

Cytotoxic and Proliferative T Cell Responses in HIV-1-infected *Macaca nemestrina*

Stephen J. Kent,* Lawrence Corey,** Michael B. Agy,[§] William R. Morton,^{||} M. Julianna McElrath,* and Philip D. Greenberg*¹

Departments of *Medicine, [†]Laboratory Medicine, [§]Microbiology, ^{||}the Regional Primate Research Center, and ¹Immunology, University of Washington, Seattle, Washington 98195

Abstract

Macaca nemestrina has been described as an animal model for acute HIV-1 infection. This animal, unlike most infected humans, appears to contain HIV-1 replication. Therefore analysis of HIV-1-specific proliferative and cytotoxic T lymphocyte (CTL) responses following HIV-1 challenge of *M. nemestrina* may provide information into the role of such responses in both the control of acute HIV infection and protective immunity. Although CD4⁺ T cell responses to HIV-1 are generally difficult to detect in HIV-1-infected humans, early and persistent CD4⁺ T cell proliferative responses to HIV-1 antigens were detected in all HIV-1-inoculated *M. nemestrina*. HIV-1-specific CD8⁺ CTL responses were evaluated in PBMC by stimulation with autologous cells expressing HIV-1 genes, limiting dilution precursor frequency analysis, and T cell cloning. CTL reactive with *gag*, *env*, and *nef* were present 4–8 wk after infection, and persisted to 140 wk after infection. The presence of both CD4⁺ and CD8⁺ T cell responses before and after clearance of HIV-1 viremia is consistent with a role for these responses in the successful control of HIV-1 viral replication observed in *M. nemestrina*. Further studies of T cell immunity in these animals that resist disease should provide insights into the immunobiology of HIV-1 infection. (*J. Clin. Invest.* 1995. 95:248–256.) Key words: HIV-1 • T lymphocytes • cytotoxic • T4 lymphocytes • *Macaca nemestrina*

Introduction

The pig-tailed macaque, *Macaca nemestrina* (*M. nemestrina*), has recently been described as an animal model of acute HIV-1 infection (1, 2). Intravenous inoculation of several strains of HIV-1 has resulted in a clinical rash and lymphadenopathy syndrome 2–12 wk after inoculation and a transient viremia lasting 4–24 wk. Long-term HIV-1 replication is restricted,

however, despite detection of persistent HIV-1 DNA in PBL and splenic lymphocytes. The cause of this inhibition in viral replication is unclear, but may be due at least in part to immunologic containment of HIV-1 replication. Antibodies to multiple HIV-1 proteins, including neutralizing antibodies to HIV-1 envelope, have been detected in HIV-1-infected *M. nemestrina* (1, 2) but to date no studies of cellular immune responses have been reported.

Cellular immune responses are critical for either the elimination or immunologic control of most viral infections and may be necessary components of a successful vaccination program to induce protection against viral challenge (3). In HIV-1-infected humans, CD4⁺ T cell proliferative responses to HIV-1 proteins are rarely present, even early in infection, and this apparent lack of CD4 cell responsiveness to HIV-1 may play a role in both the failure to generate and maintain an immune response effective at controlling HIV-1 and the etiology of the subsequent immunodeficiency (4). CD8⁺ cytotoxic T lymphocytes (CTL), specific to multiple HIV-1 proteins are readily detectable in HIV-1-infected humans, and correlative studies as well as analysis of viral escape variants suggest that such effector cells play a role in control of the infection (5, 6).

CD4- and CD8-mediated T cell responses have also been detected in recipients of candidate HIV-1 and simian immunodeficiency virus vaccines, although the definitive role of T cell immune responses in protective immunity to HIV-1 and SIV remains to be defined (7–10). Analysis of cellular immune responses in HIV-1-infected macaques, a host that appears to contain viral replication, should facilitate further definition of the parameters of protective immune responses to HIV-1. As such, we measured T lymphocyte proliferative and cytotoxic responses in PBMC from HIV-1-infected *M. nemestrina*. Our data indicate that both CD8-mediated cytotoxic and CD4-mediated proliferative T cell responses to HIV-1 proteins are present early and persist for > 100 wk in HIV-1-infected *M. nemestrina*. Moreover, limiting dilution cultures can be used to both isolate HIV-1-specific CTL clones and quantify the frequency of CTL precursors. These responses in HIV-1-infected *M. nemestrina* provide insights into the role of cellular immunity in resistance to HIV-1 disease in these primates.

Methods

Cell lines. B lymphoblastoid cell lines (BLCL) were established from each macaque by infecting 10⁵ freshly isolated PBMC in 100 μ l of

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Address correspondence to Stephen J. Kent, AIDS Pathogenesis Research Unit, Macfarlane Burnet Centre for Medical Research, PO Box 254, Fairfield, Victoria, Australia 3078. Phone: 61-3-2802823; FAX: 61-3-2802561.

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1. **Abbreviations used in this paper:** APC, antigen-presenting cells; BLCL, B lymphoblastoid cell lines; CTL, cytotoxic T lymphocytes; CTLp, cytotoxic L lymphocyte precursors; CRA, 51Cr release assay; rVV, recombinant vaccinia viruses; SIV, simian immunodeficiency virus.

R15 (RPMI 1640 supplemented with L-glutamine penicillin 100 U/ml, streptomycin 100 µg/ml, and 15% FCS [Whittaker M. A. Bioproducts, Inc., Walkersville, MD]) with 100 µl of supernatant from S394-1X1055 cells (a kind donation of Dr. Sharp, Southwest Foundation for Biomedical Research, San Antonio, TX) containing *Herpes virus papio*, a baboon herpesvirus, in a 96-well, U-bottomed plate (Costar Corp., Cambridge, MA). African green monkey kidney cells (BSC-40), maintained in DME supplemented 10% FCS, were used to generate stocks of vaccinia viruses.

