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Induction of AIDS Virus-Specific CTL Activity in Fresh, Unstimulated Peripheral Blood Lymphocytes from Rhesus Macaques Vaccinated with a DNA Prime/Modified Vaccinia Virus Ankara Boost Regimen[1]

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The observed role of CTL in the containment of AIDS virus replication suggests that an effective HIV vaccine will be required to generate strong CTL responses. Because epitope-based vaccines offer several potential advantages for inducing strong, multispecific CTL responses, we tested the ability of an epitope-based DNA prime/modified vaccinia virus Ankara (MVA) boost vaccine to induce CTL responses against a single SIVgag CTL epitope. As assessed using both ^{51}Cr release assays and tetramer staining of in vitro stimulated PBMC, DNA vaccinations administered to the skin with the gene gun induced and progressively increased p11C, C→M (CTPYDINQM)-specific CD8$^+$ T lymphocyte responses in six of six *Mamu-A*01*$^+$ rhesus macaques. Tetramer staining of fresh, unstimulated PBMC from two of the DNA-vaccinated animals indicated that as much as 0.4% of all CD3$^+$/CD8α^+ T lymphocytes were specific for the SIVgag CTL epitope. Administration of MVA expressing the SIVgag CTL epitope further boosted these responses, such that 0.8–20.0% of CD3$^+$/CD8α^+ T lymphocytes in fresh, unstimulated PBMC were now Ag specific. Enzyme-linked immunospot assays confirmed this high frequency of Ag-specific cells, and intracellular IFN-γ staining demonstrated that the majority of these cells produced IFN-γ after peptide stimulation. Moreover, direct ex vivo SIV-specific cytotoxic activity could be detected in PBMC from five of the six DNA/MVA-vaccinated animals, indicating that this epitope-based DNA prime/MVA boost regimen represents a potent method for inducing high levels of functionally active, Ag-specific CD8$^+$ T lymphocytes in non-human primates. *The Journal of Immunology,* **2000, 164: 4968–4978.**

While antiretroviral therapies have been successful in reducing viral loads in HIV-infected patients, these drugs are having little impact on the global epidemic. In 1998 alone, there were >5.8 million new cases of HIV infection worldwide (1). Reservoirs for HIV still persist in HIV-infected patients undergoing antiretroviral therapy (2), and almost 90% of the world's HIV-infected population resides within countries unable to afford these drugs. Therefore, the development of vaccines designed to prevent HIV infections, rather than treat the disease, is the best hope.

CD8$^+$ T cell responses play a key role in the containment of lentivirus infections. Strong CTL responses have been found to correlate with reduced plasma RNA viral loads in HIV-infected individuals (3). It has also been demonstrated that CTL exert selective pressure on the AIDS virus populations, as evidenced by the eventual predominance of viruses with amino acid replacements in those regions of the virus to which CTL responses are directed (4–10). Adoptive transfer of autologous HIVgag-specific CD8$^+$ CTL clones to three seropositive patients was capable of rapidly decreasing the percentage of productively infected CD4$^+$ T cells (11). Similarly, SIV-infected macaques depleted of CD8$^+$ T lymphocytes were subsequently unable to control virus replication (12–14). Together, these studies strongly support a role for CD8$^+$ T cells in controlling AIDS virus infections and emphasize the importance of HIV vaccines to induce strong CD8$^+$ T cell responses.

In comparing the various vaccine approaches designed to induce CD8$^+$ T cell responses, epitope-based vaccines offer several advantages over vaccines encoding whole protein Ags. Not only are epitope-based vaccines capable of inducing more potent responses than whole protein vaccines (15), they confer the capacity to control qualitative aspects of the immune response by simultaneously targeting multiple dominant and subdominant epitopes (16, 17). This may be particularly important to the development of HIV vaccines, because the breadth of an immune response is likely to be crucial for controlling rapidly mutating pathogens such as HIV and hepatitis C virus (18–21). The use of epitopes can also overcome any potential safety concerns associated with the vaccinating Ag, as exemplified in the case of the human papillomavirus E6 and

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E7 Ags, whose expression is clearly associated with cervical carcinoma (22, 23).

DNA vaccines are receiving considerable attention for their ability to induce cellular immune responses. They have induced immune responses to and in some cases even protected against various pathogens, such as influenza and malaria (24–27). Although DNA vaccines encoding whole protein have effectively induced immunodeficiency virus-specific cellular immune responses in non-human primates (28–33), they have been unable to reliably protect against vigorous AIDS virus challenges. This lack of protection, however, may be due to the inability of previous DNA vaccine regimens to induce potent enough cellular immune responses against HIV and SIV.

Current approaches to vaccine design have begun to take advantage of the synergistic effects of combining DNA vaccines with other approaches to induce stronger, more persistent cellular immune responses (34–39). Recently, live pox viruses, such as modified vaccinia virus Ankara (MVA),[4] have proven to be safe and effective vaccine vectors for inducing strong cellular immune responses (40–44). Combining a DNA prime with an MVA boost has generated strong protective CTL responses against malaria in mice (74). More importantly, these murine studies demonstrated that the combination of DNA and MVA induced significantly higher levels of epitope-specific immune responses than either method alone (38).

Extrapolation of the results of the immunogenicity of potential vaccines in mice to primates is not always possible. Accordingly, in our studies we have determined whether a combination DNA/MVA vaccination regimen using a single CTL epitope can induce virus-specific CTL in non-human primates. We have used the Mamu-A*01[4]-restricted SIV gag CTL epitope p11C, C→M (CTPYDINQM) (45, 46) as an immunogen to induce AIDS virus-specific CTL responses. Using tetramers, ELISPOT, intracellular IFN-γ staining, and fresh killing assays, we describe the capability of an epitope-based DNA/MVA vaccine to induce in rhesus macaques levels of functionally active epitope-specific $CD8^+$ T lymphocytes equivalent to those observed during acute SIV infection.

Materials and Methods

Animals

Rhesus macaques used in this study were identified as *Mamu-A*01*$^+$ by PCR-sequence-specific priming and direct sequencing as previously described (47). Animals were maintained in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals and under the approval of the University of Wisconsin Research Animal Resource Center review committee.

DNA vectors pTH.HW and HC,C→M

The pTH.HW vector was derived from the pTH.H vector (48), which encoded for a polyepitope of 20 HIV and 3 SIV CTL epitopes, including the Mamu-A*01-restricted CTL epitope (TPYDINQML) (49). Following construction of the pTH.H plasmid, the Mamu-A*01 CTL epitope was optimally defined and was found to require an N-terminal cysteine residue (46), now termed p11C, C→M (50). The pTH.H vector was modified to include the cysteine residue of this CTL epitope and was renamed pTH.HW (51). The hepatitis B core Ag vector WRG7262 (PowderJect Vaccines, Madison, WI) was modified to include the Mamu-A*01 CTL epitope CTPYDINQM (HC,C→M) within the immunodominant loop of the core protein between aa 80 and 81.

DNA vaccinations

Frequencies of the DNA vaccinations using the Dermal PowderJect XR gene gun (PowderJect Vaccines) are outlined in Fig. 1. Each immunization delivered 32 μg of DNA, precipitated on gold beads, unilaterally over a total of eight skin sites in the abdominal and inguinal lymph node areas at 350 or 500 psi. The first vaccination delivered to each of the six animals employed only the pTH.HW plasmid, while all subsequent booster vaccinations involved codelivery of equal molar amounts of both the pTH.HW and HC,C→M vectors.

