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Protective Efficacy of Individual CD8+ T Cell Specificities in Chronic Viral Infection

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Specific CD8+ T cells (CTLs) play an important role in resolving protracted infection with hepatitis B and C virus in humans and lymphocytic choriomeningitis virus (LCMV) in mice. The contribution of individual CTL specificities to chronic virus control, as well as epitope-specific patterns in timing and persistence of antiviral selection pressure, remain, however, incompletely defined. To monitor and characterize the antiviral efficacy of individual CTL specificities throughout the course of chronic infection, we coinoculated mice with a mixture of wild-type LCMV and genetically engineered CTL epitope-deficient mutant virus. A quantitative longitudinal assessment of viral competition revealed that mice continuously exerted CTL selection pressure on the persisting virus population. The timing of selection pressure characterized individual epitope specificities, and its magnitude varied considerably between individual mice. This longitudinal assessment of “antiviral efficacy” provides a novel parameter to characterize CTL responses in chronic viral infection. It demonstrates remarkable perseverance of all antiviral CTL specificities studied, thus raising hope for therapeutic vaccination in the treatment of persistent viral diseases. The Journal of Immunology, 2015, 194: 1755–1762.

In accordance with initial observations in lymphocytic choriomeningitis virus (LCMV)-infected mice (2, 3), CD8+ T cells (CTLs) have been shown to play a key role in containing and resolving persistent viral infections in humans. HIV and HCV viremia decline when the antiviral CD8+ T cell response emerges (4–6). HIV long-term nonprogression, as well as spontaneous clearance of HCV, is associated with so-called protective HLA molecules (7, 8), corroborating the role of MHC class I–restricted T cells in viral control. Moreover, CD8+ T cell depletion experiments in SIV-infected macaques and HBV- or HCV-infected chimpanzees underscore the importance of CTLs in the acute phase of infection, as well as in long-term control and acquired immunity (9, 10).

Infection of mice with the LCMV strain Clone 13 (Cl13) has demonstrated governing principles of CTL responses in chronic viral infection (11–13), finding subsequent confirmation in the aforementioned human diseases (1). As a common finding, persisting viral infections with continuous exposure to a high Ag burden tend to subvert specific CTL responses (1). Fig. 1A and 1B and Supplemental Fig. 1A–C provide an illustration of reduced CTL functionality in Cl13 chronically infected mice, as compared with CTLs originating from acute infection with the Armstrong strain of LCMV. If not physically deleted (11), such CTLs gradually lose IL-2 and TNF-α production but tend to retain IFN-γ secretion and CD107a expression (12–14). “Exhaustion” therefore refers to mostly monofunctional to bifunctional CTL populations with a relative paucity in polyfunctional cells, and is associated with the upregulation of inhibitory receptors such as programmed cell death 1 (Fig. 1B) (15).

The course of Cl13 infection in C57BL/6 mice has resemblance to spontaneous HBV or HCV control in humans in that the immune system eventually prevails and clears the virus from blood after 2–3 mo (16). The underlying mechanisms remain, however, incompletely understood. Specific Ab responses and T follicular helper cell–driven germinal center reactions are necessary but not sufficient to resolve protracted infection (17, 18). C57BL/6 (H-2b) mice also mount a very potent and broad antiviral CTL response,

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Abbreviations used in this article: Cl13, LCMV strain Clone 13; CTL, CD8+ T cell; HBV, hepatitis B virus; HCV, hepatitis C virus; LCMV, lymphocytic choriomeningitis virus; TDSP, time-dependent CTL selection pressure; wt, wild-type.

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coadministration in one single syringe. When given as individual infection, CI13 was given at a dose of 200 PFU (Supplemental Fig. 1E–I) or 2 × 10^7 PFU i.v. (Fig. 1 and Supplemental Fig. 1A–C), and 10^8 PFU LCMV Armstrong was administered i.p.

