

# CD4-Deficient Mice Have Reduced Levels of Memory Cytotoxic T Lymphocytes after Immunization and Show Diminished Resistance to Subsequent Virus Challenge†

MATTHIAS G. VON HERRATH,\* MASAYUKI YOKOYAMA, JANEL DOCKTER,  
MICHAEL B. A. OLDSTONE, AND J. LINDSAY WHITTON

Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037

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**Although primary antiviral CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) can be induced in mice depleted of CD4<sup>+</sup> T cells, the role of CD4<sup>+</sup> T lymphocytes in the generation and maintenance of antiviral memory CTL is uncertain. This question, and the consequences upon vaccine-mediated protection, were investigated in transgenic CD4 knockout (CD4ko) mice, which lack CD4<sup>+</sup> T lymphocytes. Infection of immunocompetent C57BL/6 mice with lymphocytic choriomeningitis virus (LCMV), or with recombinant vaccinia viruses bearing appropriate LCMV sequences, induces long-lasting protective immunity, mediated mainly by antiviral CD8<sup>+</sup> CTL. Here we report two important findings. First, LCMV-specific CD8<sup>+</sup> memory CTL are maintained at considerably lower levels in CD4ko mice than in normal C57BL/6J mice; we demonstrate a reduction in precursor CTL evident as soon as 30 days postimmunization and declining, by day 120, to levels 1 to 2 log units below those in normal mice. Thus, CD4<sup>+</sup> T cells appear to be important to the generation and maintenance of their CD8<sup>+</sup> counterparts. Second, this reduction has an important biological consequence; compared with immunocompetent mice, CD4ko mice immunized with vaccinia virus recombinants expressing nucleoprotein or glycoprotein of LCMV are less effectively protected from subsequent LCMV challenge. Thus, this study underscores the potential importance of CD4<sup>+</sup> T lymphocytes in generation of appropriate levels of CD8<sup>+</sup>-cell-mediated immunoprotective memory and has implications for vaccine efficacy in individuals with immune defects in which CD4 levels may be reduced, such as AIDS.**

CD4<sup>+</sup> T lymphocytes play a central role in regulation of the immune response. Generation of antibodies usually requires CD4<sup>+</sup> help, since CD4<sup>+</sup> cells provide important activation and costimulatory signals to B lymphocytes, although in certain instances antibody production and class switching may occur in the absence of CD4<sup>+</sup> helper cells (40). The role of CD4<sup>+</sup> T cells in the generation and maintenance of CD8<sup>+</sup> antiviral cytotoxic T lymphocytes (CTL) is less well defined. Early studies indicated that primary CTL responses to lymphocytic choriomeningitis virus (LCMV) were reduced 2-fold (1) to 5- to 15-fold (27), although the biological significance of this finding was questioned since such mice could nevertheless clear the acute virus infection. Further, observations after depletion of CD4<sup>+</sup> cells with antibodies, or using CD4 knockout (CD4ko) mice or class II-deficient mice, show that the primary CTL response generated on day 7 after LCMV infection is not significantly reduced in these mice (17, 39, 50) and the LCMV infection is cleared. Thus, in the LCMV system, CD8<sup>+</sup> CTL can be induced in the absence of CD4<sup>+</sup> T cells. In contrast, in other systems CD4<sup>+</sup> T cells were found to be required for successful induction of a primary CD8<sup>+</sup> CTL response (15).

Less is known about the role of CD4<sup>+</sup> lymphocytes in maintaining memory CTL responses. Some reports indicate an impairment of immunological memory in CD4<sup>+</sup>-deficient mice (4, 17), and in the absence of CD4<sup>+</sup> cells, LCMV can more readily establish a persistent infection (4, 29). Additionally, adoptive transfer of splenocytes from LCMV-immune CD4<sup>+</sup>-

deficient mice does not clear LCMV infection from persistently infected recipient mice (48), whereas the same procedure using normal donors results in complete eradication of the virus (35, 48). To date, however, a precise analysis of memory CTL precursors has not been done.

In this study, we wished first to establish more exactly the degree to which memory CD8<sup>+</sup> CTL populations were diminished in the absence of CD4<sup>+</sup> T cells; we show that precursor frequencies of LCMV-specific memory CTL are 1 to 2 log units lower in CD4ko mice than in normal C57BL/6 controls. Second, we wished to evaluate the possible biological consequences of such a difference. In the LCMV system, it has been clearly shown that protective antiviral immunity, induced by active immunization with either intact LCMV or recombinant vaccinia viruses, is conferred by the vaccine-induced antiviral CTL (52). We thus evaluated the ability of immunized CD4ko mice to withstand subsequent LCMV challenge and found a reduction in vaccine efficacy evident at 6 weeks postvaccination and remaining at 120 days.

## MATERIALS AND METHODS

**Mice.** CD4ko (*H-2<sup>b</sup>*) mice were obtained from D. Littman (19). C57BL/6J (*H-2<sup>b</sup>*) mice were from the rodent breeding colony at the Scripps Research Institute.

**Viral strains.** Virus stocks consisted of LCMV Armstrong (clone 5-3b) and vaccinia virus-LCMV glycoprotein (GP) and nucleoprotein (NP) recombinants (VVGp and VVNP) that expressed LCMV GP amino acids 1 to 398 and LCMV NP amino acids 1 to 558 (52). Virus was plaque purified three times on Vero cells, and virus stock was prepared by a single passage on BHK-21 cells. Recombinant vaccinia viruses were made by standard procedures and propagated and titrated on HeLa cells. MC57 (*H-2<sup>b</sup>*) and BALB/c17 (*H-2<sup>d</sup>*) cells, used as CTL targets, were grown as reported previously (50).

**Antibody-mediated in vivo depletion of CD4<sup>+</sup> T cells.** Monoclonal antibody YTS 191.1 (rat immunoglobulin G2b [IgG2b]) was injected (1 mg in 0.1 ml of

\* Corresponding author. Mailing address: The Scripps Research Institute, Department of Neuropharmacology, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-9602. Fax: (619) 554-9981. Electronic mail address: matthias@scripps.riscsm.edu.

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phosphate-buffered saline [PBS]) intravenously on days -1, +3, and +10 (with respect to LCMV challenge).

**In vitro cytotoxicity assays.** Target cells (MC57 [*H-2<sup>b</sup>*] and BALB/c17 [*H-2<sup>d</sup>*]) were infected with LCMV for 1 h at 37°C (multiplicity of infection of 1) or vaccinia virus-LCMV recombinants for 1 h (multiplicity of infection of 3). At 48 (LCMV) or 12 (vaccinia) h postinfection (p.i.), cells were labeled with <sup>51</sup>Cr and incubated for 5 h with effector cells (see below), after which the amount of chromium released was measured.

(i) **Primary effector CTL.** Splenic CTL were obtained from mice 7 days after intraperitoneal (i.p.) inoculation of 10<sup>5</sup> PFU of LCMV. Effector/target ratios used were 100:1, 50:1, 25:1, 12:1, and 6:1.