MVA inoculations

The six DNA-vaccinated rhesus macaques were inoculated with attenuated MVA encoding the same HIV/SIV polyepitope HW (MVA.HW) used in the pTH.HW vector (37, 44, 48). Each vaccination consisted of 5×10^8 PFU of MVA.HW in a volume of 150 μl of PBS delivered intradermally and dispersed over three chest sites on each animal. Animals 95045 and 95058 received their MVA inoculations 9 and 13 wk after their last DNA vaccination, animal 96031 received its inoculations 2 and 6 wk after its last DNA vaccination, and animals 94004, 96123, and 96118 received their inoculations 18 and 22 wk after their last DNA vaccination (Fig. 1). The MVA.HW was cultured on primary chick embryo fibroblasts derived from eggs of a pathogen-free stock and prepared as previously described (37). No lesions were found associated with the inoculations.

Isolation of PBMC

PBMC were isolated from EDTA-treated whole blood using Ficoll/diatrioate gradient centrifugation. Cells were then washed twice in R10 medium (RPMI 1640 supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), and 10% FBS (BioCell, Carson, CA)).

Generation of in vitro cultured CTL effector cells

PBMC were isolated from EDTA blood drawn 1, 2, or 4 wk after each DNA vaccination, and experiments were conducted using freshly isolated PBMC unless otherwise stated. CTL cultures were initiated by culturing 5×10^6 PBMC in R10 with peptide (10 μM). On day 3 half of the medium was replaced with R10 medium containing 20 U of rIL-2/ml (provided by M. Gately, Hoffmann-La Roche, Nutley, NJ). Medium was supplemented every other day with rIL-2 until day 7, when cells were stimulated with 5×10^6 peptide-pulsed γ-irradiated (3000 rad) autologous PBMC. Again, rIL-2 was added every 2 days until day 14 when the CTL activity of the cultures was assessed in a standard ^{51}Cr release assay. Peptides were obtained from Biosynthesis (Lexisville, TX) as desalted products. Lyophilized aliquots were resuspended in HBSS with 10% DMSO (Sigma) to a final concentration of 1 mg/ml.

Cytotoxicity assays

The CTL activity of in vitro stimulated CTL cultures was assessed as previously described (46). Briefly, 5×10^5 *Mamu-A*01*-transfected 721.221 cells (46) or B-LCLs derived from a Mamu-A*01$^+$ rhesus macaque were incubated for 1.5 h with 80 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Life Sciences Products, Boston, MA) and 5 μg of corresponding peptide in 200 μl of R10 medium. These target cells were plated into duplicate wells of a 96-well U-bottom microtiter plate (5×10^3 cells/well) and incubated with effector CTL for 5 h. The reported percent specific lysis represents ^{51}Cr release from CTPYDINQM peptide-pulsed targets minus ^{51}Cr release from target cells pulsed with an irrelevant SIVnef peptide (NQGQYMNTPR). Spontaneous release was always <20% of maximal release. Data reported for in vitro stimulated CTL cultures are based on single CTL assays tested at an E:T cell ratio of 20:1 unless otherwise noted. No appreciable difference in lysis was observed between either Mamu-A*01$^+$ B-LCL or *Mamu-A*01*-721.221 cells as targets in ^{51}Cr release assays.

The CTL activity of freshly isolated PBMC was assessed in a similar manner as for in vitro stimulated CTL cultures with the following exceptions: 1) target cells were Mamu-A*01$^+$ B-LCLs; 2) triplicate rather than duplicate wells were plated; 3) the irrelevant peptide (SNEGSYFF) used in these experiments was derived from the influenza virus nucleoprotein; and 4) data reported for fresh PBMCs were based on single CTL assays tested at E:T cell ratios of 150:1 and 50:1.

*Mamu-A*01/CTPYDINQM tetramers*

Soluble tetrameric Mamu-A*01 MHC class I/SIVgag CTPYDINQM peptide complexes were constructed as previously described (52) with the exception of the PCR primers required for amplification of the soluble MHC molecule. Primers Mamu-A*01–5p (5′-GGA ATT CCA TAT GGG ATC TCA TTC AAT GAA ATA TTT CTA CAC CTC CAT G-3′) and Mamu-A*01–3p (5′-CGC GGA TCC GGA CTG GGA AAA CGG CTC-3′) were designed to amplify the Mamu-A*01 heavy chain from a pKG5

[4] Abbreviations used in this paper: MVA, modified vaccinia virus Ankara; *Mamu*, *Macaca mulatta*; ELISPOT, enzyme-linked immunospot; B-LCL, B lymphoblastoid cell line; PFA, paraformaldehyde; LDA, limiting dilution analysis; BFA, brefeldin A.

vector containing a cDNA for the rhesus MHC class I molecule Mamu-A*01. Once the PCR products were cloned into an expression vector using *Nde*I and *Bam*HI cloning sites (52), the rhesus MHC molecules were expressed and then folded with human β_2m and peptide.

Tetramer staining

Lymphocytes (2×10^5) from 2-wk in vitro CTL cultures were stained in the dark for 30 min at room temperature with the Mamu-A*01-PE tetramer (0.5 μg/100 μl for in vitro cultures, 0.1 μg/100 μl for fresh PBMC) and an anti-rhesus CD3-FITC-conjugated mAb (10 μl; BioSource, Camarillo, CA) in a 100-μl volume of FACS buffer (PBS from Life Technologies with 2% FCS from BioCell). To cool down the cells quickly to 4°C, the plates were placed at −20°C for 5 min. An anti-CD8α-PECy5 Ab (1 μl; Coulter, Hialeah, FL) was added for 10 min at 4°C, and the cells were washed three times with FACS buffer and fixed with 450 μl of 2% paraformaldehyde (PFA).

For staining of fresh, unstimulated PBMC, 1×10^6 PBMC were stained as described for in vitro CTL cultures, except that cells were incubated with the anti-CD3-FITC mAb, anti-CD8α-PECy5 mAb, and Mamu-A*01-PE tetramer simultaneously for 40 min at room temperature. Sample data were acquired on a Becton Dickinson FACSCalibur instrument and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Background tetramer staining of in vitro stimulated cultures from naive Mamu-A*01$^+$ animals was routinely <0.2% and <0.08% for fresh, unstimulated PBMC (data not shown).

IFN-γ ELISPOT assay

Ninety-six-well flat-bottom plates (U-Cytech, Utrecht, The Netherlands) were coated with 5 μg/well of anti-IFN-γ mAb MD-1 (U-Cytech) overnight at 4°C. The plates were washed 10 times with PBST (PBS containing 0.05% Tween 20) and blocked with PBS containing 2% BSA for 1 h at 37°C. PBS containing 2% BSA was discarded from the plates, and freshly isolated PBMC in R5 medium were added. The R5 contained 5 μg/ml Con A (Sigma, St. Louis, MO), 1 μM p11C, C→M peptide, 1 μM of an irrelevant SIVenv peptide (ELGDYKLV), or no peptide. PBMC (5×10^4, 2.5×10^4, 1.2×10^4, and 6×10^3) were plated in triplicate (100 μl/well) and incubated for 14 h at 37°C in 5% CO_2. The cells were then removed, and 200 μl/well of ice-cold deionized water was added to lyse the remaining PBMC. Plates were incubated on ice for 15 min and washed 20 times with PBST. One microgram per well of rabbit polyclonal biotinylated anti-IFN-γ detector Ab (U-Cytech) was added, and plates were incubated for 1 h at 37°C before being washed 10 times with PBST. Fifty microliters per well of a gold-labeled anti-biotin IgG (U-Cytech) was added for 1 h at 37°C and washed 10 times with PBST. Thirty microliters per well of activator mix (U-Cytech) was then added and developed for 30 min to allow formation of silver salt precipitate at the site of gold clusters. Wells were then washed with distilled water and air-dried, and spots were counted (53).

