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CD4 T Cells Mediate Killing during Persistent Gammaherpesvirus 68 Infection▽

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CD4 T cells are critical for the control of gammaherpesvirus persistence, but their direct effector mechanisms of virus control in vivo are still poorly understood. In this study, we use murine gammaherpesvirus 68 (γHV68) in in vitro and in vivo cytotoxicity assays to show CD4-dependent killing of γHV68-loaded cells in mice persistently infected with γHV68. Our results underscore the cytotoxic capacity of CD4 T cells during γHV68 persistence.

There is mounting evidence that during chronic or persistent viral infections, effector CD4 T cells have the ability to display cytotoxic characteristics (7, 8, 18). CD4 T cells with cytotoxic potential have been characterized during persistent human immunodeficiency virus and human cytomegalovirus infections (2, 4, 19). There are also extensive reports describing antigen-specific cytolytic CD4 T-cell cultures during persistent Epstein-Barr virus infection in humans (1, 9, 10, 12, 13, 17). Whether effector CD4 T cells use cytotoxic abilities and/or other mechanisms to eliminate virus-infected cells during persistent gammaherpesvirus infection in vivo remains unknown.

We first analyzed the existence of CD4 T cells with a cytotoxic phenotype in mice persistently infected with gammaherpesvirus 68 (γHV68) (1,000 PFU in 30 μl of phosphate-buffered saline, administered intranasally). Reduced surface expression of CD27 has been used as a marker of cytotoxic T cells (2). As shown in Fig. 1, the frequency of CD4 T cells lacking cell surface expression of CD27 increases from 10% in noninfected mice to 40% in the spleens and 80% in the lungs of mice at 3 months after γHV68 infection. The loss of CD27 expression during γHV68 herpesvirus latency suggested an increase in the frequency of CD4 T cells that may have a cytolytic phenotype. Next, we sought to analyze the potential of CD4 T cells to act as killers. We used CD107a and CD107b (CD107a/b) cell surface mobilization as an indicator of in vitro degranulation associated with cytolytic function (3). Splenocytes (10^6) obtained from the spleens of naive or long-term γHV68-infected mice were incubated with anti-CD28 (clone 37.51), anti-CD49d (clone 9C10 [MRF4.B]), anti-CD107a (clone 1D4B), and anti-CD107b (clone ABL-93) and stimulated with 2 μg/ml of $gp150_{67\text{-}83}$ peptide, whole γHV68 virus (multiplicity of infection [MOI], 10), or phorbol myristate acetate/ionomycin or not stimulated (Fig. 1C). The cell suspensions were incubated for 1 h at 37°C followed by an additional 4 h in the presence of the secretion inhibitor monensin, and cell surface expression levels of CD4 (clone GK1.5), CD8 (clone 53.6.72), and CD107a/b were determined by flow cytometry. The data show that CD4 T cells isolated from the spleens of latently infected mice mobilize significantly more CD107a/b to the cell surface (4%) than their naïve counterparts (2%) (Fig. 1D). The pattern of degranulation of CD4 T cells isolated from γHV68-infected mice was similar in the presence or absence of exogenous in vitro restimulation. These results show that there is a statistically significant difference in CD107a/b mobilization between CD4 T cells isolated from naïve and γHV68-infected mice, and this difference is independent of exogenous restimulation. This pattern of degranulation is specific to CD4 T cells, as steady-state CD107a/b expression is not observed with CD8 T-cell splenocytes from γHV68-infected mice (Fig. 1E), a result which suggests that carryover of viral antigens by host cells during the in vitro incubation is not the cause of the observed CD107a/b mobilization on CD4 T cells. Collectively, the CD27 profile and the CD107a/b mobilization data suggest that during γHV68 persistence, CD4 T cells acquire a cytolytic phenotype.

