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Vaccine-Induced Immune Responses in Rodents and Nonhuman Primates by Use of a Humanized Human Immunodeficiency Virus Type 1 *pol* Gene

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A synthetic gene consisting of the reverse transcriptase (RT) and integrase (IN) domains of human immunodeficiency virus type 1 (HIV-1) *pol* was constructed using codons most frequently used in humans. The humanized *pol* gave dramatically improved levels of Rev-independent, in vitro protein production in mammalian cells and elicited much stronger cellular immunity in rodents than did virus-derived gene. Specifically, BALB/c mice were immunized with plasmids and/or recombinant vaccinia virus constructs expressing the synthetic gene. High frequencies of Pol-specific T lymphocytes were detected in these animals by the gamma interferon enzyme-linked immunospot assay against pools of short overlapping peptides. Characterization of the stimulatory peptides from these pools indicates that the optimized gene constructs are able to effectively activate both $CD4^+$ and $CD8^+$ T cells. Immunization of rhesus macaques with DNA vaccines expressing the humanized *pol* coupled to a human tissue plasminogen activator leader sequence led to pronounced in vitro cytotoxic T-lymphocyte killing activities and enhanced levels of circulating Pol-specific T cells, comparable to those observed in HIV-1-infected human subjects. Thus, optimizing the immunogenic properties of HIV-1 Pol at the level of the gene sequence validates it as an antigen and provides an important step toward the construction of a potent *pol*-based HIV-1 vaccine component.

With over 5 million new human immunodeficiency virus (HIV) infections each year (http://www.unaids.org/wac/2000/wad00/files/WAD_epidemic_report.pdf), the development of a therapeutic and prophylactic vaccine has become critical. There is increasing evidence that a vaccine which elicits anti-HIV T-cell responses might effectively control viral infection. During acute HIV-1 infection, the appearance of virus-specific cytotoxic T lymphocytes (CTLs) coincides with a decrease in plasma viremia (5, 28). Broadly cross-reactive CTLs have been detected in highly exposed Nairobi prostitutes who appear resistant to infection (39). In vivo depletion of $CD8^+$ T cells in rhesus macaques during primary SIV infection results in a rapid increase in viremia, which is reversibly suppressed with the reappearance of virus-specific CTLs (41). Wyand et al. (45) have shown that a live attenuated SIV (nef^- vpr^-) vaccine confers strong protection from a heterologous SHIV challenge in rhesus macaques, suggesting the role of factors other than anti-Env immunity (such as host T-cell responses).

The internal proteins of HIV (Gag, Pol, and Nef) represent the most highly conserved sequences of the virus and are the predominant targets of host CTL responses (19, 21; our unpublished results). As such, these proteins are logical components of an HIV vaccine that utilizes this arm of the immune response. Pol is a late-gene product of HIV and consists of protease (Pro, 99 amino acids [aa]) reverse transcriptase (RT, 550 aa), and integrase (IN, 290 aa) functions (11, 16). As the product of the largest structural gene in the HIV genome, it contains many T-cell epitopes. In fact, Pol-specific T-cell responses have been identified in infected HIV-1 patients with very high incidence (21), and over 60 CTL epitopes have been reported thus far (26, 35). For these reasons, Pol would be integral to a vaccine that is capable of inducing potent, broad cellular immunity against HIV.

Cellular immunity can be induced using a number of in vivo gene-based transfection approaches that include nonintegrating DNA plasmids (1, 2, 6, 44) and replication-defective viral vectors (3, 7, 9, 13, 22). Reports on vaccine-induced cellular immunity against Pol in model animal systems have, however, been limited (25). While nonhuman primate studies that involve immunization with DNA vaccines consisting of HIV-1 Pol have been reported (1, 6), there has been no definitive evidence that a cellular immune response to this antigen has been generated in these animal systems. The usefulness of rodent systems in assessing the immunogenicity of vaccine candidates is even more limited given that only one CTL epitope (in C3H, $H\text{-}2^k$ background) (24) is known to date. We hypothesize that the general lack of immunological determinants for HIV-1 Pol is partly attributable to the poor immunogenicity of the wild-type gene when delivered in vivo. In this article, we describe the use of human-preferred codons in maximizing the expression of HIV-1 *pol* (expressing RT and IN) in mammalian cells. We further evaluate the immunogenic

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properties of the humanized gene (with or without fusion to a human tissue type plasminogen leader sequence) in mice and in nonhuman primates.

MATERIALS AND METHODS

Construction of Pol-encoding plasmids. A synthetic gene for HIV HXB2r RT-IN (opt-*pol*) was designed to incorporate human-preferred (humanized) codons for each amino acid residue (27, 31). A *Bgl*II fragment containing the synthetic gene was constructed from 10 double-stranded synthetic oligonucleotide duplexes (ranging from 159 to 430 bp) by Midland Certified Reagent Company (Midland, Tex.) following stepwise ligation procedures (15). The sequence of the gene was confirmed by dye termination sequencing. opt-*pol* was subcloned into the *Bgl*II site of the mammalian expression vector V1R (12) or V1Jns-tpa (43) using standard methods.

The wild-type gene for the RT-IN domains (pol_{IIIB}) was extracted and amplified from the HIV IIIB genome by PCR using two primers, 5′-CAG GCG AGA TCT ACC ATG GCC CCC ATT AGC CCT ATT GAG ACT GTA-3′ and 5′-CAG GCG AGA TCT GCC CGG GCT TTA ATC CTC ATC CTG TCT ACT TGC CAC-3′, containing *Bgl*II sites. Thermocycling conditions were as follows: 20 cycles of 1 min at 95°C, 1 min at 56°C, and 4 min at 72°C with a 15-min capping step at 72°C. The digested PCR fragment was subcloned into the *Bgl*II site of the V1Jns (43) expression plasmid, its sequence was confirmed, and the plasmid was amplified in DH5 cells for large-scale preparation. All plasmid constructs for animal immunization were purified by the CsCl method (40).

Construction of recombinant vaccinia virus. Shuttle vector plasmid pSC11 (8) was kindly provided by B. Moss (National Institutes of Health, Bethesda, Md.). The opt-*pol* gene was cloned into the *Sma*I site downstream of the viral P7.5 promoter and is preceded by a Kozak (CCACC) sequence for efficient translation (29). The recombinant vaccinia virus (vacc-opt-*pol*) expressing the humanized *pol* was propagated using published protocols (17). A control vaccinia virus vector (vacc-SC) expressing only β-galactosidase under the control of the p11 promoter was also constructed.