Intracellular IFN-γ staining

Freshly isolated PBMC (1×10^6) were incubated at 37°C for 1 h with 50 ng/ml PMA and 1 μg/ml ionomycin, 5 μM p11C, C→M peptide-pulsed Mamu-A*01$^+$ B-LCL, or Mamu-A*01$^+$ B-LCL alone as a control. Cells were treated with 10 μg/ml of brefeldin A (BFA) for 4–5 h at 37°C to inhibit export of protein from the endoplasmic reticulum. Cells were washed twice with FACS buffer (PBS and 2% FCS), stained with CD8α-PerCP (Becton Dickinson) and Mamu-A*01-PE tetramers as described above, fixed with PFA overnight, and washed twice with FACS buffer. The cells were then treated with 150 μl of permeablization buffer (0.1% saponin in FACS buffer) for 5 min at room temperature, washed once with 0.1% saponin, and incubated in the dark with 1 μl of anti-human IFN-γ-FITC mAb (PharMingen, San Diego, CA) for 50 min. Cells were washed three times with 0.1% saponin buffer and once with PBS before the 100-μl cell suspension was fixed with 450 μl of 2% PFA.

Results

Induction of CTL responses following a single skin DNA vaccination

To demonstrate that a CTL response can be induced in primates using experimental vaccines encoding well-characterized CTL epitopes, we used the PowderJect XR gene delivery system (PowderJect Vaccines) to vaccinate Mamu-A*01$^+$ rhesus macaques with the pTH.HW vector encoding for the Mamu-A*01-restricted SIVgag CTL epitope p11C, C→M (46) (Fig. 1). Chest and inguinal lymph node regions provided a sufficiently large region of skin to vaccinate. Two and 4 wk following the first vaccination PBMC were isolated, cultured for 2 wk in vitro with the p11C, C→M peptide, and analyzed for CTL responses using standard ^{51}Cr release assays at an E:T cell ratio of 20:1. A p11C, C→M-specific CTL response was detected in one of the six vaccinated animals (animal 94004; 17% specific lysis) from PBMC taken 2 wk after the first vaccination (Fig. 2*A*), and in three animals (animals 96031, 96123, and 94004; 13, 19, and 19% specific lysis, respectively) from PBMC taken 4 wk after the first vaccination (data not shown). Responses from cultured PBMC taken before DNA vaccination were all <5% specific lysis (Fig. 2*A*).

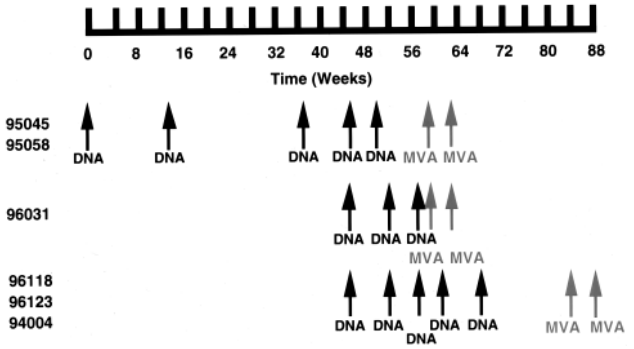

FIGURE 1. DNA and MVA vaccination schedule of six Mamu-A*01$^+$ macaques. Using the Dermal PowderJect XR gene gun six Mamu-A*01$^+$ rhesus macaques were vaccinated with DNA plasmids precipitated onto gold particles. Two different DNA plasmids were employed, each encoding the SIVgag CTL epitope CTPYDINQM. Three to five DNA vaccinations were directed to skin tissues as indicated. Nine weeks (95045 and 95058), 2 wk (96031), and 18 wk (96118, 96123, and 94004) after their last DNA vaccination these six macaques were boosted with two doses of 5×10^8 PFU of MVA.HW (MVA) spaced 4 wk apart.

In parallel, tetrameric complexes were used to determine the percentage of CD3$^+$/CD8α^+ T lymphocytes in each culture that expressed TCRs specific for the p11C, C→M peptide/Mamu-A*01 complex. Staining of the 2-wk in vitro cultures with these tetramers revealed that four of the animals (95058, 96031, 96123, and 94004) were responding with levels of tetramer-positive cells between 3 and 8% (Fig. 2*B*). These responses were well above the levels of 0.2% or less detected in cultured PBMC derived from each animal before DNA vaccination (Fig. 2*B*) or the background responses of less than 0.2% detected in cultured PBMC from three naive Mamu-A*01$^+$ macaques (data not shown). Similar analysis of the 4-wk in vitro cultures revealed that all six animals had responded to the vaccination (tetramer levels between 1 and 9%; data not shown). Taken together, the ^{51}Cr release assays and tetramer staining of in vitro cultures revealed that all six DNA-vaccinated macaques were generating low, but detectable, p11C, C→M-specific responses after a single DNA vaccination.

Booster DNA vaccinations enhanced CD8$^+$ T cell responses

Two to four booster vaccinations codelivering the pTH.HW and an additional plasmid (HC,C→M) were then administered. The HC,C→M plasmid expresses a highly immunogenic hepatitis B core Ag (HBcAg) shown to augment the cellular immune responses to inserted T cell epitopes (54–57). Initially, for the first two animals (95045 and 95058) a schedule of booster vaccinations with substantial resting periods was chosen based on previous studies in primates in which Ab responses were significantly enhanced by lengthening the resting periods between DNA vaccinations (58). When vaccinations were begun in the second set of four

