JOURNAL OF VIROLOGY, July 1994, p. 4700–4704
0022-538X/94/$04.00+0

Vol. 68, No. 7

Enhanced Establishment of a Virus Carrier State in Adult CD4$^+$ T-Cell-Deficient Mice

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Received 28 October 1993/Accepted 19 April 1994

CD4$^+$ T cells play an important role in regulating the immune response; their contribution to virus clearance is variable. Mice that lack CD4$^+$ T cells (CD4$^{-/-}$ mice) and are therefore unable to produce neutralizing antibodies cleared viscero-lymphotropic lymphocytic choriomeningitis virus (LCMV) strain WE when infected intravenously with a low dose (2×10^2 PFU) because of an effective CD8$^+$ cytotoxic T-cell (CTL) response. In contrast, infection with a high dose (2×10^6 PFU) of LCMV strain WE led to expansion of antiviral CTL, which disappeared in CD4$^{-/-}$ mice; in contrast, CD4$^+$ T-cell-competent mice developed antiviral memory CTL. This exhaustion of specific CTL caused viral persistence in CD4$^{-/-}$ mice, whereas CD4$^+$ T-cell-competent mice eliminated the virus. After infection of CD4$^{-/-}$ mice with the faster-replicating LCMV strain DOCILE, abrogation of CTL response and establishment of viral persistence developed after infection with a low dose (5×10^2 PFU), i.e., an about 100-fold lower dose than in CD4$^+$-competent control mice. These results show that absence of T help enhances establishment of an LCMV carrier state in selected situations.

CD8$^+$ cytotoxic T cells (CTL) are important effector cells recognizing viral peptides presented in association with major histocompatibility complex class I molecules on the surfaces of virus-infected cells; they cause elimination of the virus by direct lysis of infected target cells, by secreted antiviral cytokines, or by both (8, 10, 38, 49). CD4$^+$ T cells play an important role in regulating the immune response. However, it remains unclear to what extent CD4$^+$ T cells provide help for the antiviral CD8$^+$ T-cell response. Some in vivo experiments suggest a mandatory role for T-helper cells, whereas others show that T help is not necessary for antiviral major histocompatibility complex class I-restricted T-cell responses in vivo (1, 2, 7, 17, 22, 23, 37). These and other studies have addressed the role and interdependence of CD8$^+$ and CD4$^+$ T cells during viral infections, including those with noncytopathic viruses (6, 14, 16, 17, 21, 24, 39, 41, 43) (for reviews, see references 11 and 12). For example, mice without functioning class I effector T cells were able to clear vaccinia (43), influenza (14), or Sendai (17) virus, indicating that CD8$^+$ T-cell-independent mechanisms involving CD4$^+$ T cells that contribute to viral elimination exist.

CTL have been demonstrated to control virus replication efficiently during primary infection with lymphocytic choriomeningitis virus (LCMV), a poorly cytopathic or noncytopathic RNA virus (10, 49). After infection with LCMV, neutralizing antibodies normally occur late, i.e., after 20 to 60 days (5, 20), suggesting that they play no role or only a secondary role in virus clearance. During infection of adult mice with LCMV, CTL responses are induced in the absence of T-helper cells. The role of CD4$^+$ T cells in the clearance of LCMV has been analyzed in mice treated with depleting anti-CD4 antibodies (1, 22, 23) and in mice lacking CD4$^+$ T cells (37) (CD4$^{-/-}$; generated by homologous recombination). These studies revealed a normal (37) or variable, two- to ninefold-reduced (1, 22, 23) anti-LCMV CD8$^+$ T-cell function.

Recently it has been shown that overwhelming infection of adult mice with certain LCMV isolates induces CTL responses so completely that specific CTL disappear and virus persists (26, 27, 48). Possible mechanisms may include lack or dysbalance of T help or of interleukins (18, 28), unbalanced distribution of antigen on antigen-presenting cells versus other cells (31), or some sort of suppression (13, 29). In this study, we investigated whether lack of CD4$^+$ T cells in CD4$^{-/-}$ mice enhances exhaustion of CD8$^+$ T cells. The experiments reported here confirmed that CD4$^+$ helper T cells are not generally needed to induce antiviral CTL (19, 22, 23, 30) and to eliminate LCMV. However, under certain conditions of high-dose virus infection or infection with a rapidly replicating LCMV isolate, CD4$^+$ T cells seem to be important in preventing exhaustion of antiviral CTL responses and of viral persistence.

