

Impairment of CD4⁺ T Cell Responses during Chronic Virus Infection Prevents Neutralizing Antibody Responses against Virus Escape Mutants

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Abstract

We have shown previously that neutralizing antibodies (nAbs) are important contributors to the long-term immune control of lymphocytic choriomeningitis virus infection, particularly if cytotoxic T cell responses are low or absent. Nevertheless, virus escape from the nAb response due to mutations within the surface glycoprotein gene may subsequently allow the virus to persist. Here we show that most of the antibody-escape viral mutants retain their immunogenicity. We present evidence that the failure of the infected host to mount effective humoral responses against emerging neutralization-escape mutants correlates with the rapid loss of CD4⁺ T cell responsiveness during the establishment of viral persistence. Similar mechanisms may contribute to the persistence of some human pathogens such as hepatitis B and C viruses, and human immunodeficiency virus.

Key words: persistent infection • lymphocytic choriomeningitis virus • T helper cells • humoral responses • viral evasion

Introduction

Strong CD8⁺ CTL responses characterize the initial immunosurveillance of infections with poorly or noncytotoxic viruses such as hepatitis B and C viruses, HIV, and the murine RNA virus lymphocytic choriomeningitis virus (LCMV¹ [1–3]). Nevertheless, this initial clearance of viremia by CD8⁺ CTLs does not always prevent the establishment of a chronic infection. Besides CTLs, neutralizing Abs (nAbs) in association with other noncytolytic factors (IFN- γ , TNF- α , and chemokines) play a crucial role in controlling persisting virus infection (4–8). However, virus escape from the nAb response during chronic infections does occur and may contribute to viral persistence (9). We have recently demonstrated in a model system that low CTL activity during LCMV infection facilitates the detection of neutralization-escape virus variants (10). Escape was due to single point mutations within the genes coding for the envelope glycoprotein (GP)-1. The ensuing amino

acid changes affect the conformation of the neutralizing epitope (11).

The following questions have been further addressed in this study. (a) Are the envelope GPs of nAb-escape LCMV variants less immunogenic? (b) How quickly and efficiently are nAbs generated against emerging escape variants? (c) Do variants evolve that are in general neutralization resistant? (d) Is the impairment of nAb responses due to deletion or anergy of virus-specific T helper cells?

We studied the nAb response during a long-term infection of CD8^{-/-} mice with LCMV, strain WE (12). Due to the absence of CTLs in these mice, augmented virus production occurs, which is transiently controlled by polyclonal nAbs in the blood and only to a limited extent in solid organs (10). The results indicate that the broadening of nAb responses against emerging neutralization-resistant virus variants in this high viremia model infection was mainly limited by a decrease and eventual loss of virus-specific CD4⁺ T cell responses. Changes in replication properties or a decreased immunogenicity of some virus variants may additionally contribute to virus persistence.

Materials and Methods

Mice and Viruses. CD8^{-/-} mice (13), LCMV-GP61-80-specific CD4⁺ TCR transgenic (tg) SMARTA mice (14), and control C57BL/6 (B6) mice were obtained from the Institut für

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¹Abbreviations used in this paper: GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; nAb, neutralizing Ab; tg, transgenic; VSV, vesicular stomatitis virus; VSV-IND, VSV Indiana; wt, wild-type.

Labortierkunde (University of Zürich, Zürich, Switzerland). In certain experiments, B6 mice were *in vivo* depleted of CD8⁺ T cells by treatment with a tested anti-CD8 monoclonal Ab on days 3 and 1 preceding the infection, as described (10). The degree of depletion was always >95% in blood and spleen. All animals were kept under specific pathogen-free conditions.

LCMV strain WE originally was obtained from F. Lehmann-Grube (Heinrich Pette Institut, Hamburg, Germany) and propagated on L929 cells. Mice were infected with 2×10^6 PFU of LCMV-WE intravenously. LCMV titers in blood or virus titers of stock solutions were determined with an immunological focus assay (15).

LCMV nAb-escape variants were isolated from the blood of CD8^{-/-} mice (120 and 240 d after infection), grown on MC57 cells for 48 h, and subsequently plaque purified two times *in vitro* as described (16). For *de novo* infections, mice were immunized with 2×10^4 PFU of selected virus variants intravenously.

