

An Antigenic Threshold for Maintaining Human Immunodeficiency Virus Type 1-specific Cytotoxic T Lymphocytes

Xia Jin,¹ Graham Ogg,² Sabstian Bonhoeffer,³ Jeffrey Safrit,⁴ Mika Vesanen,¹ Daniel Bauer,¹ Donald Chen,¹ Yunzhen Cao,^{1, 5} Marie-Ange Demoitie,¹ Linqi Zhang,¹ Martin Markowitz,¹ Douglas Nixon,¹ Andrew McMichael,² and David D. Ho,¹

¹The Aaron Diamond AIDS Research Center, The Rockefeller University, New York, New York, U.S.A.

²Institute of Molecular Medicine, Oxford, U.K.

³Fredrich Miescher Institute, Basel, Switzerland

⁴Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia, U.S.A.

⁵Current Address: National Center for AIDS Prevention and Control, Chinese Academy of Preventive Medicine, Beijing, China.

Accepted June 1, 2000.

Abstract

Background: Using the lymphocytic choriomeningitis virus (LCMV) model in mice, a number of studies show that memory cytotoxic T-lymphocyte (CTL) responses are maintained in the presence of continuous antigenic stimulation. Yet, other groups found that memory CTL specific for LCMV could last for a lifetime in mice without viral antigens. Thus, the extent to which an antigen is required for the maintenance of virus-specific CTL remains controversial. In humans, very few studies have been conducted to investigate the relationship between the quantity of antigen and the magnitude of CTL responses.

Materials and Methods: We quantified CTL precursors (CTLp) using a limiting-dilution analysis (LDA) and CTL effectors (CTLe) using a new Major Histocompatibility Complex (MHC) class I tetramer technology in six long-term nonprogressors (LTNPs) with human immunodeficiency virus type-1 (HIV-1) infection, as well as in eight patients whose viral

loads were well suppressed by antiretroviral therapy. The viremia levels in these patients were measured using an reverse transcription polymerase chain reaction (RT-PCR) assay. The proviral DNA load in peripheral blood mononuclear cell (PBMC) was also measured by PCR in four LTNPs.

Results: The LTNPs had high levels of HIV-1-specific memory CTLp and CTLe, while maintaining a low plasma viral load. Despite also having low viral loads, patients whose plasma viremia was well-suppressed by effective therapy had low levels of CTLe.

Conclusions: Our findings suggest that a complex, rather than a monotonic, relationship exists between CTL levels and HIV-1 viremia, including what appears to be an antigenic threshold for the maintenance of CTL at a measurable level. Under conditions of "antigen excess," CTLe levels correlate inversely with viral load. On the other hand, under conditions that are "antigen limited," the correlation appears to be direct.

Address correspondence and reprint requests to: David D. Ho, The Aaron Diamond AIDS Research Center, The Rockefeller University, 455 First Avenue, 7th Floor, New York, NY, U.S.A. 10016. Phone: 212-448-5100; Email: dho@adarc.org

Introduction

Upon re-exposure to antigens, the immune system can act faster and mount a stronger recall response to invading foreign microorganisms

in vivo. This phenomenon is known as immunological memory. As of yet, however, the cellular basis for immunological memory is not clearly understood. In humans, the memory T-cell response can last for years, though the average life-span of memory cells that exhibit the CD45RO phenotype is significantly shorter (1,2). Thus, defining what factors are necessary for the maintenance of immunological memory remains an area of intense interest. One possibility under consideration is the persistence of antigen as a requirement for sustained cytotoxic T lymphocyte (CTL) memory and effector function. This idea has been explored mostly in mice, yielding inconsistent conclusions (3–6).

It has been demonstrated that CTL effector (CTLe) numbers are inversely correlated with antigen load in plasma during natural HIV-1 infection (7). Paradoxically, in individuals whose viremia levels are well-suppressed by highly active antiretroviral therapy (HAART), CTL numbers are typically low (7–9). To examine the role of antigen in sustaining CTL responses in these subjects, we carried out an assessment of the relationship between their viremia levels and the quantity of human im-

munodeficiency virus type-1 (HIV-1)-specific CTL in blood. One group of patients was comprised of HIV-1-infected long-term nonprogressors (LTNPs), the majority of whom had high CTL and low viremia levels (8,10,11). The other group represented patients with low plasma viremia levels as a result of HAART (12). Our results showed that, despite low levels of plasma viremia in both groups, LTNPs have high CTLe; whereas, the treated patients had low CTLe. The importance and implication of these findings are discussed.