(ii) **In vitro-stimulated effectors.** Spleen cells harvested from mice 30 to 120 days after primary inoculation with 10<sup>5</sup> PFU of LCMV i.p. were incubated with major histocompatibility complex (MHC)-matched, irradiated, LCMV-infected macrophages in the presence of T-cell growth factor (TCGF) and irradiated syngeneic spleen feeder cells (50). TCGF, which is supernatant from rat spleen cells stimulated for 1 day in the presence of concanavalin A, was added to all secondary splenocyte cultures at day 4. For most assays, splenocytes were cultivated for a total of 6 days, except for the experiments shown in Fig. 2, for which cells were stimulated for a total of 13 days in vitro.

**Precursor frequency analysis.** For precursor frequency analysis (limiting-dilution analysis), spleen cells were harvested on days 7, 28, 60, 90, and 120 after primary LCMV infection. These cells were diluted serially and cultivated in 96-well flat-bottom plates in the presence of TCGF and syngeneic irradiated LCMV-infected (10<sup>5</sup> PFU/ml) spleen cells (10<sup>5</sup> per well). After 6 days, each well was assayed for cytotoxic activity (described above) on target cells that were uninfected or infected with LCMV or recombinant vaccinia virus. Positive cultures were defined as specific lysis 3 standard deviations above the lysis found in negative controls (3% ± 2% for all experiments shown). The fraction of negative cultures was determined for each dilution (total of 48 cultures per dilution). Precursor CTL (pCTL) numbers were assessed as described previously (31). Briefly, the fraction of negative cultures was plotted on a semilogarithmic scale against the number of splenocytes per culture (see Fig. 3). pCTL frequencies are defined by the slope of the linear regression between at least three separate datum points. The formula used was  $f = (\ln a - \ln y)/n$ ;  $f$  is the frequency of pCTL,  $n$  is the number of splenocytes added to each culture,  $a$  is the y-axis intercept (in our experiments, 100% ± 4%), and  $y$  is the percentage of negative cultures.

**Antibody assays.** LCMV-specific antibody was determined by a solid-phase enzyme-linked immunosorbent assay (50). Secondary antibodies were peroxidase-coupled goat anti-mouse IgM and IgG (Fc), both from Pharmingen and used at a 1:5,000 dilution in PBS. Antibody levels are plotted as the log<sub>10</sub> reciprocal serum endpoint dilution.

## RESULTS

**Cytotoxic activity after in vitro stimulation remains detectable for at least 120 days in normal mice but is usually undetectable by 90 days postimmunization in CD4ko mice and in normal mice depleted of CD4 cells.** To determine for how long CTL remained detectable following immunization, splenocytes were harvested at several time points post-LCMV infection, restimulated in vitro for 6 days, and tested for cytotoxic activity. CTL activities could be easily demonstrated up to 120 days after LCMV infection in normal *H-2<sup>b</sup>* mice. In contrast, CTL activity was diminished at day 28 and was undetectable at day 60 and at later time points in CD4ko mice (Fig. 1A). This diminution in CTL activity most probably reflects the absence of CD4<sup>+</sup> T cells, rather than other possible mouse strain differences, because depletion of CD4<sup>+</sup> T cells from normal mice led to the disappearance of CTL activity at time points later than 90 days post-LCMV infection. However, if splenic lymphocytes from CD4ko mice were stimulated in vitro for a longer time than 6 days (i.e., 13 or 20 days) in the presence of interleukin-2 (IL-2) (TCGF), in many instances LCMV-specific CTL activity could be demonstrated (Fig. 2 and data not shown). This finding indicated that memory CTL were still present in CD4ko mice at time points later than day 60 p.i., but probably at low precursor frequencies, or that they lacked the appropriate activation signals (e.g., IL-2).

**Precursor frequency analyses confirm a defect in CD4ko mice.** To quantitate the defect in the CD4ko mice, precursor frequency analyses were carried out to compare normal and CD4ko *H-2<sup>b</sup>* mice at various times post-LCMV infection (Fig.

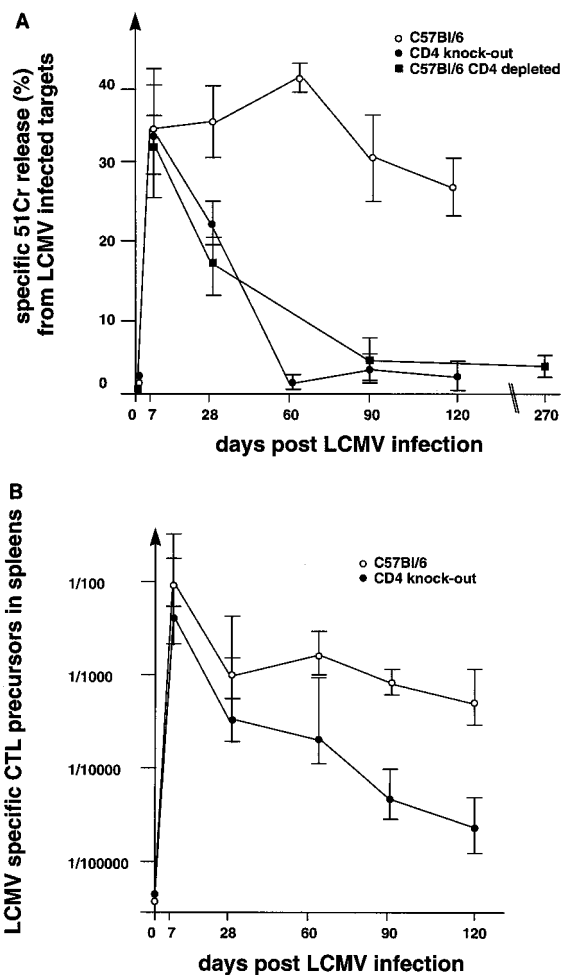


FIG. 1. (A) Memory CTL following LCMV infection are diminished in CD4ko mice. Splenocytes were harvested from C57BL/6J mice (normal or depleted by anti-CD4 antibody as described in Materials and Methods) and CD4ko *H-2<sup>b</sup>* mice at the indicated times after inoculation of 10<sup>5</sup> PFU of LCMV i.p. and stimulated in vitro for 6 days in vitro as described in Materials and Methods. Samples for each time point obtained from C57BL/6J and CD4ko *H-2<sup>b</sup>* mice were processed in parallel. Values represent means from five individual mice tested. Primary CTL activities on day 7 post-LCMV infection were assessed without in vitro stimulation. (B) Quantitation of memory CTL reduction by precursor frequency analyses in CD4<sup>+</sup> and CD4ko mice immunized with LCMV. Splenocytes were harvested from C57BL/6J and CD4ko *H-2<sup>b</sup>* mice at the indicated times after inoculation of 10<sup>5</sup> PFU of LCMV i.p., and precursor frequencies were determined as described in Materials and Methods and reference 31. C57BL/6J and CD4ko samples were processed, cultivated, and tested in parallel for each time point to allow a direct comparison and avoid introduction of artifacts through the precursor frequency analysis. Values represent means from three individual mice tested.