Vesicular stomatitis virus (VSV) Indiana (VSV-IND; Mudd-Summers isolate) was originally obtained from Dr. D. Kolakovsky (University of Geneva, Geneva, Switzerland) and was grown on BHK21 cells. Mice were infected with 2×10^6 PFU of VSV-IND intravenously.

Neutralizing Activity. Neutralizing activity against LCMV was measured in a focus reduction assay (15). The neutralizing titer was defined as the dilution causing half-maximal reduction of plaques of LCMV when compared with the same amount of virus incubated with control sera from uninfected mice. VSV neutralization assay was performed as described (17). The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer. To determine IgG Ab titers, undiluted serum was pretreated with 0.1 M β -mercaptoethanol.

Adoptive Transfers. Spleen cell suspensions were prepared from naive SMARTA tg mice previously *in vivo* depleted of CD8⁺ T cells by treatment with anti-CD8 monoclonal Abs. 5×10^5 SMARTA splenocytes were transferred intravenously into recipient B6 or CD8^{-/-} mice, 1 d before infection with 2×10^6 PFU of LCMV-WE. Both SMARTA and CD8^{-/-} mice have a pure B6 background. Clonal expansion and disappearance of transferred tg cells in the blood of recipients were monitored by FACS[®] analysis using TCR V α 2 and V β 8.3 monoclonal Abs.

Intracellular IFN- γ Staining. Splenocytes (10^6 cells/well in 96-well plates) were stimulated *in vitro* with media or with the immunodominant LCMV-specific peptide GP61-80 (1 μ g/ml). Brefeldin A (Sigma-Aldrich) was added after 1 h of culture. After a 5-h stimulation, the cells were harvested, washed once in PBS containing 2% FCS, and stained with PE-conjugated anti-CD4 monoclonal Abs (Caltag) for 30 min at 4°C. After washing of unbound Ab, the cells were fixed with PBS/4% paraformaldehyde for 10 min and then permeabilized with PBS/0.1% Saponin (Sigma-Aldrich). For intracellular staining, FITC-conjugated anti-IFN- γ monoclonal Abs were used and incubated with the cells for 30 min at 4°C. After washing twice with PBS/0.1% Saponin, the cells were resuspended in PBS containing 2% FCS and analyzed using a FACScan[™] (Becton Dickinson). To control for nonspecific intracellular staining, parallel samples of stimulated and permeabilized CD4⁺ T cells were stained with FITC-conjugated isotype-matched monoclonal Abs of irrelevant specificity, which did not result in any staining signal.

Molecular Analysis. Total RNA of MC57 cells infected either with LCMV-WE wild-type (wt) or with variant virus isolates for 48 h at an initial multiplicity of infection of 0.01 was extracted by using RNeasy kit (QIAGEN). Reverse transcription PCR was performed using the LCMV-GP1-specific primers R1 (5'-

1037TCG TAG CAT GTC ACA GAA CTC TTC_{1014-3'}) for reverse transcription and the primer pairs 001/R1 and 001/RC1 (001, 5'-₁CGC ACC GGG GAT CCT AGG CTT_{21-3'}; RC1, 5'-₉₆₅GAG CTC TGC AGC AAG GAT CAT CC_{942-3'}) for Hot Start PCR amplification. PCR products were sequenced by automated *Taq* cycle sequencing (*Taq* Dye Deoxy Terminator Cycle Sequencing kit; Applied Biosystems; Bio-Rad Laboratories) using the primers 001 and RC1.

Results

nAb Responses against Emerging nAb-escape Virus Mutants. Virus titers as well as nAb titers were sequentially determined in the blood of five LCMV-WE-infected CD8^{-/-} mice (animals M7–M11) for up to 240 d. As reported previously (10), nAb-mediated control of viremia, attained within 50–60 d after infection, was only transient and virus reappeared in the blood 2–4 wk after initial control (Fig. 1). This occurred despite the presence of relatively high titers of nAbs (Fig. 2, filled circles). Viremia was not controlled at later time points in CD8^{-/-} mice (Fig. 1), suggesting that induction of new nAb responses against emerging neutralization-resistant virus variants had failed. To assess long-term virus-specific humoral and T helper responses in these mice, we next characterized the virus variants emerging *in vivo*. Virus was isolated from the blood of mice M7–M11 after the recrudescence of viremia (day 120). Sequence analysis of the gene encoding the envelope GP1 of LCMV isolates revealed amino acid alterations of the predominant viral clone (WE-M7 to WE-M11) within the bulk virus isolated from each animal (at least 5 out of 8–10 independent clones; Table I). One to three base pair exchanges per GP1 gene were identified, leading to amino acid substitutions within the three regions of GP1 that have been shown to correlate with virus escape from the nAb response (10). These mutations affected the efficiency of variant virus neutralization by polyclonal hyperimmune serum (pooled from B6 mice immunized with LCMV-WE) and by LCMV-WE-GP1-specific mAb (data not shown).