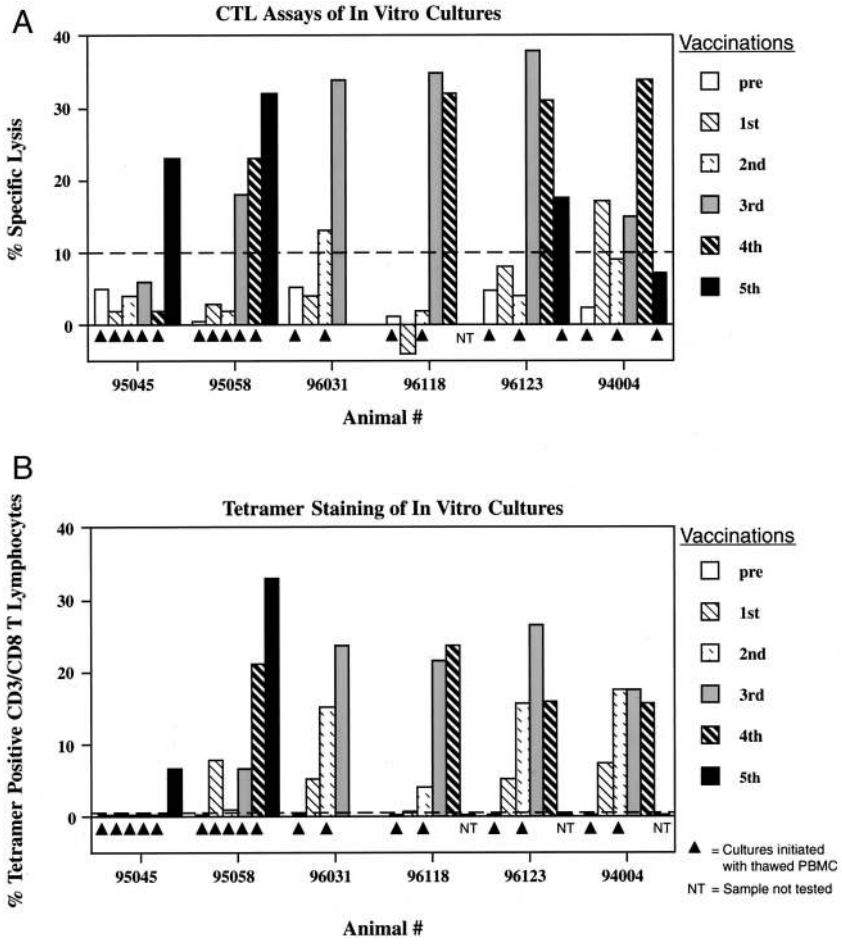

FIGURE 2. DNA vaccinations prime and boost the levels of epitope-specific $CD8^+$ T lymphocytes in Mamu-A*01 macaques. Fresh PBMC taken 2 wk after each DNA vaccination were used to derive 2 wk in vitro stimulated cultures from each animal, with the exception of the cultures indicated (▲) that were initiated using thawed PBMC. *A*, Standard ^{51}Cr release assays (E:T cell ratio, 20:1) were initiated from PBMC derived pre- and postvaccination. Mamu-A*01^+ B-LCLs were used as target cells, with the exception of samples from animal 95058 in which target cells were *Mamu-A*01*-721.221-transfected cells. Background peptide-specific lysis from naive Mamu-A*01^+ animals tested in each assay was consistently $<10\%$ (dotted line). *B*, In vitro cultured PBMC were also were stained with p11C, C→M-specific Mamu-A*01 tetramers. Background tetramer staining of in vitro stimulated cultures from naive Mamu-A*01 animals was always $<0.2\%$ (dotted line). NT, time points when in vitro stimulated cultures were not tested.

animals, the resting periods between vaccinations was shortened because the beneficial effects of the resting periods was believed to be less critical to the induction of a strong cellular immune response than to a strong humoral response. PBMC were isolated 2 wk after each DNA vaccination, and in vitro stimulated cultures were initiated. The administration of booster DNA vaccinations effectively increased responses in each of the six vaccinated macaques as measured by ^{51}Cr release assays (Fig. 2*A*) and tetramer staining (Fig. 2*B*). Following the third or fourth booster vaccination, levels of p11C, C→M-specific lysis in in vitro stimulated cultures were elevated in some animals to levels $>30\%$. Levels of tetramer-positive $CD3^+/CD8\alpha^+$ T lymphocytes were also increased to $>20\%$ in some cultures. In the majority of cultures tested these two independent measurements did roughly correspond. Following the fourth and fifth vaccinations of some animals (96118, 96123, and 94004), the responses appeared to plateau and even decline. This may be a consequence of the reduced resting period between DNA vaccinations in this group of three animals (58). Nevertheless, booster DNA vaccinations were capable of enhancing the levels of p11C, C→M-specific $CD8^+$ T cell responses in all six vaccinated macaques.

Tetramer-positive cells detected in fresh, unstimulated PBMC after DNA vaccination

Due to the strong p11C, C→M-specific T cell responses observed in in vitro stimulated cultures, we reasoned that the frequency of p11C, C→M-specific $CD8^+$ T lymphocytes might be high enough to detect low levels of tetramer-positive cells in fresh, unstimulated PBMC. To follow the time course of Ag-specific $CD8^+$ T lymphocyte induction in DNA-vaccinated animals, fresh unstimulated PBMC from animals 96118, 96123, and 94004 were stained 2, 4, 6, and 13 days following their fifth DNA vaccination. Levels of tetramer-positive $CD3^+/CD8\alpha^+$ T cells peaked 6 days after the DNA vaccination and declined by day 13 (data not shown). When the percentages of tetramer-positive cells in unstimulated PBMC were followed in all six macaques after their last two DNA vaccinations, two of the six animals showed levels of p11C, C→M-specific $CD3^+/CD8\alpha^+$ T lymphocytes as high as 0.4% (Fig. 3 and Table I). This DNA vaccine regimen, therefore, was able to induce levels of p11C, C→M-specific $CD8^+$ T lymphocytes high enough to be detected in fresh, unstimulated PBMC of some animals using tetramers.

MVA significantly boosts levels of tetramer-positive cells in fresh, unstimulated PBMC

Because MVA has proven very effective at both inducing and boosting CTL responses in mice and primates (37, 42, 51), we were interested in assessing the effects of MVA boosting the six DNA-vaccinated macaques. One week after administering MVA.HW fresh unstimulated, Ficoll-separated PBMC were assessed for the percentage of $CD3^+/CD8\alpha^+$ T lymphocytes which were specific for the p11C, C→M epitope. Tetramer analysis revealed that 0.8, 18.0, 8.3, 1.2, 1.6, and 20.0% of the $CD3^+/CD8\alpha^+$ T lymphocytes from these six macaques were specific for the p11C, C→M epitope (Fig. 4). These levels represented a significant increase over the 0.4% levels detected weeks earlier in some of the Mamu-A*01^+ macaques after DNA vaccination (Fig. 3 and Table I). Responses were then followed over the subsequent 3 wk

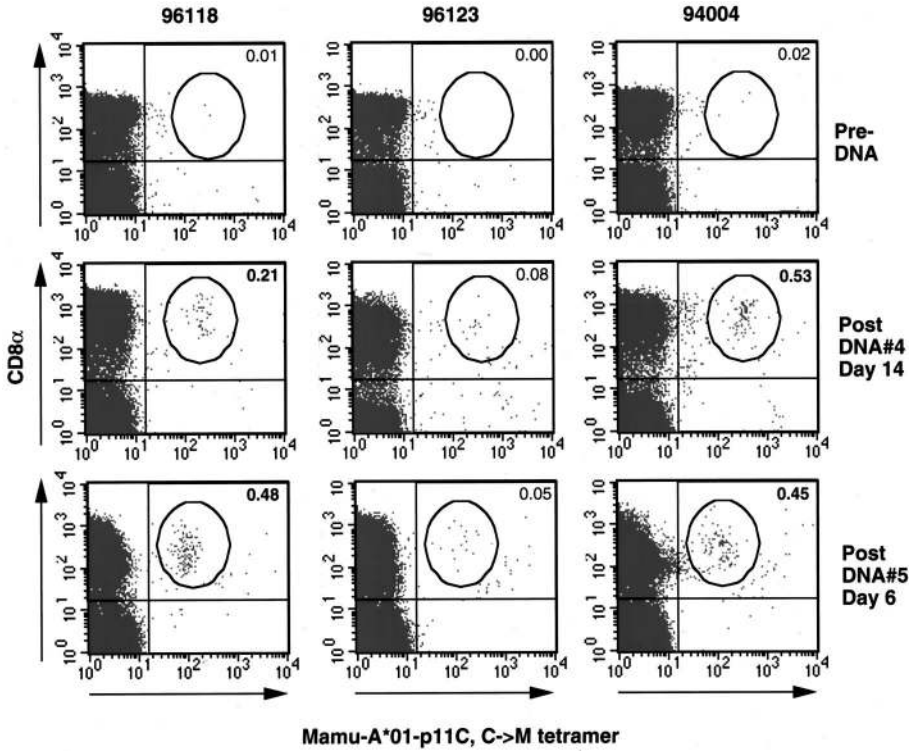

FIGURE 3. Tetramer-positive CD3$^+$/CD8α^+ T lymphocytes are detectable in fresh PBMC of some macaques after multiple DNA vaccinations. Fresh, unstimulated PBMC from six DNA-vaccinated macaques were stained 6 or 14 days after receiving their fourth or fifth DNA vaccination. The percentage of CD3$^+$/CD8α^+, p11C, C→M tetramer-positive T lymphocytes is indicated in the *top right corner* of each plot. Significant responses >0.10% are in boldface. The background level from fresh unstimulated PBMC of a naive Mamu-A*01-positive animal was <0.08% (data not shown).

and were observed to decline to levels <1% in the majority of animals. Interestingly, in animals 95058 and 94004, which had demonstrated exceptionally high responses 1 wk post-MVA (18 and 20% respectively), levels were maintained above 8 and 2%, respectively, after 2 wk. Responses in animal 95058 were maintained at this high level up to 4 wk (Fig. 4).