Lack of CD4$^+$ T cells and outcome of LCMV infection. To determine whether CD4$^{-/-}$ mice (*H-2^b*) infected with LCMV strain WE (LCMV-WE) could eliminate the virus, groups of three to five mice were infected intravenously (i.v.) (in three independent experiments) with graded doses of LCMV-WE (obtained from F. Lehmann-Grube, Hamburg, Germany). After a low-dose (2×10^2 PFU) infection, CD4$^{-/-}$ mice cleared LCMV-WE from their spleens between days 9 and 20 (Fig. 1A); virus was not measurable in blood assessed with an immunological focus assay (3). In contrast, virus persisted in the spleens, blood (Fig. 1), and thymuses (data not shown) of CD4$^{-/-}$ mice infected with 2×10^6 PFU but not in those of identically infected CD4$^+$ T-cell-competent controls (C57BL/6 *H-2^b*), which cleared the virus from their spleens by around day 20. Thus, lack of CD4$^+$ T cells enhanced viral persistence after infection with a high dose of LCMV-WE.

Enhanced virus persistence by exhaustion of antiviral CTL in CD4$^{-/-}$ mice. To determine whether a CD8$^+$ CTL response was generated in CD4$^{-/-}$ mice, groups of two to five animals were infected i.v. with 2×10^2 or 2×10^6 PFU of LCMV-WE.

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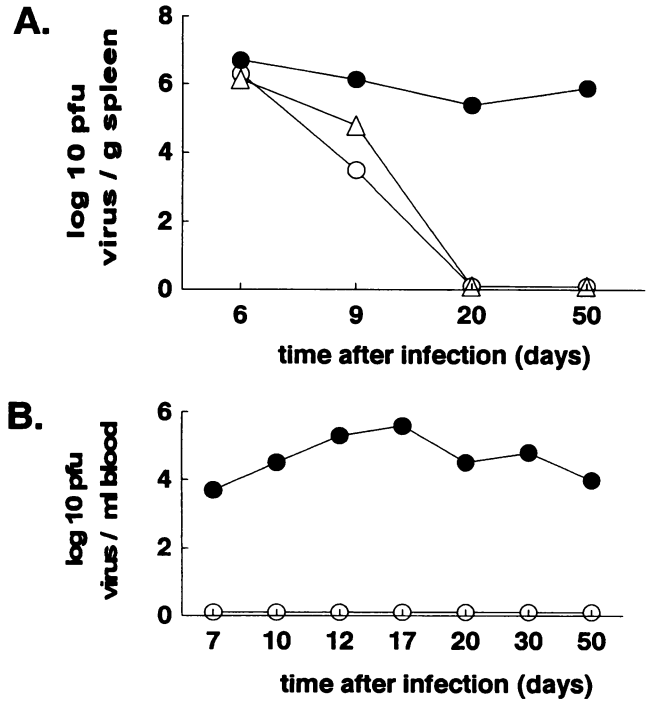

FIG. 1. Kinetics of virus titers in spleens (A) and blood (B) of $CD4^{-/-}$ and control mice. Groups of three to five $CD4^{-/-}$ mice (for each time point) were infected i.v. with 2×10^2 (○) or 2×10^6 (●) PFU of LCMV-WE. As controls, two to four C57BL/6 mice were infected i.v. with 2×10^6 PFU of LCMV-WE (△). LCMV was quantified by an immunological focus assay (3). Results of one of three similar experiments are shown. The standard error of the mean viral titer was less than ± 0.7 $\log_{10}$.