We next performed autologous serum neutralization assays with virus variants WE-M7 to WE-M11 and with

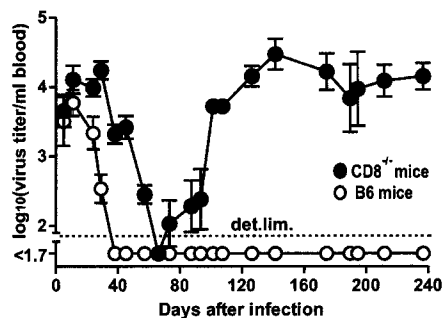


Figure 1. Transient control of viremia in CD8^{-/-} mice. CD8^{-/-} mice (animals M7 to M11; ●) and control B6 mice (○) were infected with 2×10^6 PFU of LCMV-WE-wt intravenously, and sequential blood samples were analyzed for virus titers. Data shown are the mean for five mice \pm SEM.

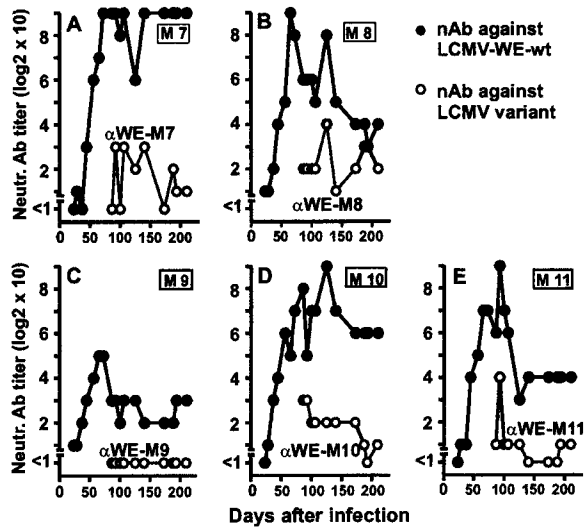


Figure 2. NAb responses against immunizing LCMV-WE-wt and emerging LCMV variants in five individual CD8^{-/-} mice. (A–E) Five CD8^{-/-} mice (animals M7–M11) were infected with 2×10^6 PFU of LCMV-WE-wt. Sequential serum samples were tested for neutralizing (Neutr.) activity against LCMV-WE-wt (●) and against the predominant LCMV variant (WE-M7 to WE-M11; ○) isolated from the corresponding mouse after viral reemergence on day 120 after infection (see characterization of variants in Table I). α , anti.

LCMV-WE-wt (Fig. 2). The strong initial nAb response against the immunizing virus (LCMV-WE-wt) peaked around day 75 and declined very slowly. In contrast, low or no neutralizing activity was detected against virus escape variants at all time points tested. Hence, these variants have

Table I. LCMV-WE nAb-escape Variants Contain Amino Acid-changing Point Mutations within the Sequence Coding for the Envelope Protein GP1 (Amino Acids 59–262)

| Mouse | Day | Virus | Amino acid substitution at indicated position | | | | |
|-------|-----|------------|-----------------------------------------------|-----|-----|-----|-----|
| | | | 122 | 177 | 182 | 211 | 212 |
| | 0 | LCMV-WE-wt | Phe | Pro | Ser | Ala | Gly |
| M7 | 120 | WE-M7 | | | Arg | | Asp |
| | 240 | | | | Arg | | Asp |
| M8 | 120 | WE-M8 | | | Asn | | |
| | 240 | | Ser | | Asn | | |
| M9 | 120 | WE-M9 | Ser | | | | Asp |
| | 240 | | Ser | | | | Asp |
| M10 | 120 | WE-M10 | Ser | Leu | | Thr | |
| M11 | 120 | WE-M11 | | | Asn | Thr | |

Virus was isolated from the blood of five CD8^{-/-} mice (M7 to M11) 120 and 240 d after infection with 2×10^6 PFU of LCMV-WE and double plaque purified. Positions of alterations in deduced amino acid residues of the envelope GP1 of the predominant viral clone (at least 5 out of 8–10 clones) from each animal are shown.

