cells stably transfected with

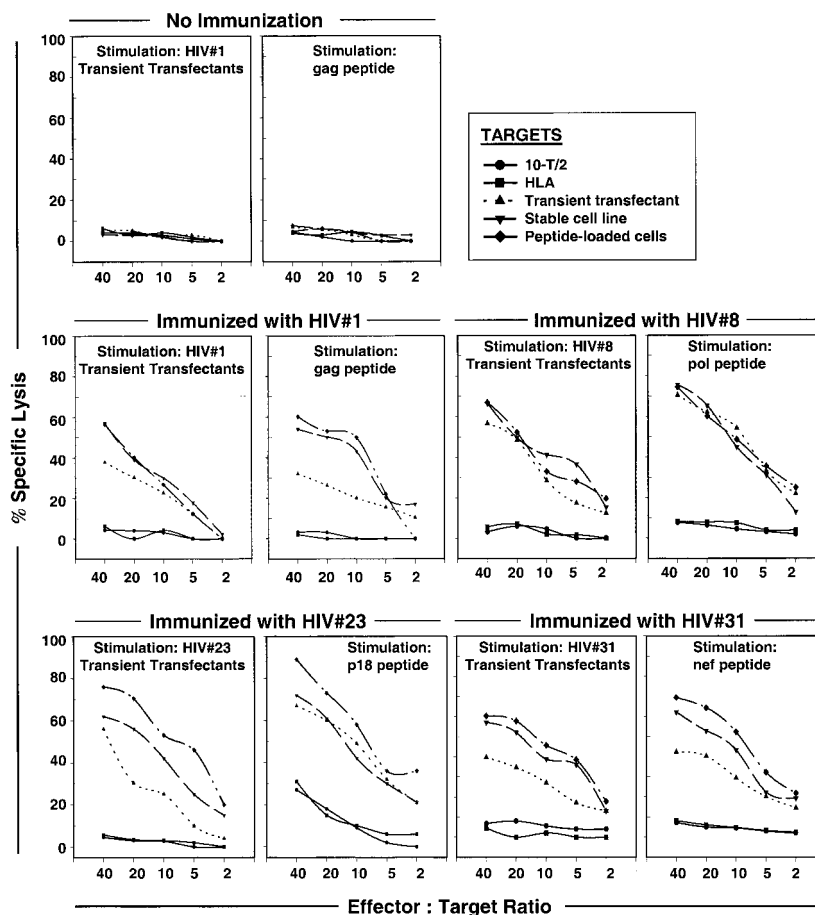
UB-HIV plasmid 1 or with cells loaded with the gag peptide (Fig. 2). Comparable CTL levels and specificity were observed in splenocytes from mice immunized with plasmids 8, 23, and 31 vs pol-, env-, and nef-displaying cells, respectively, when these cells were used as either stimulators or targets (Fig. 2).

Although, in general, the CTL activity against transiently transfected target cells was lower than that observed against stable transfectants or peptide-loaded target cells, these responses were substantially higher than those of control mice or against control cells. This demonstrates the utility of both transiently transfected and stable cell lines for use as T cell stimulators and CTL target cells. These observations were significant, because it was unclear from the literature how well transient or stable cell lines would work as stimulators and target cells. These observations indicated that the transfected cell lines could be used to screen for T cell responses generated by the rest of the library plasmids where in many cases we do not know the identity of all dominant and subdominant MHC-I-restricted T cell epitopes. These results also demonstrated that transient transfectants functioned nearly as well as stable transfectants in the assays, indicating that this approach can be used to avoid the time needed to generate stables and should also bypass problems in expressing toxic HIV proteins in stable cell lines.

*Demonstration of HLA-A*0201-restricted T cell responses against gag and pol in humanized transgenic mice using HLA-A*0201 MHC I tetramers*

The data shown above demonstrate the ability of UB-HIV-1 library vaccine plasmids to generate CTL responses against known HLA-A*0201-restricted HIV-1 epitopes in HLA-A*0201 transgenic mice. These CTL responses paralleled the levels of CD8/

FIGURE 2. UB-HIV-1 library vaccine members induce human (HLA-A*0201)-restricted CTL responses in HLA-A*0201 \times C3H mice that can be detected using transiently or stably transfected cell lines. Transgenic mice expressing the HLA-A*0201 molecule in the MHC-I K^b background were immunized by gene gun with the indicated library plasmids as shown in Fig. 1. Four weeks later splenocytes pooled from two or more identically immunized mice were in vitro stimulated for 6 days with the irradiated (6000 rad) stimulator cells expressing appropriate HIV-1 Ags or loaded with appropriate HIV-1 peptide. The responder:stimulator ratio was 10:1. On day 7 the Ag-specific CTL activity present in the culture was assayed by a 4-h ⁵¹Cr release assay using the following target cells: 1) 10T/2, 2) 10T/2 cells expressing only HLA-A*0201/K^b (HLA), 3) 10T/2 cells transiently transfected with HLA and HIV-1 library member (transient), 4) 10T/2 cells stably transfected with HLA and HIV-1 library member (stable), and 5) 10T/2-HLA cells loaded with HIV-1 peptide (peptide). CTL activity generated by plasmid 1 expressing a region of gag including the HLA-A*0201-restricted gag_{76–84} peptide. CTL activity generated by plasmid 8 expressing a region of pol including the HLA-A*0201-restricted pol_{468–476} peptide. CTL activity generated by plasmid 23 expressing a region of env including the HLA-A*0201-restricted P18 peptide from the V3 loop. CTL activity generated by plasmid 31 expressing a region of nef including the HLA-A*0201-restricted nef_{190–198} peptide. Each set of experiments was performed in triplicate and was repeated at least three times with similar results. Values shown are from one representative experiment. The SD was consistently <10% of the mean value.



IFN- γ double-positive T cells in splenocytes from mice immunized with the same constructs (Figs. 6–8 and data not shown). Given that HLA-A*0201-restricted peptides were used and that the 10T1/2 cells do not express H-2 MHC I molecules despite their C3H derivation, these T cell responses we observed were probably restricted by HLA-A*0201 in the transgenic mice. Furthermore, Ag-specific CD8/IFN- γ responses were observed only when stimulator cells coexpressed HLA-A*0201 (data not shown). No CD8 responses were observed when splenocytes were stimulated by 10T1/2 cells expressing only the Ags, suggesting that the observed responses are indeed restricted by human MHC I molecules rather than mouse MHC I.