Materials and Methods

Study Subjects

All patients included in this study were chosen from various study cohorts established at the Aaron Diamond AIDS Research Center, New York. Clinical information on these patients is given in Table 1. All studies have been approved by the Rockefeller University Institutional Review Board (IRB) and conducted in accordance to the guidelines. Informed consent was obtained from all patients included in the study.

Table 1. Clinical characteristics of the subjects

Subject	Sex	Age	Length of Infection (months)	Length of Infection (days)	Antiretroviral Treatment CD4/ μ l*	Plasma RNA (copies/ml)
LTNPs						
1	M	46	156	No	560–740	430
2	M	41	170	No	800–1000	1902
3	M	38	145	No	500–700	80
4	M	44	190	No	400–800	6148
5	F	42	195	No	279	575
6	M	40	200	No	505	6501
Others						
7	M	44	37	670	704	<50
8	M	54	30	365	569	<50
9	M	34	37	700	420	<50
10	M	46	41	538	426	<50
11	M	31	36	575	627–752	<50
12	M	32	31	862	500–809	<50
13	M	37	31	535	395–695	<50
14	M	36	29	865	1015–1033	<50

*The range of CD4 count over that period is given when data on a particular date is not available.

Quantifying HIV Load

Viral RNA copy numbers in plasma were determined by reverse-transcriptase polymerase chain reaction (RT-PCR) using the Amplicor HIV Monitor Kit (Roche Molecular Systems, Branchburg, NJ) according to the manufacturer's instructions. The lower limit of detection for this assay is 50 copies/ml (12). For viral load measurement in the LTNPs, the branched-chain DNA (bDNA) (Bayer, Emeryville, CA) assay was used, which has a detection limit of 50 copies per milliliter of plasma (13). Quantification of viral DNA in peripheral blood mononuclear cell (PBMC) was performed using an established PCR method (12), with a limit of detection of 10 copies.

Limiting Dilution Assay (LDA) for CTL

LDA plates were set up with 8 input cell dilutions ranging from 0 to 32,000 cells per well. In addition, 2.5×10^4 gamma-irradiated allogeneic PBMC and 0.1 $\mu\text{g/ml}$ anti-CD3 (clone 12F6) were added to each well. The cultures were kept for 14 days, with twice a week medium changes. A standard ^{51}Cr release assay was performed on days 12-14, with each LDA plate split four to six ways and assayed against autologous B lymphoblastoid cell lines (BLCL) that had been infected with either control vaccinia (strain NYCBH), or recombinant vaccinia expressing HIV-1 antigens (IIIB Env-gp160 , IIIB Gag-p55 and IIIB Pol). Recombinant vaccinias were provided by Therion Biologic Corporation (Boston, MA).

A well was regarded as positive for specific CTL activity only when the percentage of specific lysis exceeded 10%, as well as 10% more than that of the corresponding control target cells. The CTL precursors (CTLp) frequencies were determined using a maximum likelihood method, as performed on a Microsoft Excel spreadsheet (kindly provided by Spyros Kalams, Massachusetts General Hospital, Boston, MA), and the results were expressed as the number of CTLp per 10^6 PBMC.

MHC Class I Tetramer Staining

Cryopreserved PBMC from different time points were resurrected and stained with phycoerythrin (PE)-conjugated (MHC) class I tetramers synthesized with HIV-Gag (77-85: SLYNTVATL) and Pol (476-484: ILKEPVHGV)

peptides (7). The same cells were also costained with anti-CD8 antibody. The stained PBMC were analyzed using fluorescence-activated cell sorting (FACS), and results were presented as percentage of tetramer-positive and CD8-positive cells in the small lymphocyte population.