A second administration of MVA boosted responses moderately

A second administration of MVA.HW was given to all six macaques 4 wk after receiving their first MVA.HW. As before, responses peaked 1 wk after this second MVA.HW and increased over resting levels in the majority of animals (Fig. 4). Only in animal 95045 were stronger responses induced by this additional MVA.HW than had been induced by the first MVA.HW. In the three macaques examined, 96118, 96123, and 94004, responses again declined by the second week. Therefore, while a second administration of MVA.HW was capable of boosting levels of Ag-specific responses, these levels did not generally exceed those induced after the first MVA.HW. Longer resting periods between MVA vaccinations, as used in other studies (42), may have allowed for better induction by the second MVA.

Frequency of p11C, C→M-specific IFN-γ-producing T cells determined by ELISPOT

The ELISPOT assay represents an effective method for measuring the frequency of Ag-specific IFN-γ-producing T cells in the circulation (59–61). Fresh, unstimulated PBMC were tested at 1 and 2 wk following administration of their first MVA.HW with Con A, the p11C, C→M peptide, an irrelevant SIV envelope peptide (ELGDYKLV), or no peptide. ELISPOT indicated that 1 wk following the first MVA.HW vaccination, strong Ag-specific responses were observed in three of the DNA/MVA-vaccinated animals tested. Animals 96118 and 96123 had 92 p11C, C→M-specific SFCs/50,000 input cells (effector frequency 1, 840/10^6

Table I. *Percentage of tetramer-positive CD3$^+$/CD8α^+ T lymphocytes detected in fresh, unstimulated PBMC from DNA-vaccinated macaques*[a]

No. of DNA Vaccinations	Days Post-DNA	95045[b]	95058[b]	96031[b]	96118	96123	94004
0[c]		0.02	0.04	0.01	0.01	0.00	0.02
4	14	–	–	–	**0.21**[e]	0.08	**0.53**
	28	0.03	0.05	–	–	–	–
5	6	–	–	–	**0.48**	0.05	**0.45**
	13	0.02	–	–	**0.21**	0.08	**0.34**
	28	0.01	0.07	0.03[d]	–	–	–

[a] Tetramers typically stain <0.08% of fresh, unstimulated CD3$^+$/CD8α^+ T lymphocytes from PBL of naive Mamu-A*01$^+$ animals.
[b] Thawed PBMC were used for samples from animals 95045, 95058, and 96031.
[c] Thawed PBMC from each animal were used to test levels of pre-DNA tetramer-positive cells.
[d] PBMC isolated 4 wk following animal 96031's last (third, not fifth) DNA vaccination were tested.
[e] Significant responses >0.10% are in boldface.

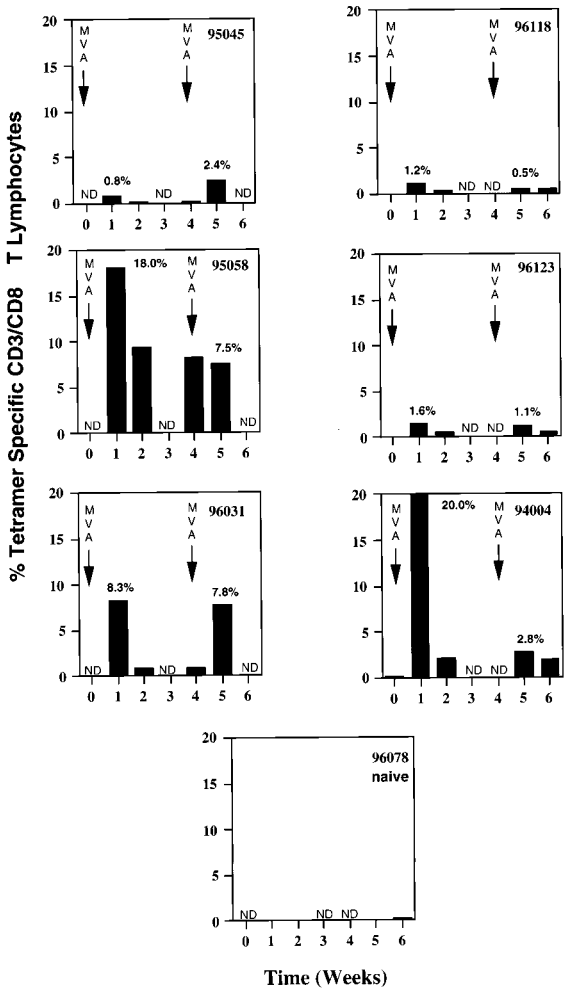

FIGURE 4. MVA.HW vaccination of DNA-primed macaques significantly boosts levels of epitope-specific $CD3^+/CD8\alpha^+$ T lymphocytes in fresh, unstimulated PBMC. Nine weeks (95045 and 95058), 2 wk (96031), and 18 wk (96118, 96123, and 94004) after their last DNA vaccination six macaques were boosted with two doses of 5×10^8 PFU of MVA.HW spaced 4 wk apart. Fresh PBMC from DNA/MVA-vaccinated macaques were isolated and tetramer stained to determine the percentage of p11C, C→M-specific $CD8^+$ T cells. Background staining of fresh, unstimulated PBMC taken from a naive Mamu-A*01^+ macaque (animal 96078) was <0.08%. Levels detected in fresh PBMC taken 1–4 wk before the MVA.HW vaccination were <0.2% (data not shown).

PBMC) and 44 p11C, C→M-specific SFCs/50,000 input cells (effector frequency, 880/10^6 PBMC), respectively (Fig. 5*A*). SFC responses in each of the animals titrated according to the number of input cells per well and were similar to values induced by Con A stimulation. Animal 94004, with 20.0% tetramer-positive cells, was capable of demonstrating a better response to the p11C, C→M peptide, 206 SFCs/50,000 cells (effector frequency, 4,120/10^6 PBMC), than to Con A (effector frequency, 1,940/10^6 PBMC). None of the animals demonstrated significant responses to the irrelevant peptide or to wells without any peptide even at 50,000 cells/well (less than five spots per well).

Samples derived 2 wk following the first MVA.HW were also analyzed (Fig. 5*B*). Although the levels of tetramer-positive cells had declined slightly in animals 96118 and 96123, the number of IFN-γ-producing cells detected in each of these animals at this 2 wk point were now reduced by almost 90%. This may suggest that by 2 wk, in the setting of declining levels of Ag, in addition to being deleted some of these Ag-specific cells also lost their ability to produce IFN-γ in response to the p11C,C→M epitope. In 94004, the drop in tetramer-positive cells by 2 wk post-MVA.HW was reflected by a more comparable decline in IFN-γ-producing cells. Nonetheless, detectable levels of IFN-γ-producing cells were still present 2 wk after administration of MVA.HW. Further analysis conducted 21 wk after the first MVA.HW indicated that positive ELISPOT responses were still detectable in 96118, 96123, and 94004 at effector frequencies of 125/10^6, 35/10^6, and 230/10^6 PBMC, respectively (data not shown).