indeed escaped the original nAb response, and failed to induce specific nAbs over a period of 120 d (Fig. 2). The failure of the variants to induce an effective nAb response could have several reasons. Theoretically, we cannot exclude that novel nAbs are generated, as this could be masked by outgrowth of newly evolving escape mutants. We therefore sequenced day 240 isolates from the blood of three test animals (M7, M8, and M9). The GP1 sequence of the predominant clone (at least 5/8 isolates) is shown in Table I. No sequence changes were observed when viral isolates derived on day 120 and 240 after infection were compared in mice M7 and M9, respectively. In mouse M8, the predominant virus clone had one additional amino acid-changing mutation at position 122 on day 240, compared with day 120. Nevertheless, a clone with this genotype was already present in the viral quasispecies on day 120 (1/8 clones; data not shown). Overall, the escape mutants were relatively stable, which suggests the absence of a specific newly induced immune selection pressure. Selection of predominant virus variants within the quasispecies at late time points, as seen in animal M8, might therefore be influenced by viral fitness (18).

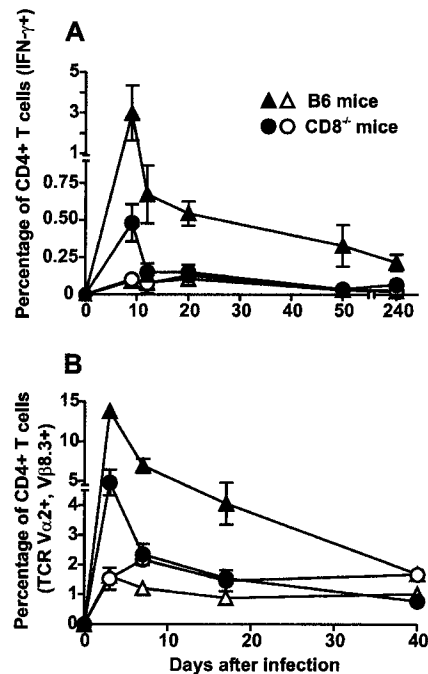


Figure 3. Induction of specific CD4⁺ T cell unresponsiveness in LCMV-infected CD8^{-/-} mice. B6 mice (triangles) and CD8^{-/-} mice (circles) were infected with 2×10^6 PFU LCMV-WE intravenously. (A) Splenocytes from the indicated days after infection were stimulated in vitro with the immunodominant LCMV class II-restricted epitope (GP61–80; filled symbols) or with no peptide (open symbols), and the percentage of peptide-specific CD4⁺ T cells expressing intracellular IFN- γ was then assessed. (B) 5×10^5 splenocytes from in vivo CD8-depleted, LCMV-GP61–80-specific CD4⁺ TCR tg SMARTA mice (B6 background) were adoptively transferred intravenously to recipient B6 mice that were either infected with LCMV-WE 1 d later (filled symbols) or were not infected (open symbols). The percentage of tg CD4⁺ T cells (TCR V α 2⁺; V β 8.3⁺) in the blood was then sequentially determined by FACS[®] analysis. Data shown are the mean for four mice \pm SEM.

Loss of CD4⁺ T Cell Responsiveness Precedes the Emergence of nAb-escape Virus Mutants. Failure of the hosts to elicit new nAb responses against emerging nAb-escape virus variants could be due to insufficient CD4⁺ T helper responses at late time points. This could be the consequence of CD4⁺ T cell unresponsiveness induced during establishment of persistent infection (19), T helper epitope variation, or a decrease of infected CD4⁺ T cells.