Results

LTNPs Have High Memory CTL Numbers and Low Plasma Viral Load

Four of the LTNPs were chosen for the initial screening of CTL activity. These LTNPs were enrolled in an earlier study after 10–12 years of HIV-1 infection (14), and have been followed for an additional 4–5 years. The plasma viremia levels in these patients range from 80 to 6,148 copies/ml. To measure CTLp, LDA were set up according to a protocol previously described (15,16) using cryopreserved PBMC from each of the patients. Results showed that CTL responses to more than one of the three major HIV-1 structural antigens (Env, Gag, and Pol) were detectable in all four patients. In addition, the magnitude of CTLp responses was high, with frequencies in some as high as 10,000 per 10^6 PBMC (Fig. 1). These high CTLp frequencies were similar to that of one other LTNP cohort (11), but a log higher than that observed in others with progressive HIV-1 infection (15,17).

LTNPs Have High Levels of Effector CTL and Low Levels of Proviral DNA

Although the absolute quantity of HIV-1 antigens may be an important determinant, the kinetics of viral load changes may also be a factor for maintaining the number of CTL. To further analyze the correlation between viral load and CTL responses, we performed a longitudinal assessment of viral load in five LTNPs. In addition, viral DNA in PBMC was also measured in four of the LTNPs. Since the five LTNPs had HLA-A*0201, their CTL levels were measured using MHC class I tetramers. Only one patient (#6), who had sustained high plasma viremia levels (Fig. 2A) and progressive increase in proviral DNA levels (Fig. 2B), had low CTL numbers. All the others were found to have moderate to high levels of CTL (between 0.1% to 1% of CD8+ T cells), with corresponding low levels of plasma viremia

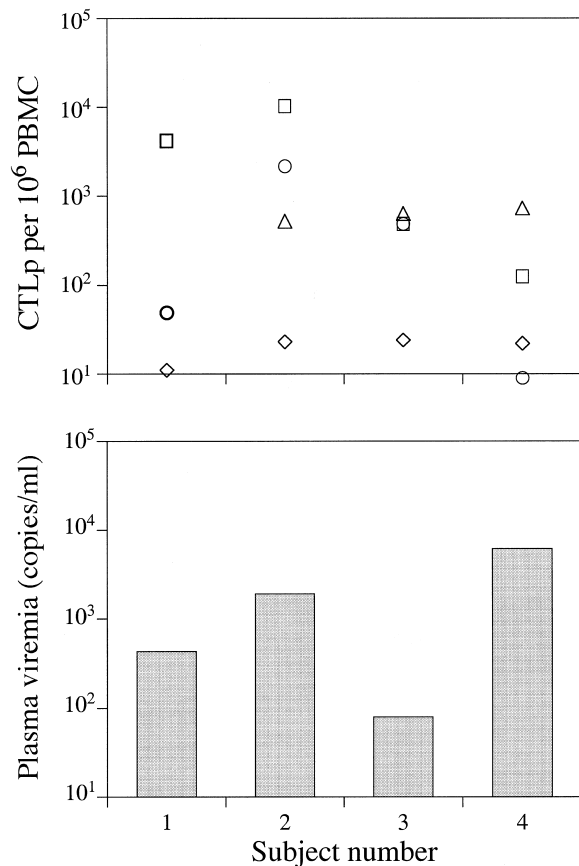


Fig. 1. CTLp frequency and viral load in long-term nonprogressors (LNTNs). (A) Cytotoxic T lymphocyte precursor (CTLp) frequency in each subject was determined using limiting-dilution analysis (LDA). Effector cells were peripheral blood mononuclear cell (PBMC) cultured under standard LDA conditions. Target cells were autologous B lymphoblastoid cell lines (BLCL) that had been infected with either control vaccinia (open diamond) or recombinant vaccinia expressing human immunodeficiency virus (HIV) antigens: vAbT 299 (II-Benv-gp160; open circle), vAbT 141 (II-Bgag-p55; open square), and vAbT 204 (II-Bpol; open triangle). (B) The corresponding viremia levels were measured using a reverse transcription polymerase chain reaction (RT-PCR) assay.

(Fig. 2A), and without a consistent increase in proviral loads (Fig. 2B).

HAART-treated Patients Have Both Low CTLe and Low Plasma Viral Load

To further investigate the influence of antigen quantity on the number of memory and effector CTL, we next examined eight patients whose viral loads were even lower than the LNTNs after receiving HAART. Some patients were acutely and others were chronically infected by HIV-1. The viremia levels of these selected pa-

tients were well-suppressed by HAART (<50 copies/ml). All patients treated with HAART had either low or undetectable CTLe numbers (from less than 0.02% to 0.1% of CD8+ T cells); whereas, the LNTNs had high average CTLe numbers, but only slightly higher viremia levels (Fig. 3).