In vitro expression in mammalian cells. A total of 1.5×10^6 293 cells were transfected with 1 or 10 μg of V1R-opt-*pol* and V1Jns-pol_{IIIB} using the Cell Phect kit (Pharmacia, Uppsala, Sweden) and incubated for 48 h at 37°C under 5% CO_2 at 90% humidity. Supernatants and cell lysates were prepared and assayed for protein content using the Pierce (Rockford, Ill.) protein assay reagent. Aliquots containing equal amounts of total protein were loaded into a 10 to 20% Tris glycine gel (Novex, San Diego, Calif.) along with the appropriate molecular weight markers. The Pol product was detected using 1:1,000-diluted antiserum from a seropositive patient (Scripps Clinic, San Diego, Calif.), and the bands were developed using goat anti-human immunoglobulin G (IgG) conjugated to horseradish peroxidase (IgG) (Bethyl, Montgomery, Tex.) at a 1:2,000 dilution and a standard ECL reagent kit (Pharmacia).

Immunization and sample collection. All vaccines were prepared in 6 mM sodium phosphate–150 mM sodium chloride (pH 7.2). In mice, the total dose was injected to both quadricep muscles in 50-μl aliquots using a 0.3-ml insulin syringe with a 28.5-gauge needle (Becton Dickinson, Franklin Lakes, N.J.). Three separate rodent immunization protocols were set up. In the first protocol, four separate cohorts ($n = 15$ mice/group) were immunized by different triple-dosing regimens: (i) three immunizations with 20-μg doses of V1R-opt-*pol*, (ii) three vaccinations with 10^7 PFU of vacc-opt-*pol* virus, (iii) two immunizations with 20-μg doses of V1R-opt-*pol* followed by 10^7 PFU of recombinant vacc-opt-*pol* virus, or (iv) two immunizations with 10^7 PFU of vacc-opt-*pol* virus followed by 20 μg of V1R-opt-*pol*. The three immunizations were administered 5 weeks apart. As a subset of this protocol, new sets of three mice per strain were vaccinated with two doses, administered 6 weeks apart, of 10^7 PFU of vacc-SC, a vaccinia virus not expressing the *pol* gene. In a second protocol aimed to characterize the $CD8^+$-specific peptides, BALB/c mice ($n = 40$) were vaccinated with two doses of V1R-opt-*pol* and one dose of 10^7 PFU of vacc-opt-*pol* as described above. In the third protocol, cohorts of 10 BALB/c mice (Taconic, Germantown, N.Y.) were vaccinated with V1Jns-pol_{IIIB} (10 or 100 μg doses), 30 μg of V1R-opt-*pol*, or 30 μg of V1Jns-tpa-opt-*pol* plasmid. At 5 weeks after dose 1, half the cohort was boosted with the same dose of plasmid they initially received. Serum samples were collected by bleeding the rodents from their tail veins; 6 to 8 drops of blood was fractionated using Microtainer separator tubes (Becton-Dickinson). Spleens were collected aseptically following terminal surgery and pooled for each cohort.

Cohorts of three rhesus macaques (5 to 10 kg each) were vaccinated with a 5-mg dose of either V1Jns-opt-*pol* or V1Jns-tpa-opt-*pol*. The vaccine was administered to anesthetized monkeys (10 mg of ketamine per kg) by needle injection, into both deltoid muscles, of 0.5-ml aliquots of 5-mg/ml plasmid solution. The animals were immunized three times at 4-week intervals ($t = 0$, 4, and 8 weeks). Blood samples were collected at $t = 0$, 4, 8, 12, 16, and 18 weeks. All animal care and treatment was in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council.

HIV-1-seropositive patients. Peripheral blood mononuclear cell (PBMC) samples isolated from 20 adult patients infected with HIV-1 were collected for evaluation of *pol*-specific T-cell responses. These patients had been treated with highly active antiretroviral therapy for more than 3 years, and their plasma viral loads were below 400 copies/ml. Their $CD4^+$ T-cell counts were above 400/μl during the study.

ELISPOT assay. The enzyme-linked immunospot (ELISPOT) assay was used to enumerate antigen-specific gamma interferon (IFN-γ)-secreting cells from mouse spleens (36), macaque PBMCs (30), and human PBMCs (20). Mouse spleen cells were prepared using pooled tissues from three to five mice per cohort. Rhesus and human PBMCs were prepared from EDTA-treated blood following standard Ficoll gradient separation (10). Multiscreen opaque plates (Millipore, Molsheim, France) were coated at 100 μl per well with either 5 μg of purified rat anti-mouse IFN-γ IgG1, clone R4-6A2 (Pharmingen, San Diego, Calif.) per ml, 15 μg of mouse anti-human IFN-γ IgG2a (no. 1598-00; R&D Systems, Minneapolis, Minn.) per ml, or 10 μg of anti-IFN-γ monoclonal antibody mAb, clone 1-D1K (Mabtech, Nacka, Sweden) per ml in phosphate-buffered saline PBS at 4°C overnight for murine, monkey, and human assays, respectively. To each well, 100 μl of cell suspensions (1×10^5 to 5×10^5 cells) and 100 μl of antigen solution were added. To the control well, 100 μl of the medium was added; for specific responses, either selected peptides or peptide pools (4 μg/ml per peptide final concentration) were added. For overall response, cells were stimulated using pools of 20-aa peptides that encompassed the entire Pol sequence and overlapped by 10 aa. Each sample-antigen mixture was used in triplicate wells for murine samples or in duplicate wells for rhesus and human PBMCs. Following incubation at 37°C under 5% CO_2 at 90% humidity for 20 to 24 h, the plates were washed and incubated at 100 μl per well with 1.25 μg of biotin-conjugated rat anti-mouse IFN-γ MAb, clone XMG1.2 (Pharmingen), per ml for mouse cells, 0.1 μg of biotinylated anti-human IFN-γ goat polyclonal antibody (R&D Systems) per ml for monkey cells, or 1 μg of biotinylated anti-IFN-γ MAb 7-B6-1 (Mabtech) per ml for human cells at 4°C overnight. The plates were developed using established procedures. The number of spots in each well was determined under a dissecting microscope, and the data were normalized to an input of 10^6 cells.