T cell assays

Intracellular cytokine assays were performed on splenocytes as previously described (30, 32). For the assessment of degranulation, anti-CD107a Ab was added to the restimulation culture at a concentration of 1 μg/ml. Peptides (90% pure) of the sequences displayed in Fig. 1D and Supplemental Fig. 1E were purchased from ProImmune. MHC class I tetramers (GP33: H-2K^d/KA VYNFATC; GP276: H-2K^d/ SGVENPGGYCL) were supplied by Beckman Coulter. Abs were from BD Biosciences and Biolegend. Flow cytometry was performed on BD FACS Calibur and Beckman Coulter Gallios flow cytometers.

Assessment of viral loads by TaqMan RT-PCR

Mouse serum was collected into microtainer tubes (BD Biosciences) and viral RNA was extracted using the Qia-gen 96 DNA blood kit. TaqMan RT-PCR was performed using Invitrogen SuperScript III One Step Platinum Taq. Primers and probes were ordered through Sigma Aldrich. To detect the natural CI13 NP sequence (present in epitope-escape variants), we used forward primer 5′-AGCTAGAGGCTACAAGCGG-3′, reverse primer 5′-CAAGTACTCCACACCGCATGGA-3′, and the probe 5′-FAM- CTTGCCAC-CTTCTCAATGCGCA-BHQ1-3′ (33). For detection of the “tagged” sequence (in epitope-sufficient wt virus, used in competition experiments), we designed forward primer 5′-GTCTCTGTAACCTGTGFTT-3′, reverse primer 5′-TAAACGACACAGCAATATAGCTATG-3′, and the probe 5′-HEX- caflTrtGTagCgc-BHQ1-3′ (lowercase letters indicate locked nucleotides). Measurements were performed on an ABI TaqMan device. Absolute viral RNA copies per 3.3 μl serum were calculated by comparison with in vitro transcript RNAs (cf. Supplemental Fig. 1F–L), which served as reference in all TaqMan assays. Further details on cycling conditions and the generation of in vitro transcripts are available from the authors upon request.

Quantification of epitope-specific CTL selection pressure

Mathematical model to determine selection. To infer the selection pressure exerted by CTLs specific for individual epitopes, we used the following mathematical model published by Ganusov and De Boer (34). Wild-type, wt(t), and epitope-mutant virus, m(t), are assumed to grow at a time-dependent rate, r(t), and die at a common time-dependent death rate, d(t). In addition, the wt virus can be killed at a time-dependent rate, k(t), denoting the epitope-specific CTL response. The change of wt and epitope-mutant virus over time was then described by the following system of ordinary differential equations:

\[
dw/dt = r(t)w - d(t)w - k(t)w \tag{1}
\]

\[
dm/dt = r(t)m - d(t)m \tag{2}
\]

By considering the ratio of wt and epitope-mutant virus we could directly estimate the time-dependent CTL selection pressure (TDSP), k(t), exerted on the epitope in question. Defining \(z(t) = w(t)/m(t)\), we obtained

\[
k(t) = -d(t)\frac{dz(t)}{dt} \tag{3}
\]

Thus, the selection pressure could be obtained from the change over time of the log ratio of wt and epitope-mutant virus. Because we were only interested in the change of \(\ln(z(t))\) over time, the initial ratio between wt and epitope-mutant was irrelevant. The parameter \(k(t)\) denotes the net-selection pressure, that is, the difference between the selective advantage of the epitope-mutant compared with the wt, because of the escaped CTL response, and the disadvantage due, for example, to fitness costs. Hence negative values of \(k(t)\) could indicate that the fitness cost of the epitope-mutant outweighs its selective advantage over the wt. In most instances, however, negative \(k(t)\) values are due to viral loads falling below detection limits, thus preventing an accurate determination of wt/mutant virus ratios at the respective time points (outlined in more detail in Calculating the Average Selection Coefficient \(s\)). It is equally conceivable that mechanisms such as transient and compartmentalized CTL selection pressure result in periods of negative \(k(t)\).

Application to the data. The time-dependent selection curve \(k(t)\) for each individual mouse was determined by using Eq. 3. A smooth cubic spline function was fitted to the individual data points given by \(-\ln(w(t)/m(t))\), where \(w(t)\) and \(m(t)\), \(i = 1, \ldots, n\) denote the measured viral load for wt and epitope-mutant virus, respectively, at time point \(i\). The time-dependent

Materials and Methods

Ethics statement

Animal experiments were performed at the Universities of Geneva and Zurich, and have been approved by the Direction générale de la santé (permissions 1005/3312/2 and 1005/3312/2-R) of the Canton of Geneva, and the Cantonal Veterinary Office of the Canton of Zurich (permission 176/2005), respectively. All animal experiments were performed in accordance with the Swiss law for animal protection.