Discussion

CD8+ T cell-mediated immunity plays a pivotal role in controlling retroviral infections in vivo (18,19). Understanding the mechanisms governing priming and maintenance of CTL responses is crucial for the rational design of a vaccine against AIDS. In an attempt to address the correlation between antigen and CTL responses in human subjects, we examined the number of CTL in two groups of patients whose viremia levels were low. A newly developed tetramer technology (7,20), which allows direct visualization of circulating CTL without the need of in vitro expansion, was utilized in conjunction with a LDA method. We found the two groups of patients had very different levels of CTLe, despite both having low levels of viremia.

To select patients suitable for this study, we initially examined CTL activity in LNTNs with a standard LDA. The magnitude of CTLp frequencies in this cohort of LNTNs was found to be higher than that of patients with progressive HIV-1 infection (17), with acute HIV-1 infection (21), and infected pregnant women (15). The values are, however, of the same order of magnitude as those found in other LNTN cohorts (10,11). Predictably, the majority of the LNTNs we studied also had high levels of circulating CTLe. Notably, the level of plasma viremia alone did not necessarily predict the number of CTLe. For example, one LNTN who had persistently low CTLe levels not only had high plasma viremia, but also had PBMC proviral DNA that increased steadily over time (patient #6 in Figs. 2A & B). In contrast, in the absence of a gradual increase in proviral load, another patient had high CTLe levels, in spite of high plasma viremia (patient #4 in Figs. 2A & B).

In contrast to the LNTNs, patients who had been successfully treated with HAART not only had low plasma viremia (even lower than that of the LNTNs), but also low numbers of CTLe. Thus, the correlation between viral load and CTLe levels may not be a simple linear one