1B). In normal mice, the frequencies remain stable after day 28, while in CD4ko mice, a decrease in pCTL frequency is evident by day 28, and the numbers diminish with time over the range measured. Compared with normal mice, CD4ko mice have 1- to 2-log-units fewer LCMV-specific memory CTL precursors at 120 days p.i.

**Anti-LCMV memory CTL are undetectable in most CD4ko mice 120 days after immunization with recombinant vaccinia viruses.** To evaluate memory CTL in mice immunized with recombinant vaccinia viruses and to determine differences between normal and CD4ko mice, splenic CTL from normal and CD4ko *H-2<sup>b</sup>* mice were obtained 120 days after infection with LCMV, VVGP, or VVNP and were stimulated in vitro for 13

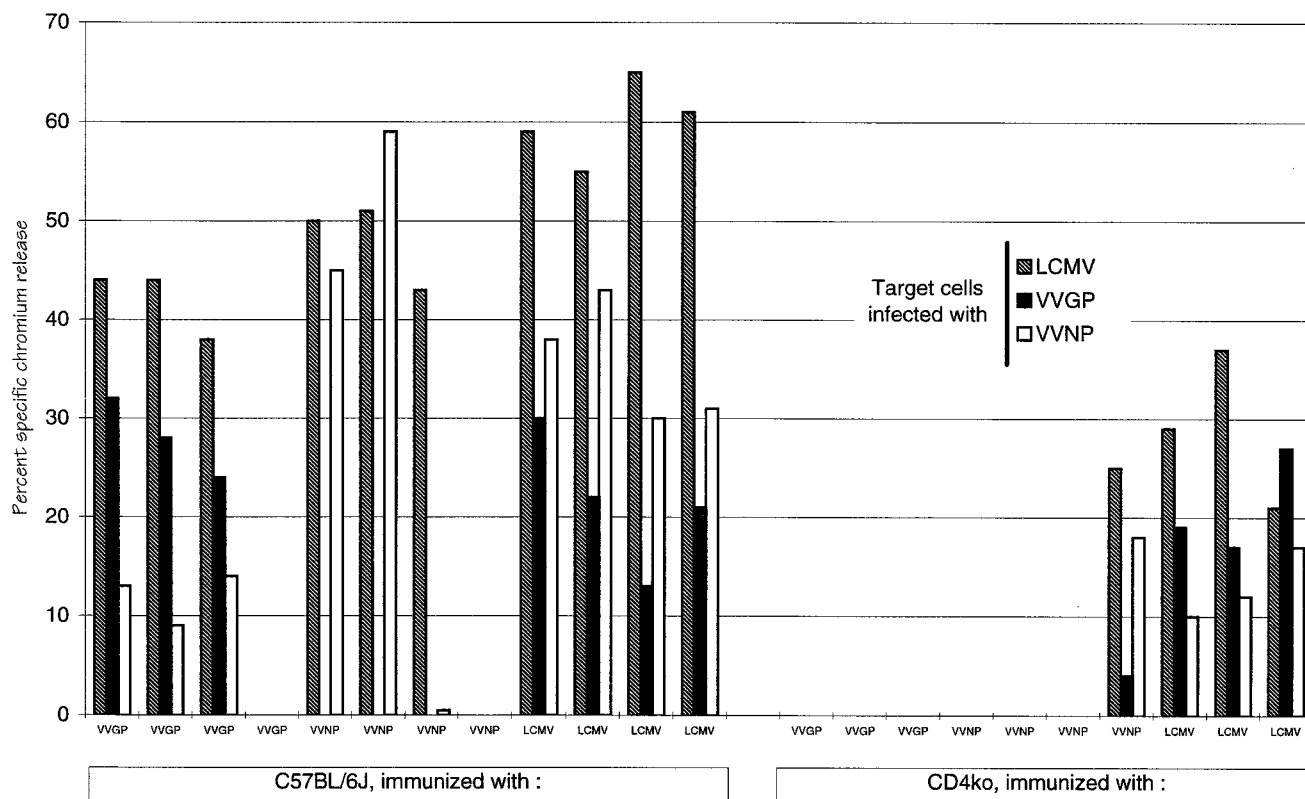


FIG. 2. Quantitation of CTL activities in CD4<sup>+</sup> and CD4ko mice immunized with recombinant vaccinia viruses. Normal or CD4ko mice were immunized with LCMV, VVNP, or VVGP, as shown on the x axis. Effector cells were taken at 120 days postimmunization, subjected to secondary *in vitro* stimulation for 13 days, and used in an *in vitro* cytotoxicity assay against target cells infected with LCMV, VVGP, or VVNP. Effector-to-target ratios displayed were 5:1, and data from individual mice are shown.

days in the presence of TCGF prior to assay of cytotoxic activities (Fig. 2). CTL activity was demonstrated in all seven LCMV-immunized mice, regardless of their CD4 status. In contrast, following immunization with vaccinia virus recombinants expressing LCMV proteins, anti-LCMV memory CTL were undetectable in >85% (six of seven) of CD4ko mice, although they remained detectable in 75% (six of eight) of normal mice (specific lysis, 45%  $\pm$  8%). Shorter stimulation times (6 days) did not result in detectable CTL activities of LCMV-specific CTL in CD4-deficient mice (Fig. 1). This apparent difference indicates that *in vitro* stimulation in the presence of TCGF (IL-2) can rescue CD8<sup>+</sup> memory CTL activities in CD4-deficient mice, as described previously (27).

**CD4ko mice have normal levels of primary LCMV-specific CTL responses 7 days following infection with either LCMV or VVGP.** The absence of detectable anti-LCMV CTL in CD4ko mice following recombinant vaccinia virus immunization might reflect a poorer primary response in CD4ko mice than in normal animals. However, results displayed in Fig. 1 indicated that primary CTL activities found 7 days after LCMV infection in normal and CD4-deficient mice were very similar by comparison of direct <sup>51</sup>Cr release values. To confirm and extend these findings, precursor frequencies of LCMV-specific and LCMV GP-specific CTL were assessed in normal and CD4-deficient *H-2<sup>b</sup>* mice 7 days after infection with LCMV or VVGP (Fig. 3). Numbers of pCTL in normal mice were similar to those in CD4ko mice. These conclusions may be drawn for tests against targets expressing whole LCMV or LCMV GP and for responses induced by either whole LCMV or the VVGP recombinant. Thus, at 7 days p.i., CD4<sup>+</sup> and CD4ko

mice mount indistinguishable responses both to LCMV and to VVGP.