Mice

C57BL/6, H-2Db^+/−, and H-2K^b^+/− (29) mice were bred at the Institute for Laboratory Animal Sciences of the University of Zurich, Switzerland, and BALB/c mice were obtained from Harlan Laboratories. All mice were maintained in specific pathogen-free conditions.

Generation and titration of viruses, Ab neutralization, and infection of mice

CI13 and variants thereof were generated from cDNA by reverse genetic techniques (30). To generate a “tagged” virus for discrimination from a competitor virus by TaqMan RT-PCR (Fig. 1C, 1E) and to introduce single amino acid changes resulting in deletion of select CTL epitopes (Fig. 1D and Supplemental Fig. 1E–I), we used standard two-way site-directed mutagenesis on the LCMV S segment expression plasmid pL-S-C113(-)* (30) that was used for the generation of LCMV CI13 from cDNA (primer sequences are available from the authors upon request). The codons of the mutated amino acids were chosen to be as different from the wt amino acid’s codons as possible, to avoid viral reversion by a single nucleotide change. All viruses were grown on BHK-21 cells. Infectious titers and the potency of antiviral Abs were assessed by immunofocus assay and focus reduction neutralization tests, respectively, as previously described (30, 31). Mice were infected with a 10:1 ratio of wt/epitope-mutant virus, that is, 2 × 10^6 and 2 × 10^5 PFU, respectively, mixed for i.v.
selection curves $k(t)$ were then estimated by the first derivative of the obtained spline function. Measurements of wt and epitope-mutant virus below the detection limit (100 copies/3.3 µl) were set to half of the detection limit (50 copies/3.3 µl). Using other values for the replacement, for example, setting measurements below the detection limit to 99 copies/3.3 µl, did not change the results overall.

Calculating the average selection coefficient $\kappa$. We assumed that the observed variation in the log-ratio between wt and epitope-mutant virus in BALB/c mice represented effects unrelated to the epitope-specific CTL response. Therefore, for each epitope, we used the BALB/c mice to calculate a function, $k_{\text{BALB/c}}(t)$, representing background noise in $k(t)$ due to fluctuation of ratios of wt and epitope-mutant virus in the absence of selection and also reflecting potential fitness costs for the epitope-mutant viruses. To this end, a smooth cubic spline function was fitted to the pooled data of all BALB/c mice tested for a given epitope, resulting in the same translation product. The “background-selection” function, $k_{\text{BG}}(t)$, was then determined by performing a bootstrap analysis with 10,000 replicates resampling from the data of the BALB/c mice and taking the time-wise 97.5% upper limit of the obtained values of $k_{\text{BALB/c}}(t)$.

To calculate the average selection coefficient over time for each individual mouse, we considered only those parts of the individual selection curves where the individual selection was larger than the estimated background noise, $k(t) > k_{\text{BG}}(t)$. Hence the average selection coefficient $\kappa$ until time point $T$ was calculated by

$$\kappa = \frac{1}{T} \int_0^T k^+(t) \, dt$$

with $k^+(t) = k(t) - k_{\text{BG}}(t)$ if $k(t) > k_{\text{BG}}(t)$ and 0 otherwise. Hereby, $t^+$ denoted the total length of the time intervals in $[0,T]$ where $k(t) > k_{\text{BG}}(t)$. By only considering the time intervals where the individual selection was larger than the background selection, $k(t) > k_{\text{BG}}(t)$, we accounted for the fact that negative selection can be due to viral loads falling below detection limits (see, e.g., wt virus in strongly selecting C57BL/6 mice; top panels of Fig. 2). This prevented an accurate determination of wt/mutant virus ratios at the respective time points, with TDSF artificially turning negative. By considering only $k(t) > k_{\text{BG}}(t)$ values, our method to calculate the average selection coefficient $\kappa$ focused on unambiguous signals of selection.