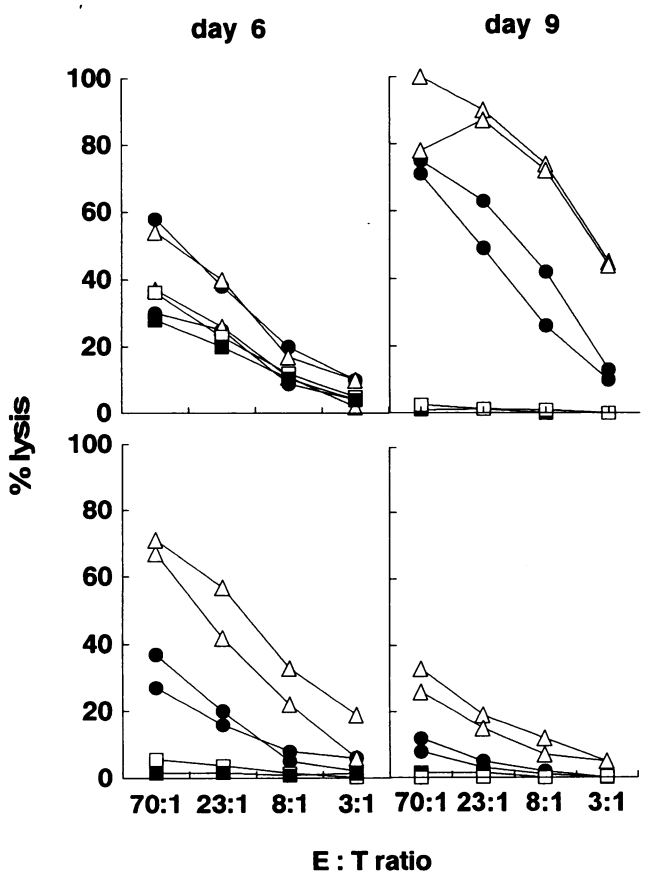

FIG. 2. Primary ex vivo CTL response measured in a ^{51}Cr release assay on days 6 and 9 after i.v. infection with 2×10^2 PFU (upper panels) or 2×10^6 PFU (lower panels) of LCMV-WE. Spleen cells of infected $CD4^{-/-}$ (●) and C57BL/6 (△) mice were tested at the indicated effector-target (E:T) cell ratios on infected and uninfected MC57G cells ($H\text{-}2^b$). Lysis of uninfected target cells by $CD4^{-/-}$ (■) or C57BL/6 (□) effector cells is shown. Each curve represents a single mouse. Spontaneous ^{51}Cr release was <17%.

After low-dose infection, $CD4^{-/-}$ and C57BL/6 mice exhibited primary CTL activity (Fig. 2, upper panels), as measured in a ^{51}Cr release assay (23), on day 6 and particularly on day 9, as well as a strong and long-lasting memory CTL response (Fig. 3). The antiviral memory CTL activities of spleen cells were monitored at different time points in spleens after in vitro restimulation in interleukin 2-enriched medium for 5 days as described previously (27). $CD4^{-/-}$ mice infected with 2×10^6 PFU exhibited a substantial CTL response on day 6 (Fig. 2, lower panels, and Fig. 3), which decayed rapidly thereafter, i.e., on day 9. As shown in Fig. 3, memory CTL eventually disappeared by day 20 and were absent at all later time points (tested up to day 200); correspondingly, LCMV persisted (Fig. 1). In parallel, spleen cells from C57BL/6 mice infected i.v. with 2×10^6 PFU of LCMV-WE were used as controls. Even though the primary CTL activity was relatively low in these mice on day 9 (Fig. 2, lower panels), memory CTL activity persisted (Fig. 3) and LCMV was cleared (Fig. 1A). Absence of CTL precursors has been earlier shown to be specific (27) and was not due to suppressive effects of persisting virus; mixing of CTL precursor-positive and CTL precursor-negative spleen cells in secondary cultures revealed no suppression by the latter (data not shown).

Search for persistence of mutant virus. To determine whether the observed absence of cytotoxicity was due to virus mutants that had escaped the CTL response (35), we infected groups of three C57BL/6 mice i.v. with either 2×10^2 PFU of virus isolated from spleens of persistently infected $CD4^{-/-}$ mice (day 50 after LCMV-WE infection) or with 2×10^2 PFU of LCMV-WE. Day 8 effector spleen cells from both groups of mice efficiently lysed target cells (MC57G $H\text{-}2^b$) infected with LCMV-WE, as well as targets infected with the virus obtained from persistently infected $CD4^{-/-}$ mice (Fig. 4, left graph). Also, day 8 CTL obtained from C57BL/6 mice immunized with 2×10^2 PFU of LCMV-WE or with the same dose of the persisting virus efficiently lysed target cells labeled with LCMV peptides recognized in the context of $H\text{-}2^b$ (glycoprotein amino acids 32 to 42 and 275 to 289 and nucleoprotein amino acids 391 to 408 [35, 42, 47]) (Fig. 4, right graph). Taken together, these results indicate that after infection with a high dose of LCMV-WE, lack of $CD4^+$ T cells caused LCMV to persist in C57BL/6 mice because of exhaustion of $CD8^+$ T cells and not because of virus mutants that escaped the antiviral CTL immune response.