T-cell depletion. Murine splenocytes were depleted of either $CD4^+$ or $CD8^+$ T cells using Dynabeads mouse CD4 or Dynabeads mouse CD8 (Dynal, Oslo, Norway), respectively, at bead/cell ratio of 20:1 by incubation at 4°C for 30 to 60 min with gentle rocking. The mixture was placed on a Dynal MPC-6 instrument for 2 min, and the unbound cells were collected. The cells were pelleted at 300 $\times$ *g* for 10 min at room temperature, and the cell density was adjusted with complete medium. The efficiency of depletion was greater than 96.0%, as confirmed by flow-cytometric analysis of antibody-stained aliquots (fluorescein isothiocyanate-conjugated rat anti-mouse CD4, clone GK1.5, and phycoerythrin-conjugated rat anti-mouse CD8, clone 53-6.7 [Pharmingen]). Then 5×10^5 cells/well were placed on the ELISPOT plate as described above.

Rhesus macaque and murine CTL assay. Murine CTL activity was measured by sodium chromate (^{51}Cr) release assay as previously described (10, 23). CTLs were generated by a 6-day restimulation of a mixture of splenocytes derived from BALB/c vaccinees and irradiated syngeneic spleen cells in the presence of 10 U of recombinant human interleukin-2 (Cellular Products, Inc., Buffalo, N.Y.) per ml and all $CD8^+$-epitope containing 20-mer peptides (2 μg/ml) at 37°C under 95% humidity at 5% CO_2. A total of 2×10^6 of P815 cells (American Type Culture Collection, Manassas, Va.) were pulsed with each relevant antigenic peptide at 10 μg/ml for 2 h with 200 μCi of ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, N.J.). Target cells (10^4) were incubated with CTLs (10^4 to 10^6) in a U-bottom 96-well plate (Costar 3799) at 37°C for 3 h. The ^{51}Cr release CTL assay for rhesus macaques was conducted by following a protocol detailed by Fu et al. (18). Generally, effector cells were prepared from a 2-week incubation with vaccinee PBMCs infected previously with recombinant vaccinia virus, vacc-tpa-opt-*pol*. The harvested effector cells were tested against autologous B-lymphoid cell lines (BLCL) sensitized overnight with peptide pools (at 4 μg/ml for each peptide). Then 30 μl of supernatants per well was transferred into a Luma Plate-96 (Packard, Meriden, Conn.), and radioactivity was determined using a TopCount microplate scintillation counter (Packard). The percent specific lysis was calculated using the formula percent specific lysis = $(E - S)/(M - S)$, where E represents the average cpm released from target cells in the presence of

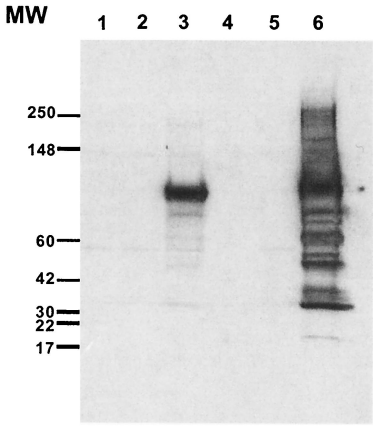

FIG. 1. Detection of in vitro *pol* expression from cell lysates of 293 cells transfected with V1Jns-pol_{IIIB} or V1R-opt-*pol*. Bands were detected using antiserum from an HIV-infected human subject. Lanes: 1, mock or no-vector control; 2 and 3, extracts from cells transformed with 1 μg of V1Jns-pol_{IIIB} or V1R-opt-*pol*, respectively; 4, blank; 5 and 6, extracts from cells transformed with 10 μg of V1Jns-pol_{IIIB} or V1R-opt-*pol*, respectively. MW, molecular weight (in thousands).

effector cells, *S* is the spontaneous cpm released in the presence of medium only, and *M* is the maximum cpm released in the presence of 2% Triton X-100.

RESULTS

Enhanced in vitro expression using the humanized gene. A synthetic gene (opt-*pol*) for the RT-IN domains of Pol (aa 154 to 1003) was designed to incorporate human-preferred (humanized) codons for each amino acid residue (31); 73.5% of the codons were affected by changing 26.6% of the nucleotides. The encoded protein sequence is based on that of HXB2r, a clonal isolate of IIIB; this sequence is closest to the consensus HIV clade B sequence, with only 16 nonidentical residues out of 848 (27). The gene was subcloned into the V1R vector (3.5 kb) (12), placing it in front of a Kozak sequence (5′-CTACC-3′) (29) and under the control of the human cytomegalovirus/intron A promoter plus the bovine growth hormone terminator. For comparison, the equivalent wild-type gene was extracted from the HIV IIIB genome and subcloned into V1Jns (4.9 kb) (43), a vector containing the same regulatory elements as V1R, to give V1Jns-pol_{IIIB}. It should be noted that for other viral antigens (unpublished results), the expression efficiency and immune responses are comparable when they are cloned into either V1R or V1Jns; V1Jns was used as an alternative because of increased bacterial amplification yields with V1Jns-based vectors (unpublished results).

Rev-independent expression of the opt-*pol* plasmid in 293 cells (Fig. 1) yielded a single polypeptide of the predicted molecular size (90 kDa) for the RT-IN fusion product. In contrast, no expression could be detected when cells were transfected with 1 and 10 μg of the V1Jns-pol_{IIIB}.

Identification and characterization of epitope-containing peptides for direct enumeration of Pol-specific T cells. The evaluation of cellular immune responses induced by Pol immunization requires quantitative assays in the appropriate animal model systems. One such method, the ELISPOT assay (36), relies on the epitope-dependent induction of effector cytokine secretion from T cells for direct detection of these cells. In BALB/c mice, sequences of HIV-1 Pol T-cell epitopes especially for $CD8^+$ cells have yet to be identified. For this purpose, highly immunized animals had to be generated to provide the maximum potential for detecting responses to individual peptides from a series that encompasses the entire HIV-1 Pol sequence. Recent studies have indicated that high levels of cellular immunity can be induced by combining DNA and poxvirus vectors for alternate priming and boosting (22). To explore this possibility, mice were immunized intramuscularly by different regimens: (i) three doses of V1R-opt-*pol* plasmid (DNA+DNA+DNA), (ii) three doses of vacc-opt-*pol* (vacc+vacc+vacc), (iii) two doses of V1R-opt-*pol* plasmid followed by a vacc-opt-*pol* dose (DNA+DNA+vacc), or (iv) one dose of vacc-opt-*pol* followed by two doses of the plasmid construct (vacc+DNA+DNA); the immunizations were 5 weeks apart.