T cell proliferative responses. Freshly separated PBMC were incubated in quadruplicate replicate cultures at 10^5 cells per well in 96-well U-bottomed plates for 6 d with 10 µg/ml preparations of HIV-1 antigens, including psoralen-inactivated whole HIV-1_{LAI} and HIV-1_{SF2}, and recombinant HIV-1_{SF2} gp120 produced in Chinese hamster ovary cells (kindly provided by Dr. K. Higgins, Chiron, Emeryville, CA), in a total of 200 µl. PBMC were incubated with media alone to assess unstimulated control responses, and stimulated with PHA (Sigma Chemical Co., St. Louis, MO, 10 µg/ml) and Candida antigen (Casta, Greer Labs, Lenoir, NC, 20 µg/ml) as positive mitogenic and antigenic control responses. Wells were pulsed with 2.5 µCi of [³H]thymidine (NEN Products, Boston, MA) 18 h before harvesting with a cell harvester (Cambridge Technology, Inc., Watertown, MA). Proliferation is expressed as stimulation index (mean [³H]thymidine incorporation of cells stimulated with antigen/mean incorporation in absence of stimulation).

Recombinant vaccinia viruses (rVV). The entire *gag* and *env* genes of HIV-1_{LAI} were inserted into wild type vaccinia (New York City Board of Health strain) as previously described (11, 12) (kindly donated by S.-L. Hu, Bristol Meyers, Squibb, Seattle, WA). The entire *nef* gene of HIV-1_{LAI} was inserted into wild type vaccinia, as previously described (13) (vTF*nef*, kindly donated by S. Koenig, MedImmune, Inc., Gaithersburg, MD). The rVV were amplified and titered on BSC-40 cells.

In vitro generation of CTL. CTL activity in PBMC was assessed in vitro by sequential bulk stimulation cycles as a modification of previously described methods (14). For cycle 1, freshly isolated PBMC for use as stimulators were infected with rVV, expressing either *gag*, *env*, or *nef* at an moi of 10 for 90 min in 200 µl of R10 (similar to R15, except 10% human serum pooled from screened volunteers was used instead of FCS), washed twice, and placed 4 cm from an ultraviolet light source for 10 min; the stimulator cells were incubated with freshly isolated PBMC cells at a ratio of 10 responder : 1 stimulator cell at 37°C in 5% CO₂ for 7 d at 10^6 cells/ml. Cycle 2 and subsequent weekly stimulators were prepared using autologous BLCL infected with rVV at an moi of 10 for 16 h, and inactivated with an ultraviolet light source and γ-irradiation (8,000 rad). These stimulator cells were added to the responder population at a ratio of 10 responder : 1 stimulator cell. Freshly isolated autologous γ-irradiated (3,000 rad) PBMC filler cells were also added at a ratio of 1 responder : 1 filler cell to the culture. Each well, containing 10^6 responder cells in R10 media, was supplemented 2 d after stimulations with 2 U/ml of rIL-2 (Hoffman-La Roche, Nutley, NJ).

Assay for cytolytic activity. Cytolytic activity was measured in a standard 4-h ⁵¹Cr release assay (CRA). Autologous BLCL targets were infected with an rVV at an moi of 10, labeled with 0.1 µCi Na⁵¹CrO₄ (NEN Products) for 16 h, gently washed three times with HBSS (Whittaker M. A. Bioproducts, Inc.) with 2% FCS, resuspended in R10, and plated at 5×10^3 BLCL targets per well in U-bottomed 96-well plates (Costar Corp.). Effector cells were washed and added to targets at varying E/T ratios to a total volume of 200 µl. After a 4-h incubation 100 µl of each well was sampled and counted (Beckman Instrs., Inc., Fullerton, CA; γ-5,500B). Spontaneous release was determined by harvesting supernatant from targets cultured in media only and maximum release from targets lysed with 1% NP-40 detergent, and percent specific release calculated by standard formula. Standard deviation of replicate wells was always less than 10% and spontaneous release was always less than 30% of the maximal release.

Analysis of lymphocyte subpopulations. Phenotypic analysis of cells present in bulk stimulated cultures of PBMC was determined by two-color flow cytometry with either FITC or phycoerythrin (PE)-conju-

gated mAbs directed against CD8 (Leu-2a-PE) and CD4 (Leu-3a-FITC). The T cell lineage of CD4⁺ and CD8⁺ populations being studied was demonstrated by two-color flow cytometry using a second mAb to CD3 (Leu-4-PE or FITC, all from Becton Dickinson, Mountain View, CA).

To subsequently determine the specific subset of cells present in PBMC cultures mediating the observed proliferative and cytolytic responses, subpopulations were isolated before assay by adherence to a 25-cm² flask coated with an αCD8 mAb (A.I.S., Menlo Park, CA) for 1 h. The nonadherent population (CD4⁺-enriched cells) was aspirated, and the adherent population (CD8⁺-enriched cells) subsequently gently removed with a cell scraper (Costar Corp.). The relative enrichment of CD8⁺ and CD4⁺ T cells achieved by these methods was determined by analyzing each population using two-color flow cytometry following staining with CD4-FITC and CD8-PE mAbs as described above.

Cloning of CTL to HIV-1 gag. Cloning of T cells was performed as a modification of previously described methods (15). Briefly, following generation of bulk-stimulated PBMC cultures containing HIV-1 gag-specific cytolytic activity, cells from these cultures were plated at 1 effector cell per well in 96-well U-bottomed plates (Costar Corp.). Each well was stimulated with 10^4 vac-gag-infected autologous BLCL (ultraviolet, and γ-irradiated), 10^5 autologous PBMC filler (γ-irradiated), and rIL-2 100 U/ml. Wells in which growth was present were expanded by specific antigen restimulation as described above, tested for cytolytic activity, and analyzed for phenotype by flow cytometry with mAbs to CD8, CD4, CD3 (as described above), and CD16 (Leu-11a-FITC, Becton Dickinson).

Limiting dilution analysis of cytotoxic T lymphocyte precursor (CTLp) frequencies. Analysis of CTLp frequencies in the PBMC of macaques to HIV-1 gag was performed by a modification of previously described methods (16). Briefly, fresh PBMC were plated in 96-well plates at dilutions of 8×10^5 – 5×10^4 cells/well in 24 replicates. Each well was stimulated with 10^4 vac-gag-infected autologous BLCL (ultraviolet- and γ-irradiated) and supplemented with 25 U/ml rIL-2 every 3 d to ensure that adequate helper function was present without the requirement for helper cells. After 10 d of culture, cells in each well were divided and assayed for cytolytic activity against a panel of three BLCL targets. The targets expressed either no antigens other than those derived from the *H. papio* virus used to transform the B cells (uninfected BLCL), vaccinia virus antigens (vaccinia-infected BLCL), or HIV-1 gag antigen (vac-gag-infected BLCL). Wells were considered positive against a particular target if cytotoxicity exceeded by three standard deviations the mean spontaneous release from 24 replicates of the target cultured in media alone. CTLp frequencies and confidence intervals were determined by the statistical method of χ² minimization and assumes a single hit model and Poisson distribution as described by Taswell (17). Data was expressed on a log scale as proportion of negative wells and graphed against cells/well.