Time-dependent selection bands for C57BL/6 mice. Analogously to the function representing background noise in $k(t)$ (due to fluctuation in ratios of wt and epitope-mutant virus in the absence of selection), time-dependent selection bands were calculated for the C57BL/6 mice, individually for each epitope. A smooth cubic spline function was fitted to the pooled data of all C57BL/6 mice for one epitope, and the first derivative was estimated to obtain $k_{\text{C57BL/6}}(t)$. The “background-selection” function, $k_{\text{BG}}(t)$, was then determined by performing a bootstrap analysis with 10,000 replicates resampling from the data of the C57BL/6 mice and taking the time-wise 97.5% upper limit and 2.75% lower limits of the obtained values of $k_{\text{C57BL/6}}(t)$.

$\text{FIGURE 1. Characterization of exhausted CTLs and experimental approach for assessing their antiviral efficacy in protracted infection. (A and B)}$ We infected C57BL/6 mice with LCMV Armstrong (acute) or LCMV strain Clone 13 (chronic). On day 20, we assessed GP33-specific CTLs by intracellular cytokine/marker combinations displayed (complete data sets in Supplemental Fig. 1A–C). Mean fluorescence intensity in (B) is displayed as mean ± SEM of three mice per group. Frequent cytokine/marker combinations are displayed in (A) and surface programmed cell death 1 expression in (B). Bars in (A) represent the mean ± SEM of three mice per group. Frequency cytokine/marker combinations are displayed (complete data sets in Supplemental Fig. 1A–C). Mean fluorescence intensity in (B) is displayed as mean ± SD of three mice. (C) Experimental approach: mice were coinfected with CTL epitope-mutant (MUT) and wt virus at the indicated dose. (D) Amino acid sequences of the wt and mutant CTL epitopes. (E) Target sequences of the TaqMan RT-PCR assays used to individually quantify epitope-mutant virus (normal viral nt sequence) and wt virus (tagged viral nt sequence) resulting in the same translation product. (F) We infected C57BL/6 mice with $2 \times 10^5$ PFU of either “tagged” wt Cl13 or “normal” wt Cl13 and monitored viremia over time. (G–I) Schematic of viral load curves (G) with epitope-mutant/wt virus ratios (H) and time-dependent selection coefficients $k(t)$ of prototypic selecting (C57BL/6, H-2b) or nonselecting (BALB/c, H-2d) hosts. Shaded gray area in (I) denotes the 95% confidence interval of background $k(t)$ determined in BALB/c mice.
$k_{C57BL/6}(t)$. For these latter analyses, only those C57BL/6 mice were considered, where the average selection pressure, $k$, was larger than the maximal selection pressure determined for BALB/c mice.

Results

To determine the protective efficacy of individual CTL specificities in protracted LCMV infection, we designed viral in vivo competition experiments (Fig. 1C). We infected C57BL/6 mice with $2 \times 10^6$ PFU wt cDNA-derived Cl13 virus, in combination with $2 \times 10^5$ PFU of genetically engineered CTL epitope-mutant virus (see later). In this setting, a relative enrichment of the epitope-mutant virus over time would be evidence of CTL selection pressure on the epitope in question. We engineered CTL epitope-mutant viruses, differing from the wt virus by only one amino acid substitution in the GP276, GP33, or NP396 CTL epitope, respectively (Fig. 1D). Each of these mutations were known and/or predicted to prevent MHC presentation to CD8+ T cells (H-2D$^b$ for GP276 and NP396; H-2D$^b$ and H-2K$^b$ for GP33) (35). Conversely, these mutations did not affect their neutralization by mAbs or virus-specific antiserum (Supplemental Fig. 1D). This was expected because neutralizing Abs are uniformly targeted against the outer globular GP-1 domain of the viral glycoprotein complex, whereas the mutations introduced were situated in the membrane-proximal glycoprotein stalk (GP276), signal peptide (GP33), and virion-internal nucleoprotein (NP396). We validated the epitope-mutant viruses by verifying their inability to induce CTL responses against the wt and mutated epitope sequences, and we also tested the lack of recognition of the mutant epitopes by wt virus-induced CTLs (Supplemental Fig. 1E-I). To individually quantify wt and epitope-mutant viruses in coinfected animals, we “tagged” the wt virus by introducing noncoding changes in the target sequence of our TaqMan RT-PCR assay (Fig. 1E). As expected, this “tagged” wt virus reached normal titers and persisted in the blood of mice analogously to nontagged wt virus (Fig. 1F). The respective TaqMan assays were validated for accuracy and discrimination of the two viral target sequences (Supplemental Fig. 1J-L; see Materials and Methods), and we used them to individually follow serum loads of epitope-mutant and wt virus in coinfected animals over time (Fig. 1G). From these values, we calculated ratios of epitope-mutant/wt virus (Fig. 1H) and derived a time-dependent selection coefficient $k(t)$, which quantifies the TDSP CTLs exert on the epitope under study (Fig. 1I; see also Materials and Methods). Given the MHC molecules presenting these epitopes, CTL-driven selection of epitope-mutant virus was only expected in C57BL/6 (H-2b) but not in BALB/c mice of the H-2$^d$ haplotype (control). Cohorts of 26, 15, and 12 inbred C57BL/6 mice were subject to coinfection experiments with GP276-, GP33-, or NP396-deficient epitope-mutant and wt competitor virus, respectively. We collected serum samples over time to monitor viral