To examine the γHV68-specific cytotoxic activity of CD4 T cells from persistently γHV68-infected mice, we used an in vitro chromium release assay. L fibroblasts (H-2^k) stably expressing IA^b molecules on their cell surface (L-IA^b) were used as antigen-presenting target cells. L-IA^b cells were pulsed with γHV68, labeled with ^{51}Cr, and incubated at various ratios with purified CD4 T-cell splenocytes (95% purity) isolated from long-term γHV68-infected mice. CD4 T cells were purified to avoid killing mediated by other cell populations. In addition, to determine whether the killing was major histocompatibility complex (MHC) class IA^b mediated, we used purified CD4 T cells isolated from persistently infected C57BL/6J mice (H-2^b, matched) or from persistently infected BALB/c mice as the control (H-2^d, mismatched). In addition, we also used L-IA^b cells incubated without γHV68 to determine nonspecific lysis by both MHC-matched and mismatched CD4 T cells and subtracted these values from the values obtained with virus-loaded targets. The data show that incubation of purified CD4 T cells from γHV68-infected C57BL/6J mice with virus-pulsed L-IA^b fibroblasts resulted in measurable and dose-dependent specific lysis (Fig. 2). However, CD4 T cells purified from γHV68-

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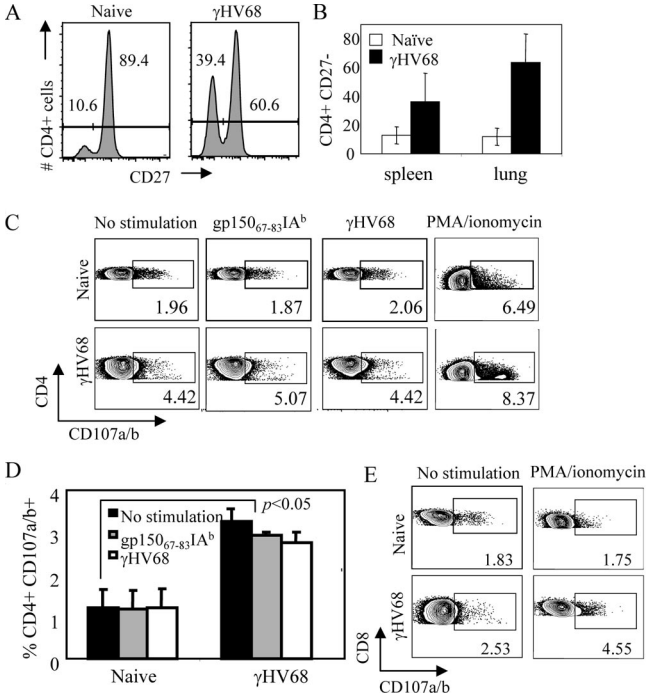

FIG. 1. CD4 T cells acquire a cytolytic phenotype and degranulate during persistent gammaherpesvirus infection. (A) Representative histogram of CD4 T-cell splenocytes isolated from naïve or long-term (>3 months postinfection [mpi]) γHV68-infected C57BL/6J mice and stained with anti-CD27 and anti-CD4 antibodies. (B) Bar diagram of the frequency of $CD27^-$ CD4 T cells in the spleens and lungs of naïve or long-term (>3 mpi) γHV68-infected mice. (C) Representative fluorescence-activated cell sorter (FACS) dot plots show increased surface expression of CD107a/b on CD4 T-cell splenocytes isolated from long-term (>3 mpi) γHV68-infected C57BL/6J mice. (D) Bar diagram of CD4 T-cell splenocytes stained with anti-CD107a/b show increased CD107a/b expression on CD4 T cells from long-term (>3 mpi) γHV68-infected C57BL/6J mice compared with noninfected mice. (E) Representative dot plots of CD8 T splenocytes stained with CD107a/b. The bar diagrams indicate the means for three mice per group from one experiment, which is representative of at least two independent experiments. Error bars represent standard deviations. #, number; PMA, phorbol myristate acetate.