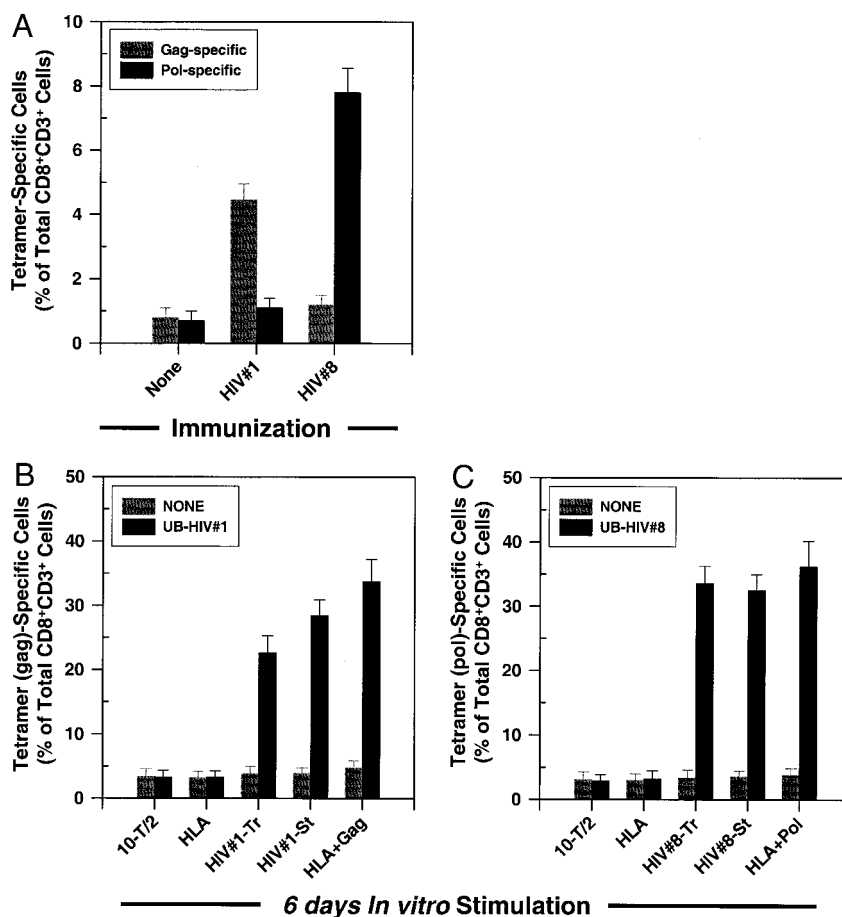
The HLA specificity of these responses was further confirmed by assaying specific binding of gag- and pol-specific TCR⁺ T cells with HLA-A*0201 tetramers loaded with the gag and pol peptides (35). Binding of these gag and pol HLA tetramers was tested against primary T cells from HLA-A*0201 \times C3H mice immunized with plasmids 1 and 8 (Fig. 3A). The frequency of tetramer-positive T cells provoked by the HIV-1 UB fusion plasmids were quite robust (4.8% gag-specific and 8.2% pol-specific) given that mice were only immunized a single time 4 wk before this assay, and this frequency was comparable to the levels of CD8/IFN- γ T cells observed under the same conditions (Fig. 4). Six-day *in vitro* stimulation of the splenocytes with peptides or transfected cells similarly generated robust expansion of the tetramer-positive effector T cells in the population to levels comparable to the observed numbers of CD8/IFN- γ T cells (Fig. 3, B and C). The specificity of HLA-A*0201 tetramer staining was demonstrated by the low level of gag tetramer-specific T cells in the primary splenocytes from mice immunized with plasmid 8 that expresses a region

of pol (Fig. 3A). Similarly, a low level of pol tetramer-specific staining was observed in the splenocytes from mice immunized with plasmid 1 expressing a region of gag. In addition, splenocytes from mice immunized with HLA and *in vitro* expanded with HLA-A*0201-transfected cells showed only background staining with gag and pol tetramer (Fig. 3, B and C), confirming that these tetramers bind to only gag- and pol-specific TCR⁺ T cells. These data demonstrate that the human-restricted CD8 T cell responses are produced in HLA-A*0201 transgenic mice by UB-HIV-1 library vaccine members.

Effects of plasmid dilution and Ag dominance and interference in the complex UB-HIV-1 library vaccine

In expression library immunization the entire genome of a pathogen is captured in an expression library to generate multivalent immune responses or to identify vaccine candidates by genetic immunization (28). The number of plasmids in a given library is directly proportional to the size of the genome in this process. While the HIV-1 library vaccine represents one of the smallest libraries (32 plasmids vs 50,000 for some bacterial pathogens), this multigene vaccine still represents a complex mixture of plasmids for transfection and a diverse mixture of Ags for immune stimulation. Given the complexity of the vaccine, two primary issues needed to be clarified. First, how would dilution of an individual library member in the complex mixture of the other 31 plasmids of the library affect the level of T cell responses? Secondly, would overt Ag dominance and interference be observed, as suggested previously (36), when the immune system is exposed to a large repertoire of T cell epitopes presented by the HIV-1 library vaccine?

FIGURE 3. HLA-A*0201 MHC I tetramer staining of CD8⁺ T cells from HIV-1 library vaccine member-immunized HLA-A*0201 \times C3H mice. Mice were immunized by gene gun with the indicated library plasmids, and their splenocytes (1×10^6 lymphocytes/sample) were assayed for the presence of gag_{76–84}-specific and pol_{468–476}-specific T cells by flow cytometry with Abs against CD3, CD8, and with HLA-A*0201 MHC I tetramers presenting either gag_{76–84} or pol_{468–476}. CD3/CD8 double-positive cells ($5–10 \times 10^3$ /sample) were analyzed for the presence of tetramer-positive cells on a FACS-can using CellQuest software. A, Primary memory CD8⁺ T cells tetramer staining of fresh lymphocytes. B, Tetramer-positive effector CD8⁺ T cells expanded by 6-day *in vitro* stimulation with the indicated peptides and cells. Each set of experiments was repeated at least three times. Values shown are the mean \pm SD.