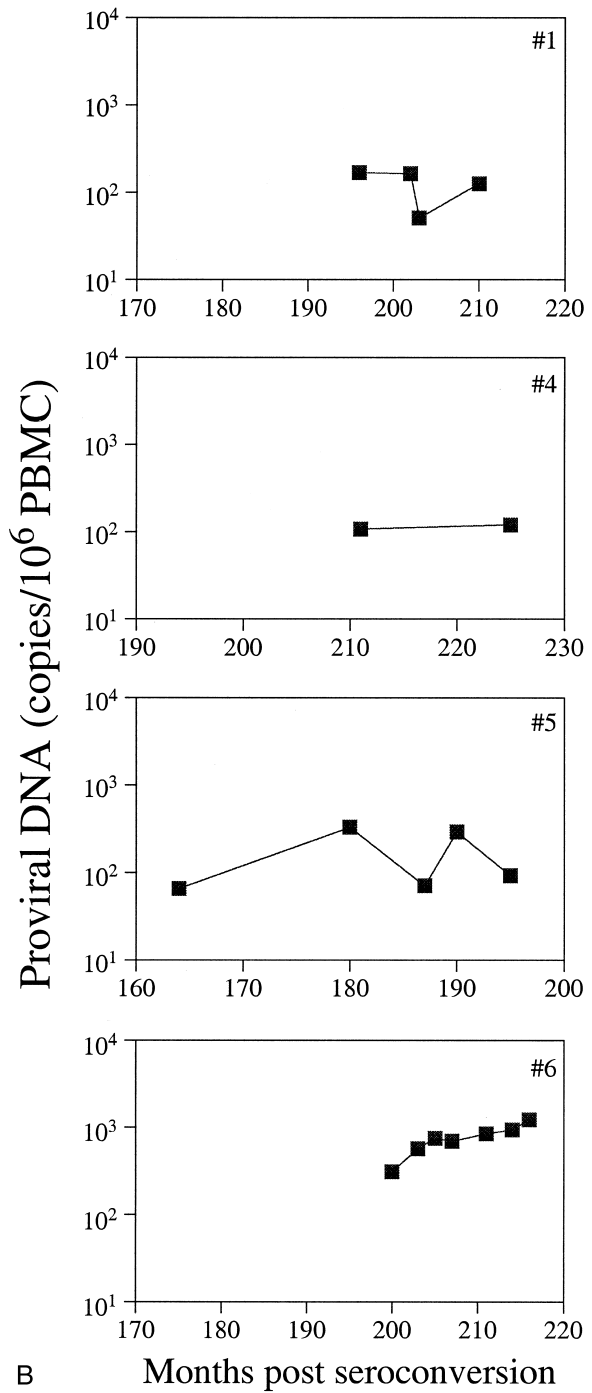
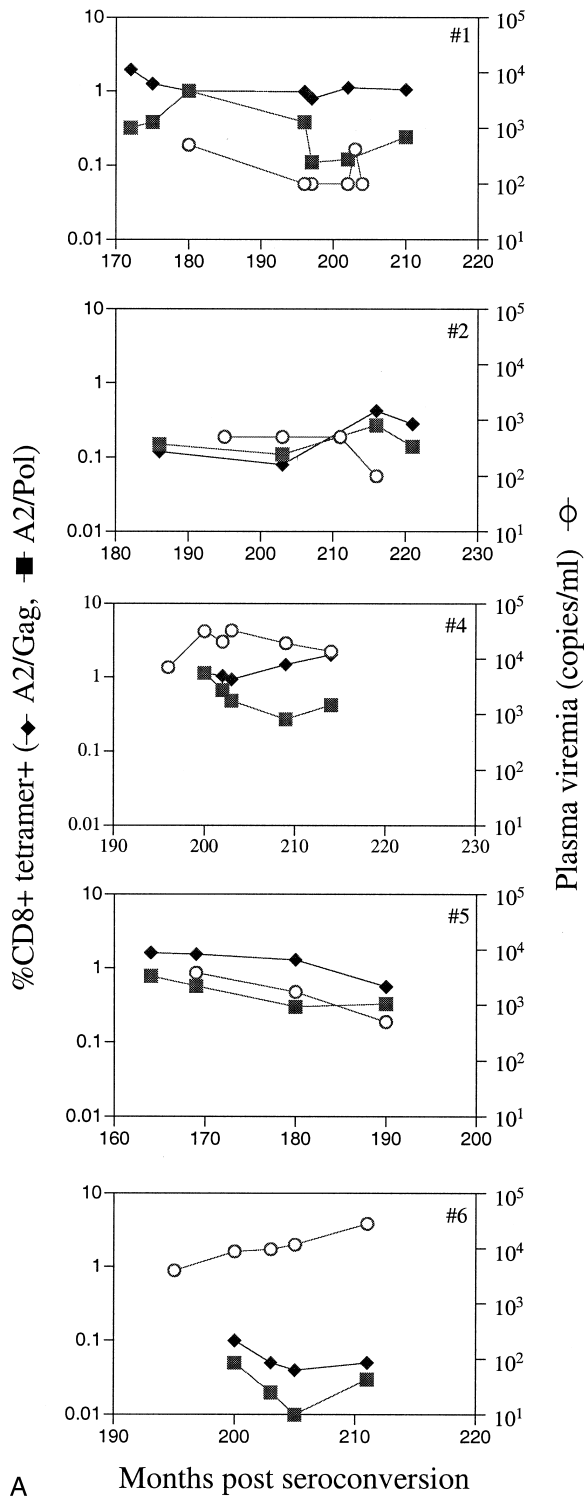


Fig. 2. Immunological and virological assessments in LNTPs. (A) Sequential cryopreserved peripheral blood mononuclear cell (PBMC) samples from five long-term nonprogressors (LNTPs) were stained with human immunodeficiency virus

(HIV)-Gag (closed diamond) and HIV-Pol (closed square) tetramers, and expressed as percentage positive for CD8+ T cells. The plasma viremia in the same patients was expressed as open circles. (B) The proviral DNA load in PBMC in four of the LTNPs.