**Recombinant vaccinia viruses induced lower levels of anti-LCMV CTL in both normal and CD4ko mice.** CTL numbers generated after infection with VVGP were 1 to 2 log units lower than those generated after LCMV infection in both normal and CD4ko *H-2<sup>b</sup>* mice (Fig. 3). This difference between LCMV and recombinant vaccinia viruses may explain why, in CD4ko mice, anti-LCMV memory cells are detected 120 days after LCMV immunization but not vaccinia virus immunization. We attempted to evaluate precursor frequencies in normal and CD4ko mice at later time points following infection with VVGP or VVNP (analogous to those shown in Fig. 1B for LCMV-induced precursor frequencies), but precise quantitation proved impossible even in normal mice, probably because the memory CTL, although detectable following bulk restimulation, are at or below the limit of our precursor frequency analysis. Thus, recombinant vaccinia viruses induce CTL which remain detectable after *in vitro* restimulation, but only in normal mice, not in CD4ko mice (Fig. 2). Even in CD4<sup>+</sup> mice, the levels are too low to reliably quantitate by precursor frequency assay. However, in all cases, the immunizing viruses were cleared 10 to 14 days p.i. (data not shown).

**LCMV-specific antibodies are reduced in CD4ko mice.** We analyzed LCMV-specific antibody titers in normal and CD4ko mice following LCMV infection (data not shown). No significant amount of LCMV-specific IgG antibodies were detected in CD4ko mice. Some IgM antibodies were generated, but the titers were low (1:100 in CD4ko mice, compared with 1:5,000

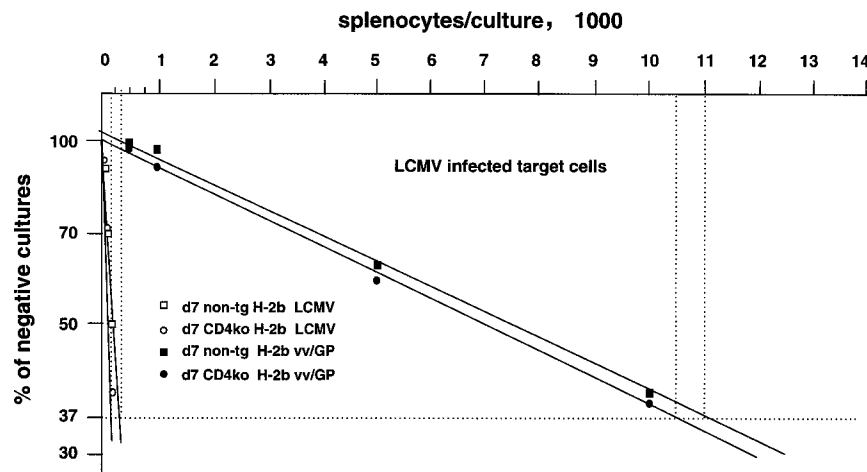


FIG. 3. Quantitation of memory CTL reduction by precursor frequency analyses in CD4<sup>+</sup> and CD4ko mice immunized with recombinant vaccinia viruses. Splenocytes were harvested from C57BL/6J and CD4ko *H-2<sup>b</sup>* mice 7 days after inoculation of 10<sup>5</sup> PFU of LCMV or 10<sup>7</sup> PFU of VV/GP i.p., and precursor frequencies of LCMV-specific CTL were determined as described in Materials and Methods and reference 31, using LCMV-infected targets. Data are from one representative experiment; similar data were obtained in three separate experiments. The means  $\pm$  1 standard deviation for pCTL were as follows: normal C57BL/6J mice infected with LCMV, 1:200  $\pm$  122; normal mice infected with VVGP, 1:11,000  $\pm$  2,500; CD4-deficient mice infected with LCMV, 1:380  $\pm$  175; and CD4-deficient mice infected with VVGP, 1:10,300  $\pm$  2,600. For infection with VVGP, only the day 7 time point was assayed, as at later times the levels of memory CTL in mice receiving recombinant vaccinia viruses was too low for reliable quantitation using this technique (see text).

in normal *H-2<sup>b</sup>* mice). Thus, as expected, CD4ko mice do not generate significant levels of LCMV-specific antibodies.

**Compared with normal mice, CD4ko mice are less effectively protected from lethal choriomeningitis when vaccinated with VVGP or VVNP.** In this study, vaccine efficacy is determined by resistance to intracerebral (i.c.) LCMV challenge. To ensure that both mouse strains were susceptible to LCMV i.c. challenge, nonimmune CD4ko mice and *H-2<sup>b</sup>* mice were challenged at five LCMV doses, differing by threefold in virus concentration. The highest dosage was 100 50% lethal doses (LD<sub>50</sub>; approximately 250 PFU), and the lowest dosage was 81-fold lower. All mice, normal and CD4ko, succumbed at all doses. Interestingly, at the lower viral doses, the CTL-mediated death was delayed in the CD4ko animals. This may reflect subtle differences in the development of biologically relevant acute CTL responses in normal and CD4ko mice.

Ten LD<sub>50</sub>, a lethal amount by day 7 in all mice regardless of CD4 phenotype, was selected as the challenge dose to evaluate vaccine efficacy. Two postvaccination time points were evaluated. At 42 days postimmunization, all mice (normal and CD4ko) immunized with LCMV or with VVNP are protected from this normally lethal challenge (Fig. 4A). A small difference is noted between normal and CD4ko mice immunized with VVGP; 75% of normal mice, but only 50% of CD4ko mice, survive the challenge. This apparent difference is confirmed at the later time point (120 days p.i.), when 7 of 17 (41%) of the vaccinia virus-immunized animals succumb to challenge, while all of the 16 normal mice survive (Fig. 4B). This difference is seen only in vaccinia virus-immunized mice; all LCMV-immunized CD4ko *H-2<sup>b</sup>* mice were protected against LCMV i.c. challenge.

## DISCUSSION

CD8<sup>+</sup> memory CTL are critical protective factors induced by most antiviral vaccines. In the LCMV model system, we (23, 52, 54) and others (42) have clearly demonstrated that a single CTL epitope can, by inducing anti-LCMV memory CTL, confer complete protection against subsequent LCMV challenge. Similar studies with murine cytomegalovirus (16) and influenza

virus (49) indicate that this observation may be generally applicable. The availability of specific immunologic reagents and of transgenic knockout mice has allowed evaluation of the antiviral activities of the various arms of the immune system. For example, in the absence of CD8<sup>+</sup> T cells, mice are less able to cope with various viral challenges and often clear the infection more slowly (8, 13, 26) if at all; often, persistent infection results (7, 29). In human infection, too, these cells play an important role. Agammaglobulinemic children withstand measles virus infection, mounting a good T-cell response (18). In contrast, in T-cell-deficient children the disease is often fatal (11, 34, 43). Agammaglobulinemic children clear the virus and, if reexposed, are immune, indicating that memory T cells provide protection (9). Therefore, it is important to identify elements involved in the generation of primary CTL responses and in the generation and maintenance of memory CTL. One potential contributor to CD8<sup>+</sup> CTL generation is the CD4<sup>+</sup> helper T cell.