The cellular immunity induced by the different regimens was assessed using the IFN-γ ELISPOT at 3 weeks after the third dose (Fig. 2A). Antigen-specific cytokine secretion from splenocytes was stimulated by adding one of two peptide pools that collectively encompass the entire protein sequence. Pool 1 contains a set of 20-mer peptides that overlap by 10 aa and cover aa 154 to 573 of the sequence; pool 2 contains a comparable set that covers aa 564 to 1003. After the third dose, the most vigorous responses were observed from the DNA+DNA+vacc cohort; they were about two- to threefold higher than for the other mixed-modality regimen; the responses were biased toward the N-terminal half of the antigen (pool 1). Hence, the DNA+DNA+vacc cohort provided an excellent source of animals for further analyses of the immune responses.

To determine the components of the peptide pools eliciting the responses, spleens from three mice of the DNA+DNA+vacc cohort were analyzed in an IFN-γ ELISPOT assay against each of the 84 Pol peptides. The results are shown in Fig. 2B, and the positive peptides are listed in Table 1. Pol-directed responses were mapped to a total of 10 stimulatory peptides, two pairs of which (aa 464 to 483 plus aa 474 to 493, and aa 924 to 943 plus aa 934 to 953) have overlapping sequences. The epitopes contained in these peptides were confirmed to be specific for Pol and not the vaccinia virus backbone. In mice vaccinated with two doses of a noncoding vacc-SC control, all peptides gave counts which were not significantly above the levels seen in untreated animals (data not shown). All positive peptides were also tested against unfractionated, $CD4^+$-depleted, or $CD8^+$-depleted cells prepared from spleens of another set of *pol* vaccinees. In all cases, abrogation of spot-forming cells was observed after depletion of one or the other of the T-cell subsets (representative data are shown in Fig. 3A); the results are summarized in Table 1. Of the 10 peptides, 4 (aa 354 to 373, 464 to 483, 474 to 493, and 934 to 953) were $CD8^+$ responsive. The first three peptides dominate the cellular responses (Fig. 2B), consistent with the observed biased of the responses toward pool 1 (Fig. 2A).

Cytotoxic properties associated with the $CD8^+$-specific epitopes were verified by restimulating vaccinee splenocytes with a mixture of all four $CD8^+$-specific 20-mer peptides and analyzing the ability of the resulting effector cells to kill syngeneic P815 cells pulsed with each of the four $CD8^+$-specific 20-mer peptides (Fig. 3B). All but one (aa 474 to 493) of the

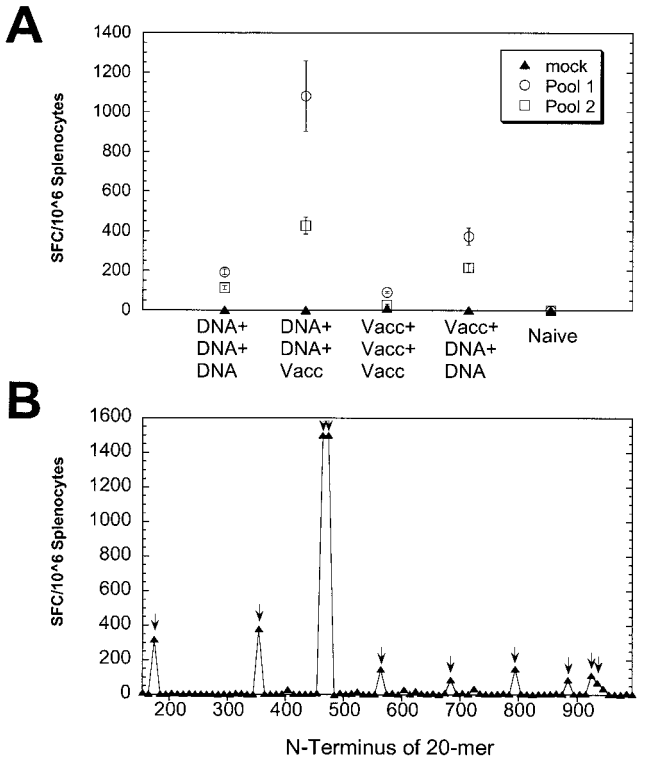

FIG. 2. Groups of BALB/c mice were immunized with different combinations of three doses of V1R-opt-*pol* (20 μg/dose) and/or vacc-opt-pol (10^7 PFU). (A) Frequencies of Pol-specific T cells in splenocytes of BALB/c mice at the end of the multidose regimen (3 weeks after dose 3). Spleens were collected from three representative mice per cohort and pooled. The numbers of IFN-γ-secreting SFC per 10^6 splenocytes were determined in the absence (mock) or presence of pools of Pol sequence-derived 20-mer peptides (Pool 1 and 2). The arithmetic means of the counts from triplicate wells are reported, along with the standard errors of the mean. The analysis was repeated 9 weeks after dose 3 and yielded similar trends. (B) Mapping of T-cell responses to select Pol peptides. Pooled splenocytes from three mice from the DNA+DNA+vacc cohort were assayed for IFN-γ secretion against each of the 20-mer peptides. ELISPOT data were plotted as a function of the number of amino acids of the N-terminal residue of the 20-mer peptide. Arrows are indicated above each peptide that reproducibly stimulated cytokine secretion in succeeding T-cell depletion studies. A conservative count of 1,500 per 10^6 cells was assigned to sample wells where the spots could not be quantified because of high spot density. A total of 84 peptides were tested; the aa 784 to 803 peptide is missing because of difficulties in synthesis. Reported are the means of triplicate well normalized to a 10^6 cell input; the standard errors did not exceed 20% of the mean values.

20-mer peptides induced killer function and sensitized target cells. Of the four 20-mer peptides, aa 464 to 483 and 474 to 493 stimulated the largest number of lymphocytes from the BALB/c vaccinees for cytokine secretion (Fig. 2B); it is likely that both peptides interact with the same T-cell clone.

To delineate the location of the $CD8^+$ epitope(s) within the each of the three 20-aa CTL peptides, a series of 10 11-aa peptides shifted by 1 residue (37) were synthesized and tested against vaccinee splenocytes. Four contiguous 11-mers were positive for each 20-mer parental sequence in an ELISPOT assay (Table 2). All positive 11-aa analogs also provided effective targets for the pool-restimulated effectors (Table 2). The

TABLE 1. Sequences of epitope-containing peptides (20 aa) for HIV-1 Pol in BALB/c mice

Position (aa)	Sequence	T-cell specificity
RT 174–193	PKVKQWPLTEEKIKALVEIC	$CD4^+$
RT 354–373	RTKIEELRQHLLRWGLTTPD	$CD8^+$
RT 464–483	ILKEPVHGVYYDPSKDLIAE	$CD8^+$
RT 474–493	YDPSKDLIAEIQKQGQGQWT	$CD8^+$
RT 564–583	TWIPEWEFVNTPPLVKLWYQ	$CD4^+$
RT 684–703	EKVYLAWVPAHKGIGGNEQV	$CD4^+$
IN 794–813	VASGYIEAEVIPAETGQETA	$CD4^+$
IN 884–903	AEHLKTAVQMAVFIHNFKRK	$CD4^+$
IN 924–943	QTKELQKQITKIQNFRVYYR	$CD4^+$
IN 934–953	KIQNFRVYYRDSRNPLWKGP	$CD8^+$

shorter peptides are at least as effective as the parental 20-mer in presentation for CTL killing, probably due to improved peptide processing or major histocompatibility complex class I binding (representative data for aa 472 to 482 shown in Fig. 3B).