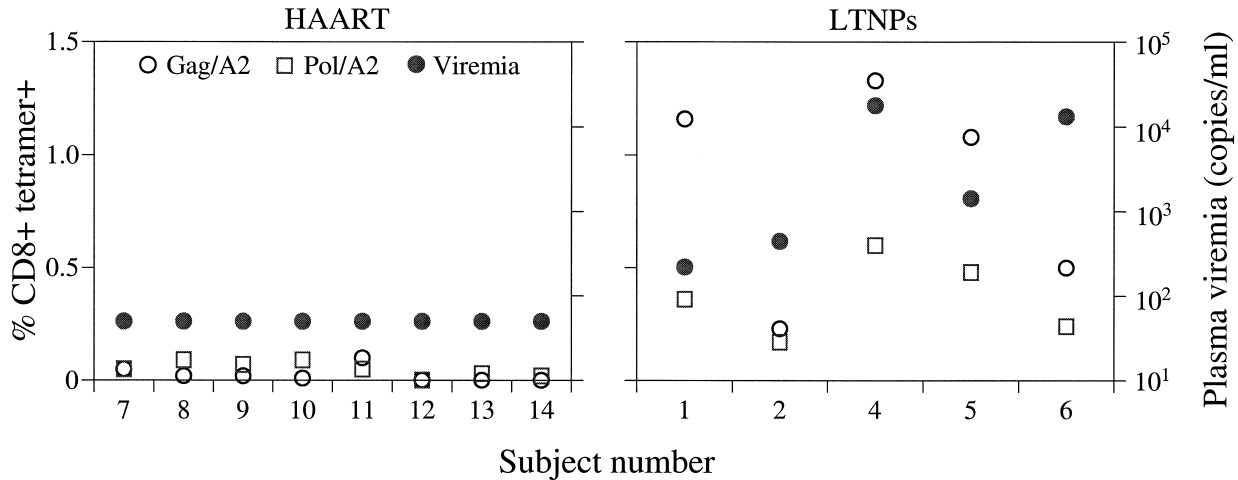


Fig. 3. Cytotoxic T lymphocyte effector (CTL) numbers in the patients whose viremia is well-suppressed by highly active antiretroviral therapy (HAART) and the average CTL levels in long-term nonprogressors (LTNPs).

for all patients. Furthermore, the correlation may vary according to the stage of viral infection, during which the quantity of antigen fluctuates. In the (LCMV) model, for example, the magnitude of memory CTL responses is determined by the amount of virus introduced into the mice during priming (22). Alternatively, CTLs may arise from memory cells that can be maintained in the absence of antigen (4). Whether these conclusions hold true in HIV-1 infection is yet to be determined.

To reconcile the apparent contradictory observations made regarding the correlation between plasma viremia and CTLs, we now hypothesize that a complex correlation must exist between these two parameters. In a previous study, we demonstrated that there was an in-

verse correlation between plasma viral load and CTLs during natural HIV-1 infection (7), which could be re-plotted as phase A of Fig. 4. These patients were untreated and, thus, were probably in the state of “antigen excess.” In the present study, however, we provide evidence that most LTNPs have high levels of CTL response (measured by either LDA or tetramers) in the presence of relatively low viral load, thus informing phase B of Fig. 4. In contrast, patients whose viral loads are well-suppressed by HAART generally have undetectable levels of CTLs, supporting phase D of Fig. 4. Phase C is inferred partly from phases B and D, and partly from the knowledge that CTLs decline after HAART (7,8). Based on the experimental evidence, we hypothesize that the threshold of HIV-1 antigen necessary to maintain measurable CTL in blood lies in between viral levels found in subjects successfully treated with HAART and those typically observed in LTNPs, such as between phases B and D in an “antigen limited” state. Therefore, to maximize the protective role of antigen-specific immune responses, we should try to bring patients into an antigenic range slightly above this threshold through clinical manipulations that include the use of HAART and therapeutic immunizations.

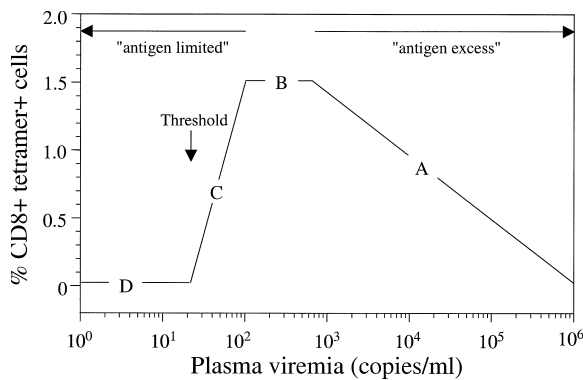


Fig. 4. Schematic presentation of proposed relationship between viral load and the number of measurable cytotoxic T lymphocyte effectors (CTLs). See text for explanation, but the values shown should not be taken literally.