Analysis of macaques infected with HIV-1. All animals were housed in stainless steel containment cages in a BSL-3 facility at the University of Washington Regional Primate Research Center, and anesthetized with ketamine (10 mg/kg i.m.) before blood draws. Eight HIV-1-infected and four uninfected control *M. nemestrina* were studied. Animals F89111, F89115, T92051, J92076, 93086, and 93089 were infected with cell-associated and cell-free stocks of HIV-1_{LAI}, animal J90402 was infected with cell-free HIV-1_{LAI}, animal M74046 was infected with cell-free and autologously infected lymphocytes from a previously described clinical isolate (1, 2) denoted as HIV-1_{RO}. Infection in all animals was confirmed by assay for anti-HIV antibodies by a whole virus EIA (Genetic Systems, Redmond, WA), virus isolation by co-culture with PHA-stimulated donor human PBMC, and PCR of PBMC DNA using the HIV-1 gag-specific primers SK38/39 (1, 2). All eight animals were healthy and had normal CD4 and CD8 lymphocyte counts, with no change in the CD4/CD8 ratio, at all time points evaluated after infection.

Results

Proliferative responses. PBMC proliferative responses were measured as an initial reflection of T cell recognition of HIV-

I proteins. PBMC from infected or control uninfected animals were stimulated with either whole inactivated HIV-1 virus, purified gp120, Candida Ag, or PHA. None of three control uninfected animals proliferated to the HIV-1 antigens (stimulation index < 1.7), but did respond to PHA (stimulation index > 20) and Candida Ag (stimulation index > 5). All five infected animals studied demonstrated significant proliferative responses (stimulation index > 5) to two strains of whole inactivated HIV-1 (LA1 and SF2) and to recombinant gp120, as well as to PHA and Candida. Each of the five animals was evaluated at a minimum of three distinct time points after infection, and proliferative responses to HIV-1 antigens were detected on every assay. Responses were present at the first time points measured (three animals evaluated at 4 wk postinfection) and persisted in two animals that were monitored as far as 140 wk postinfection. Representative lymphoproliferative assays to HIV-1 antigens from two uninfected control animals and two infected animals, one assayed early and one late postinfection, are shown in Fig. 1 A.

CD4⁺ T cells are the predominant cells responsible for the proliferative response to HIV-1 in humans (4, 18, 19), and we evaluated the relative role of T cell subsets in the proliferative response to HIV-1 in infected *M. nemestrina*. PBMC from animal F89115 were separated into CD8-enriched and CD4-enriched populations by panning on an α CD8 mAb-coated flask and tested as above. Enrichment for CD8⁺ and CD4⁺ cell populations was confirmed by FACS analysis. Of the adherent cells removed from the α CD8-coated flask, 88–94% expressed CD8, and 2–6% of this CD8-enriched population expressed CD4. The CD4-enriched population, represented by the cells nonadherent to the flask, contained 67–82% CD4⁺ cells, as compared to 35–45% in the unfractionated PBMC. The CD4-enriched population demonstrated a stronger proliferative response to HIV-1 than the unfractionated population, whereas the CD8-enriched population demonstrated a substantially lower response than the unfractionated population. These results suggest that CD4⁺ T cells were the predominant cell responding by proliferation (Fig. 1 B).

HIV-1 gag-specific cytolytic activity in bulk-stimulated cultures. HIV-1-specific CTL have been detected in humans in fresh PBMC and in PBMC stimulated with autologous cells infected with rVV-expressing HIV-1 genes (5). Fresh PBMC were evaluated in all eight HIV-1-infected *M. nemestrina*, and no direct HIV-1-specific lytic activity was detected. We therefore adapted a method that we had previously reported to improve the sensitivity of detection of virus-specific CTL (14, 20), in which the responder cells are stimulated for sequential cycles with cells expressing HIV-1 antigen. The initial stimulation was performed with autologous rVV-infected PBMC as stimulators. At weekly intervals thereafter, the cells were harvested and restimulated with rVV-infected autologous BLCL (Note: autologous *H. papio*-transformed BLCL were not used for the initial stimulation to avoid enrichment of T cells potentially reactive with *H. papio* products). 2 d after the second and subsequent stimulations, 2 U/ml rIL-2 were added to cultures to supplement helper function for CD8⁺ responses.

Specific cytolytic activity (% specific lysis $> 10\%$) to HIV-1 gag from bulk-stimulated PBMC cultures was detected in four of five HIV-1-infected *M. nemestrina* following three to five sequential stimulations, but was not detected in PBMC cultures from three uninfected control *M. nemestrina* stimulated in an identical manner (five or more experiments from each