FIGURE 2. Epitope-specific CTL selection pressure in protracted LCMV infection. We infected C57BL/6 (top and center) and BALB/c mice (bottom) with $2 \times 10^5$ PFU of either $\Delta$GP276, $\Delta$GP33, or $\Delta$NP396 virus, in combination with $2 \times 10^5$ PFU wt LCMV (see schematic in Fig. 1C). Each virus was individually quantified in serum by TaqMan RT-PCR. Time-dependent selection coefficients $k(t)$ (red lines) were calculated (see Materials and Methods and schematic in Fig. 1G-I). Gray shaded area denotes the 95% confidence interval of background $k(t)$ in the absence of epitope-specific selection pressure. Representative C57BL/6 mice with either strong (top) or weak/absent selection pressure (center) are shown in comparison with nonselecting BALB/c mice (bottom). Analogous plots for all mice tested are displayed in Supplemental Figs. 2–4.
loads and calculate TDSP by GP276-, GP33-, and NP396-specific CTLs. Viral load curves and deducted selection coefficients are shown in Fig. 2 (representative animals) and Supplemental Figs. 2–4. Six to 10 BALB/c mice per epitope were included as controls. As expected, wt and epitope-mutant viruses showed largely parallel clearance kinetics in these H-2d nonselecting mice. There were minor residual fluctuations in epitope-mutant/wt virus ratios. These were used to calculate a time-dependent background noise in the selection coefficient $k(t)$ for each epitope, which we defined as being unrelated to specific CTL selection pressure (gray area in Fig. 2 and Supplemental Figs. 2–4). Unlike in BALB/c mice, C57BL/6 mice tended to clear the wt virus more readily than GP276-, GP33-, or NP396-deleted viruses, indicating selection pressure on the respective epitopes. The timing and magnitude of