Tetramer-positive lymphocytes produce IFN-γ in response to antigenic stimulation

Given the surprisingly high levels of Ag-specific lymphocytes detected by the tetramer and ELISPOT assays, we used an additional assay to confirm these results. Fresh PBMC from animals 95045, 95058, and 96031 taken 1 wk after the second MVA.HW and fresh PBMC from a naive Mamu-A*01^+ animal (95084) were incubated with BFA in the absence or the presence of mitogenic or Ag-specific stimulation and stained for CD8α, Mamu-A*01/p11C, C→M tetramers, and IFN-γ. In the absence of stimulation no IFN-γ was produced (Fig. 6*A*). As a control, treatment of cells with mitogens PMA/ionomycin induced the production of IFN-γ in tetramer-positive as well as tetramer-negative cells (Fig. 6*B*). Treatment of Ag-specific cells with the cognate peptide has been shown to internalize the TCRs on the cell surface (62). Because the cells have been treated with BFA, egress of newly synthesized TCRs to the cell surface is prevented, abolishing tetramer staining. Treatment of PBMC from each of the vaccinated animals with the p11C, C→M peptide resulted in a reduction in the fraction of tetramer-positive cells as expected (Fig. 6*C*). However, the fraction of cells that demonstrated the production of intracellular IFN-γ after p11C, C→M peptide stimulation was nearly equivalent to the fraction of untreated cells that was previously tetramer positive (Fig. 6*A*). Treatment of fresh PBMC from these animals with an irrelevant peptide induced no internalization of TCR and little or no production of IFN-γ (data not shown). These results indicate that the majority of tetramer-positive $CD8^+$ cells in these DNA/MVA-vaccinated macaques are functionally active and capable of responding specifically to the p11C, C→M peptide through the production of intracellular IFN-γ.

Direct ex vivo cytotoxic activity in fresh PBMC from DNA/MVA-vaccinated macaques

Because we induced high levels of peptide p11C, C→M-specific $CD8^+$ T lymphocytes in our DNA/MVA-vaccinated animals as detected by tetramer assays (Fig. 4), and those cells produced IFN-γ upon activation (Figs. 5 and 6), we were interested in determining whether we could detect direct ex vivo CTL activity from fresh, unstimulated PBMC. Normally, CTL activity from PBMC of vaccinated macaques, and even in chronically SIV infected animals, is only detectable after in vitro stimulation with peptide. PBMC were tested in ^{51}Cr release assays at E:T cell ratios of 150:1 and 50:1 against target B-LCLs pulsed with either peptide p11C, C→M or an irrelevant peptide. Although these experiments used unconventionally high E:T ratios, similar ratios were required to detect ex vivo CTL activity in intestinal intraepithelial lymphocytes of SIV-infected macaques (63), as well as in PBMC of HIV-infected patients (64, 65).

Thawed PBMC from animals 95045, 95058, and 96031 taken 1 wk after their first MVA.HW, and fresh PBMC from a naive Mamu-A*01^+ macaque 96078, were initially tested. The percent specific lysis from thawed PBMC of animals 95045 and 96031 at

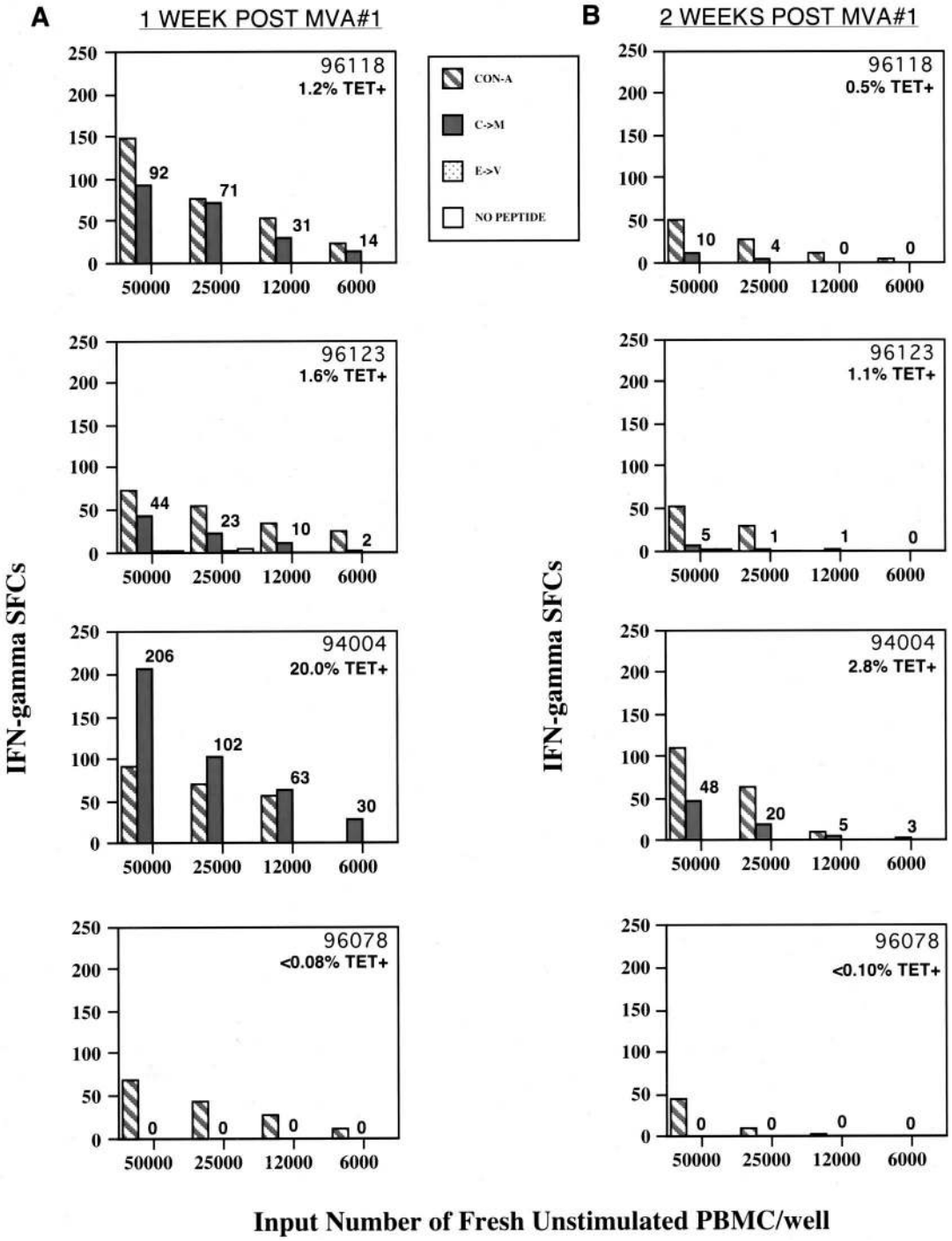

FIGURE 5. Frequency of p11C, C→M-specific $CD8^+$ T cells as measured by an IFN-γ ELISPOT assay. Freshly isolated PBMC were assessed for their ability to produce IFN-γ in response to Con A, peptide p11C, C→M (1 μM), an irrelevant SIVenv 9 mer (ELGDYKLV; 1 μM), or no peptide. PBMC taken 1 wk (*A*) and 2 wk (*B*) following the first MVA.HW were plated at varying dilutions of input cells.

the higher E:T ratio were low, 5 and 10%, respectively, over background levels in the control animal (Fig. 7). 95058, however, which had 18.0% tetramer-positive $CD3^+/CD8\alpha^+$ T cells at this time point, demonstrated a more significant level of 15% specific lysis. These levels of specific lysis were reproducible in a replicate experiment (data not shown).