The role of CD4<sup>+</sup> T cells in generation of primary and memory CD8<sup>+</sup> CTL responses has been controversial. Early work (1, 28) indicated that there was no absolute requirement for CD4<sup>+</sup> cells in generating primary CD8<sup>+</sup> CTL responses. Nevertheless, one study, using LCMV, reported a 5- to 15-fold reduction in CTL activity (27), and in other viral models, CD4<sup>+</sup> T cells may be required for the generation of primary responses (15). The situation is, however, complex, as the requirement for CD4<sup>+</sup> help may be mitigated by more efficient antigen presentation (32), and some work suggests that CD4<sup>+</sup> and CD8<sup>+</sup> T cells can function largely independently (17). In this study, we used the well-characterized LCMV model (2, 22, 23, 37, 41, 52, 55, 56) and found that (i) levels of memory cells induced by LCMV infection are 1 to 2 log units lower in CD4ko mice than in normal mice, (ii) anti-LCMV memory cells induced by recombinant vaccinia viruses are diminished to almost undetectable levels in CD4ko mice, and (iii) the reduced levels of memory CTL correlate with reduced protection against LCMV challenge. Thus, our data demonstrate that vaccine efficiency has to be carefully evaluated when applied in immunocompromised situations where CD4 cells are lacking,

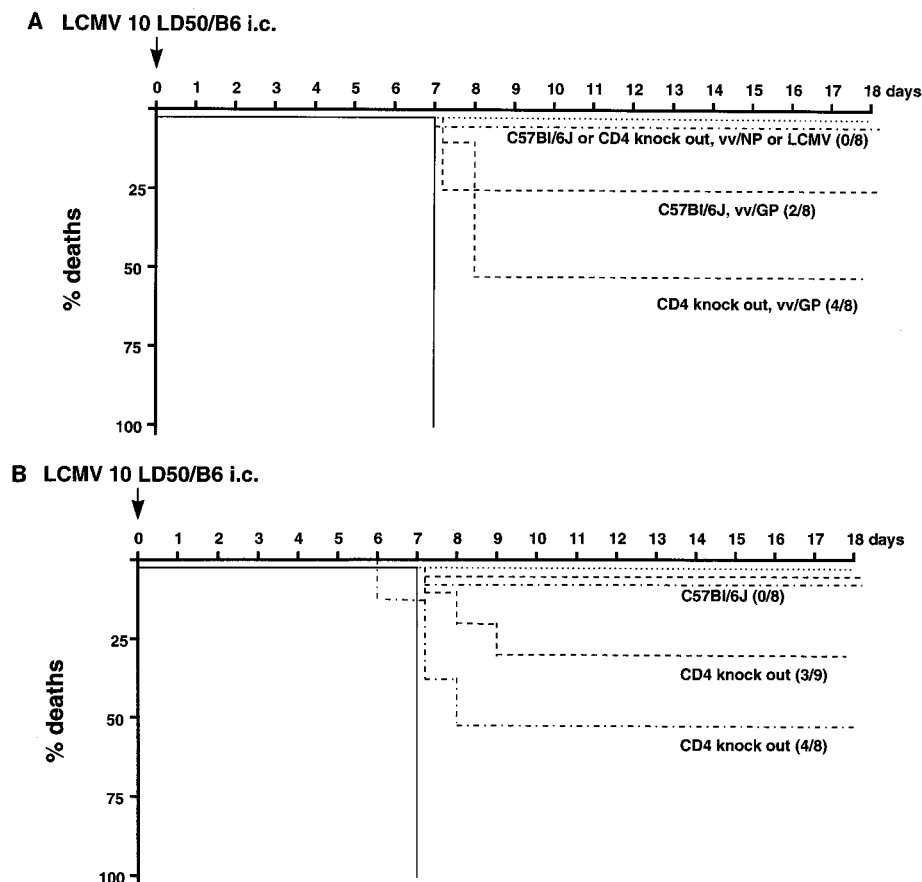


FIG. 4. (A) Protective immunity induced 42 days following immunization of CD4<sup>+</sup> or CD4ko mice. C57BL/6J and CD4ko *H-2<sup>b</sup>* mice were immunized 42 days earlier with LCMV (10<sup>5</sup> PFU i.p.) or VVGP or VVNP (10<sup>7</sup> PFU i.p.) and were injected with 10 LD<sub>50</sub> of LCMV i.c. on day 0. —, no vaccination, C57BL/6J mice; ·····, day 42 after vaccination with 10<sup>5</sup> PFU of LCMV, both C57BL/6J and CD4ko mice; - - - - - , day 42 after vaccination with 10<sup>7</sup> PFU of VVNP, C57BL/6J or CD4ko mice as indicated; - - - - - , day 42 after vaccination with 10<sup>7</sup> PFU of VVGP, C57BL/6J or CD4ko mice as indicated. LCMV-vaccinated animals did not get sick; vaccinia virus recombinant-vaccinated animals were all sick on days 5 to 10. (B) Protective immunity induced 120 days following immunization of CD4<sup>+</sup> or CD4ko mice. C57BL/6J and CD4ko *H-2<sup>b</sup>* mice were immunized 120 days earlier with LCMV (10<sup>5</sup> PFU i.p.) or VVGP or VVNP (10<sup>7</sup> PFU i.p.) and were injected with 10 LD<sub>50</sub> of LCMV i.c. on day 0. Groups of 10 mice were analyzed. —, no vaccination, C57BL/6J mice; ·····, day 120 after vaccination with 10<sup>5</sup> PFU of LCMV, both C57BL/6J and CD4ko mice; - - - - - , day 120 after vaccination with 10<sup>7</sup> PFU of VVNP, C57BL/6J or CD4ko mice as indicated; - - - - - , day 120 after vaccination with 10<sup>7</sup> PFU of VVGP, C57BL/6J or CD4ko mice as indicated. LCMV-vaccinated animals did not get sick; vaccinia virus recombinant-vaccinated animals were all sick on days 5 to 10.

such as later stages of human immunodeficiency virus infection.

We demonstrate that in the absence of CD4<sup>+</sup> T cells, the levels of CD8<sup>+</sup> memory cells induced by LCMV infection are decreased by up to 1 to 2 log units (Fig. 1). The reduction is evident by 28 days p.i. and becomes increasingly marked up to 120 days p.i. At least two possible mechanisms might explain this outcome. First, the number of CD8<sup>+</sup> cells induced in the primary response may dictate subsequent memory levels. Against this argument is our finding that precursor frequency analysis at 7 days p.i. showed indistinguishable numbers in normal and CD4ko mice (Fig. 3). Second, CD4<sup>+</sup> cells may provide help to maintain memory CTL levels, for example by producing IL-2 or gamma interferon (12, 38, 48). Although there is *in vivo* evidence that IL-2 may prevent deletion of mature CD8<sup>+</sup> T cells (20), little evidence is available to indicate a role in maintenance of memory. Several investigations have shown that memory cells are fairly long lived (25, 46), but there is no universal agreement, as some postulate a more rapid turnover of memory cells (45). The mechanism of memory persistence in normal mice is controversial; it may result from long-lived memory cells, which are maintained in the

absence of antigen (14, 25) and maybe through cross-activation by other viral infections (30), or conversely from antigen-driven short-lived or cycling cells (10). Our results show that memory CTL activities in CD4ko mice can at least partially be rescued by *in vitro* cultivation in the presence of TCGF (IL-2). This finding indicates a potential for CD4-dependent IL-2 in maintaining CD8 memory and extends earlier observations by Leist and colleagues (27).