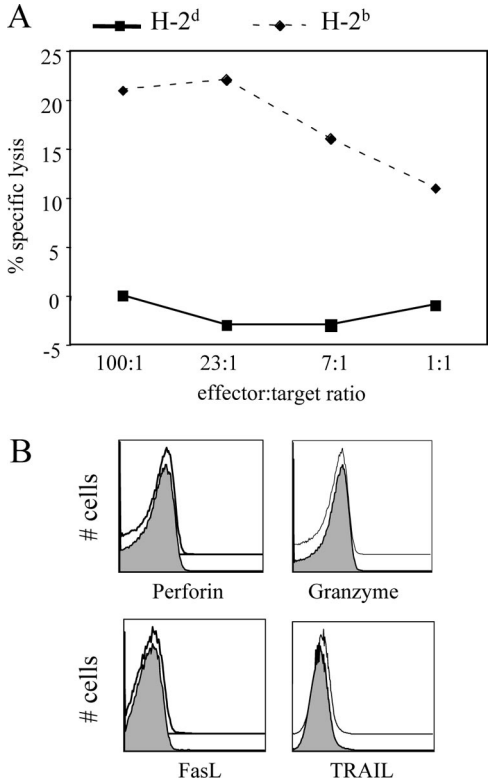

FIG. 2. Purified CD4 T cells elicit direct cytotoxic activity in vitro. (A) CD4 T-cell splenocytes were purified from long-term (>3 mpi) γHV68-infected C57BL/6J ($H\text{-}2^b$) or mismatched naïve BALB/c ($H\text{-}2^d$) mice using CD4 columns (purity 95%; R&D Systems). L-IA^b fibroblasts were pulsed with γHV68 (MOI, 10) overnight and labeled with 100 μCi Na_2CrO_4 for 1 h at 37°C. Effector and target cells were incubated at different ratios in complete medium for 18 h, and an aliquot of the supernatant was harvested and chromium release was measured with a Wallac 1450 MicroBeta TriLux plate counter. Spontaneous release was determined by incubating labeled L-IA^b targets with medium alone, and maximum release was determined by incubating L-IA^b labeled targets with a 3% NP-40 solution. Percent specific lysis was calculated by the following formula: [(counts per min of experimental release − counts per min of spontaneous release)/(counts per min of maximum release − counts per min of spontaneous release)] × 100. (B) Representative histograms of intracellular staining for perforin and granzyme B and surface staining for FasL and TRAIL show no change in expression levels in comparisons of results for long-term γHV68-infected mice (>3 mpi, shaded histograms) and naïve controls (unshaded histograms). The results are the means for three mice from one experiment, which is representative of at least two independent experiments. Data are from two independent experiments each; three individual mice were analyzed. #, number.

infected BALB/c mice were not able to induce specific lysis of γHV68-loaded L-IA^b cells at any of the ratios tested (100:1 to 1:1). These findings indicate that CD4 T cells from γHV68-infected mice are capable of killing γHV68-loaded target cells in vitro by an MHC-restricted process. Next, we used flow cytometry staining to analyze the expression of the cytotoxicity mediators perforin, granzyme B, FasL, and TRAIL (*t*umor necrosis factor-*r*elated *a*poptosis-*i*nducing *l*igand). The data (Fig. 2B) show that expression of these molecules could not be detected by flow cytometry on CD4 T cells isolated from the spleens of γHV68-infected mice.

To determine the biological significance of these in vitro findings and whether CD4 T cells could act as killers in mice, we performed in vivo cytotoxicity assays using γHV68-latently infected mice. To confirm that any observed cytotoxicity is not due to the presence of CD8 effector functions, CD8 T cells were depleted prior to the transfer of target cells. To do so, γHV68-infected and naïve control mice were depleted of CD8 T cells in vivo by intraperitoneal injections of 250 μg of anti-CD8 antibody (clone 2.43) on alternate days for 1 week prior to the transfer of target cells (Fig. 3A). The efficiency of the CD8 T-cell depletion was confirmed using a different anti-CD8 antibody (clone 53-6.72) (Fig. 3B). Antigen-presenting cells obtained from the spleens of naïve mice were pulsed with γHV68 (MOI, 10) or not (nonspecific killing control) and labeled with different concentrations of carboxyfluorescein succinimidyl ester (CFSE), mixed at a 1:1 ratio and intravenously injected into naïve control mice or into mice persistently infected with γHV68 that had been depleted of CD8 T cells. Recipient mice were sacrificed 40 h later, and the frequency of CFSE-positive target cells was measured to determine the per-