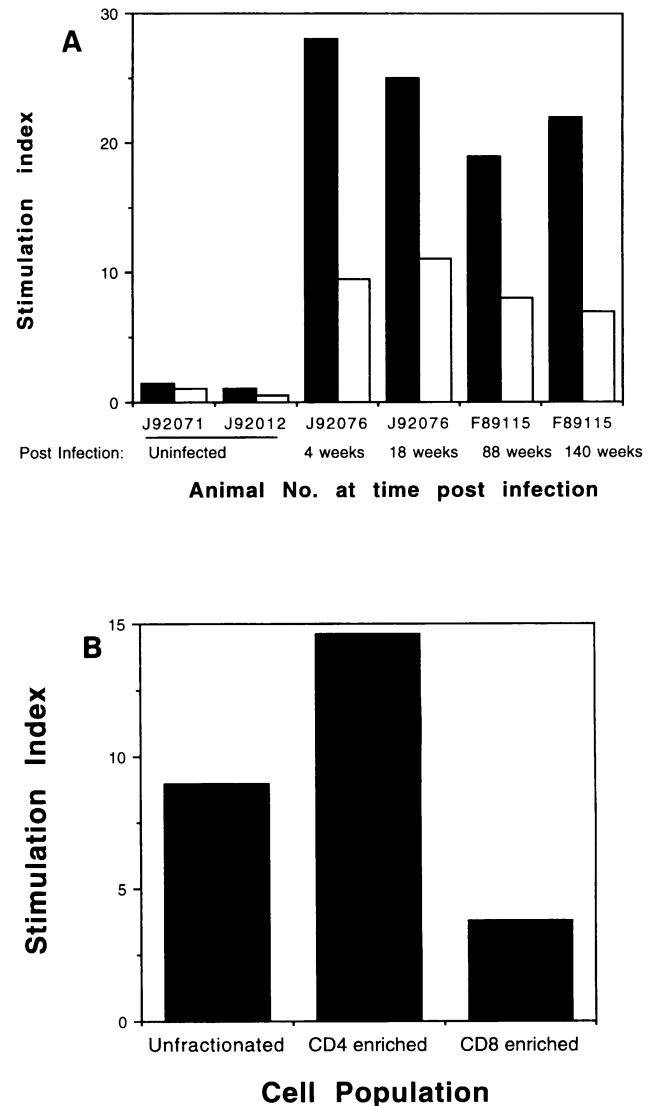


Figure 1. (A) The proliferation of PBMC from *M. nemestrina* on exposure to whole inactivated HIV-1 or gp120. Fresh PBMC from either uninfected control *M. nemestrina* or HIV-1-infected *M. nemestrina* were incubated for 7 d with either whole psoralen-inactivated HIV-1_{SF2} (■) or recombinant HIV-1_{SF2} gp120 (□) at 10 μ g/ml and [³H]thymidine incorporation measured. Animals F89115 (88–140 wk after infection) and J92076 (4 wk after infection) were infected with HIV-1_{LA1} and animals J92071 and J92012 were uninfected controls. (B) Determination of the cell subset mediating proliferative response in HIV-1_{LA1}-infected *M. nemestrina* animal F89115 (120 wk after infection). Cells were separated into CD8- or CD4-enriched populations using an α -CD8 mAb-coated flask and proliferation to whole psoralen-inactivated HIV-1_{SF2} at 10 μ g/ml was measured after 7 d by [³H]thymidine incorporation.

animal). Background lysis of vaccinia-infected or uninfected BLCL was always less than 6%. Representative experiments from two infected animals (Fig. 2, A and B) and one control animal (Fig. 2 C) are shown. HIV-1 gag-specific lysis was detected at two or more separate time points in four of five HIV-1-infected animals (Table I). The animal with no discernable response was tested only on one occasion. One animal (T92051) was assayed for HIV-1 gag-specific lysis before and

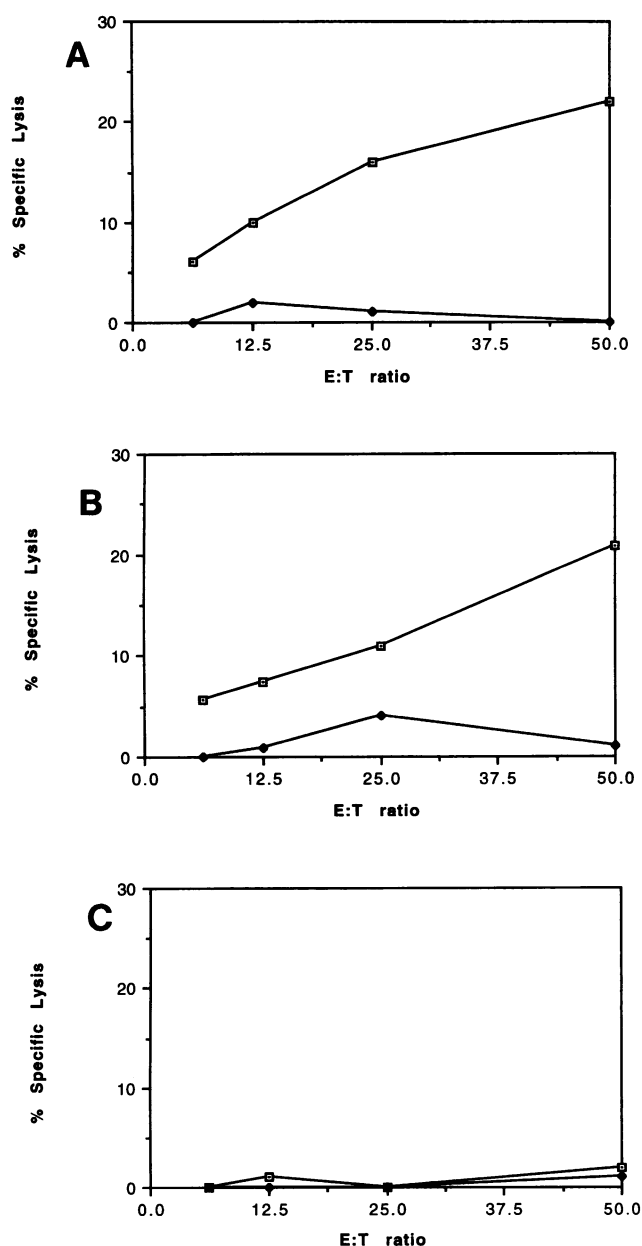


Figure 2. Lysis of *vac-gag*-infected autologous BLCL by antigen-stimulated PBMC from *M. nemestrina*. PBMC which underwent 3–5 sequential stimulations, initially with autologous *vac-gag*-infected PBMC and subsequently with autologous *vac-gag*-infected γ -irradiated BLCL and autologous filler, were tested in a standard 4-h CRA against *vac* (♦)- or *vac-gag* (□)-infected autologous ^{51}Cr -labeled BLCL targets. Representative experiments are shown from two HIV-1 infected animals (A, animal F89111 and B, animal M74046) and a control uninfected animal (C).

after inoculation with HIV-1, and specific lysis was only detected after infection (Table I).