Prevention of exhaustion of CTL by transfer of neutralizing antibodies but not by transfer of nonneutralizing antibodies. Since antibody responses to LCMV are strictly T help dependent, it was not surprising that $CD4^{-/-}$ mice did not mount a neutralizing antibody response (measured with a focus reduction assay [5]) after infection with LCMV-WE, irrespective of the virus dose. This result confirmed that relatively low doses of LCMV could be eliminated by CTL without $CD4^+$ T cells and without neutralizing antibodies (16, 22, 23, 25).

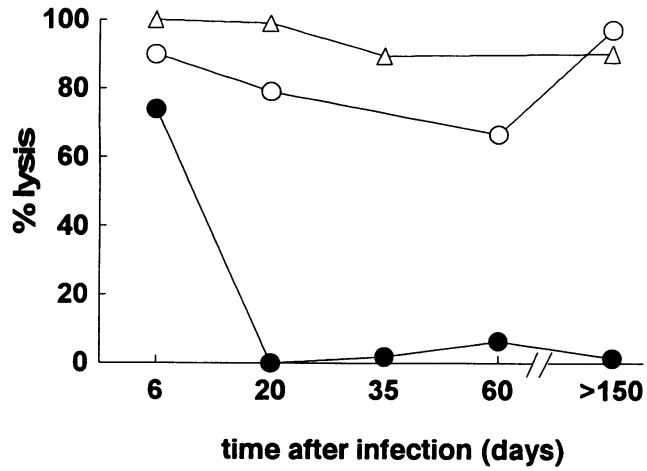

FIG. 3. Secondary cytotoxic anti-LCMV T-cell responses in $CD4^{-/-}$ mice. At different time points after infection and after in vitro restimulation in interleukin 2-enriched medium for 5 days (27), lytic activity was assessed in a ^{51}Cr release assay. $CD4^{-/-}$ mice were infected with either 2×10^2 (○) or 2×10^6 (●) PFU of LCMV-WE. Control C57BL/6 mice were infected with 2×10^6 PFU of LCMV-WE (△). For each time point, four or five mice were tested in each group and the standard error of the mean was less than ±10% lysis. A second experiment at days 6, 20, and 60 yielded comparable results.

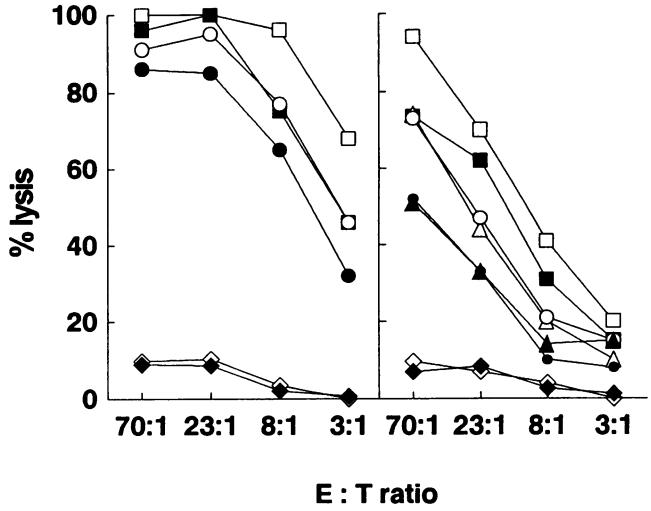

FIG. 4. Cytotoxic T-cell activity of postinfection day 8 splenic CTL from C57BL/6 mice (open symbols) infected i.v. with 2×10^2 PFU of LCMV-WE or LCMV from persistently infected $CD4^{-/-}$ mice (isolated on day 50 after LCMV-WE infection) (filled symbols). Target cells were infected or incubated with peptide as follows. Left-graph symbols: ■ and □, LCMV-WE; ● and ○, virus from persistently infected $CD4^{-/-}$ mice (day 50 after LCMV-WE infection); ◆ and ◇, uninfected target cells. Right-graph symbols: ■ and □, glycoprotein 32; ● and ○, glycoprotein 275; ▲ and △, nucleoprotein 391; ◆ and ◇, uninfected target cells. The standard error of the mean was less than ±12% lysis for all points. Spontaneous ^{51}Cr release was <15% for all targets. Results of one of two similar experiments are shown. E:T ratio, effector-target cell ratio.