We next assessed for ex vivo CTL activity in fresh, unstimulated PBMC from animals 96118, 96123, and 94004 1 wk after receiving their first MVA.HW, a point at which levels of tetramer staining were between 1.2 and 20.0%. More dramatic levels of fresh killing of 36, 13, and 77% were detected in these animals at the highest E:T cell ratio over background levels in the control animal. Samples from animals 96118, 96123, and 94004 taken 1 wk after the second MVA.HW vaccination were also assessed, although the levels of tetramer-positive cells were lower at this time point. These lower levels of tetramer-positive cells were paralleled by a comparable reduction in the levels of specific lysis (Fig. 7). Nonetheless, significant responses were still detectable in PBMC from animal 94004 (27% and 18%, respectively, at E:T cell ratios of 150:1 and 50:1), compared with a background level of <10% lysis

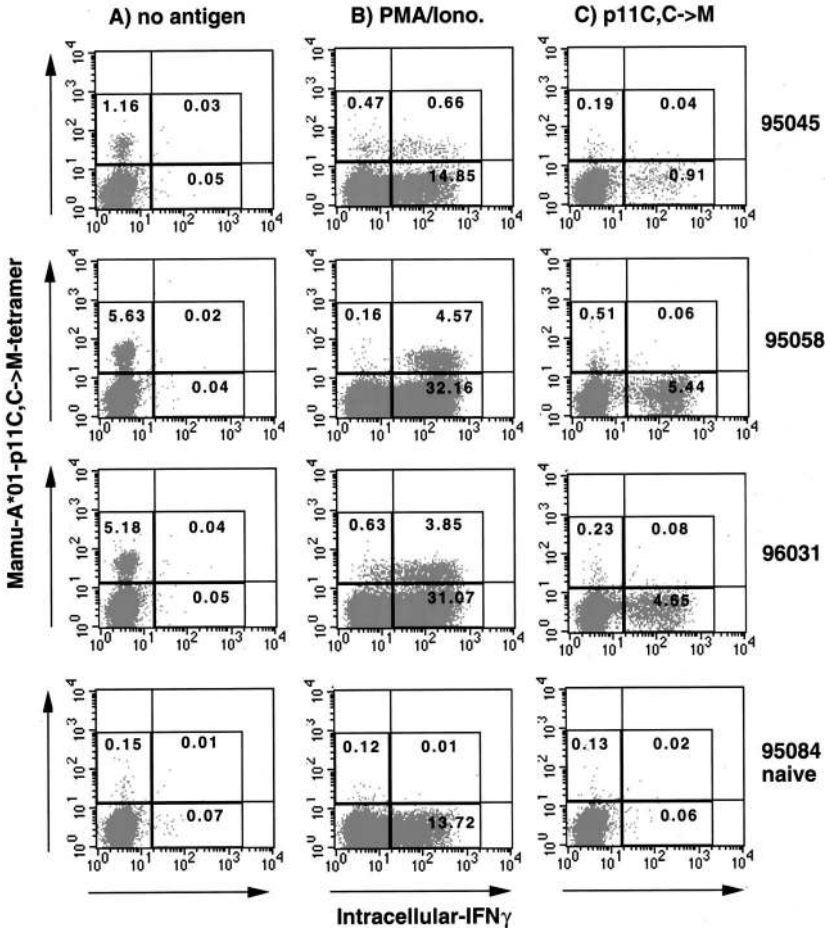

FIGURE 6. The majority of tetramer-positive cells isolated 1 wk following the second MVA.HW produce IFN-γ in response to stimulation with the p11C, C→M peptide. Fresh Ficoll-purified PBMC purified 1 wk after the second MVA.HW were stained for CD8α, intracellular IFN-γ production, and tetramers to determine the percentage of tetramer-positive cells capable of producing IFN-γ in response to the p11C, C→M peptide. *A*, Unstimulated PBMC. *B*, PMA/ionomycin-stimulated cells. *C*, p11C, C→M peptide (5 μM)-stimulated cells, which in response to the peptide have internalized their TCR (therefore tetramer negative) and produced intracellular IFN-γ. The percentage of positively staining cells is indicated in each quadrant. The levels of tetramer-positive cells illustrated in Fig. 6 are slightly lower than those reported in Fig. 4, since the percentage of $CD8\alpha^+$ (not $CD3^+/CD8\alpha^+$) lymphocytes is reported.

in the control animal. Therefore, not only was this epitope-based DNA/MVA vaccine capable of inducing high levels of Ag-specific $CD8^+$ T cells, the levels of induced CTL were sufficient to exhibit cytolytic activity in fresh, unstimulated PBMC in the majority of animals.

Discussion

We have demonstrated the ability of an epitope-based DNA/MVA vaccine to induce the highest reported levels of Ag-specific $CD8^+$ T lymphocyte responses detected in a mammalian species. Gene gun DNA vaccinations targeted to the skin proved effective at priming p11C, C→M-specific $CD8^+$ T cell responses in six Mamu-A*01$^+$ rhesus macaques. Tetramer staining of fresh, unstimulated PBMC revealed that some DNA-vaccinated animals had levels of epitope-specific $CD3^+/CD8\alpha^+$ T lymphocytes as high as 0.4%. Boosting DNA-vaccinated animals with recombinant MVA expanded the percentage of epitope-specific $CD3^+/CD8\alpha^+$ T lymphocytes in fresh PBMC to levels between 1.2 and 20%. ELISPOT and intracellular IFN-γ staining indicated that these $CD8^+$ cells were functionally active. Furthermore, in five of six DNA/MVA-vaccinated animals the levels of Ag-specific T lymphocytes were sufficiently high to detect, for the first time, direct ex vivo vaccine-induced cytotoxic activity in non-human primates. The levels of Ag-specific $CD8^+$ T lymphocytes induced by this epitope-based DNA/MVA vaccination regimen are equivalent to those observed in acutely SIV-infected rhesus macaques (66).

Before the advent of tetramers, estimation of precursor CTL levels was typically determined by limiting dilution analysis (LDA) assays. LDA typically underestimates the levels of Ag-specific $CD8^+$ T cells by 10- to 100-fold compared with tetramer or ELISPOT analysis (67–69). Corrected LDA values for SIV-specific $CD8^+$ T lymphocytes from rhesus macaques immunized with a vaccinia virus-based subunit vaccine (70) or immune-stimulating complexes (iscoms) (71) suggest that these vaccines were capable of inducing between 100 and 2000 protein-specific $CD8^+$ T lymphocytes/10^6 PBMC. In our study, tetramer staining indicated that 0.4% of $CD3^+/CD8\alpha^+$ T cells from two of our DNA-vaccinated animals were specific for a single CTL epitope, which would extrapolate to $1000/10^6$ PBMC. Therefore, the levels of Ag-specific responses induced by the DNA vaccinations in this study were equivalent to some previously published levels of SIV-specific CTL induced in non-human primates.

The induction of p11C, C→M-specific $CD8^+$ responses in DNA/MVA-vaccinated macaques has also recently been reported by Hanke et al. (38, 51). In these experiments, tetramer staining of 1.3, 4.9, and 1.5% of $CD8\alpha^+$ T lymphocytes in frozen PBMC were induced in three Mamu-A*01$^+$ macaques following a regimen of two DNA and two MVA vaccinations. In our DNA/MVA-vaccinated animals, responses following the first MVA (1.2–20.0%) were in most cases substantially higher. Hanke et al. (51) also reported no significant lytic activity in 2-wk in vitro stimulated cultures derived from PBMC of the DNA-vaccinated macaques compared with this study in which responses were detected after a single DNA vaccination. These differences were probably due to the vaccine regimens, which in our experiments delivered greater amounts of DNA and included a second DNA vector. This additional hepatitis B core Ag vector was included to