Analyses of the positive 11-mer analogs suggest that the epitopes are located within aa 359 to 372, 470 to 483, and 938 to 951. The aa 470 to 483 region contains two overlapping sequences ($V\mathbf{Y}_{473}$YDPSKD$\mathbf{L}_{480}$ and $\mathbf{YY}_{474}$DPSKDL$\mathbf{I}_{481}$; anchor residues are in bold) that conform with the canonical *H-2K*^{d} motif (14). The aa 938 to 951 region contains a characteristic *H-2K*^{d} motif in the sequence $\mathbf{YY}_{942}$RDSRNP$\mathbf{L}_{949}$; however, the cytotoxic activity observed with the aa 938 to 948 peptide suggests an alternative epitope of $V\mathbf{Y}_{941}$YRDSRN$\mathbf{P}_{948}$. Epitope clustering is rather common, especially in regions of high hydrophobicity (26). The RT aa 359 to 372 sequence does not have any sequence that could be associated with the classical *H-2*^{d} motifs. A core 8-aa sequence of Q_{362}HLLRWGL$_{369}$ can be deduced from the four positive 11-mer analogs of this peptide.

It is important to note that at this stage, we have identified and validated peptide reagents that can now be used to quantify circulating levels of $CD8^+$ and $CD4^+$ T cells from immunized mice. The $CD8^+$-specific peptides have been confirmed as epitopes for CTLs; two of the three peptides were further shown to contain putative clusters of known major histocompatibility complex class I binding motifs. To definitively determine the anchor residues and secondary recognition residues for all $CD8^+$ T-lymphocytic epitopes, experiments involving alanine scanning and peptide titrations are under way and will be the subject of a separate report.

Comparative T-cell immunity using humanized and virus-derived gene. To compare the levels of cellular immunity generated by the different plasmid vaccines in a quantitative manner, BALB/c mice (n = 10 per group) were immunized with either 10 or 100 μg of V1Jns-*pol*$_{\text{IIIB}}$, 30 μg of V1R-opt-*pol*, or 30 μg of V1Jns-tpa-*pol*. At week 5, 5 of 10 animals were randomly boosted with the same vaccine and dose that they initially received. A human secretory plasminogen activator leader sequence (tpa) (38) was attached to opt-*pol* to determine if the immunogenicity could be improved by altering the trafficking of the transgene product; the resulting tpa-opt-*pol* was introduced into V1Jns. N-terminal fusion of the human tpa sequence was shown to enhance both humoral and cell-mediated immune responses to select vaccine-encoded bacterial

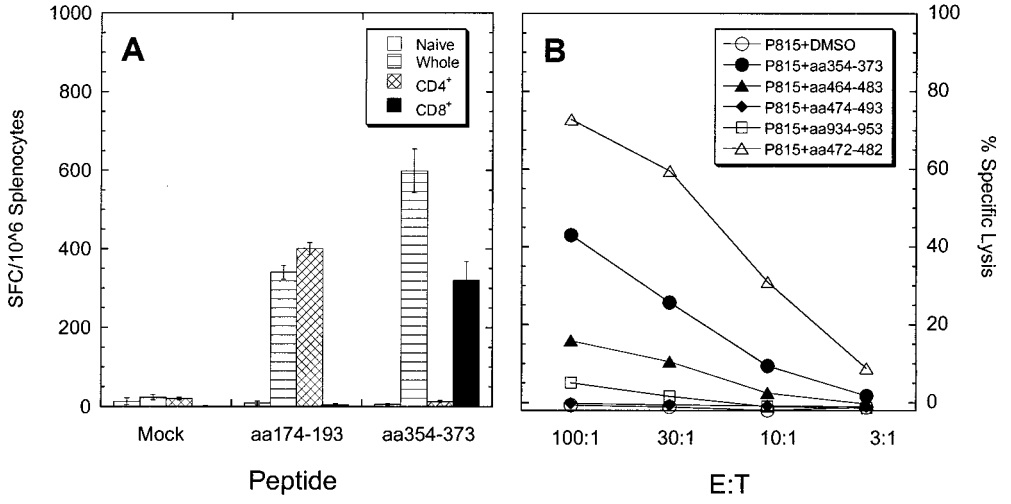

FIG. 3. (A) Frequencies of antigen-specific IFN-γ-secreting cells in unfractionated (whole) and $CD4^+$- and $CD8^+$-depleted splenocytes from BALB/c vaccinees. Each antigenic peptide was also tested with splenocytes from untreated (naïve) mice. (B) ^{51}Cr release CTL killing by effector cells (from BALB/c vaccinees) of P815 cells pulsed with each of the $CD8^+$ 20-mer peptides. Effector cells were prepared from splenocytes of 10 BALB/c mice immunized following DNA+DNA+vacc regimen (at 1 week after dose 3) and were restimulated with a mixture of aa 354 to 373, 464 to 483, 474 to 493, and 934 to 953 for 6 days in the presence of IL-2. E:T, effector/target ratio.

antigens (33, 34). Pooled spleen cells from the 5 BALB/c vaccinees per cohort were analyzed for IFN-γ secretion following stimulation with mixtures of either all $CD4^+$ peptide epitopes or all $CD8^+$ peptide epitopes (Fig. 4). No nonhumanized *pol* vaccinees exhibited any detectable cellular immunity to HIV-1 Pol above the untreated controls. In contrast, strong and boostable antigen-stimulated IFN-γ secretion was observed in animals vaccinated with 30 μg of the opt-*pol* construct. Pol-specific $CD4^+$ and $CD8^+$ responses were detected, with the $CD8^+$ T cells dominating the response, with as many as 600 per 10^6 splenocytes after two vaccine doses. Addition of the tpa leader sequence led to a further twofold increase in frequencies of both Pol-specific cytotoxic and helper T cells compared to the non-tagged *pol* gene.