LCMV-specific CD4⁺ T cell responses were compared between LCMV-infected CD8^{-/-} mice, which show a high and sustained viremia, particularly in solid organs, and control B6 mice, which rapidly control the virus (10; Fig. 1). We monitored the number of splenic CD4⁺ T cells specific for the LCMV-immunodominant epitope GP61–80 and expressing intracellular IFN- γ after antigenic stimulation at different time points after infection (Fig. 3 A). In agreement with previous studies (20–22), this response peaked at day 9 in control B6 mice (\sim 3% of CD4⁺ T cells) and decreased to 0.4% by day 50. This percentage was stable in the memory phase (up to 240 d). By contrast, only 0.6% of CD4⁺ T cells stained positive for intracellular IFN- γ at the peak of the response in CD8^{-/-} mice. This percentage rapidly dropped to background levels by day 20. In CD8^{-/-} test animals M7 to M11, only background levels of virus-specific CD4⁺ T helper activity were detected on day 240 (Fig. 3 A), irrespective of the ability of the mice to produce low or high nAb titers against the immunizing LCMV-WE-wt virus (Fig. 2). We were not able to monitor an additional T helper response against the nucleoprotein NP309–328 epitope because on day 8 after infection, frequencies of specific CD4⁺ T cells were below background levels in LCMV-WE-infected CD8^{-/-} mice. To ascertain that the responses measured in the *in vitro* assays were not affected by the absence of CD8⁺ T cells, we performed the following control experiment. LCMV-WE-immune B6 mice (70 d after infection) were either depleted of CD8⁺ T cells or left untreated. We then evaluated the number of splenic LCMV-specific, IFN- γ -expressing CD4⁺ T cells. Similar percentages of GP61–80-specific CD4⁺ T cells were measured in the spleens of mice of the two groups (data not shown). As indicated by sequence analysis, failure to detect GP61–80-specific CD4⁺ T cells in CD8^{-/-} mice was not due to epitope variation (Table I, and data not shown).

As mentioned above, several factors could account for the low levels of CD4⁺ T cells in the high viremia CD8^{-/-} model system. Loss of total CD4⁺ T cell population due to infection and/or immunopathology, specific unresponsiveness of the LCMV-specific T helper subset, but also a deficiency in generating the initial response, have to be considered. To specifically study the effects of persistent high viremia on CD4⁺ T cell expansion, we performed adoptive transfer experiments with LCMV GP61–80 epitope-specific splenocytes from *in vivo* CD8-depleted SMARTA TCR tg mice. This approach allowed for monitoring of the transferred LCMV-specific T helper cells via the tg TCR V α and V β chains (Fig. 3 B). Compared with B6 mice, clonal expansion of the transferred tg CD4⁺ T cells

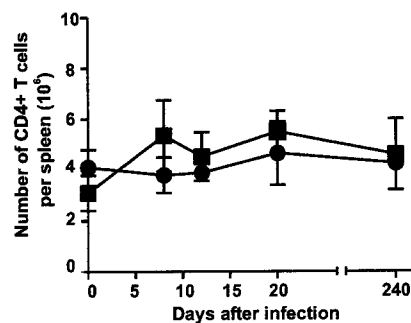


Figure 4. The CD4⁺ T cell population is not depleted during LCMV infection. B6 mice and CD8^{-/-} mice were infected with 2×10^6 PFU of LCMV-WE. Splenocytes at various time points after infection were stained for CD4. Numbers of CD4⁺ T cells were determined by flow cytometry. Data shown are the mean of three mice per group \pm SEM.

was compromised in LCMV-infected CD8^{-/-} mice. Moreover, we found that these cells disappeared more rapidly from the blood of recipient LCMV-infected CD8^{-/-} mice. The tg CD4⁺ T cells were still present in high numbers on day 16 in the blood of LCMV-infected B6 mice and were still above background levels by about day 40. No obvious differences were seen in uninfected B6 and CD8^{-/-} mice, suggesting that the effects seen were a consequence of high level viral replication.

To investigate whether the reduction of LCMV-specific T helper cells was due to an overall decline of the CD4⁺ T cell population in the high viremia model, we assessed the absolute number of splenic CD4⁺ T cells by flow cytometry before and after LCMV infection. No depletion of the general CD4⁺ T cell pool was detected after infection of CD8^{-/-} mice with LCMV-WE (Fig. 4).

To test whether loss of CD4⁺ T cells during high viral replication was restricted to LCMV-specific cells or whether unrelated, non-LCMV responses were also affected, we evaluated CD4 T cell help against VSV. VSV induces a very early neutralizing IgM Ab response that is largely T cell help independent; the subsequent IgM switch