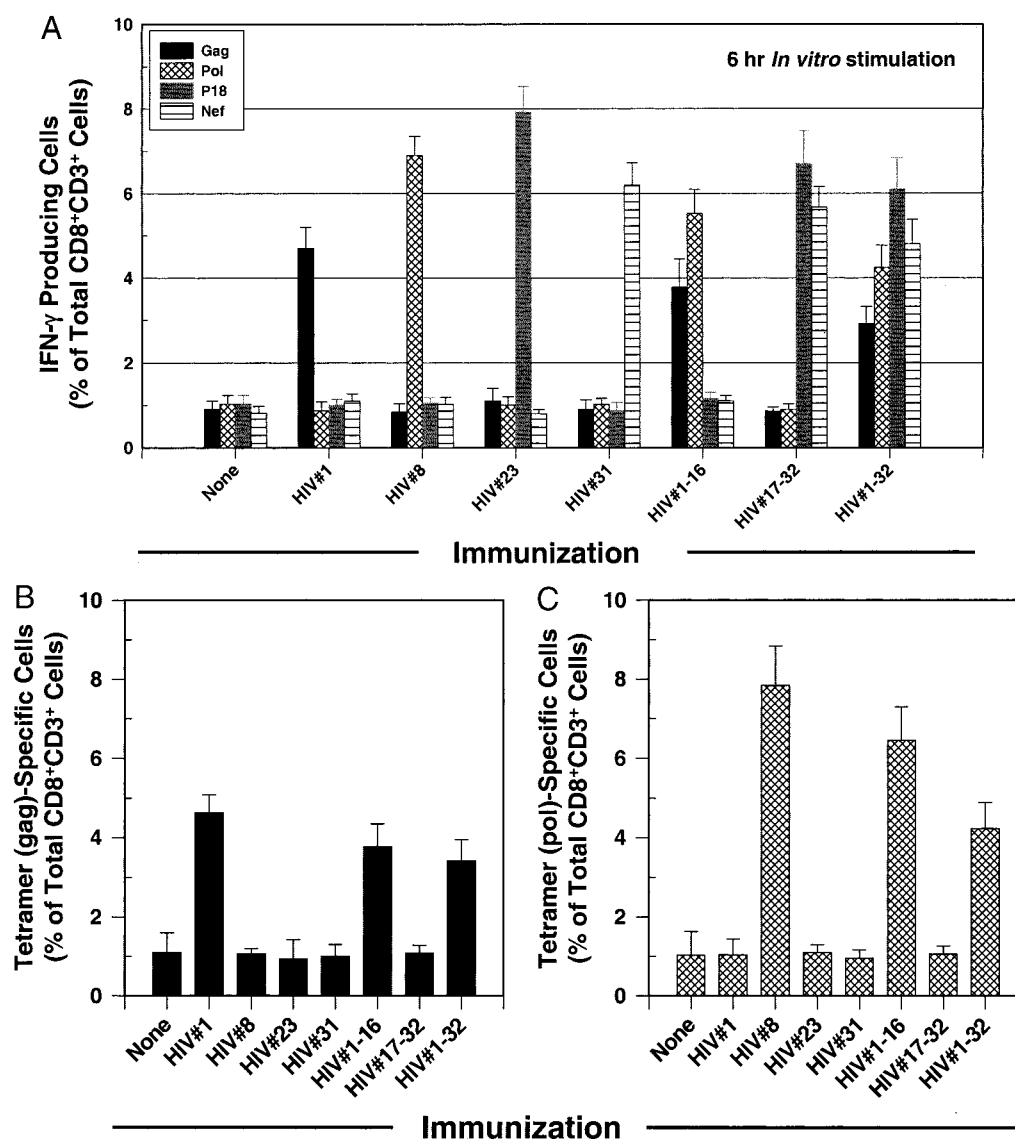


FIGURE 4. Effects of plasmid dilution and increased number of Ag-expressing plasmids on HLA-A*0201-restricted, Ag-specific CD8 T cell responses. Transgenic mice were immunized by gene gun with the indicated library plasmids. Mice labeled HIV#1, #8, #23, and #31 were immunized with 5 μ g of DNA from only one plasmid. Mice labeled HIV#1-16 and HIV#17-32 were immunized with 16 different plasmids (a total of 5 μ g of DNA) representing 800 ng of DNA from an individual plasmid. Mice labeled HIV#1-32 were immunized with all 32 members of the HIV-1 library vaccine (a total of 5 μ g of DNA) representing 400 ng of DNA from an individual member. One month later their splenocytes were cultured for 6 days with 1 μ M of the indicated HLA-A*0201-restricted peptide. For the Ag-specific CD8/IFN- γ T cell response, fresh or cultured lymphocytes (1×10^6 /sample) were stimulated for 6 h with 5 μ g of peptide in the presence of brefeldin A. For the CD8/tetramer T cell response, fresh and 6-day cultured splenocytes (1×10^6 /sample) were used without further stimulation. CD3/CD8 double-positive cells ($5\text{--}10 \times 10^4$ /sample) were analyzed for the presence of IFN- γ ⁺ and tetramer⁺ cells on a FACScan using CellQuest software. **A**, Primary memory CD8⁺ T cells producing IFN- γ after immunization with the indicated plasmids and 6-h stimulation with matched and mismatched HLA-A*0201-restricted peptides. **B**, HLA-A*0201-gag tetramer staining of unstimulated primary memory CD8⁺ T cells after immunization with the indicated plasmids. **C**, HLA-A*0201-pol tetramer staining of unstimulated primary memory CD8⁺ T cells after immunization with the indicated plasmids. **D**, Effector CD8⁺ T cells producing IFN- γ after 6-day expansion in vitro and 6-h stimulation with matched and mismatched HLA-A*0201-restricted peptides. **E**, HLA-A*0201-gag tetramer staining of effector CD8⁺ T cells after immunization with the indicated plasmids and 6-day expansion in vitro with the indicated matched and mismatched HLA-A*0201-restricted peptides. **F**, HLA-A*0201-pol tetramer staining of effector CD8⁺ T cells as in **E**. Each set of experiments was repeated at least three times. Values shown are the mean \pm SD.