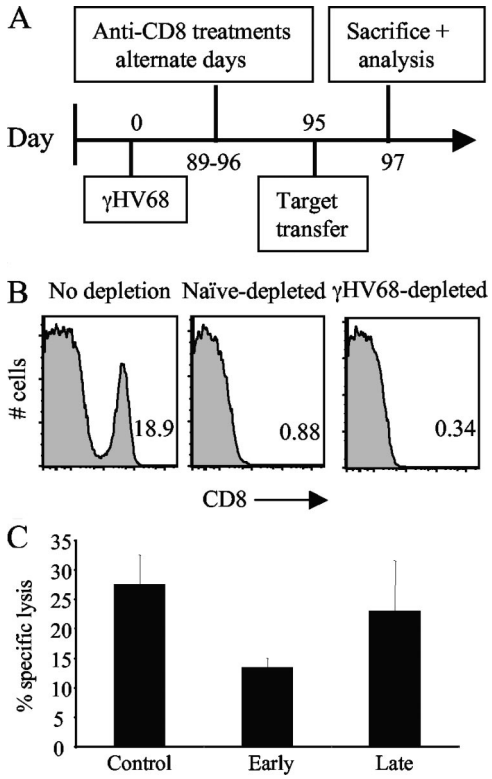

FIG. 3. In vivo cytotoxic activity in the absence of CD8 T cells. (A) Experimental timetable for long-term infection. γHV68-infected and naïve control mice were depleted of CD8 T cells in vivo by intraperitoneal injection of anti-CD8 antibody (clone 2.43) or control IgG on alternate days for 1 week prior to the transfer of target cells. (B) CD8 T-cell depletion was confirmed by FACS analysis of CD8 expression (clone 53.6.72) on splenocytes from undepleted (left), naïve/depleted (middle), and γHV68-infected/depleted (right) mice. #, number. (C) The bar diagram indicates the percentage of specific lysis during early (20 days) and late (90 days) latency after CD8 depletion relative to results for control mice (no depletion). The results are the means for three mice per group from one experiment representative of at least two independent experiments. Error bars represent standard deviations.

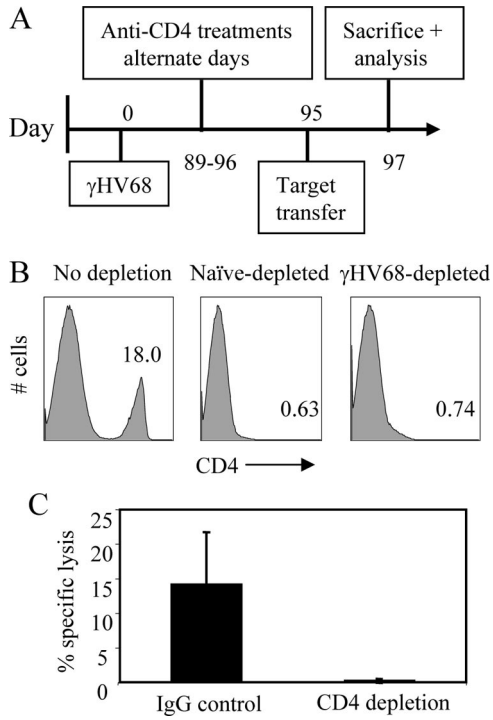

FIG. 4. Antigen-specific cytotoxic activity during latent γHV68 infection is mediated by CD4 cells. (A) Experimental timetable for long-term infection. γHV68-infected and naïve control mice were depleted of CD4 T cells in vivo by intraperitoneal injection of anti-CD4 antibody (clone GK1.5) or control IgG on alternate days for 1 week prior to the transfer of target cells. (B) CD4 T-cell depletion was confirmed by FACS analysis of CD4 expression (clone RM4-5) on splenocytes from undepleted (left), naïve/depleted (middle), and γHV68-infected/depleted (right) mice. (C) The bar diagram indicates the percentage of specific lysis of CD4-depleted or IgG control-treated mice. The results are the means for three mice per group from one experiment representative of at least two independent experiments. Error bars represent standard deviations.