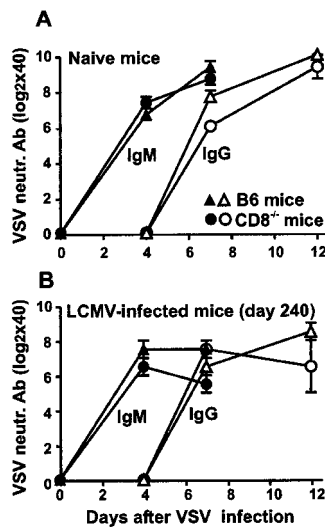


Figure 5. Functional VSV-specific T cell help in LCMV-WE infected CD8^{-/-} mice. B6 mice (triangles) and CD8^{-/-} mice (circles) infected with 2×10^6 PFU of LCMV-WE 240 d previously (B), as well as naive control animals (A), were immunized with 2×10^6 PFU of VSV-IND. Sequential serum samples were obtained for quantitation of VSV-neutralizing (neutr.) IgM Ab titers (filled symbols) or IgG Ab titers (open symbols), distinguished from IgM by reduction with 0.1 M of β -mercaptoethanol. The results are given as the mean \pm SEM of three to four mice per group.

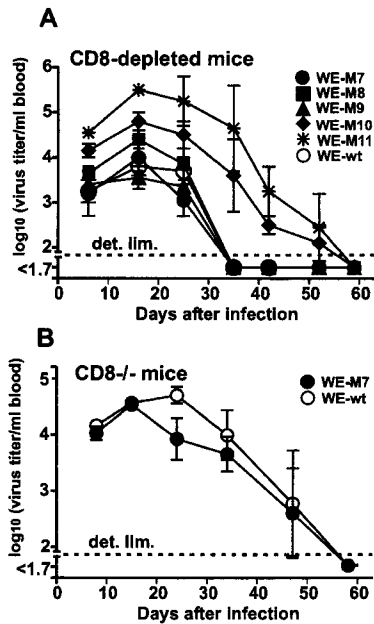


Figure 6. In vivo replication of nAb-escape variants. CD8-depleted B6 mice (A) and CD8^{-/-} mice (B) were infected with 2×10^4 PFU of LCMV-WE-wt or of the indicated nAb-escape LCMV variant (for CD8^{-/-} mice only variant WE-M7). Sequential blood samples were obtained for quantitation of virus titers. Data shown are the mean of three to four mice per group \pm SEM. The experiment shown in A was performed twice with similar results.

to IgG is in contrast strictly dependent on functional CD4⁺ T cell help (23). CD8^{-/-} and B6 mice, 240 d after LCMV infection, and uninfected control animals were immunized with 2×10^6 PFU of VSV-IND intravenously. Anti-VSV IgM and IgG Ab titers were assessed in sequential serum samples (Fig. 5, A and B). LCMV-infected CD8^{-/-} mice were able to switch their anti-VSV IgM Ab responses to IgG, proving that functional VSV-specific CD4⁺ T cell help was present (Fig. 5 B). Hence, the time-dependent induction of T helper unresponsiveness in CD8^{-/-} mice was LCMV specific.

Immunogenicity of nAb-escape Virus Variants. In addition to insufficient CD4 T help, nAb responses against escape viruses might be low as a consequence of decreased immunogenicity of their envelope proteins. To study the immunogenicity of nAb-escape variants in vivo, we have chosen to use the model infection of mice depleted in vivo of CD8⁺ T cells by treatment with anti-CD8 monoclonal Abs. In contrast to CD8^{-/-} mice, earlier virus control through the antiviral activity of reappearing CTLs takes place in this model (10). Therefore, due to the lower viremia, less Ab is masked by excess antigen. Escape mutants were tested in infections with 2×10^4 PFU, a dose available for all variants. Two isolates (WE-M10 and WE-M11) persisted for longer periods in CD8-depleted mice, whereas WE-M7, WE-M8, and WE-M9 were eliminated with similar kinetics as LCMV-WE-wt (Fig. 6 A). In contrast to our previous study (10), in which neutralization-resistant variants that showed enhanced persistence induced