Several factors may explain the reduced level of anti-LCMV CTL induced by recombinant vaccinia viruses. First, LCMV and vaccinia viruses have different cell tropisms and therefore probably express their antigens in different cell types and in different amounts. Second, vaccinia virus encodes a number of proteins which help the virus evade host immunity (33, 44, 47). Third, the recombinant-encoded LCMV epitopes must compete for MHC presentation with a large excess of vaccinia virus epitopes. Nevertheless, in normal mice a single inoculation of recombinant vaccinia virus is sufficient to induce anti-LCMV CTL, including a detectable memory response at 120 days p.i. (Fig. 2), although 10 days of *in vitro* restimulation are required. In fact, CTL are detectable in normal mice up to 1 year post-vaccination (51). In contrast, in vaccinated (VVGP or VVNP)

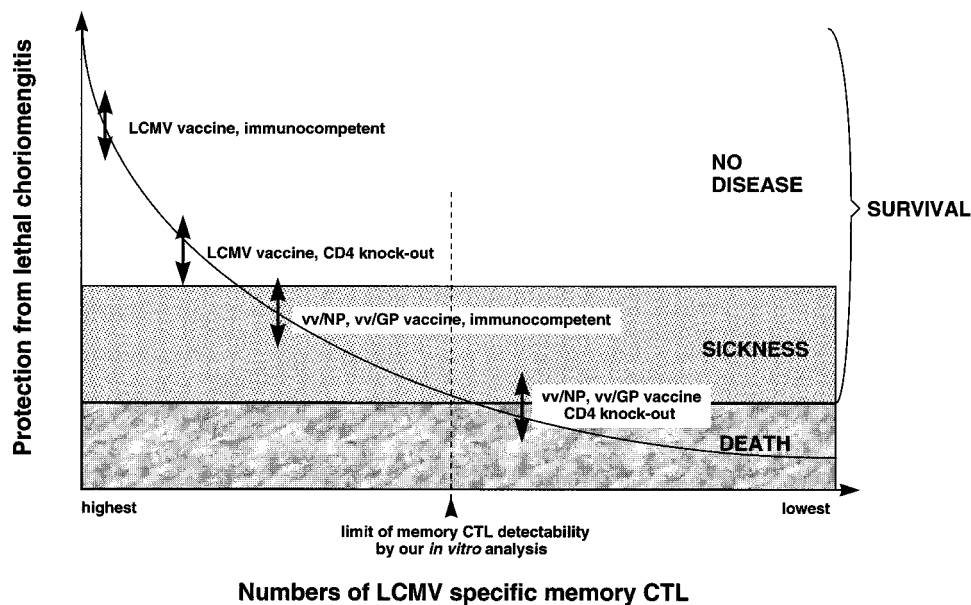


FIG. 5. Possible scenarios following i.c. LCMV infection of mice with differing vaccine histories. The correlation between memory CD8<sup>+</sup> CTL numbers and challenge outcome is shown for C57BL/6J and CD4ko *H-2<sup>b</sup>* mice treated with different vaccines that induce different numbers of memory CTL.

CD4ko mice, the anti-LCMV CTL are undetectable after 28 days postvaccination and cannot be rescued by *in vitro* cultivation in the presence of IL-2. Potentially there is a critical low level of CD8<sup>+</sup> memory precursors, beyond which memory is lost irrevocably.

It is possible but unlikely that loss of CD8<sup>+</sup> memory in CD4-deficient mice is due to direct killing of LCMV-infected CD8<sup>+</sup> cells by CTL. Although it has been reported that CTL can kill CTL after addition of MHC class I-restricted peptides *in vitro* (6, 24), there is good evidence that the number of infected CD4 and CD8 lymphocytes is very low after LCMV infection (5, 36).

Having demonstrated a reduction in vaccine-induced memory CTL, we evaluated the biological consequence. Intracranial inoculation of LCMV leads to death at around 7 days p.i.; death is immunopathological, being mediated by antiviral CD8<sup>+</sup> CTL responses to the disseminated expression of viral antigens in the leptomeninges, ventricles, and choroid plexus. Protection from this lethal choriomeningitis is, like the disease itself, mediated by CD8<sup>+</sup> memory CTL. These CTL can be induced by prior immunization with LCMV, vaccinia virus recombinants expressing LCMV proteins, or minigenes encoding LCMV CTL epitopes (21, 53, 54). The vaccine-induced memory CTL respond more rapidly than naive T lymphocytes to i.c. LCMV inoculation, thus quickly restricting viral replication and spread. As a consequence, the immune system-mediated central nervous system pathologic effect is reduced and the mice survive. Using this i.c. LCMV challenge protocol, at two time points (days 42 and 120 postimmunization), we found that CD4ko mice were less well protected by prior immunization. No difference was seen when mice had been immunized with whole LCMV, consistent with our observation (Fig. 2) that under these circumstances, anti-LCMV CTL can be rescued and detected up to 120 days p.i. However, when recombinant vaccinia viruses were used as immunogens, CD4ko mice were markedly susceptible to subsequent LCMV challenge (11 of 25 mice died). Vaccine efficacy as determined by protection therefore correlated directly with the levels of CTL detected. Survival of approximately 56% of the CD4ko

mice may reflect the presence of memory CTL at levels capable of altering the outcome *in vivo* but too low to detect by our *in vitro* assays.

This study demonstrates the fine-tuned quantitative mechanisms which determine vaccine efficacy in protecting against a usually lethal viral infection and underscores the importance both of host immunocompetence (normal or CD4 deficient) and of the vaccine used (LCMV or recombinant vaccinia virus). The interactions between these variables, and the consequent clinical picture following i.c. LCMV challenge, are summarized in Fig. 5. Normal and CD4ko mice immunized with LCMV remain healthy at all times following challenge. In contrast, mice immunized with recombinant vaccinia viruses invariably appear ill between days 5 and 10 p.i.; normal mice usually recover completely and eradicate the virus, while CD4ko mice often succumb. The illness seen in vaccinia virus-immunized mice probably reflects the CTL-based nature of most of our vaccinia virus vaccines; the protective anti-LCMV CTL can recognize cells only after they become infected, and so some replication and spread of virus may occur, leading to limited disease. The superiority of the LCMV-induced immunity may be attributable to induction of anti-LCMV antibodies, which can prevent infection. However, antibody responses are of secondary importance in control of LCMV infection, although they can alter the outcome of i.c. challenge (3, 57). Our data indicate that other factors are involved, as LCMV-immunized CD4ko mice, which produced only low levels of antiviral antibodies, did not become sick following i.c. challenge. At least two additional mechanisms may explain the improved outcome following LCMV immunization. First, the use of whole virus induces responses to all virus proteins, while the recombinant vaccinia viruses induce a limited response, to a single LCMV protein. Second, higher levels of anti-LCMV memory CTL are induced. The relative importance of each of these factors has not been determined.