Pol-specific responses in DNA-vaccinated macaques and HIV-1-infected patients. To determine the efficacy of these optimized *pol* genes in a primate system, groups of three macaques were vaccinated intramuscularly with multiple doses of V1Jns-tpa-opt-*pol* or V1R-opt-*pol*. An immunization schedule of 0, 4, and 8 weeks was adopted; at selected time points, PBMCs were analyzed for cytokine secretion and cytolytic activity using the two peptide pools. Immunization with V1R-opt-*pol* resulted in moderate levels of antigen-specific IFN-γ-secreting PBMCs in the vaccinees (Fig. 5). CTL killing of peptide-pulsed autologous cells was observed in one of three animals at 8 weeks after the last dose (Fig. 6), indicating the presence of $CD8^+$ T cells that recognized epitopes within the carboxy-terminal half of Pol. Immunization with V1Jns-tpa-

TABLE 2. Reactivities of unfractionated murine splenocytes (DNA+DNA+vacc) to overlapping 11-aa peptide analogs in an ELISPOT assay

Peptide sequence			Relative ELISPOT response[a]	CTL[b]
354	RTKIEELRQHLLRWGLTTPD	373	ND[c]	+++
359	ELRQHLLRWGL	369	1.00 (1,400 ± 168)	+++
360	LRQHLLRWGLT	370	0.88 ± 0.11	+++
361	RQHLLRWGLTT	371	0.95 ± 0.12	+++
362	QHLLRWGLTTP	372	0.17 ± 0.02	+++
464	ILKEPVHGVYYDPSKDLIAE	483	ND	++
470	HGVYYDPSKDL	480	1.00 (>1,500)	+++++
471	GVYYDPSKDLI	481	1.00	+++++
472	VYYDPSKDLIA	482	1.00	+++++
473	YYDPSKDLIAE	483	1.00	+++++
934	KIQNFRVYYRDSRNPLWKGP	953	ND	+
938	FRVYYRDSRNP	948	0.87 ± 0.12	+
939	RVYYRDSRNPL	949	1.00 (129 ± 17)	+
940	VYYRDSRNPLW	950	0.84 ± 0.10	+
941	YYRDSRNPLWK	951	0.95 ± 0.12	+

[a] The frequencies of antigen-specific IFN-γ-secreting cells along with standard errors (mock subtracted) are reported relative to the strongest response (1.00) among the family of 11-mer analogs. The triplicate mean values for the SFC per 10^6 cells of the peak response are indicated in parentheses.
[b] +, 5 to 10%; ++, 5 to 20%; +++, 10 to 40%; +++++, 30 to 70% specific lysis at effector/target ratios of 10:1 to 30:1.
[c] ND, not done.

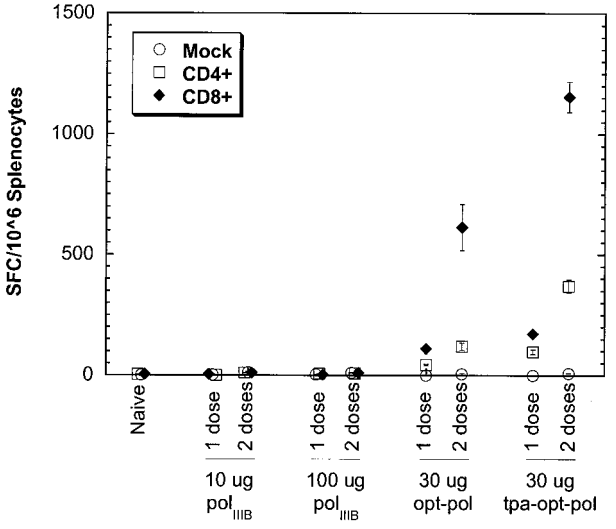

FIG. 4. Cellular immune responses in BALB/c mice vaccinated intramuscularly with one or two doses of different amounts of either pol_{IIIB} or opt-*pol* plasmids. At 3 weeks after the second immunization, frequencies of IFN-γ-secreting splenocytes (shown with the standard errors) were determined for boosted and unboosted cohorts (using pools of five spleens per cohort) against mixtures of either $CD4^+$ peptides (aa 174 to 193, 564 to 583, 684 to 703, 794 to 813, 884 to 903, and 924 to 943) or $CD8^+$ peptides (aa 354 to 373, 464 to 483, and 934 to 953) at a final concentration of 4 μg/ml per peptide.

opt-*pol* produced T-cell responses from two vaccinees that were substantially higher than those in vaccinees treated with the non-tagged *pol* vaccine (Fig. 5); the responses in monkey 920078 reached as high as 660 spot-forming cells (SFC) per 10^6 PBMCs against both peptide pools. These were consistent with the trend observed in the rodent study. Two macaques, 920078 and 94R028, exhibited bulk cytotoxic activity against pool 2-pulsed targets, consistent with the presence of residual pool 2-specific IFN-γ-secreting cells in $CD4^+$-depleted PBMCs from both vaccinees (data not shown).

To compare these vaccine-induced responses with the levels of host immunity in patients infected with HIV-1, PBMCs from 20 HIV-1-seropositive individuals whose infection had been controlled by antiretroviral therapy for more than 3 years were collected and analyzed by a human IFN-γ ELISPOT assay against the same pair of peptide pools (Table 3). Based on the validation results for the human assay, a positive response must have at least 55 SFC/10^6 PBMC and at least a fourfold rise above the mock value (our unpublished results). Thirteen of these individuals (65%) exhibited an antigen-specific response to one of the peptide pools with levels as high as 654 SFC/10^6 PBMCs. The peak immune responses in the vaccinated monkeys overlapped substantially (although noticeably lower) with those measured in infected individuals against the same peptide pools.