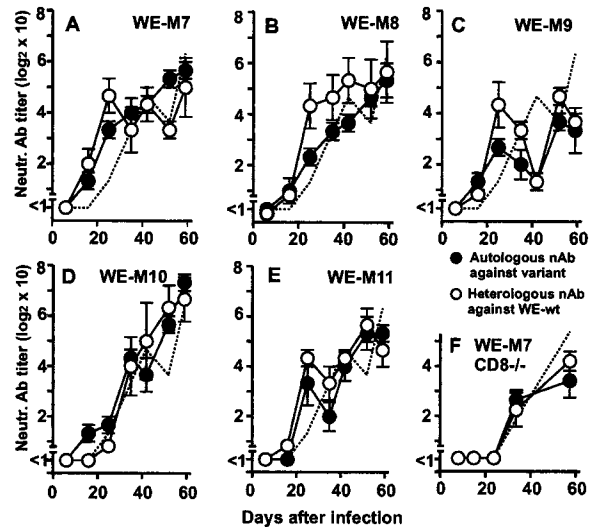


Figure 7. Immunogenicity of LCMV-WE nAb-escape variants. (A–E) Sequential blood samples from CD8-depleted mice infected with 2×10^4 PFU of five nAb-escape variants WE-M7 (A), WE-M8 (B), WE-M9 (C), WE-M10 (D), and WE-M11 (E) were obtained for quantitation of nAb titers against the immunizing virus (●) and of nAb titers against LCMV-WE-wt (○). The dotted lines represent the autologous nAb response after infection of CD8-depleted mice with an equivalent dose of LCMV-WE-wt. The experiment was repeated in CD8^{-/-} mice for variant WE-M7 (F). Data shown are the mean for three mice per group \pm SEM. Neutr., neutralizing.

lower autologous nAb titers, all variants studied here raised autologous nAb responses similar to LCMV-WE-wt (Fig. 7, A–E). Interestingly, heterologous nAb titers against LCMV-WE-wt in mice infected with variant viruses were at least as high as autologous titers, demonstrating a broad neutralizing response in these mice (Fig. 7, A–E).

To assess whether differences in immunogenicity are dependent on the animal model used, we performed de novo infection experiments with 2×10^4 PFU of WE-M7 isolate and LCMV-WE-wt in CD8^{-/-} mice. Infection with the variant strain was cleared with similar kinetics (within 60 d) as wt virus in these animals (Fig. 6 B). Moreover, both variant and wt virus induced an equally strong neutralization response (Fig. 7 F). Of note is that the nAb response in CD8^{-/-} mice was, for both wt and escape virus, lower than in CD8-depleted mice.

Discussion

We have previously introduced a LCMV infection model in CD8-deficient mice that is characterized by a high persistent viremia, and that only transiently is controlled by nAb, as neutralization-resistant variants are generated very rapidly (10). This model of prolonged absence of CD8⁺ T cells is reflected in several virus diseases in which low or absent CTL activity during establishment of persistent infections occurs. CTL responses may exhaust or become unresponsive after overwhelming infection with LCMV (24, 25), may physically or functionally disappear during HIV-1 infection (26–28), and become scarce once

infection with hepatitis C virus is established (3). In addition, viral escape from CTL responses through selection of mutations in the relevant epitopes has been documented (29–32), and may even occur early in the course of infection (33, 34).

As documented here, several factors may allow the persistence of emerging neutralization-resistant variants. A weaker immunogenicity of the escape variants seems not to be a general phenomenon, as most of the variants were able to induce autologous nAb responses after *de novo* infections similar to wt LCMV-WE. However, we observed an asymmetric pattern of cross-reactivity between neutralizing responses induced by wt and escape virus isolates. Whereas the original wt strain induced a response against itself but not against the emerging escape variants, the mutant viruses were able to induce nAbs that inhibited both wt and escape viruses. Thus, the variant viruses reveal via nAbs a recapitulation of their evolution and exhibit a new sort of coevolutionarily directed connectivity that one might call “archetypical”. In some aspects, this recall of genetic history is reminiscent of findings during influenza virus infections in the context of preexisting immune memory: after infection with an escape mutant after antigenic drift, influenza-immune individuals will generate higher Ab titers against the influenza surface hemagglutinin experienced during the original infection than against the mutated hemagglutinin of the drifted variant. This has been called original antigenic sin (35, 36).

Other factors may contribute to the long-term persistence of nAb-resistant viruses, depending on the variant analyzed. Some variants may have better intrinsic replication capacities *in vivo*. Alternatively, changes in cell or tissue tropism, as well as possible differences in resistance to interferons, may account for the observed enhanced persistence. All these alterations may be the consequence of some of the documented mutations in the GP1 gene, as demonstrated at several occasions for different LCMV strains (37–39). In addition, mutations in other genes cannot be excluded, particularly on the L strand which encodes the viral polymerase and which was not sequenced in this study.