The evidence from the above-described experiments indicates that T help reduces exhaustion of CTL and thereby contributes to viral clearance. $CD4^+$ T cells may support $CD8^+$ T cells by helping B cells produce nonneutralizing antibodies or very small amounts of neutralizing antibodies. Therefore, we examined whether serum from C57BL/6 mice obtained 10 days after i.v. infection with 10^4 PFU of LCMV-WE could prevent enhanced exhaustion in $CD4^{-/-}$ mice (Fig. 5). $CD4^{-/-}$ mice were infected with 2×10^6 PFU of LCMV-WE and treated daily from days 6 to 10 and on days 14 and 16 after infection with a polyclonal antiserum (0.2 ml undiluted, given intraperitoneally; neutralization titer, <1:10; enzyme-linked immunosorbent assay [ELISA] anti-glycoprotein titer, 1:7,290; ELISA anti-nucleoprotein titer, 1:65,610 [4]). Memory CTL were determined in the spleen on day 25. Despite transfer of day 10 immune serum, $CD4^{-/-}$ mice infected with 10^6 PFU of LCMV-WE lacked secondary CTL activity (Fig. 5). Thus, exhaustion was not prevented with this early serum. In addition, we tested the effects of serum with a high titer of neutralizing activity against LCMV-WE. The hyperimmune serum was obtained from C57BL/6 mice and had a neutralizing capacity of 1:2,560 against LCMV-WE when tested in a focus reduction assay (as described in detail elsewhere [4, 5]). In all hyperimmune serum-treated $CD4^{-/-}$ mice, restimulatable LCMV-specific cytotoxicity was maintained (Fig. 5) and virus was eliminated from the blood, spleen, and thymus by 25 days after infection (data not shown). In $CD4^{-/-}$ mice left untreated or treated with nonneutralizing antibodies, memory CTL were not detectable at this time point (day 25) and LCMV persisted (data not shown). Serum with a high level of neutralizing activity prevented exhaustion of CTL, probably simply by helping to reduce virus titers from day 6 on, when treatment was started.

Enhanced establishment of a virus carrier state in $CD4^{-/-}$ mice after low-dose infection with LCMV strain DOCILE. Similar results were obtained with $CD4^{-/-}$ mice infected i.v. with a low dose (5×10^2 PFU) of rapidly replicating LCMV strain DOCILE (provided by C. J. Pfau, Rensselaer Polytechnic Institute, Troy, N.Y. [33]). Memory CTL that could be restimulated in vitro were not detectable 35 days after infection of $CD4^{-/-}$ mice, whereas $CD4^+$ competent mice exhibited a normal secondary CTL response in vitro (Table 1). Again, virus persisted only in $CD4^{-/-}$ mice, as assessed in blood and spleens. Since neutralizing antibodies were undetectable in both $CD4^{-/-}$ and control C57BL/6 mice, the difference in virus elimination is likely to be due to a helper effect of $CD4^+$ T cells on CTL.

After primary infection with LCMV, viral persistence is established in adult mice, dependent upon the virus strain, the virus dose, and the genetics of the host (26, 27, 48). Recently, it was shown that overwhelming LCMV infection caused by

TABLE 1. Enhanced establishment of persistent infection in $CD4^{-/-}$ mice after infection with 5×10^2 PFU of LCMV strain DOCILE

Mouse strain	Neutralizing antibody titer, day 30 p.i.[a]	Mean virus titer[b] ± SEM in:		Secondary CTL activity,[c] day 35 p.i.
		Blood, day 30 p.i.	Spleen, day 35 p.i.	
$CD4^{-/-}$	<1:4	4.8 ± 0.27	5.9 ± 0.15	0–1.3
C57BL/6	<1:4	—[d]	—[e]	80–84[f]

[a] Measured in a focus reduction assay. p.i., postinfection.
[b] Log_{10} PFU per milliliter of blood or gram of spleen tissue.
[c] Percent lysis obtained in $CD4^{-/-}$ mice with the highest concentration of effector cells (i.e., 1:1 standard culture after in vitro restimulation). The range of CTL activities of four individual mice in one of two similar experiments is shown.
[d] —, No detectable virus (<250 PFU) in any of three mice.
[e] —, No detectable virus (<500 PFU) in any of three mice.
[f] For C57BL/6 mice, additional dilution steps produced the following results: 1:3, 75 to 82% lysis; 1:9, 56 to 72% lysis; 1:27, 30 to 48% lysis.