In conclusion, a vaccine given to a recipient suffering from CD4<sup>+</sup> T-cell deficiency may confer transient, but not prolonged, protection. This finding may be particularly relevant to the use of subunit vaccines that induce CD8<sup>+</sup> T-cell responses.

Such vaccines should include MHC class II-restricted epitopes, to induce CD4<sup>+</sup> T cells. Such a combination might best ensure maintenance of CD8<sup>+</sup> T-cell memory.

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#### REFERENCES

- Ahmed, R., L. D. Butler, and L. Bhatti. 1988. T4<sup>+</sup> T helper cell function in vivo: differential requirement for induction of antiviral cytotoxic T-cell and antibody responses. *J. Virol.* **62**:2102–2106.
- Ahmed, R., J. A. Byrne, and M. B. A. Oldstone. 1984. Virus specificity of cytotoxic T lymphocytes generated during acute lymphocytic choriomeningitis virus infection: role of the H-2 region in determining cross-reactivity for different lymphocytic choriomeningitis virus strains. *J. Virol.* **51**:34–41.
- Baldridge, J. R., and M. J. Buchmeier. 1992. Mechanisms of antibody-mediated protection against lymphocytic choriomeningitis virus infection: mother-to-baby transfer of humoral protection. *J. Virol.* **66**:4252–4257.
- Battegay, M., D. Moskopidhis, A. Rahemtulla, H. Hengartner, T. W. Mak, and R. M. Zinkernagel. 1994. Enhanced establishment of a virus carrier state in adult CD4<sup>+</sup> T-cell-deficient mice. *J. Virol.* **68**:4700–4704.
- Borrow, P., C. Evans, and M. B. A. Oldstone. 1995. Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression. *J. Virol.* **69**:1059–1070.
- Borrow, P., A. Tishon, and M. B. A. Oldstone. 1991. Infection of lymphocytes by a virus that aborts CTL activity and establishes persistent infection. *J. Exp. Med.* **174**:203–212.
- Doherty, P. C., S. Hou, and P. J. Southern. 1993. Lymphocytic choriomeningitis virus induces a chronic wasting disease in mice lacking class I MHC glycoproteins. *J. Neuroimmunol.* **46**:11–17.
- Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P. C. Doherty. 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8<sup>+</sup> T cells. *J. Exp. Med.* **174**:875–880.
- Good, R. A., and S. J. Zak. 1956. Disturbance in gamma-globulin synthesis as "experiments of nature." *Pediatrics* **18**:109–149.
- Gray, D. 1994. Regulation of immunological memory. *Curr. Opin. Immunol.* **6**:425–430.
- Gray, M. M., I. M. Hann, S. Glass, O. B. Eden, P. M. Jones, and R. F. Stevens. 1987. Mortality and morbidity caused by measles in children with malignant disease attending four major treatment centres: a retrospective review. *Br. Med. J. Clin. Res.* **295**:19–22.
- Horvat, B., J. A. Loukides, L. Anandan, E. Brewer, and P. M. Flood. 1991. Production of interleukin 2 and interleukin 4 by immune CD4-CD8<sup>+</sup> and their role in the generation of antigen-specific cytotoxic T cells. *Eur. J. Immunol.* **21**:1863–1871.
- Hou, S., P. C. Doherty, M. Zijlstra, R. Jaenisch, and J. M. Katz. 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8<sup>+</sup> T cells. *J. Immunol.* **149**:1319–1325.
- Hou, S., L. Hyland, K. W. Ryan, A. Portner, and P. C. Doherty. 1994. Virus-specific CD8<sup>+</sup> T-cell memory determined by clonal burst size. *Nature (London)* **369**:652–654.
- Jennings, S. R., R. H. Bonneau, P. M. Smith, R. M. Wolcott, and R. Chervenak. 1991. CD4-positive T lymphocytes are required for the generation of the primary but not the secondary CD8-positive cytolytic T lymphocyte response to herpes simplex virus in C57BL/6 mice. *Cell Immunol.* **133**:234–252.
- Jonjic, S., M. del Val, G. M. Keil, M. J. Reddehase, and U. H. Koszinowski. 1988. A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. *J. Virol.* **62**:1653–1658.
- Kasaian, M. T., K. A. Leite-Morris, and C. A. Biron. 1991. The role of CD4<sup>+</sup> cells in sustaining lymphocyte proliferation during lymphocytic choriomeningitis virus infection. *J. Immunol.* **146**:1955–1963.
- Kernahan, J., J. McQuillin, and A. W. Craft. 1987. Measles in children who have malignant disease. *Br. Med. J. Clin. Res.* **295**:15–18.
- Killeen, N., S. Sawada, and D. R. Littman. 1993. Regulated expression of human CD4 rescues helper T cell development in mice lacking expression of endogenous CD4. *EMBO J.* **12**:1547–1553.
- Kirberg, J., L. Bruno, and H. von Boehmer. 1993. CD4<sup>+</sup> help prevents rapid deletion of CD8<sup>+</sup> cells after a transient response to antigen. *Eur. J. Immunol.* **23**:1963–1967.
- Klavinskis, L. S., M. B. A. Oldstone, and J. L. Whitton. 1989. Designing vaccines to induce cytotoxic T lymphocytes: protection from lethal viral infection, p. 485–489. *In* F. Brown, R. Chanock, H. Ginsberg, and R. Lerner (ed.), *Vaccines 89*. Modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Klavinskis, L. S., J. L. Whitton, E. Joly, and M. B. A. Oldstone. 1990. Vaccination and protection from a lethal viral infection: identification, incorporation, and use of a cytotoxic T lymphocyte glycoprotein epitope. *Virology* **178**:393–400.
- Klavinskis, L. S., J. L. Whitton, and M. B. A. Oldstone. 1989. Molecularly engineered vaccine which expresses an immunodominant T-cell epitope induces cytotoxic T lymphocytes that confer protection from lethal virus infection. *J. Virol.* **63**:4311–4316.
- Kyburz, D., D. Speiser, T. Aebischer, H. Hengartner, and R. M. Zinkernagel. 1993. Virus-specific CTL mediated lysis of lymphocytes in vitro and in vivo. *J. Immunol.* **150**:5051–5058.
- Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature (London)* **369**:648–652.
- Lehmann-Grube, F., J. Lohler, O. Utermohlen, and C. Gegin. 1993. Antiviral immune responses of lymphocytic choriomeningitis virus-infected mice lacking CD8<sup>+</sup> T lymphocytes because of disruption of the  $\beta$ 2-microglobulin gene. *J. Virol.* **67**:332–339.
- Leist, T. P., M. Kohler, and R. M. Zinkernagel. 1989. Impaired generation of anti-viral cytotoxicity against lymphocytic choriomeningitis and vaccinia virus in mice treated with CD4-specific monoclonal antibody. *Scand. J. Immunol.* **30**:679–686.
- Liu, Y., and A. Mullbacher. 1989. The generation and activation of memory class I MHC restricted cytotoxic T cell responses to influenza A virus in vivo do not require CD4<sup>+</sup> T cells. *Immunol. Cell Biol.* **67**:413–420.
- Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4<sup>+</sup> T cells are required to sustain CD8<sup>+</sup> cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**:8056–8063.
- Matzinger, P. 1994. Memories are made of this? *Nature (London)* **369**:605–606.
- McDonald, H. R., J. C. Cerottini, J.-E. Ryser, J. L. Maryanski, C. Taswell, M. Widmer, and K. T. Brunner. 1980. Quantitation and cloning of CTL and their precursors. *Immunol. Rev.* **51**:93–123.
- Mercadal, C. M., S. Martin, and B. T. Rouse. 1991. Apparent requirement for CD4<sup>+</sup> T cells in primary anti-herpes simplex virus cytotoxic T-lymphocyte induction can be overcome by optimal antigen presentation. *Viral Immunol.* **4**:177–186.
- Mossman, K., C. Upton, R. M. Buller, and G. McFadden. 1995. Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. *Virology* **208**:762–769.
- Nahmias, A. J., D. Griffith, C. Salsbury, and K. Yoshida. 1967. Thymic aplasia with lymphopenia, plasma cells, and normal immunoglobulins. Relation to measles virus infection. *JAMA* **201**:729–734.
- Oldstone, M. B. A., P. Blount, P. J. Southern, and P. W. Lampert. 1986. Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature (London)* **321**:239–243.
- Oldstone, M. B. A., M. Salvato, A. Tishon, and H. Lewicki. 1988. Virus-lymphocyte interactions. III. Biologic parameters of a virus variant that fails to generate CTL and establishes persistent infection in immunocompetent hosts. *Virology* **164**:507–516.
- Oldstone, M. B. A., J. L. Whitton, H. Lewicki, and A. Tishon. 1988. Fine dissection of a nine amino acid glycoprotein epitope a major determinant recognized by lymphocytic choriomeningitis virus-specific class I-restricted H-2Db cytotoxic T lymphocytes. *J. Exp. Med.* **168**:559–570.
- Palladino, G., P. A. Scherle, and W. Gerhard. 1991. Activity of CD4<sup>+</sup> T-cell clones of type 1 and type 2 in generation of influenza virus-specific cytotoxic responses in vitro. *J. Virol.* **65**:6071–6076.
- Rahemtulla, A., W. P. Fung-Leung, M. W. Schilham, T. M. Kundig, S. R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C. J. Paige, and R. M. Zinkernagel. 1991. Normal development and function of CD8<sup>+</sup> cells but markedly decreased helper cell activity in mice lacking CD4. *Nature (London)* **353**:180–184.
- Rahemtulla, A., T. M. Kundig, A. Narendran, M. F. Bachmann, M. Julius, C. J. Paige, P. S. Ohashi, R. M. Zinkernagel, and T. W. Mak. 1994. Class II major histocompatibility complex-restricted T cell function in CD4-deficient mice. *Eur. J. Immunol.* **24**:2213–2218.
- Riviere, Y., and M. B. A. Oldstone. 1986. Genetic reassortants of lymphocytic choriomeningitis virus: unexpected disease and mechanism of pathogenesis. *J. Virol.* **59**:363–368.
- Schulz, M., P. Aichele, M. Vollenweider, F. W. Bobe, F. Cardinaux, H. Hengartner, and R. M. Zinkernagel. 1989. Major histocompatibility complex-dependent T cell epitopes of lymphocytic choriomeningitis virus nucleoprotein and their protective capacity against viral disease. *Eur. J. Immunol.* **19**:1657–1668.
- Siegel, M. M., T. K. Walter, and A. R. Ablin. 1977. Measles pneumonia in childhood leukemia. *Pediatrics* **60**:38–40.
- Smith, G. L. 1993. Vaccinia virus glycoproteins and immune evasion. The sixteenth Fleming Lecture. *J. Gen. Virol.* **74**:1725–1740.
- Sparshott, S. M., and E. B. Bell. 1994. Membrane CD45R isoform exchange on CD4 T cells is rapid, frequent and dynamic in vivo. *Eur. J. Immunol.* **24**:2573–2578.
- Sprent, J. 1993. Lifespans of naive, memory and effector lymphocytes. *Curr. Opin. Immunol.* **5**:433–438.
- Spriggs, M. K., D. E. Hruby, C. R. Maliszewski, D. J. Pickup, J. E. Sims,