However, the most important mechanism leading to the persistence of emerging nAb-escape variants is the induction of specific CD4⁺ T cell unresponsiveness, as demonstrated here. The absence of an adequate T helper response at the moment of viral escape impaired the induction of new nAb responses against the virus mutants and allowed them to persist. Interestingly, the primary nAb response in CD8^{-/-} mice after infection with LCMV-WE was very potent, despite the fact that the CD4⁺ T cell expansion was much lower than in normal mice and the helper response was rapidly lost. Several factors may account for this finding. First, virus-specific B cells, infected through the LCMV-GP1-recognizing receptor, are not killed in the absence of CTL responses (40). Second, the antigen load is much higher in CD8^{-/-} mice. On the other hand, memory B cell responses may be less dependent on functional T help (41), explaining how high nAb titers can be main-

tained in CD8^{-/-} mice against the immunizing LCMV-WE-wt despite vanishing T helper responses.

The CD4⁺ T cell dysfunction in CD8^{-/-} mice does not affect the overall CD4⁺ T cell population and is only confined to the LCMV-specific compartment. Furthermore, as exemplified by the adoptive transfer of tg SMARTA splenocytes into LCMV-infected CD8^{-/-} mice, LCMV-specific CD4⁺ T cell numbers remain equal to levels in uninfected control animals for at least 3 wk, indicating that the cells might undergo a phase of functional unresponsiveness before being physically deleted.

It has to be emphasized that CD8^{-/-} mice are able to mount vigorous CD4⁺ T cell responses when infected with a low dose of a slowly replicating LCMV strain (LCMV-Armstrong; up to 14% at day 9; reference 21). An influence of elevated virus titers on the fate of T helper responses is indicated by the fact that a similar low percentage of LCMV-specific CD4⁺ T cells was reached during infection with the clone 13 strain of LCMV (21), which is able to establish persistent infections in immunocompetent mice (37). The high viral replication level achieved in CD8^{-/-} mice (but not B6 mice) infected with LCMV-WE is comparable to the overwhelming infection of immunocompetent hosts by the rapidly replicating Docile strain of LCMV, where virus-specific T helper cell loss has also been shown (19). As CD8⁺ T cells may also contribute positively to T helper cell responsiveness via bystander factors (42), their absence in CD8^{-/-} mice might also impact on the loss of specific CD4⁺ T cells.

Decrease of virus-specific T helper cells seems to be a critical evolutionary phenomenon which allows the survival of the host by preventing lethal immunopathology during persisting noncytopathic virus infection (19). The molecular mechanisms involved in the loss of T cell function remain unclear. As hypothesized for the described exhaustion of CTLs (24, 43), high antigenic load after wide viral spread may induce a general activation of all available virus-specific CD4⁺ T cells. These cells then would die of activation-induced apoptosis preceded by a period of anergy. Replenishment of virus-specific T helper cells would not be possible as a consequence of negative T cell selection in the infected thymus (44). Other contributing factors like interleukin starvation have been postulated, but their involvement has not been conclusively demonstrated.

The relevance of this *in vivo* model of inactivation of specific CD4⁺ T cells is based on increasing evidence for a role of T helper cells in the immune control of noncytopathic viral infections, by supporting not only nAb responses, but also CTLs (45). Studies of LCMV-infected mice deficient in CD4⁺ T cells either by *in vivo* treatment with anti-CD4 monoclonal Ab or by gene targeting have demonstrated a loss of long-term functional CTL responses and the enhancement of viral persistence in the absence of T helper cells (4, 5, 25, 46–49). Low CD4⁺ T cell responses have also been shown to correlate with high viral load (50, 51) and with low CTL responses (52) during HIV infection. Similarly, hepatitis C virus-specific CD4⁺ T cells seem to be essential not only for initial viral clearance, but

also for long-term viral control (53). With regard to the impact of early viral load on subsequent impairment of T helper responses, it has been recently shown that early suppression of HIV-1 by highly active antiretroviral therapy may preserve CD4⁺ and CD8⁺ T cell function and may be associated with immune control (54, 55). The preserved T helper cell function may allow multiple rounds of nAb-escape and may therefore lead to the observed broadly cross-reactive nAb responses in HIV-infected long-term nonprogressors (56).

Together with earlier observations demonstrating an influence of nAb-producing B cells on LCMV long-term control (4, 5, 10, 57), this study provides further evidence for the interactions between cellular and humoral immune responses for efficient virus control. However, an adequate balance between the two arms of the acquired immune system is needed in order to avoid virus escape due to virus variant selection through a unilateral immune response.

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