Persistence of HIV-1 *gag*-specific CTL. HIV-1 *gag*-specific CTL activity was evaluated at several time points following infection in four animals to determine the durability of responses (Table I). Persistent *gag*-specific CTL activity in animals F89111 and M74046 was detected from bulk-stimulated PBMC cultures assayed as described on five occasions from 80 to 140

Table I. HIV-1 *gag*-specific CTL in HIV-1-infected Macaques

Animal No.	Target cell infection*	Percent specific lysis at time after infection					
		–2 wk	4 wk	12–20 wk	52–80 wk	112 wk	140 wk
T92051	<i>vac</i>	0 [†]	0	0	—	—	—
(infected)	<i>vac-gag</i>	0	11	10	—	—	—
M74046	<i>vac</i>	—	1	3	2	—	—
(infected)	<i>vac-gag</i>	—	21	11	17	—	—
F89111 [§]	<i>vac</i>	—	—	—	0	1	0
(infected)	<i>vac-gag</i>	—	—	—	22	10	10
F89115 [§]	<i>vac</i>	—	—	—	3	3	1
(infected)	<i>vac-gag</i>	—	—	—	12	7	14

		Percent specific lysis at time after initial monitoring					
		0 wk	4 wk	10–12 wk	16–18 wk	24–28 wk	42–54 wk
86092	<i>vac</i>	3	0	2	2	1	0
(uninfected)	<i>vac-gag</i>	0	0	1	2	1	2
89218	<i>vac</i>	0	0	0	4	0	0
(uninfected)	<i>vac-gag</i>	1	0	0	1	0	0

* Target cells were *vac* and *vac-gag*-infected autologous ^{51}Cr -labeled BLCL. [†] Percent specific lysis by Ag-specific stimulated cultures of HIV-1 inoculated *M. nemestrina* PBMC at three time points at E/T ratios of 50:1. —, not tested. Infected animals were each analyzed at three separate time points, as presented. [§] PBMC from animals F89111 and F89115 were unavailable for study before 80 wk after infection. ^{||} PBMC of uninfected animals were tested on multiple occasions in an identical manner to the infected animals.

wk after infection and 4–52 wk after infection, respectively. Animal F89115 demonstrated *gag*-specific CTL activity at weeks 92 and 140 after infection, but not at week 112 after infection and animal T92051 showed *gag*-specific CTL at weeks 4 and 12 after infection, but not before infection or at week 8 after infection.

Effector T cell population mediating cytolytic activity to HIV-1 *gag*. Four bulk-stimulated cultures of PBMCs from HIV-1-infected *M. nemestrina* containing *gag*-specific cytolytic activity were evaluated by flow cytometric analysis. A mean of 93% of cells were $\text{CD}3^+$ (range 91–96%), 66% were $\text{CD}8^+\text{CD}3^+$ (range 54–74%) and a smaller subset of 20% were $\text{CD}4^+\text{CD}3^+$ (range 14–32%). FACS analysis confirmed that > 96% of $\text{CD}8^+$ or $\text{CD}4^+$ cells were $\text{CD}3^+$. The predominance of cells expressing CD8 in these cultures suggested that this population might be responsible for the observed cytolytic activity, but a significant participation by the $\text{CD}4^+$ subset could not be excluded. $\text{CD}8^+$ cells were therefore purified from the bulk-stimulated PBMC cultures by panning on an $\alpha\text{CD}8$ monoclonal antibody-coated flask. To determine the efficiency of this panning method for enriching $\text{CD}8^+$ cells from macaques, the cells were analyzed on a FACS. The adherent population was 94% $\text{CD}8^+$, whereas the nonadherent population was largely depleted of $\text{CD}8^+$ cells, containing only 4% $\text{CD}8^+$ cells. Bulk-stimulated PBMC cultures that were separated into $\text{CD}8$ -enriched and $\text{CD}8$ -depleted populations were assessed for HIV-1 *gag*-specific lytic activity (Fig. 3), and the $\text{CD}8$ -enriched population contained nearly all of the detectable lytic activity.

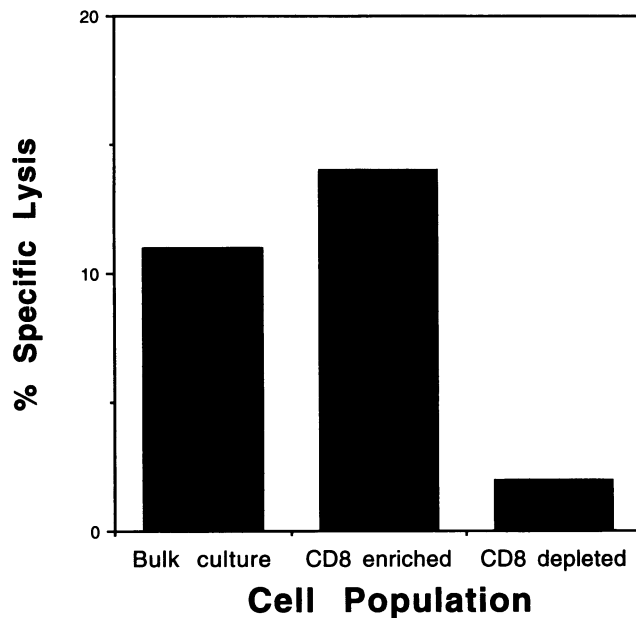


Figure 3. Determination of the cell subset mediating CTL response to HIV-1 *gag* in HIV-1-infected *M. nemestrina* animal M74046. Cells from a bulk culture containing HIV-1 *gag*-specific lysis were separated into CD8-enriched or CD8-depleted populations using an α -CD8 mAb-coated flask and lysis of autologous vac-*gag*-infected ^{51}Cr -labeled BLCL determined in a standard 4-h CRA. The E/T ratio was 25:1.

HIV-1 *env*- and *nef*-specific cytolytic activity in bulk-stimulated cultures. To assess CTL responses to HIV-1 proteins other than *gag*, PBMC were also examined by similar methods for the presence of *env*- and *nef*-specific CTL. Specific lytic activity (lysis > 10%) to *env* was detected in two of four HIV-1-infected *M. nemestrina*. Animal M74046 was evaluated three times and animal F89111 was evaluated twice, and both animals had *env*-specific lysis detectable at each occasion tested, as described below. At an E/T ratio of 50:1, the lytic activity from animal M74046 for targets infected with vac-*env* was 12% at 8 wk after infection, 11% at 40 wk after infection and 14% at 52 wk after infection, with background vaccinia lysis of 4%, 2%, and 3%, respectively. Animal F89111 had lytic activity at an E/T ratio of 50:1 for vac-*env* targets of 16% at 100 wk after infection and 10% at 132 wk after infection, with background vaccinia lysis of 7 and 0%, respectively. Two animals, T92051 and F89115, tested on a single occasion each, failed to demonstrate *env*-specific lytic activity. Two uninfected control animals were evaluated on three separate occasions, and neither animal ever demonstrated greater than 2% *env*-specific cytolytic activity following stimulation in an identical manner.