centage of specific lysis. The percent specific killing was calculated according to the following formula: percent specific killing =[1− (ratio of naive recipients/ratio of infected recipients) × 100], where ratio = (number of $CFSE_{low}$/number of $CFSE_{high}$). We analyzed mice during early latency (20 days after infection) and late latency (90 days after infection). The data show that during both the early and the late phase of viral latency, persistently infected CD8-depleted mice are capable of specifically killing γHV68-loaded targets (Fig. 3C). The analysis of in vivo cytotoxicity in CD8-depleted mice using shorter intervals of time (6 h) did not yield specific target lysis (data not shown). Importantly, the percent specific killing was calculated by using the ratio of γHV68-loaded cells/unloaded cells in both noninfected and γHV68-infected mice. This calculation implies that the specific lysis is not observed in noninfected CD8-depleted mice or with target cells that have not been loaded with γHV68, indicating that non-γHV68-specific or indirect mechanisms cannot account for the lysis of target cells. It should also be noted that during late latency, the percentage of specific lysis in vivo in the absence of CD8 T cells increases modestly, from 12% to 23%, as latent infection progresses (Fig. 3C), which is in stark contrast to the pattern of CD8 T-cell-mediated cytotoxicity, which peaks early during infection and is followed by a dramatic reduction by 3 months postinfection (6, 16). These experiments indicate that CD8 T cells do not make a significant contribution to the killing of γHV68-loaded targets during an in vivo cytotoxicity assay.

To further confirm that γHV68-specific lysis is mediated by CD4 T cells in vivo, we performed similar cytotoxicity assays in which CD4 T cells were depleted in the mouse. Intraperitoneal injections of 250 μg of anti-CD4 antibody (clone GK1.5) on alternate days for 1 week (Fig. 4A) resulted in a sufficient depletion of CD4, which was confirmed by using a different anti-CD4 antibody (clone RM4-5) (Fig. 4B). Antigen-presenting cells obtained from the spleens of naïve mice were pulsed with γHV68 (MOI, 10) or not (nonspecific killing control) and labeled with different concentrations of CFSE, mixed at a 1:1 ratio, and intravenously injected into naïve control mice or into mice persistently infected with γHV68 that had been depleted of CD4 T cells. The percent specific killing was calculated as indicated above. The data show that the killing of γHV68-

loaded targets was abrogated in the mice depleted of CD4 T cells but not in the mice that received control immunoglobulin G (IgG) treatment (Fig. 4C). These data demonstrate that CD4 T cells are responsible for the in vivo killing of γHV68-loaded targets.

In summary, our results provide compelling phenotypic and functional evidence for the existence of virus-specific cytolytic CD4 T cells during persistent γHV68 infection. In addition, they provide the first in vivo evidence for CD4 T-cell-mediated cytotoxicity during gammaherpesvirus infections. It has been demonstrated that CD4 T cells can control γHV68 infection independently of CD8 T cells, B cells, or antibodies (11, 15). Our data indicate that CD4 T-cell control of γHV68 infection not only is mediated by IFN-γ (5, 14) but also can be mediated by direct cytotoxicity. Several mechanisms have been implicated during in vitro killing by CD4 T cells, and further studies will be necessary to clarify the mechanisms employed in vivo during γHV68 infection.