To address these questions, mice were immunized under three conditions: 1) with 5 μ g of DNA of only one UB-HIV-1 library plasmid; 2) with half of the UB-HIV-1 library vaccine consisting of plasmids 1-16 or plasmids 17-32, such that 800 ng of each individual plasmid was delivered to one mouse; and 3) with the entire 32-plasmid UB-HIV-1 library vaccine, where 400 ng of each library member was delivered diluted in all other library members expressing all HIV-1 T cell epitopes. Mice were immunized with these plasmid combinations without boosting, and 1 mo later

splenocytes were analyzed for the presence of Ag-specific CD8 T cells by intracellular IFN- γ and HLA-A*0201 tetramer staining (Fig. 4). Intracellular IFN- γ staining of fresh splenocytes stimulated for 6 h with peptide demonstrated that immunization of the mice with half the library or with the full 32-plasmid library generated robust CD8 responses comparable to the responses generated in mice immunized with only the single Ag-expressing plasmids (Fig. 4A). These responses were Ag specific, as demonstrated by the peptide specificity of IFN- γ responses and by the fact that

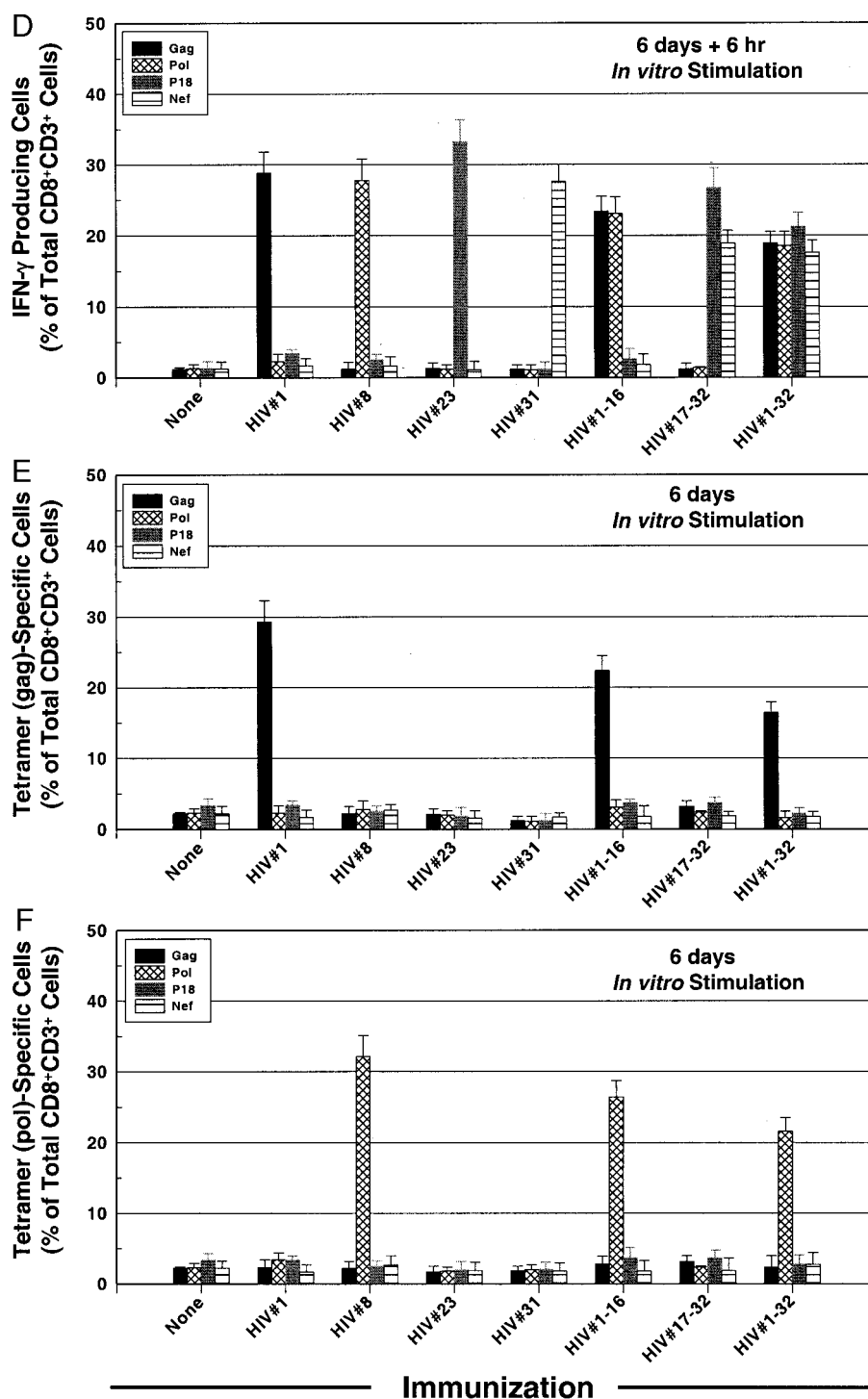


FIGURE 4. (continued)

gag- and pol-specific responses were observed by immunization with plasmids 1–16, but not by plasmids 17–32. The same specificity was observed after gag and pol HLA tetramer staining of the T cells (Fig. 4, B and C). Similarly, env- and nef-specific responses were observed after immunization with plasmids 17–32, but not after immunization with plasmids 1–16. These results, testing the specificity of memory T cells from mice, were essentially identical with the specificity of CD8/IFN- γ responses of effector T cells expanded *in vitro* by 6-day stimulation (Fig. 4, D–F). These data demonstrate robust Ag-specific CD8 responses after immunization with single plasmids, with half the library, and with the full 32-plasmid UB-HIV-1 library vaccine.