Env-specific CTL in HIV-1-infected humans has been reported to be mediated by both CD4⁺ and CD8⁺ cells (21), and we therefore sought to determine whether CD4⁺ CTL contributed significantly to the lytic activity detected in HIV-1-infected macaques. Bulk-stimulated PBMC, obtained from animal M74046 at 52 wk after infection, were separated into CD8⁺- and CD4⁺-enriched populations, as described in Methods, and assayed for *env*-specific lytic activity. The unfractionated population exhibited 14% *env*-specific lytic activity at an E/T ratio of 50:1, the CD8-enriched population exhibited 19% *env*-specific lysis, and the CD4-enriched population exhibited 4% *env*-spe-

cific lytic activity. Lysis of vaccinia-infected control targets was < 4% in all populations. Thus, the majority of the lytic activity to *env* was mediated by CD8⁺ cells, with little contribution from CD4⁺ cells.

Nef-specific CTL have been reported to be of potential importance in clearance of HIV-1 in humans (22, 23), and we therefore examined the infected macaques for the presence of *nef*-specific CTL. Two macaques were evaluated at 8 wk after infection. Bulk-stimulated PMBC cultures of animal 93089 exhibited 13% lysis of vac-*nef*-infected autologous BLCL versus 2% lysis of vaccinia-infected autologous BLCL at an E/T of 25:1. Animal 93086 exhibited weaker lytic activity towards *nef*, with 7% lysis of vac-*nef* targets and 2% lysis of vac targets at an E/T of 25:1. By contrast, two uninfected animals had less than 1% lysis of *nef*-expressing targets.

Estimation of frequency of HIV-1 *gag* CTLp. *Gag*-specific CTL in HIV-1-infected *M. nemestrina* was not present in fresh PBMC, as has been described in humans, but rather required several in vitro stimulations for detection. This suggested that *gag*-specific CTL precursors might be present in frequencies relatively lower than those in humans who have chronic viremia. Therefore, the frequency of *gag*-specific CTLp was determined by limiting dilution culture. PBMC were plated at limiting dilutions and stimulated for 10 d with autologous vac-*gag*-infected BLCL in wells supplemented with 25 U/ml rIL-2, and the responding cells were tested against autologous BLCL targets expressing vac-*gag*. Frequencies of CTLp specific for *gag* were estimated in two HIV-1-infected animals and two control uninfected animals at two time points (Fig. 4). The relative frequency in animal M74046, infected with both cell-free and cell-associated HIV-1, was estimated at 1/327,000 (95% confidence interval [CI] 1/249,000–1/476,000) at 24 wk after infection (Fig. 4 A) and 1/380,000 (95% CI 1/290,000–1/536,000) at 32 wk after infection. Animal J90402, infected with only cell-free HIV-1, was tested at 42 wk after infection (Fig. 4 B), with the *gag*-specific CTLp frequency estimated as 1/134,000 cells (95% CI 1/101,000–1/199,000), and at 52 wk after infection, with the frequency estimated at 1/170,000 cells (95% CI 1/130,000–1/246,000). The CTLp frequency to targets infected with vaccinia alone (range: 1/1,300,000–1/2,600,000 in four experiments) or uninfected BLCL targets (range: 1/1,900,000–1/4,900,000) was significantly lower in the two infected animals. The CTLp to vac-*gag*-infected targets from two control uninfected *M. nemestrina* in four experiments each were lower than in infected animals (range: 1/1,700,000–1/3,700,000, and had similar CTLp frequencies as the HIV-infected animals to vaccinia or uninfected targets (range: 1/2,300,000–1/13,000,000).

Generation of an HIV-1 *gag*-specific CTL clone. Analysis of HIV-1-specific CTL responses in primates would be facilitated by the availability of CD8⁺ T cell clones. To determine if HIV-specific CD8⁺ T cell clones could be isolated and expanded, cells from a bulk-stimulated culture of PBMC from macaque M74046 which contained *gag*-specific CTL activity were plated at 1 cell/well and stimulated with vac-*gag*-infected BLCL, autologous irradiated PBMC as filler cells, and rIL-2 as described in Methods. Cell growth was observed in 6 of 960 wells plated, and cells from these wells were expanded and tested for cytolytic activity. Cells derived from one of these six positive wells demonstrated HIV-1 *gag*-specific lysis following screening against vac- and vac-*gag*-infected targets. Cells from this well were expanded by repetitive cycles of specific stimula-

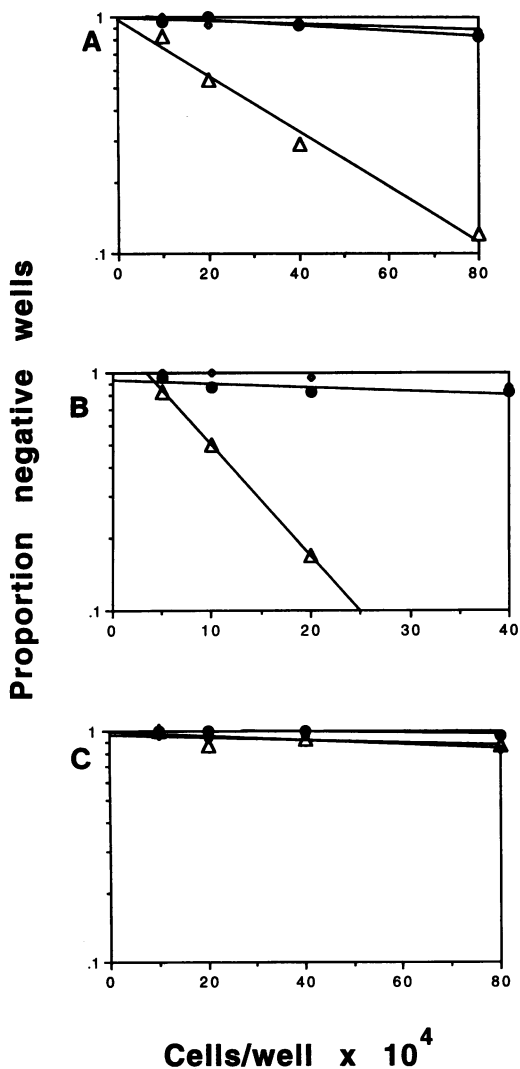


Figure 4. CTLp frequencies of HIV-1 *gag*-specific CTL were estimated by limiting dilution analysis. Fresh PBMC were plated in increasing dilutions of 24 replicates ranging from 5×10^4 to 80×10^4 cells/well and stimulated with autologous vac-*gag*-infected BLCL and 25 U/ml rIL-2. Cells from each well were harvested after 10 d and split for assay of cytolytic activity against multiple targets. Targets used were ⁵¹Cr-labeled mock infected autologous BLCL (•), vaccinia-infected autologous BLCL (◊) or vac-*gag*-infected autologous BLCL (Δ). Wells were considered positive if cytolytic activity exceeded that spontaneous release by 3 SD. Representative experiments on two HIV-1-infected animals (A, animal M74046 and B, animal J90402) and an uninfected animal (C) are shown.