**FIGURE 3.** Interindividual diversity, magnitude, and timing of epitope-specific CTL selection pressure. (A) Superimposition of $k(t)$ curves from individual C57BL/6 (left) and BALB/c (right) mice, assessing selection pressure on the respective epitopes. (B) Symbols represent the average selection pressure, $\kappa$, for individual C57BL/6 (○) and BALB/c mice (●) on the indicated epitopes over 100 d. Horizontal dashed lines denote the respective highest values recorded in BALB/c mice. (C) Symbols represent the average epitope-specific selection pressure, $\kappa$, for individual C57BL/6 (white symbols) and BALB/c mice (black symbols) calculated for the period from days 9 to 20 or from days 20 to 100, as indicated. For pairwise comparisons, paired $t$ tests (C57BL/6 days 9–20 versus C57BL/6 days 20–100) and unpaired $t$ tests (C57BL/6 days 20–100 versus BALB/c days 20–100) were used. The resulting $p$ values were subject to Bonferroni correction for multiple comparisons. (D) Time windows during which the TDSP $k(t)$ curves of individual mice (Fig. 2 and Supplemental Figs. 2–4) are above the 95% confidence interval of background noise (see Materials and Methods). Individual mice are displayed as vertical lines, with thick segments denoting detectable selection pressure. Only C57BL/6 mice with average selection coefficients above BALB/c backgrounds (dashed lines in B) were included in the analysis. (E) Derived TDSP based on all mice shown in (D). The mean (red line) and 95% confidence interval (gray shaded) of 10,000 bootstrap replicates are shown. The dashed line indicates the zero reference line, above which selection is recorded.
selection pressure (red lines in Fig. 2 and Supplemental Figs. 2–4) exhibited, however, extensive diversity between individual animals. Exemplary C57BL/6 mice with either strong or weak to undetectable selection pressure as shown in Fig. 2 represented the extremes of a spectrum (Supplemental Figs. 2–4). This finding matched earlier observations that CI13 control in the late phase of infection varies considerably within cohorts of genetically identical mice (17, 20). The resulting diversity of individual TDSP curves is illustrated in Fig. 3A. Conversely, very minor fluctuations of TDSP in BALB/c mice attest to the technical accuracy and overall reliability of the measurements and experimental setup. Note that periods of negative TDSP in C57BL/6 mice were mostly due to viral loads falling below detection limits (see, e.g., Fig. 2, top panels, and Materials and Methods). The LCMV polymerase error prone, and viral variants with increased fitness can be selected for in chronic infection. Such random mutation and selection occurs, however, irrespective of the MHC haplotype of the host. Consistently low TDSP values in BALB/c mice indicate, therefore, that the selection of epitope-mutant viruses, as observed in C57BL/6 mice, was not due to random mutations but resulted from MHC-restricted CTL pressure on the very epitope, which had been deliberately mutated. Next, we calculated the area under each mouse’s individual selection curve (striped in Fig. 1f; see Materials and Methods). Thereby we obtained an average value for epitope-specific CTL pressure (κ) during time intervals the TDSP curve was above background. The resulting values showed that ~60–75% of C57BL/6 mice exerted CTL selection pressure on GP276, GP33, and NP396 above BALB/c backgrounds (16/26, 11/15, and 9/12 mice tested for GP276, GP33, and NP396, respectively; Fig. 3B). When broken down in early and late phases of chronic infection (days 9–20 versus days 20–100), the average epitope-specific CTL selection pressure (κ) on GP276 and GP33 did not differ significantly between the two time windows, yet for NP396 was higher in the late phase of infection (Fig. 3C). Furthermore, the average CTL selection pressure in the late phase of infection (days 20–100) was significantly above BALB/c backgrounds for all three epitopes tested. These results documented that antiviral CTL selection pressure was not lost during the chronic phase of infection, which is in line with earlier reports on at least partially retained CTL killing capacity during chronic CI13 infection (20, 27).

Next, we analyzed the timing of CTL selection pressure at the level of individual C57BL/6 mice. We confirmed the trend for early-onset CTL pressure on the GP276 and GP33 epitopes, whereas NP396-specific CTL selection tended to occur later (Fig. 3D). A bootstrap analysis was performed to investigate over-arching patterns in TDSP, which characterize its timing across all mice despite considerable interindividual variability. The TDSP 95% confidence interval (Fig. 3E, gray shading) confirmed that C57BL/6 mice, on average, exerted GP276-specific CTL pressure during the first period of persistent infection up until day 50. The average GP33-specific selection pressure was biphasic, with early and late peaks. This pattern may be reflective of two distinct GP33-specific CTL populations restricted by H2-Kb and H2-Db, respectively, yet prevented a meaningful statistical assessment of CTL timing. In concordance with the results of Fig. 3C, NP396-specific CTL efficacy was significant at the cohort level in a later phase between around days 30 and 80 of infection (Fig. 3E). Measurements of GP276-specific CTL frequencies in peripheral blood have failed to reveal a correlation with normalized epitope-specific CTL pressure (κ; Fig. 4). This finding was compatible with earlier observations that the quantitative assessment of virus-specific CTLs in peripheral blood of HIV patients showed little correlation to viral loads (36, 37).