When the levels of these responses were compared, it was evident that no overt Ag dominance or interference was observed between the dominant HLA-A*0201-restricted epitopes of the vaccine, because responses against all four peptides were observed after immunization with the full library (Fig. 4). The level of responses against each epitope after immunization with the whole library was actually better than one would predict due to simple plasmid dilution. For example, in the whole library immunization, 400 ng of each plasmid was delivered, in contrast to the 5 μ g of DNA used with the single plasmids, resulting in a 12.5-fold reduction in the amount of plasmid delivered. Because the pol epitope is present twice in the library (in library members 8 and 9;

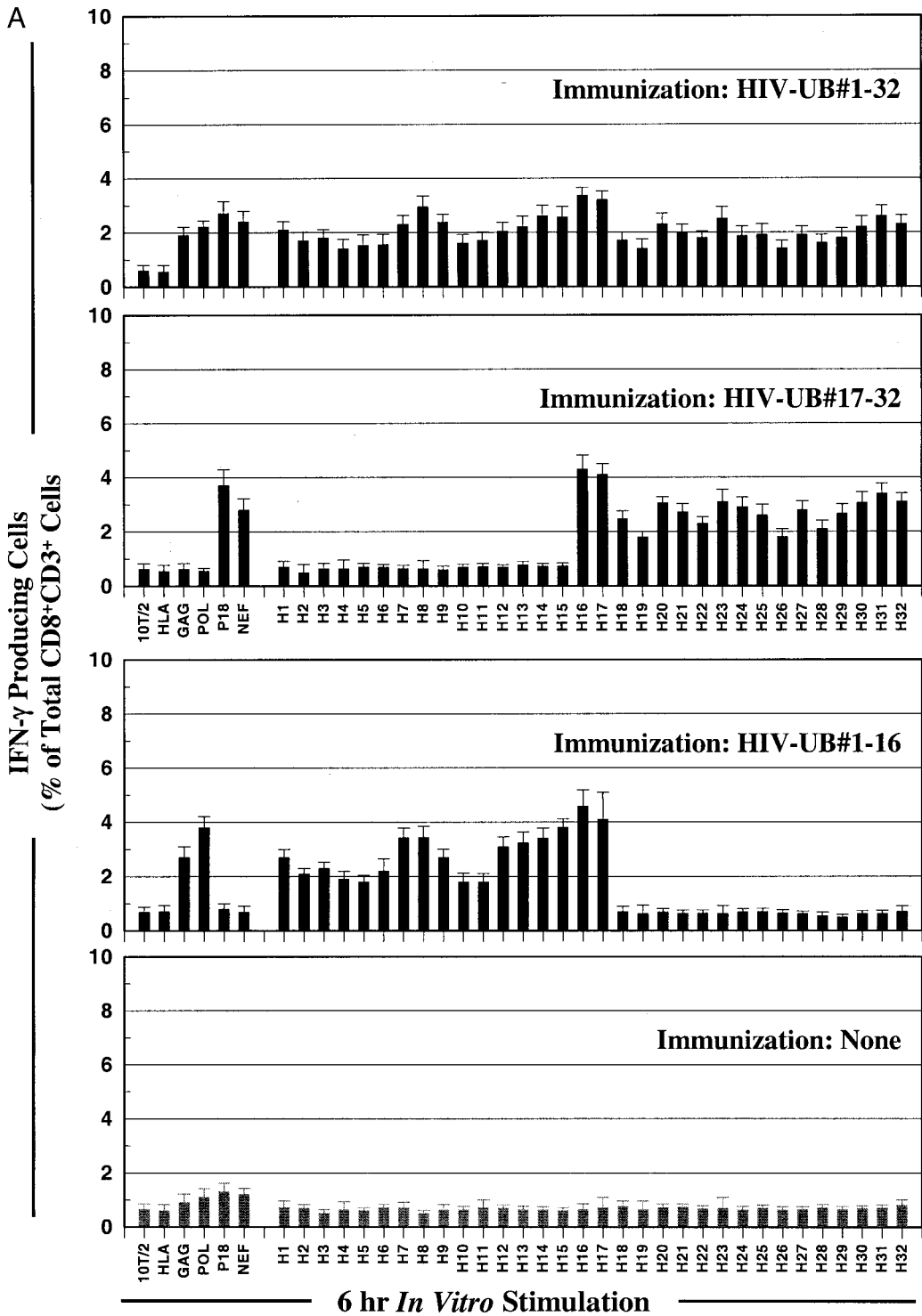


FIGURE 5. Genome-wide stimulation of CD8⁺ T cells by the UB-HIV-1 library vaccine. Mice were immunized by gene gun with the indicated half-library (left half, HIV-UB 1–16; right half, HIV-UB 17–32) or whole library (HIV-UB 1–32), and their splenocytes were stimulated with the indicated HLA-A*0201-restricted peptides or cell lines expressing UB-HIV-1 library vaccine member before flow cytometric measurement for CD8⁺ T cells producing IFN- γ . CD3/CD8 double-positive cells ($5\text{--}10 \times 10^4$ /sample) were analyzed for intracellular IFN- γ production on a FACScan using CellQuest software. A, Primary Ag-specific memory T cells producing IFN- γ . B, Effector CD8 T cells expanded by 6-day culture followed by 6-h stimulation before IFN- γ measurement. Each set of experiments was repeated at least three times. Data shown are the mean \pm SD.

Fig. 1), this T cell epitope is diluted ~ 6 -fold with respect to the single plasmid immunization. Therefore, even though there is an 80–90% reduction of each epitope in the whole library immunization vs the single plasmid immunization, T cell responses did not fall to the same degree, decreasing only 20–50% for the different dominant epitopes (Fig. 4).

These data demonstrate the ability of the UB-HIV-1 library vaccine to generate multivalent CD8 T cell responses against dominant HLA-A*0201-restricted epitopes and further demonstrate that the levels of these responses are reduced less than predicted by simple plasmid dilution. This also suggests that overt Ag dominance or interference between dominant epitopes will not be a