tion with cells expressing *gag* plus supplemental IL-2. Following expansion, the cell population demonstrated 60% specific lysis of HIV-1 *gag* at an E/T ratio of 10:1 (Fig. 5). By FACS analysis, the cells derived from this single well were 99% CD3⁺4⁺8⁺16⁺. A control uninfected animal had no clonal growth when stimulated in an identical manner.

Definitive evidence that the *gag*-reactive cells from this well were clonal would require reagents for directly analyzing T cell receptor gene rearrangements in macaques, which are not available. However, the likelihood that this reactive cell population is clonal can be calculated by relative probabilities of plating and detecting reactivity from more than one cell. Assuming

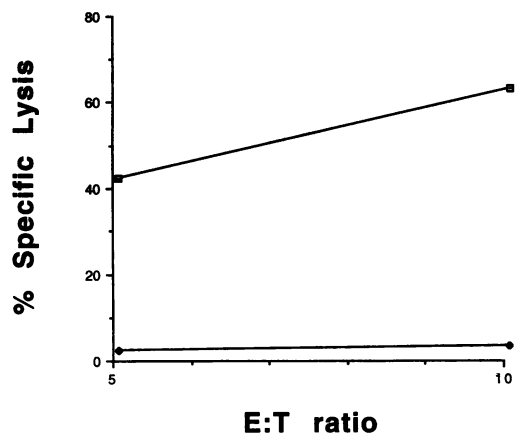


Figure 5. Cells from a bulk culture of PBMC from animal M74046 in which HIV-1 *gag*-specific CTL were present were cloned in limiting dilution by stimulating with autologous vac-*gag*-infected BLCL, autologous filler, and rIL-2. Wells which contained cell growth were expanded and tested for cytolytic activity against vac (♦)- or vac-*gag* (□)-infected autologous ⁵¹Cr-labeled BLCL targets.

a Poisson distribution, the probability that one or more cells is plated in a well when plated from a suspension diluted to 1 cell/100 μ l is 0.63 (Minitab Statistics for MS-DOS). Therefore, of the 960 wells plated, (960×0.63) or 605 wells are likely to have contained at least one cell. Of the wells that contained at least one cell, the probability that ≥ 2 cells were added to a well when plated at this dilution is 0.41. Since only 1/605 of the wells plated that contained cells demonstrated growth and was reactive for HIV-1 *gag*, the likelihood that the well exhibiting the *gag*-specific cytolytic activity reflected the outgrowth of two or more cells is $(0.41 \times 1/605)$, or 1/1,475. Thus, the probability that this population is derived from a single cell and thus is clonal is $> 99.9\%$.

Discussion

Our study is the first to demonstrate HIV-1-specific proliferative and cytotoxic T cell responses in HIV-1-infected *M. nemestrina*. T cell proliferative responses to HIV-1 envelope protein and inactivated HIV-1 were readily detectable in all animals tested at time points from 4 to 140 wk postinfection. These HIV-1-specific proliferative responses were predominantly mediated by CD4⁺ T cells. Cytotoxic T cell responses to HIV-1 were detected to HIV-1 *env*, *gag*, and *nef* proteins. CTL responses were present from 4 to 140 wk after infection, were mediated by CD8⁺ T cells, and were seen in animals inoculated with both cell-associated and cell-free HIV-1.

Persistent CD4⁺ T cell proliferative responses were observed in all animals to HIV-1_{LAI} (the infecting strain in four of the five animals), the antigenically related SF-2 strain, and purified recombinant gp120. The stimulation indices to these HIV-1 antigens were similar or greater than that observed to SIV in macaques vaccinated with SIV envelope preparations, and to HIV-1 in chimpanzees and humans vaccinated with various HIV-1 envelope preparations (24–26) but, unlike the response to those vaccine preparations, did not appear to decline over time. The persistence of CD4⁺ T cell responses has been reported in experimental systems to require the persistence of stimulating antigen, typically on antigen-presenting cells such

as follicular dendritic cells (27). However, this does not necessarily require the persistence of replicating virus. For example, we have previously reported that CD4⁺ T cell responses to vaccinia virus persist for > 15 yr after immunization (28), despite the fact that this virus has no demonstrable latency in the host. Thus, immunization with or exposure to viable viruses may more efficiently provide antigen reservoirs for maintaining CD4⁺ memory responses.

Humans infected with HIV-1 typically demonstrate poor proliferative responses to HIV-1, even at early time points when the host is asymptomatic and the number of CD4⁺ cells is normal (4, 19). In distinction to these findings, the macaques infected with HIV-1 demonstrated strong and persistent proliferative responses to HIV-1. The maintenance of normal CD4⁺ T cell numbers and a proliferative response to HIV-1 are consistent with our previous observations in this model that infected animals do not develop immunosuppression (1, 2). Indeed, the ability of HIV-1-infected macaques to generate and maintain CD4⁺ T cell proliferative responses may be important in the observed clearance of viremia in this model.

Unlike humans, HIV-1-specific CTL were not detected directly from *M. nemestrina* PBMC. The failure to detect circulating activated CTL in fresh PBMC is similar to observations made in SIV-infected macaques (29) and HIV-1-infected chimpanzees (30). Therefore, to determine if CTL were present, we adapted methods we have previously reported to enhance detection of primed virus-specific CTL responses in cytomegalovirus-infected humans and in SIV-infected macaques (14, 20), and employed sequential in vitro antigen-specific stimulation with autologous cells infected with rVV containing HIV-1 genes. Using this approach, HIV-reactive CD8⁺ CTL was detected in bulk-stimulated PBMC cultures and HIV-1-specific clones were subsequently isolated.