DISCUSSION

HIV-1 Pol is a prominent target for CTL responses in infected individuals, and, as such, a potentially important vaccine component. Haas et al. (21) have observed memory CTL responses against HIV-1 Pol in 78% of patients (77 of 98) at different stages of the disease, with the majority of the responders being able to recognize determinants within the RT (81%) and IN (54%) domains. Only 24% of the subjects were

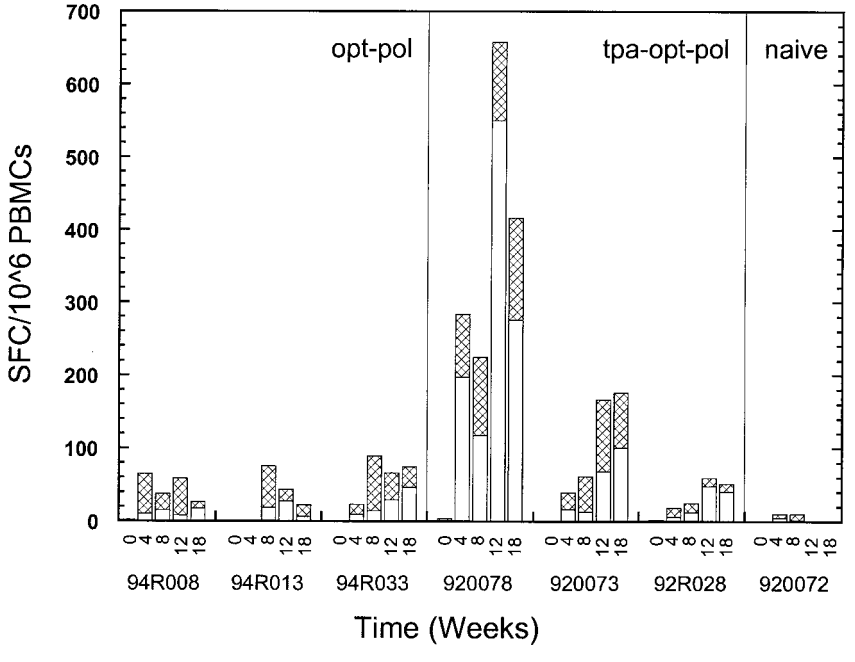

FIG. 5. Frequencies of Pol-specific IFN-γ-secreting cells (as measured by the ELISPOT assay) in rhesus macaques vaccinated with 5 mg of V1Jns-tpa-opt-*pol* and V1R-opt-*pol* DNA vaccines at 0, 4, and 8 weeks. Numbers of SFC per 10^6 PBMCs against pool 1 (cross-hatched portion of the bars) and pool 2 (open portion of the bars) are reported for each monkey at different assay times; mock responses to dimethyl sulfoxide (no peptide) ranged from 0 to 24 per 10^6 PBMCs and were subtracted from these reported responses.

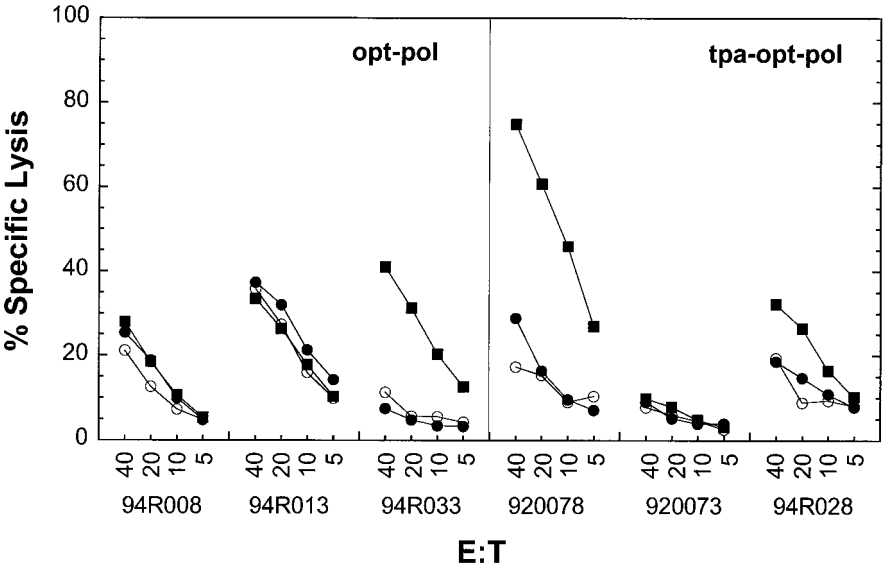

FIG. 6. Bulk CTL killing activity of Pol-peptide-pulsed BLCLs by effector cells derived from rhesus PBMCs following a 2-week restimulation with vacc-tpa-opt-*pol*. PBMCs were collected 8 weeks after dose 3. As background control, BLCLs were pulsed only with the dimethyl sulfoxide solvent (no peptide). The same responders were observed in the CTL assay conducted 4 weeks earlier. E:T, effector/target ratio.

positive for Pro, which may not be surprising since it accounts for only 10% of the size of Pol. Hence, a vaccine comprising RT and IN should, in principle, confer a level of diversity in antigen recognition comparable to that of the full-length protein. Exclusion of Pro also has the advantage of inactivating the enzymatic functions of RT and IN, which both require posttranslational Pro-mediated cleavage (16).

Several groups have reported immunizations of nonhuman primates with DNA and live attenuated vectors encoding virus-derived *pol* genes. However, there is no convincing evidence for a strong vaccine-induced T-cell immunity against Pol when using these constructs. In the only work directly related to HIV-1 Pol, chimpanzees were treated with DNA vaccines for HIV-1 *gag-pol* fusion products (1, 6). Bulk-culture proliferative and CTL activities were observed on stimulation with *gag-pol*-expressing poxviruses, but the extent to which these responses are directed at *pol* remains unclear. In more distantly related studies, rhesus macaques were immunized with MVA (42) and NYVAC (3) viruses expressing the SIV *gag-pol*; however, only anti-*gag* responses were definitively observed. Others have immunized macaques with a quadravalent cocktail of recombinant bacille Calmette-Guérin (BCG) vectors that encoded SIV Gag, Pol, Env, and Nef (32) and measured CTL activities to all but Nef. However, none of these data demonstrated the induction of a cellular immune response to HIV-1 Pol in nonhuman primates. To unambiguously address this critical issue, studies must be designed to examine HIV-1 *pol* in isolation.

In this study, we demonstrated that the virus-derived HIV-1 *pol* gene is, in fact, not fully optimized as an immunogen for in vivo delivery using nonreplicating vectors (DNA or recombinant viruses). When subcloned into a standard mammalian transfection plasmid with a strong hCMV/IA promoter, HIV-1 *pol* derived from a IIIB strain was expressed poorly both in vitro and in vivo. Examination of the gene indicates a highly AT-rich sequence that does not favor frequently used genes in highly expressed human proteins. In view of this, we constructed a synthetic gene for the RT-IN fusion protein that incorporated human-biased codons. The humanized construct remarkably gave improved in vitro mammalian expression compared to the viral gene. Similar results have been reported previously for humanized genes of other proteins (12, 46). The enhanced *pol* expression translated into elevated, detectable levels of circulating antibodies in mice (data not shown).