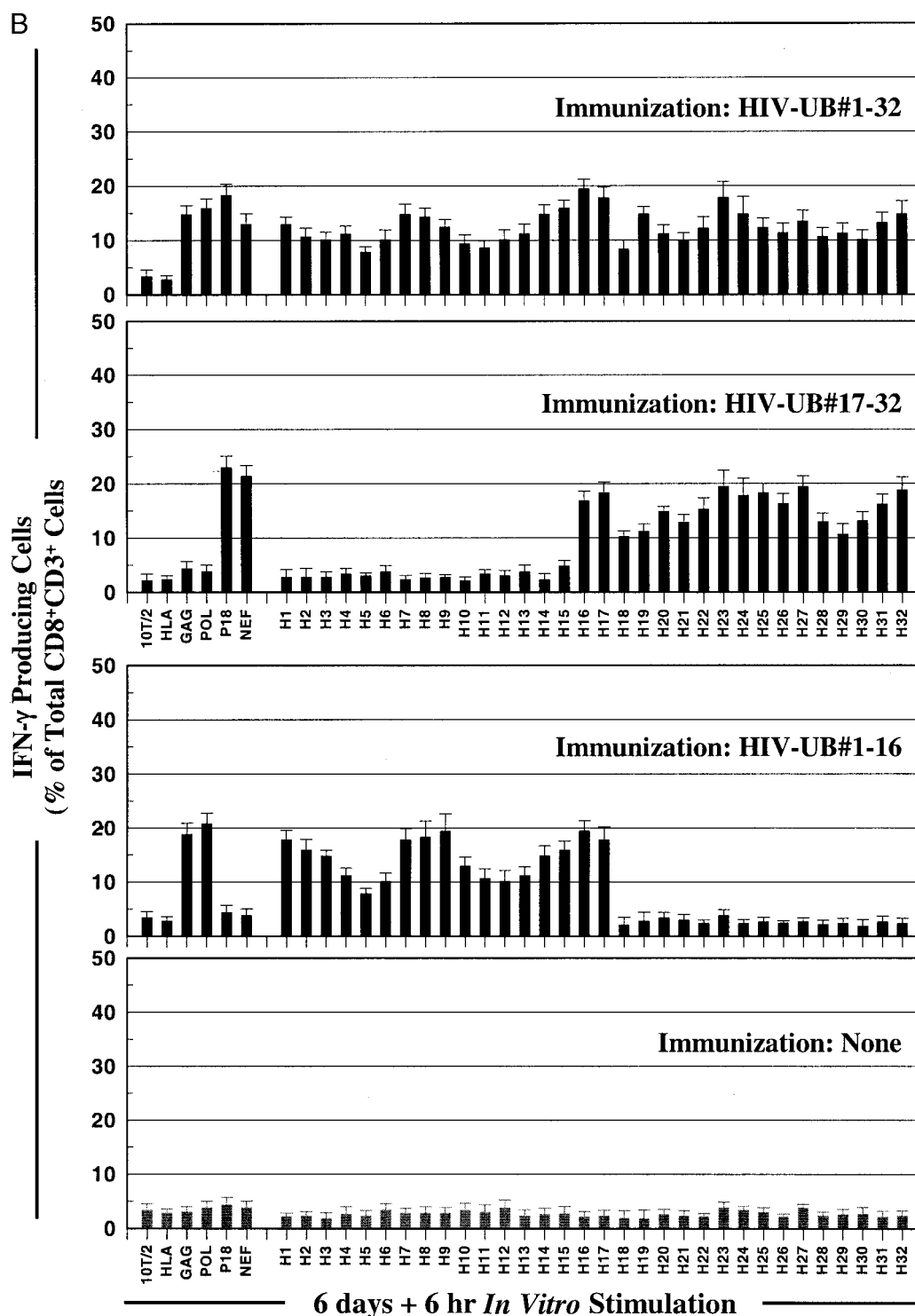


FIGURE 5. (continued)

problem in the complex UB-HIV-1 library vaccine, because mice immunized with either half the library or the whole library generated CD8 T cells responses against all tested immunodominant epitopes, showing that the presence of one or more immunodominant epitopes does not interfere with the immunogenic ability of the second or third immunodominant epitope.

Genome-wide CD8 T cell responses generated by the UB-HIV-1 library vaccine

Once the functionality of the assay system was verified in the HLA-A*0201 mouse system, the full repertoire of immune stim-

ulation by the UB-HIV-1 library vaccine was analyzed to determine how many library members actually generate CD8 T cell responses. It was of particular interest to determine whether CD8 T cell responses were only produced by plasmids expressing the known HLA-A*0201-restricted dominant epitopes or whether many or all of the other plasmids also generated productive CTL responses in this multigenic genetic vaccine.

To test this, three groups of mice were immunized by gene gun in a single round with either half the library or all 32 members of the UB-HIV-1 library vaccine. The first and second groups of mice were immunized with a DNA mixture corresponding either to the

left side of the HIV-1 genome (plasmids 1–16) or to the right side of the HIV-1 genome (plasmids 17–32). Both groups of mice received 800 ng of DNA from each library member. The third group of mice was immunized with the entire HIV-1 library. This group of mice received 400 ng of each library plasmid. All three groups received the same total amount of DNA.

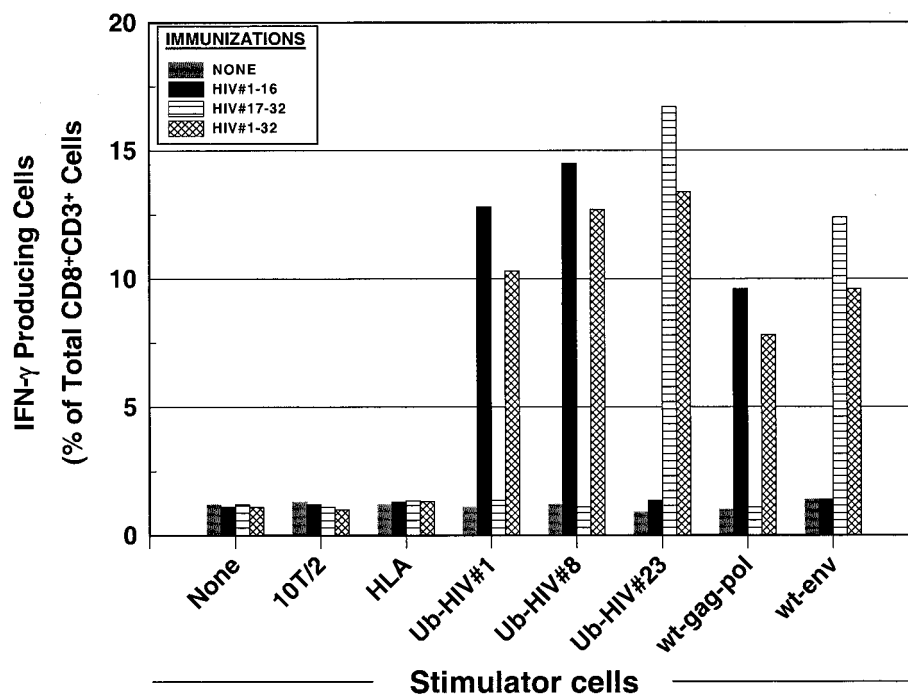
One month after immunization splenocytes from nonimmunized and immunized mice were stimulated *in vitro* with a panel of 32 cell lines, each expressing HLA-A*0201 and one library member from the library vaccine. As a positive control, splenocytes were also stimulated with the four different HLA-A*0201-restricted peptides from gag, pol, env, and nef. Stimulation of fresh splenocytes from the immunized mice for 6 h with the Ag-expressing cell lines demonstrated the generation of robust CD8 memory T cell responses (Fig. 5A). Although each library member generated different levels of CD8/IFN- γ responses, immunization with half the library or all 32 plasmids of the library generated CD8 T cell responses against all Ags encoded by the delivered portions of the library (Fig. 5). The Ag specificity of the responses generated by the half-libraries were notable, since immunization with plasmids 1–16 generated T cells that could react against the individual Ags 1–16, but did not generate responses against Ags 18–32. Similarly, immunization with plasmids 17–32 generated responses against the individual Ags 17–32, but not against 1–15. In these cases the mismatched library members each represent 15 negative control Ags, where each expresses a different HIV Ag, but all share the same plasmid backbone as the library members that provoked responses. Therefore, the lack of responses against the 15 negative control Ags in the half-library immunizations demonstrates that the responses are not directed against the UB protein fused to all Ags or to any cryptic Ags expressed from the bacterial sequences of the plasmid. It is also noteworthy that mice immunized with library members 1–16 generated CD8/IFN- γ T cells that cross-reacted against cells expressing plasmid 17 and that mice immunized with plasmids 17–32 generated CD8/IFN- γ T cell that cross-reacted against cells expressing Ag 16. This is probably due to overlap in the HIV-1 DNA sequences of the two plasmids, allow-