The demonstration of CD8⁺ cytolytic activity to HIV-1 proteins following experimental challenge with HIV-1 is consistent with the virologic evidence that HIV-1 replicates in macaques (1, 2). In vivo priming of CD8⁺ virus-specific CTL requires the presentation of appropriate viral peptide antigens in the context of MHC class I, and thus presumably requires HIV-1 infection of host cells. This has been most directly demonstrated in animal J90402, which was inoculated with only cell-free HIV-1. Both plasma viremia and cell-associated HIV-1 infection were present at 16 wk after inoculation (2), and *gag*-specific CTL were detectable at multiple subsequent time points tested. Of note, the animal became culture negative from PBMC after week 24. The finding of CTL specific for the *nef* protein, which is a non-structural, regulatory gene product expressed only in infected cells, is also consistent with in vivo viral replication. The presence of virus-specific CTL responses at time points long after virus is recoverable does not define the virologic status of the host, since this finding is consistent with either long-lived CD8⁺ memory cells that can persist in the absence of replicating virus or with limited persistent viral replication in the host (31–33).

We detected CTL to multiple HIV-1 proteins (*env*, *gag*, and *nef*) in this animal model. CTL responses to proteins which contain conserved CTL epitopes, such as *gag* and *nef*, have been suggested to be of potential utility in HIV-1 vaccine design (22, 34). Additionally, studies in humans exposed to HIV-1 who did not exhibit continued viral replication demonstrated CTL responses to *gag* and *nef* (23, 35), suggesting a potential role of such CTL in clearing HIV-1 infection. Our ability to

detect CTL responses to *gag* and *nef* in HIV-1-inoculated *M. nemestrina*, who also clear HIV-1 viremia, supports the contention that these responses may aid in viral clearance, and the role of such responses should be directly analyzable in this animal model.

The frequency of CTLp in all our HIV-1-infected *M. nemestrina* was lower than that reported in persons with chronic HIV-1 infection (36–38). Using limiting dilution assays, *gag*-specific CTLp frequency estimates in these HIV-1-infected animals ranged from 1 per 1.34×10^5 to 1 per 3.8×10^5 cells, which were ~ 100-fold lower in frequency than that described in humans (37). The reduced frequency of *gag*-reactive CTLp from *M. nemestrina* may reflect restricted viral replication of HIV-1 within *M. nemestrina* as compared to humans. Alternatively, these differences may be in part methodologic, reflecting more efficient culture conditions for stimulating human cells. Additionally, the sites of HIV-1 replication in macaques have not been well described, and analysis of lymphocytes from other compartments, such as lymph nodes and spleens from infected animals, might reveal different CTLp frequencies.

Limited data on the kinetics of HIV-1-specific CTLp in humans with acute HIV-1 infection have suggested that cytotoxic responses to HIV-1 may play a role in controlling acute HIV-1 infection (39). Kinetic studies in humans with acute HIV-1 infection are limited, however, as it is often difficult to precisely define the time of infection, and there can be a substantial delay from the time of infection until lymphocytes from infected individuals can be examined. In HIV-1-infected macaques, CTL responses to multiple HIV-1 proteins were detected as early as 4–8 wk after infection, suggesting a potential role of CD8⁺ responses in controlling viral replication and clearing virus as reported in humans. The definition of CTLp frequencies to various HIV-1 proteins at very early time points after acute HIV-1 infection of *M. nemestrina* may provide insights into the comparative role of specific CTL responses in initial viral clearance and the subsequent virologic course. The only alternative primate model for studying immunity to HIV-1 is chimpanzees. However, very limited numbers of these primates are available for study and HIV-specific CD8⁺ CTL have been difficult to detect in HIV-infected chimpanzees, suggesting chimpanzees may not be as useful as macaques for studying CD8⁺ immunity (30, 40).

A potential causative role of CD8⁺ CTL in HIV-1 immunopathogenesis, by analogy with the cause of disease following a noncytopathic viral infection in mice in which virus-infected antigen-presenting cells (APCs) are lysed by CD8⁺ CTL, has recently been speculated by Zinkernagel and Hengartner (41). Our finding that these macaques who do not develop immunodeficiency but do develop HIV-1-specific CD8⁺ CTL responses suggests that persistent viral replication, rather than CD8⁺ CTL responses, may be a more important cause of HIV-induced immunopathology. HIV-1-specific CD4⁺ T cells with cytolytic activity have also been implicated in HIV-1 immunopathogenesis by mediating lysis of uninfected APCs or CD4⁺ cells (42). However, we could not detect significant HIV-1-specific CTL activity mediated by CD4⁺ cells in bulk cultures of macaque PBMC. Such CD4⁺ CTL have been reported in HIV-1-infected humans (21), and appear to be less readily demonstrable in macaques. The use of selective depletion or adoptive transfer of T cells with defined functions in this model should allow a more definitive analysis of the role of CD4⁺ and CD8⁺ T cells in HIV-1 immunopathogenesis.

We were able to generate a CTL clone from an HIV-1-infected *M. nemestrina* that had high levels of specific lysis for HIV-1 *gag* proteins at low E/T ratios. *Gag*-reactive CTL epitopes have been well characterized in HIV-1-infected humans (34), HIV-2-infected humans (43) and SIV-infected macaques (44). A similar portion of the *gag* gene from SIV and HIV-1 elicit CTL responses in macaque and humans, respectively (45). This suggests that macaques may recognize a similar region of HIV-1 *gag* as humans, and studies to determine the epitope specificity of HIV-1-specific cellular immune responses in macaques are now possible. If similar regions are recognized, this model may prove useful for evaluating the mechanisms by which CTL select escape variants, as suggested with HIV-1-specific CTL responses (6).

In summary, this study demonstrates that HIV-1-infected *M. nemestrina* develop persistent T cell proliferative and cytotoxic responses to HIV-1 proteins. These animals which resist HIV-1-induced disease demonstrate persistent HIV-specific CD4⁺ T cell responses in contrast to HIV-1-infected humans. The combination of HIV-1-specific CD4⁺ and CD8⁺ responses may be important in the control of replication seen in this animal, a finding which if confirmed has implications for immunity to HIV-1 infection and the design of protective vaccines.

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