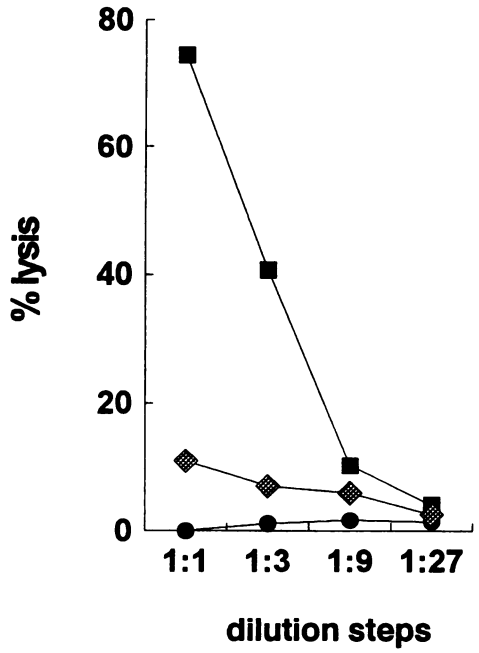

FIG. 5. Neutralizing antibodies may prevent exhaustion of CTL activity. In two independent experiments, groups of three $CD4^{-/-}$ mice were infected i.v. with 2×10^6 PFU of LCMV-WE and treated with a polyclonal neutralizing antiserum (neutralizing titer, 1:2,560; ■) (the corresponding standard error of the mean was 13% lysis) or nonneutralizing (day 10; ◆) anti-LCMV serum (neutralizing titer, <1:10; ELISA titer, >1:1,000 [5]) from day 6 until day 10 plus days 14 and 16 after infection (the standard error of the mean was 7% lysis), or $CD4^{-/-}$ mice were infected with the same virus dose but left untreated (●) (the standard error of the mean was 4% lysis). Spleen cells taken on day 25 after infection were restimulated in vitro for 5 days and tested against LCMV-WE-infected target cells at the indicated dilution steps of unit cultures.

rapidly replicating LCMV or a high virus dose can induce exhaustion of anti-LCMV-specific CTL, leading to virus persistence (26, 27, 48). The presented results demonstrate that mice lacking $CD4^+$ T cells, and therefore also neutralizing antibodies, clear a low dose of LCMV-WE as well as do control mice. However, lack of $CD4^+$ T cells leads to virus persistence after high-dose infection with LCMV-WE or after low-dose infection (5×10^2 PFU) with rapidly replicating LCMV strain DOCILE. CTL escape mutant virus is apparently not involved in this process. The present study showed that treatment slowing down further virus replication, e.g., administration of neutralizing antibodies starting on day 6 after the virus infection, could lower the viral burden sufficiently to prevent CTL exhaustion. Interestingly, adoptive transfer of nonneutralizing antibodies which occur early during LCMV infection in normal mice (5) did not prevent exhaustion of CTL. Our results therefore suggest that $CD4^+$ T cells may contribute to viral clearance by enhancing generation of $CD8^+$ effector T cells rather than by supporting B-cell responses. One possible mechanism is that $CD4^+$ T cells secrete lymphokines which support $CD8^+$ CTL precursors (12, 48). Alternatively and under special circumstances, such as in β_2-microglobulin-deficient mice, $CD4^+$ T cells may contribute more directly to virus clearance (36).

Our results may offer explanations for why loss of $CD4^+$ T-cell function and $CD4^+$ T-cell depletion impair the elimination of certain viral infections and possibly enhance the overwhelming spread of some viruses. Whether the presented findings may be taken to support the notion that some LCMV variants infect few $CD4^+$ T cells in mice and that this directly impairs immune responses remains to be shown (9, 45). Our data may apply directly to human immunodeficiency virus infection (40), in which $CD8^+$ T-cell-mediated immunity is apparently instrumental in virus control (15, 46) and the virus load may increase because of development of CTL escape mutants (34) or because of overwhelming immunopathology (29, 44). Our results may suggest that the decrease or lack of $CD4^+$ T cells and neutralizing antibodies could enhance the exhaustion of human immunodeficiency virus-specific CTL responses, as observed late during human immunodeficiency virus infection (31, 32).

We thank Alana Althage for excellent technical assistance.

This work was supported by grants from the Swiss National Science Foundation (31-32195.91), the Canton of Zürich, and the EMDO Stiftung Zürich.

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