ing expression of a common epitope between the two library members (Fig. 1).

It is also notable that the CD8 T cell responses were ~30% lower after immunization with the whole library vs the half-libraries despite the fact that 50% less of each plasmid was delivered in the whole library (Fig. 5). This again indicates that overt Ag antagonism or interference is not observed after immunization with the complex library vaccine. These responses after full library immunization in which half as much of each plasmid was delivered were higher than expected when considering plasmid dilution compared with immunization with half the library (Fig. 5). This observation is consistent with those presented in Fig. 4. However, in this case each animal receiving the half or whole library vaccination, received the same total amount of plasmid DNA. This suggests that these better than expected CD8 T cell responses were not due to the nonspecific adjuvant effects of bacterial DNA and CpG motifs (37).

While T cell responses were observed against all 32 Ags of the library, five peaks of CD8/IFN- γ T cell activity were detected (Fig. 5). Four of these corresponded with library members 1, 8, 23, and 32 and their adjacent library members expressing the dominant HLA-A*0201-restricted CTL epitopes characterized previously (Figs. 1–4). The responses from library members 16 and 17 were actually slightly higher than the responses against the well-characterized HLA-A*0201-dominant epitopes from gag, pol, env, and nef. This observation not only demonstrates the utility of the library for generating multivalent responses, but confirms the application of using the library to scan whole pathogen genomes to identify new candidate vaccines that may be missed by conventional approaches (28). While the peaks of CD8/IFN- γ responses were higher than the responses mediated by the other library members, these other subdominant library members still provoked Ag-specific responses in the range of 1.4–2.4% of total primary CD8 cells from the mice after single immunization (Fig. 5A). These genome-wide CD8 responses compare well with the background responses observed in the samples from unimmunized mice and the mismatched Ags in the half-library immunizations, in which all

FIGURE 6. CD8⁺ T cells induced by the UB-HIV-1 library vaccine respond to wild-type HIV-1 Ags. Mice were immunized by gene gun with the indicated half-library (left half, HIV 1–16; right half, HIV 17–32) or whole library (HIV 1–32). Two weeks later, their splenocytes were stimulated for 6 h with the 10T/2-HLA-A*0201 stimulator cells transfected with UB-HIV-1 library members or with wild-type (wt) HIV-1 sequences (gag-pol or env) as indicated. Intracellular IFN- γ staining of the lymphocytes (1×10^6 /sample) was performed as explained in *Materials and Methods*. CD3/CD8 double-positive cells ($5\text{--}10 \times 10^4$ /sample) were analyzed for intracellular IFN- γ production on a FACScan using CellQuest software. Data shown are representative of three independent experiments with similar results.



these control CD8/IFN- γ responses averaged $<0.7\%$ when stimulated with Ag-expressing cells.

The HLA-A*0201-restricted, HIV-1 Ag-specific T cells produced in the transgenic mice were also checked for their ability to recognize and mount an immunological response against wild-type HIV-1 Ags. For that, a new set of stimulator 10T/2 cells was prepared that were transfected with plasmids expressing wild-type gag-pol or wild-type env in the context of HLA-A*0201. In contrast to the library member-expressing cells, these cells expressed the HIV-1 Ags in full-length form without any fusion to UB. Therefore, the epitopes presented by these stimulator cells are analogous to their presentation in an HIV-1-infected host. The results demonstrate that CD8 T cells generated by the UB-HIV-1 plasmid immunization recognize and respond to cells expressing wild-type gag-pol and env epitopes (Fig. 6). However, as expected from previous results (29), these responses were slightly less than those induced by stimulator cells transfected with library members expressing proteasome-targeted fragmented HIV-1 Ags (Fig. 6). These observations demonstrate the ability of the HIV-1 library vaccine to generate robust CD8 T cell responses against HLA-restricted viral epitopes derived from wild-type HIV proteins.

These data demonstrate the ability of this prototype UB-HIV-1 library vaccine to generate simultaneous robust CD8 T cell responses against epitopes spanning the entire genome of HIV-1. These CD8 T cell responses were not restricted to library members expressing well-defined dominant HLA-A*0201-restricted peptides, but were concomitantly generated by every plasmid of the UB-HIV-1-library library. This work provides further proof of the principle for the application of library vaccines against HIV-1 to generate robust multivalent immune responses as an approach to vaccinate against a broad array of HIV-1 variants with the goal of targeting multiple conserved epitopes throughout the genome.