Discussion
The present quantification and characterization of antiviral CTL efficacy adds to our understanding how the immune system can prevail in chronic infection. Immunotherapeutic interventions bear considerable potential for treating persistent viral diseases, notably in HBV (38), and should aim for mimicking successful immune responses in spontaneous controllers. The mechanisms underlying differential epitope-specific timing of selection pressure remain to be investigated. Several contributing factors can be envisioned including: 1) viral protein abundance (39) and resulting peptide ligand availability on target cells, 2) peptide–MHC binding affinity, (23) and 3) the relative susceptibility of responding CTL populations to clonal deletion (12, 13). A better understanding thereof should help in the choice of target Ags and epitopes for antiviral immunotherapy.

Interindividual variability of CTL selection pressure in genetically identical animals, both in terms of strength and timing, matches earlier reports from TCR spectratype analyses in CI13-infected mice (40). Differences in T cell repertoire caused by stochastic events in TCR rearrangement and subsequent thymic selection may account for this, and were reported to influence HIV control (41). Similarly, de novo recruitment of thymic emigrants into the ongoing CTL response may contribute to time-dependent fluctuations in CTL selection pressure of individual mice (42).

Our observations suggest that therapeutic induction of antiviral CTL responses in chronic infection, even if partially dysfunctional by commonly used ex vivo criteria, can represent a correlate of immune control and a valuable goal of such interventions. This interpretation is in line with earlier reports that viremic CI13-infected mice reject GP33- or NP396-pulsed syngeneic splenocytes in so-called in vivo CTL assays. Similarly, NP396-specific CTLs were found to resurge in the late phase of infection (20, 27), matching the timing of selection pressure on this epitope found in this study. When adoptively transferred and rested in an Ag-free environment, phenotypically “exhausted” CTLs can afford protection against subsequent virus challenge (28). Our report adds to these earlier observations by establishing and characterizing the protective antiviral efficacy of CTL populations in the chronically infected host.
Exhaustion is a gradual process (1, 13) and individual cells of exhausted CTL populations cover a wide range of differential functionality (compare Fig. 1A, 1B and Supplemental Fig. 1A–C). Hence it seems plausible that subsets of more functional cells contributed overproportionally to the epitope-specific CTL pressure. It also is possible that fluctuations in CTL functionality account for the observed “waves” of TDSP.

The novel approach outlined in this article provides a means to directly determine the impact of defined CTL specificities on viral loads, thus lending itself to future studies aimed at validating immune correlates of CTL efficacy. Specific CTL numbers are likely of relevance, but their abundance in peripheral blood seems insufficient as a predictor for antiviral efficacy (Fig. 4) (36, 37, 43). Potential qualitative correlates of CTL efficacy comprise, among others, functional avidity (44), proliferative capacity (45), polyfunctionality (46), lytic granule loading (47), perforin expression (48), resistance to immunoregulation (49–51), and TCR clonotype composition (41). Unfortunately, peripheral blood may not represent the most relevant compartment to monitor in this context, albeit clearly the only option in longitudinal studies. This possibility is suggested by a dramatic skewing of virus-specific CTLs to LCMV-infected tissues such as bone marrow, liver, lungs, and brain, in combination with compartment-related differences in CTL functionality (13). A timely assessment of such tissue compartments would require the experimental animal to be euthanized. This conflicts with the assessment of CTL efficacy, relying on the longitudinal sampling of serum from the same animals until CTL-mediated control is evident in reduced viral loads. Conversely, an analysis of CTL populations at such a later stage of the experiment would be confounded by differential Ag compartments would require the experimental animal to be euthanized. This conflicts with the assessment of CTL efficacy, relying on the longitudinal sampling of serum from the same animals until CTL-mediated control is evident in reduced viral loads. Conversely, an analysis of CTL populations at such a later stage of the experiment would be confounded by differential Ag loads, thus lending itself to future studies aimed at validating qualitative correlates of CTL efficacy, comprising, among others, insufficient as a predictor for antiviral efficacy (Fig. 4) (36, 37, 43).

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