- R. M. Buller, and J. VanSlyke. 1992. Vaccinia and cowpox viruses encode a novel secreted interleukin-1-binding protein. *Cell* **71**:145–152.
48. Tishon, A., H. A. Lewicki, G. Rall, M. G. von Herrath, and M. B. A. Oldstone. 1995. An essential role for type I interferon-gamma in terminating persistent viral infection. *Virology* **212**:244–250.
49. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**:1745–1749.
50. von Herrath, M. G., J. Dockter, and M. B. Oldstone. 1994. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. *Immunity* **1**:231–242.
51. Whitton, J. L. Unpublished data.
52. Whitton, J. L. 1990. Lymphocytic choriomeningitis virus CTL. *Semin. Virol.* **1**:257–262.
53. Whitton, J. L. 1994. Induction of protective immunity using minigenes. *Ann. N.Y. Acad. Sci.* **730**:107–117.
54. Whitton, J. L., N. Sheng, M. B. A. Oldstone, and T. A. McKee. 1993. A “string-of-beads” vaccine, comprising linked minigenes, confers protection from lethal-dose virus challenge. *J. Virol.* **67**:348–352.
55. Whitton, J. L., P. J. Southern, and M. B. A. Oldstone. 1988. Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus. *Virology* **162**:321–327.
56. Whitton, J. L., A. Tishon, H. Lewicki, J. R. Gebhard, T. Cook, M. S. Salvato, E. Joly, and M. B. A. Oldstone. 1989. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. *J. Virol.* **63**:4303–4310.
57. Wright, K. E., and M. J. Buchmeier. 1991. Antiviral antibodies attenuate T-cell-mediated immunopathology following acute lymphocytic choriomeningitis virus infection. *J. Virol.* **65**:3001–3006.