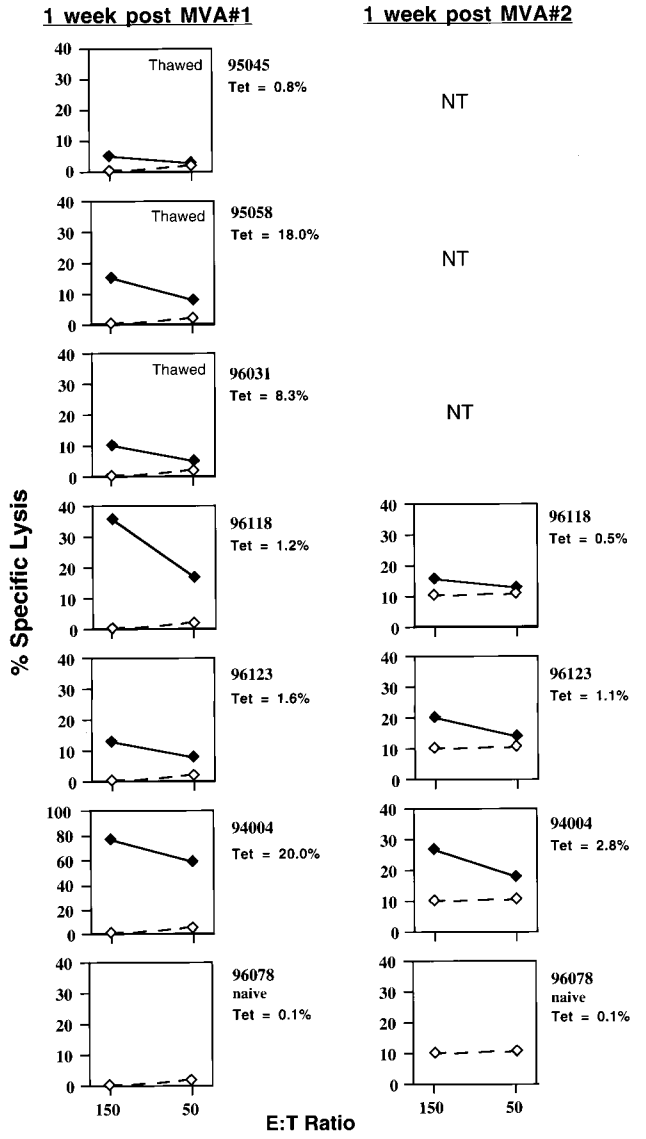

FIGURE 7. Direct ex vivo cytotoxic activity detected in fresh PBMC from DNA/MVA-vaccinated macaques. Freshly isolated PBMC were tested in a ^{51}Cr release assay using peptide-pulsed B-LCL from a Mamu-A*01$^+$ rhesus macaque. PBMC were tested at E:T cell ratios of 150:1 and 50:1 (◆). For comparison, levels of specific lysis detected from a naive Mamu-A*01$^+$ animal (96078) tested concurrently are reproduced with each graph (◇). Note that the *y*-axis for the animal 94004 graph at 1 wk post-MVA extends to 100% specific lysis. Responses 10% above those detected in fresh PBMC from a naive Mamu-A*01$^+$ macaque in the same assay were considered significant.

provide Th cell responses during the DNA vaccination. In some animals, responses to the hepatitis B core Ag appeared to correlate with the induction of good CTL responses (data not shown). Therefore, the more effective priming of CTL responses by our DNA vaccinations may account for the higher levels of p11C, C→M-specific CD8$^+$ cells induced after the MVA boost. The induction of p11C, C→M-specific responses has also recently been reported in Mamu-A*01$^+$ macaques vaccinated with MVA.HW alone (51) and MVA expressing gag/pol of SIV (42). Tetramer staining levels in these animals ranged between 1 and 5% of CD3$^+$/CD8β^+ cells. These responses, however, were not detectable until after the second MVA administration compared with only a single MVA in our study and those of Hanke et al. (51), where a DNA prime was included, illustrating the beneficial effects of priming with DNA.

Tetramer analysis of p11C, C→M-specific T lymphocytes in Mamu-A*01$^+$ macaques chronically infected with SIV indicates that between 0.7 and 10.3% of CD3$^+$/CD8$^+$ T cells are epitope specific (50, 66). The tetramer levels detected in our DNA/MVA-vaccinated animals (1.2–20.0%) were equivalent to and in some cases even greater than these levels in SIV-infected macaques.

In this study, the levels of Ag-specific cells measured in in vitro stimulated cultures using tetramers and ^{51}Cr release assays were not always in complete agreement. Such discrepancies have been observed in other studies (50, 51, 72), although the reason for these differences is not understood. A similar discordance was also observed in this study when unstimulated PBMC were analyzed, suggesting that this phenomenon is not unique to in vitro stimulated cultures.

Although MHC class I tetramers are effective at determining the levels of Ag-specific CD8$^+$ T lymphocytes in fresh PBMC, they do not reveal the functional state of the cells. In this study the ability to measure p11C, C→M-specific induction of IFN-γ in fresh PBMC by intracellular IFN-γ staining was critical to assessing the functionality of the vaccine-induced CD8$^+$ T lymphocytes. This analysis, which was not undertaken in other rhesus macaque vaccination studies in which high levels of tetramer-positive cells were induced (42, 51), revealed that the majority of tetramer-positive cells were functionally active.

Comparisons of the number of p11C, C→M-specific cells detected by ELISPOT and tetramer staining revealed that ELISPOT generally underestimated levels by between 1.3- and 10-fold. This discrepancy was not due to these cells being nonfunctional as verified by intracellular IFN-γ staining. Furthermore, because the tetramer staining of the fresh, unstimulated PBMC was conducted at room temperature rather than at 4°C, which has been observed to allow for binding of tetramers to CTLs with minimal avidity (73), it is unlikely that this discrepancy was due to an overestimation by the tetramers. Rather, the 1 μM concentration of peptide used in our ELISPOT experiments may have been insufficient to induce the maximal number of Ag-specific SFCs as has been observed in other studies (Ref. 60).[5] It is noteworthy that the results from the fresh killing assays in animals 96118, 96123, and 94004 examined 1 wk after the first MVA.HW corresponded much better with the ELISPOT values than the tetramer values at this time point. This was especially evident with animal 96118, which while possessing the lowest levels of tetramer-positive cells (1.2%), possessed intermediate levels of SFCs (92/50,000 cells) in ELISPOT and intermediate levels of specific lysis (36% at an E:T cell ratio of 150:1) in fresh killing assays. While the reasons for these discrepancies were not addressed by this study, these results nonetheless suggest that analyses in addition to tetramer staining, i.e., intracellular cytokine staining, ELISPOT, and fresh killing assays, may prove crucial in our understanding of the role of these vaccine-induced cells in viral containment. To this end, in a similar study in which DNA/MVA-vaccinated animals were challenged with SIV (51), these complementary assays were not undertaken, and there was little or no correlation between the levels of tetramer-positive cells and the containment of viral loads.

Although HIV- and SIV-specific CTL activity has been detected in fresh, unstimulated PBMC of seropositive humans (64, 65), and small intestine intraepithelial lymphocytes of chronically SIV-infected macaques (63), such responses have never been detected in

[5] T. M. Allen, B. R. Mothe, J. Sidney, P. Jing, J. L. Dzuris, M. E. Liebl, T. U. Vogel, D. H. O'Connor, X. Wang, M. C. Wussow, J. A. Thomson, J. D. Altman, D. I. Watkins, and A. Sette, Submitted for publication.

the PBMC of vaccinated or SIV-infected macaques. Detectable levels of fresh killing were present in five of our six DNA/MVA-vaccinated animals, which serves to illustrate the potency of this vaccine regimen.

Whether vaccine-induced $CD8^+$ T cell responses can control HIV and SIV infections remains unknown. Careful attention to the strength and breadth of a vaccine-induced $CD8^+$ T cell response and proper assessment of these responses using assays that measure the levels of functionally active lymphocytes will be critical to the development of an effective HIV vaccine. The ability of this epitope-based DNA/MVA regimen to induce high levels of functionally active Ag-specific CTL against a single CTL epitope in non-human primates represents a first step toward addressing this issue. These findings now facilitate the immunizing of rhesus macaques with multiple CTL epitopes (both immunodominant and subdominant) to explore the role of vaccine-induced $CD8^+$ T cell responses in controlling virus replication after SIV challenge.

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