Discussion

This work was directed at determining the breadth of immune responses mediated by the UB-HIV-1 expression library immunization vaccine with the particular goal of determining whether only a subset of the library generates CTL responses or if this immune-targeting genomic vaccine can generate a broad repertoire of responses. In this work we demonstrate the ability of this multigene genetic vaccine to simultaneously provoke robust CD8 T cell responses in humanized mice against all HIV-1 library members. Single immunization by gene gun with the entire HIV-1 library mediated quite robust CD8 T cell responses in HLA-A*0201 transgenic mice, as demonstrated by HLA-A*0201 tetramer staining, CD8/IFN responses, as well as functional CTL activity from in vitro-stimulated splenocytes. These observations are notable, since this vaccine uses HIV-1 wild-type sequences without any codon optimization, in contrast to some of the most successful preclinical HIV and SIV vaccine approaches (10). This suggests that this already potent vaccine can be made even more effective by codon optimization. These CD8 T cell responses include CTL responses against epitopes in gag, pol, env, and nef defined previously in humans (33, 34), including responses directed at gag and pol epitopes that are conserved in divergent HIV-1 strains (32). The library vaccine also generated CD8 responses in the HLA-A*0201 transgenic mice against a spectrum of previously undefined epitopes expressed by all remaining plasmids of the library. This work therefore demonstrates the ability of this UB-HIV-1 library vaccine to simultaneously provoke robust genome-wide, human-restricted CD8 T cell responses. This work provides additional evidence supporting the library vaccine approach to generate multivalent CTL responses against HIV-1 as an approach to control this highly mutable and immunoevasive pathogen.

The robust levels of multivalent CD8 T cell responses demonstrated here were produced by a single round of genetic immunization using biolistic gene gun delivery. These high level CTL responses were probably due in part to the efficiency of gene gun delivery and its ability to directly transfect epidermal dendritic cells, which have the largest concentration in the dermis (38). Gene gun delivery was selected for this study, because this method generates more efficient library-mediated immune responses in mice for screening efforts (28). Work is underway to compare the level of multivalent HLA-restricted CD8 responses in mice generated by the HIV-1 library vaccine delivery by gene gun, naked DNA injection, and mucosal-targeting approaches (39). Because naked DNA injection of full-length HIV and SIV genes has proven more effective in non-human primates (40), work is also underway to determine which method of genetic immunization is most effective for the proteasome-targeting, fragmented format of the HIV-1 library vaccine in macaques.

The strong CD8 T cell responses we observed are also probably related to the format of HIV-1 Ag expression, where each Ag is expressed as a subgene fragment to eliminate HIV protein toxicity and break down the secondary structure of the proteins. Further, each HIV Ag fragment was fused to UB to target the Ag to the proteasome to increase CTL responses (28, 29, 41). This combination of immune targeting and fragmented Ag fusion protein in the HIV-1 library vaccine appears to combine to display HIV Ags to the immune system better than wild-type HIV Ags, as evidenced by an increased level and expanded repertoire of CTLs against both dominant and subdominant epitopes from the HIV Ags (29). The robust genome-wide CD8 responses demonstrated here by CTL assay, CD8/IFN- γ staining, and HLA-A*0201 tetramer staining add further support for the library or genetic live vaccine approach (29) to produce multivalent and enhanced immune responses against HIV.

This study was directed at determining how many of the HIV library plasmids provoke CD8 CTL responses in humanized mice. Toward this goal, T cell responses provoked by library vaccination were tested against known HLA-A*0201-restricted peptides and against the same UB fusion proteins from the library expressed in stimulator and target cells. This work with the Ag-expressing cell lines was purposely performed to compare library member-specific responses. Further, the optimized UB fusion Ags were expressed in the target cells to insure that all CD8 T cell responses were identified regardless of whether these would be targeted on cells expressing wild-type HIV Ags. This approach was admittedly biased toward positive responses to most effectively scan the HIV genome for potentially effective dominant and subdominant epitopes. Therefore, the ability of UB-HIV-1 library-induced CD8 T cells to recognize and mount an immunological response against unmodified wild type HIV-1 Ags was also characterized. Lymphocytes from UB-HIV-1 library-immunized mice mounted fairly high CD8/IFN- γ responses against wild-type gag-pol and env Ags that were presented by 10T/2 cells in the absence of their targeting to the proteasome, which is similar to their presentation in an infected host (Fig. 6). Work is underway to determine how many of the subdominant epitopes in the wild-type Ags are being recognized by library-induced CD8 T cells.

This work also addresses in part one theoretical concern regarding the library technique for generating simultaneous multivalent immune responses (36). The concern relates to previous observations of Ag dominance and antagonism, where potent Ags can reduce or ablate responses against other epitopes when multiple epitopes or Ags are delivered simultaneously. While Ag dominance and antagonism may theoretically occur, in this case we did not observe any overt negative effects on CD8 T cell responses

when these were compared after immunization with single plasmids, with either half the library or the whole library. Therefore, we conclude that Ag dominance and antagonism are not fundamental impediments to the simultaneous generation of multivalent T cell responses by this UB-HIV-1 library vaccine. To address the issues of antagonism and immunodominance more critically, we have tested an OVA-based agonist-antagonist pair in C57BL/6 mice, where it has been observed that when delivered simultaneously to the immune system this pair fails to antagonize each other in vivo and generates both agonist- and antagonist-specific CD8 T cells (our unpublished observations). Therefore, we conclude that simultaneous exposure of immune system with UB-HIV-1 library vaccine circumvents Ag dominance and antagonism.

In summary, these data demonstrate the ability of the UB-HIV-1 library vaccine to provoke robust CD8 T cell responses spanning the genome of HIV-1 without any overt evidence of Ag antagonism or interference. These CD8 T cell responses included HLA-A*0201-restricted responses against the gag and pol epitopes, which are fairly conserved between different variants of HIV-1, indicating that this prototype vaccine or newer updated versions of HIV-1 library vaccines should cross-react against a broad array of HIV-1 variants. These CD8 T cell responses were generated by every plasmid of the UB-HIV-1 library, demonstrating that each member has the potential to generate CTL responses against a different set of HIV Ags. This supports the application of the entire 32-plasmid library as a genomic vaccine to elicit multivalent CD8 T cell responses against HIV-1. These data therefore provide further proof of the principle for the library vaccine approach to generate multivalent immune responses against HIV-1 to better control the diverse spectrum of viral mutants already present in the human population. Work is underway to test this vaccine in rhesus macaque models for protection and to determine whether the immune-targeting, fragmented Ags of the library applied here in plasmid format have utility if expressed from vaccine vectors for potentially synergistic DNA prime-viral vector boost strategies.

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