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REFERENCES

1. **Adhikary, D., U. Behrends, A. Moosmann, K. Witter, G. W. Bornkamm, and J. Mautner.** 2006. Control of Epstein-Barr virus infection in vitro by T helper cells specific for virion glycoproteins. J. Exp. Med. **203:**995–1006.
2. **Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-Jones, and A. D. Kelleher.** 2002. Characterization of $CD4^+$ CTLs ex vivo. J. Immunol. **168:**5954–5958.
3. **Betts, M. R., and R. A. Koup.** 2004. Detection of T-cell degranulation: CD107a and b. Methods Cell Biol. **75:**497–512.
4. **Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup.** 2006. Acquisition of direct antiviral effector functions by CMV-specific $CD4^+$ T lymphocytes with cellular maturation. J. Exp. Med. **203:**2865–2877.
5. **Christensen, J. P., R. D. Cardin, K. C. Branum, and P. C. Doherty.** 1999. $CD4^+$ T cell-mediated control of a γ-herpesvirus in B cell-deficient mice is mediated by IFN-γ. Proc. Natl. Acad. Sci. USA **96:**5135–5140.
6. **Cush, S. S., K. M. Anderson, D. H. Ravneberg, J. L. Weslow-Schmidt, and E. Flano.** 2007. Memory generation and maintenance of $CD8^+$ T cell function during viral persistence. J. Immunol. **179:**141–153.
7. **Jellison, E. R., S. K. Kim, and R. M. Welsh.** 2005. Cutting edge: MHC class II-restricted killing in vivo during viral infection. J. Immunol. **174:**614–618.
8. **Krensky, A. M., C. S. Reiss, J. W. Mier, J. L. Strominger, and S. J. Burakoff.** 1982. Long-term human cytolytic T-cell lines allospecific for HLA-DR6 antigen are $OKT4^+$. Proc. Natl. Acad. Sci. USA **79:**2365–2369.
9. **Landais, E., X. Saulquin, E. Scotet, L. Trautmann, M. A. Peyrat, J. L. Yates, W. W. Kwok, M. Bonneville, and E. Houssaint.** 2004. Direct killing of Epstein-Barr virus (EBV)-infected B cells by CD4 T cells directed against the EBV lytic protein BHRF1. Blood **103:**1408–1416.
10. **Long, H. M., T. A. Haigh, N. H. Gudgeon, A. M. Leen, C. W. Tsang, J. Brooks, E. Landais, E. Houssaint, S. P. Lee, A. B. Rickinson, and G. S. Taylor.** 2005. $CD4^+$ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines. J. Virol. **79:**4896–4907.
11. **McClellan, J. S., S. A. Tibbetts, S. Gangappa, K. A. Brett, and H. W. Virgin IV.** 2004. Critical role of CD4 T cells in an antibody-independent mechanism of vaccination against gammaherpesvirus latency. J. Virol. **78:**6836–6845.
12. **Misko, I. S., J. H. Pope, R. Hutter, T. D. Soszynski, and R. G. Kane.** 1984. HLA-DR-antigen-associated restriction of EBV-specific cytotoxic T-cell colonies. Int. J. Cancer **33:**239–243.
13. **Paludan, C., K. Bickham, S. Nikiforow, M. L. Tsang, K. Goodman, W. A. Hanekom, J. F. Fonteneau, S. Stevanovic, and C. Munz.** 2002. Epstein-Barr nuclear antigen 1-specific $CD4^+$ Th1 cells kill Burkitt's lymphoma cells. J. Immunol. **169:**1593–1603.
14. **Sparks-Thissen, R. L., D. C. Braaten, K. Hildner, T. L. Murphy, K. M. Murphy, and H. W. Virgin IV.** 2005. CD4 T cell control of acute and latent murine gammaherpesvirus infection requires IFNγ. Virology **338:**201–208.
15. **Sparks-Thissen, R. L., D. C. Braaten, S. Kreher, S. H. Speck, and H. W. Virgin IV.** 2004. An optimized CD4 T-cell response can control productive and latent gammaherpesvirus infection. J. Virol. **78:**6827–6835.
16. **Stevenson, P. G., G. T. Belz, J. D. Altman, and P. C. Doherty.** 1999. Changing patterns of dominance in the $CD8^+$ T cell response during acute and persistent murine gamma-herpesvirus infection. Eur. J. Immunol. **29:**1059–1067.
17. **Su, Z., M. V. Peluso, S. H. Raffegerst, D. J. Schendel, and M. A. Roskrow.** 2001. The generation of LMP2a-specific cytotoxic T lymphocytes for the treatment of patients with Epstein-Barr virus-positive Hodgkin disease. Eur. J. Immunol. **31:**947–958.
18. **Suni, M. A., S. A. Ghanekar, D. W. Houck, H. T. Maecker, S. B. Wormsley, L. J. Picker, R. B. Moss, and V. C. Maino.** 2001. $CD4^+CD8^{dim}$ T lymphocytes exhibit enhanced cytokine expression, proliferation and cytotoxic activity in response to HCMV and HIV-1 antigens. Eur. J. Immunol. **31:**2512–2520.
19. **Zaunders, J. J., W. B. Dyer, B. Wang, M. L. Munier, M. Miranda-Saksena, R. Newton, J. Moore, C. R. Mackay, D. A. Cooper, N. K. Saksena, and A. D. Kelleher.** 2004. Identification of circulating antigen-specific $CD4^+$ T lymphocytes with a $CCR5^+$, cytotoxic phenotype in an HIV-1 long-term nonprogressor and in CMV infection. Blood **103:**2238–2247.