Since host T-cell immunity (primarily CTL) has been strongly implicated as a correlate for protection against HIV (2, 5, 28, 39, 41, 45), it is important to be able to measure this type of immune response quantitatively in animal models. In general, mice remain an indispensable tool for vaccine development. However, the application of mouse models to HIV-1 Pol has been hampered by the lack of highly sensitive immunological markers. Prior to this work, only one murine CTL epitope (C3H, $H\text{-}2^k$) had been identified (24); however, it was only weakly recognized, providing poor assay sensitivity (unpublished results). In this study, strong T-cell responses (0.17% of circulating lymphocytes) to pools of HIV-1 Pol peptides were induced in BALB/c mice by immunization with the humanized gene in the context of a heterologous DNA-vaccinia virus prime-boost regimen. Further dissection of the responses to individual peptides revealed HIV-1 Pol epitopes for both $CD8^+$ and $CD4^+$ T cells in the $H\text{-}2^d$ background (Table 1). Three peptides containing CTL epitopes and six others for helper epitopes were identified. Of these, only two $CD4^+$ T-cell determinants (aa 566 to 580 and aa 683 to 697) had been reported previously (4) following immunization of BALB/c mice with complete Freund's adjuvant-emulsified RT protein or peptide derivatives. The $CD8^+$ T-cell epitopes are contained within the aa 361 to 369 and aa 472 to 481 sections of the RT enzyme and the carboxy-terminal aa 940 to 949 region of IN. These cytotoxic peptides dominate the overall responses following vaccination with *pol*-expressing vectors.

TABLE 3. Frequencies of Pol-specific T cells in HIV-1-infected patients and DNA-vaccinated macaques (at week 12)

Subject	T-cell frequency (SFC/10^6 PBMC) in response to:		
	Mock	Pool 1	Pool 2
HIV-1 patients			
1	15	138	125
2	24	39	283
3	35	44	50
4	11	175	140
5	6	36	75
6	44	51	61
7	21	6	36
8	3	90	159
9	14	154	88
10	10	70	76
11	5	143	331
12	31	54	105
13	10	461	654
14	13	20	33
15	15	291	48
16	18	509	179
17	24	434	170
18	44	84	59
19	64	175	649
20	16	31	38
Mean	21	150	168
Range	3–64	6–509	33–654
SE[a]	3	34	41
Macaques			
94R008	11	61	19
94R013	9	25	36
94R033	14	51	43
920078	11	119	561
920073	15	113	83
94R028	18	29	66
Mean	13	66	135
Range	9–18	25–119	19–561
SE	1	17	86

[a] SE, standard error.

We have developed a BALB/c model system for the quantitative analyses of both cytotoxic and helper responses elicited by vaccination with the HIV-1 *pol* gene. The use of defined antigenic peptides for HIV-1 Pol offers the potential advantages of (i) being cost-effective compared to the use of complete peptide pools, and of (ii) providing improved assay sensitivity and consistency compared to stimulation approaches that rely on direct transfection of antigen-presenting cells with recombinant vaccinia viruses. We have demonstrated that the helper and cytotoxic T cells induced by immunization with HIV *pol* genes can be enumerated independently by using $CD4^+$ and $CD8^+$ epitope-containing peptides, respectively. Significant increases in the numbers of Pol-activated T cells were detected in rodents by (i) humanizing the *pol* gene and (ii) attaching a tpa leader sequence to the synthetic gene.

Immunization of rhesus macaques with multiple doses of the two humanized DNA constructs (tpa-opt-*pol* and opt-*pol* plasmid) resulted in significant T-cell responses to the Pol peptide pools. A significant fraction of the vaccinees also developed cytotoxic activity against peptide-pulsed autologous cell lines. Given the rodent results, it is rather unlikely that any substantial immunity could be detected in primates by using a plasmid encoding the virus-derived gene sequence. These results are promising because the level of immunity in these animals following a simple DNA vaccination overlapped with that measured during natural human infection. We recognize that host immunity can be further improved by incorporating the humanized gene into recombinant viral vectors and/or combining DNA and viral modalities for immunization.

While this paper was being written, a report by Huang et al. (25) appeared on the use of DNA vaccines containing synthetic *gag*/*pol* genes for eliciting HIV-1-specific CTL responses in BALB/c mice. While this study also illustrated the improved in vitro expression and immune responses to Pol, our data expand on these observations by showing that a DNA vaccine based on protease-deleted HIV-1 *pol* attached to a leader tpa sequence (and independent of a fusion to *gag*) can elicit strong $CD4^+$ and $CD8^+$ T-cell responses in nonhuman primates that approximate those observed in seropositive human patients. Where the results of the two studies overlap, the data differ in several key aspects. First, in the work of Huang et al., cellular immune responses were evaluated primarily by measuring CTL killing by vaccinated BALB/c splenocytes following a 2-week restimulation with a mixture of seven 15-aa Pol peptides (encompassing aa 300 to 326, 348 to 366, and 492 to 506). We distinguish our work by the use of methods that allow ex vivo enumeration of Pol-specific T cells in mouse, macaque, and human subjects. Those methods provide reliable means of detecting quantitative differences in vaccine efficacy like the effects of tpa fusion. Second, while the sequences of stimulatory peptides employed by Huang et al. match those in the *pol* gene product used in this study (except for the incorporation in the former of an alanine residue in place of a threonine at position 501), none of these peptides overlap with the $H\text{-}2^d$ epitopes for HIV-1 Pol that we have identified (Tables 1 and 2). It is also unclear from Huang et al. which peptide(s) from the pool contains a CTL epitope. It is likely that inefficient processing of the 20-aa peptides precluded the detection of certain epitopes; this possibility and others will have to be examined in greater detail.

In summary, optimizing the magnitude of the T-cell response induced to HIV antigens will facilitate the development of an HIV vaccine. Herein, we have shown that maximizing *pol* expression by modifying the coding sequence results in a much improved T-cell immunogen compared to the wild-type gene. The humanized gene is capable of inducing both CTL and helper responses in mice and, more importantly, in monkeys. While previous vaccine approaches employed *gag-pol* precursor genes, this work clearly supports the notion that *pol* is a potent immunogen as a single gene entity and can be utilized as such. Finally, this synthetic gene represents a valid immunogen, whose intracellular processing can be altered and which can be incorporated into any nonreplicating viral vector system (or combination thereof) to optimize host immunity. As increasing numbers of Pol T-cell epitopes are identified in seropositive human subjects (26, 35), it becomes apparent that the inclusion of a *pol* component in an HIV vaccine will improve the breadth of the cellular immune responses and will increase the likelihood of establishing protection against an HIV infection.

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