Acknowledgments

We would like to thank Wendy Chen for graphics and Deborah Gurner for editorial assistance. Human IL-2 was generously provided

by M. Gately of Hoffman-La-Roche. This work was supported by NIH grants AI41534, AI40387, and AI42848 (CFAR), as well as a General Clinical Research Center grant M01-RR00102. G.O. and A.J.M. are funded by the MRC, U.K.

References

1. Michie CA, McLean A, Alcock C, Beverley PC. (1992) Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature* **360**: 264–265.
2. Mohri H, Bonhoeffer S, Monard S, Perelson AS, Ho DD. (1998) Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science* **279**: 1223–1227.
3. Jamieson BD, Ahmed R. (1989) T cell memory. Long-term persistence of virus-specific cytotoxic T cells. *J. Exp. Med.* **169**: 1993–2005.
4. Ke Y, Ma H, Kapp JA. (1998) Antigen is required for the activation of effector activities, whereas interleukin 2 is required for the maintenance of memory in ovalbumin-specific, CD8+ cytotoxic T lymphocytes. *J. Exp. Med.* **187**: 49–57.
5. Lau LL, Jamieson BD, Somasundaram T, Ahmed R. (1994) Cytotoxic T-cell memory without antigen. *Nature* **369**: 648–652.
6. Oehen S, Waldner H, Kundig TM, Hengartner H, Zinkernagel RM. (1992) Antivirally protective cytotoxic T cell memory to lymphocytic choriomeningitis virus is governed by persisting antigen. *J. Exp. Med.* **176**: 1273–1281.
7. Ogg GS, Jin X, Bonhoeffer S, et al. (1998) Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**: 2103–2106.
8. Gray CM, Lawrence J, Schapiro JM, et al. (1999) Frequency of class I HLA-restricted anti-HIV CD8+ T cells in individuals receiving highly active antiretroviral therapy (HAART). *J. Immunol.* **162**: 1780–1788.
9. Kalams SA, Goulder PJ, Shea AK, et al. (1999) Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. *J. Virol.* **73**: 6721–6728.
10. Dyer WB, Ogg GS, Demoitie M-A, et al. (1999) Strong human immunodeficiency virus (HIV)-specific cytotoxic T lymphocytes activity in Sydney Blood Bank Cohort patients infected with nef-defective HIV type 1. *J. Virol.* **73**: 436–443.
11. Rinaldo C, Huang XL, Fan Z, et al. (1995) High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T lymphocytes activity and low viral load are associated with lack of disease in HIV-1-infected long-term non-progressors. *J. Virol.* **69**: 5838–5842.
12. Markowitz M, Vesanen M, Tenner-Racz K, et al. (1999) The effect of commencing combination antiretroviral therapy soon after human immunodeficiency virus type 1 infection on viral replication and antiviral immune responses. *J. Infect. Dis.* **179**: 527–537.
13. Krogstad PA, Zack JA (ed.). (1995) *Detection of Viral DNA by PCR*. Oxford Univ. Press, Oxford.
14. Cao Y, Qin L, Zhang L, Safrin J, Ho DD. (1995) Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **332**: 201–208.
15. Jin X, Roberts CG, Nixon DF, et al. (1998) Longitudinal and cross-sectional analysis of cytotoxic T lymphocyte responses and their relationship to vertical human immunodeficiency virus transmission. *J. Infect. Dis.* **178**: 1317–1326.
16. Koup RA, Safrin JT, Cao Y, et al. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**: 4650–4655.
17. Carmichael A, Jin X, Sissons P, Borysiewicz L. (1993) Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein Barr virus in late disease. *J. Exp. Med.* **177**: 249–256.
18. Jin X, Bauer DE, Tuttleton SE, et al. (1999) Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* **189**: 991–998.
19. Schmitz JE, Kuroda MJ, Santra S, et al. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**: 857–860.
20. Altman JD, Moss PAH, Goulder PJR, et al. (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**: 94–96.
21. Musey L, Hughes J, Schacker T, Shea T, Corey L, McElrath MJ. (1997) Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **337**: 1267–1274.
22. Murali K, Altman JD, Suresh M, et al. (1998) Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**: 177–187.