Absence of CTL Responses to Early Viral Antigens Facilitates Viral Persistence

Anita Schildknecht, Sarah Welti, Markus B. Geuking, Lars Hangartner and Maries van den Broek

J Immunol 2008; 180:3113-3121; doi: 10.4049/jimmunol.180.5.3113
http://www.jimmunol.org/content/180/5/3113

References
This article cites 35 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/180/5/3113.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Absence of CTL Responses to Early Viral Antigens Facilitates Viral Persistence¹

Anita Schilddknecht,² Sarah Welti, Markus B. Geuking,³ Lars Hangartner,⁴ and Maries van den Broek²

CD8⁺ T cells are crucial for the control of intracellular pathogens such as viruses and some bacteria. Using lymphocytic choriomeningitis virus (LCMV) infection of mice—the prototypic arenavirus evolutionarily closely related to human Lassa fever and South American hemorrhagic fever viruses, we have shown previously that the kinetics of Ag presentation determine immunodominance of the LCMV-specific CTL response due to progressive exhaustion of LCMV nucleoprotein (NP)-specific CTL upon increasing viral load. In this study, we provide evidence that CTL against early LCMV NP-derived epitopes are more important in virus control than those against late glycoprotein-derived epitopes. We show that mice that are tolerant to all NP-derived T cell epitopes are severely compromised in their ability to control larger inocula of LCMV, supporting our hypothesis that CD8⁺ T cells specific for early viral Ags play a major role in acute virus control. Thus, the kinetics with which virus-derived T cell epitopes are presented has a strong impact on the efficacy of the antiviral immunity. This aspect should be taken into consideration for the development of vaccines. The Journal of Immunology, 2008, 180: 3113–3121.

Institute of Experimental Immunology, University Hospital Zurich, Zurich, Switzerland
Received for publication October 10, 2007. Accepted for publication December 26, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by the Swiss National Science Foundation and the European Community (MUGEN LSHG-CT-2005-005203).
² Address correspondence and reprint requests to Dr. Anita Schilddknecht at the current address, Clinical Immunology, University Hospital Zurich, Hägelweg 4, CH-8044 Zurich, Switzerland. E-mail address: anita_schilddknecht@bluewin.ch or Dr. Maries van den Broek, Experimental Immunology, University Hospital Zurich, Schmelbergstrasse 12, CH-8091 Zurich, Switzerland. E-mail address: maries@vanden-broek.ch
³ Current address: Intestinal Disease Research Program, Department of Medicine, McMaster University, Hamilton, Ontario, Canada.
⁴ Current address: Department of Immunology, IMM2, The Scripps Research Institute, La Jolla, CA 92037.

Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; moi, multiplicity of infection; VV, vaccinia virus; DC, dendritic cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ICS, intracellular cytokine staining.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
pGEM-T-NPA1.1fw containing the cDNA sequence of LCMV-NP with primers NcoINPfwd (2) (5′-GGC ATG GCC GTC TCC AAA GGA GTC AAA AGC-3′) and BamHIINPre (5′-GAC GGA TCT TAA GAG TGT CAC AAC ATT G-3′). The PCR consisted of 35 cycles (94° 1 min, 50° 1 min, 72° 1 min, 30 s) followed by 10 min at 72°C. LCMV-NP cDNA was generated originally by reverse transcription of total RNA isolated 48 h after LCMV-WE infection (multiplicity of infection (moi) 0.01) of L929 cells with primers WE365 (5′-GCC ACA GTG GAT CCT AGG CA-3′) and WE365r (5′-GCC ACA GTG GAT CCT AGG CA-3′) for NP-specific cDNA synthesis and primers WE1630f (5′-CGC ACA GTG GAT CCT AGG CA-3′) and WE1630r (5′-CGC ACA GTG GAT CCT AGG CA-3′) for NP cDNA amplification. To allow inductive expression of LCMV-NP, the loxP-flanked STOP cassette was PCR amplified from the pBS302 plasmid (Invitrogen Life Technologies). During this process, the STOP cassette was freed of start codons (before the first and after the second loxP site) and a Kozak-optimized NcoI restriction site was inserted at the 3′ end. The 1.5-kb STOP cassette was then SfiI/Ndel-digested, blunted, and inversely ligated into blunted SfiI/SalI-digested pфact plasmid (16) to obtain pфact-STOP. A 4.9-kb NcoI-NolI fragment containing an ampicillin-resistance gene and a poly(A) sequence (derived from the GptM vector; provided by W. Beyer, Heinrich Pette Institute, Hamburg, Germany) was cloned into the SfiI/Ndel-digested pфact-STOP plasmid fragment. The entire cassette was then digested with pфact-STOP-ScI (provided by C. Pette Institute, Hamburg, Germany) (21) and was ligated with one step with the NcoI-BamHI 1.7-kb PCR fragment encoding LCMV-NP result in the pфact-STOP plasmid.

To analyze functionality of the pфact-STOP-NP construct, it was passed through Escherichia coli strain 294, Cre-expressing bacteriophage, P1-derived Cre recombinase (provided by R. Kuehn, Institute for Genetics, University of Cologne, Cologne, Germany) for excision of the STOP cassette and was tested for LCMV-NP expression by transfecting MC57G cells followed by immunofluorescence and FACS analysis, PCR and cytotoxicity assays.

Oligonucleotides

Oligonucleotides were purchased PAGE purified from Microsynth. Enzymes and buffers for enzymatic reactions

(Restraction) enzymes and buffers were purchased from New England Biolabs, Roche Diagnostics, and Sigma-Aldrich.

Peptides

Peptides were purchased synthesized by NeoMPS in immunograde quality.

Cell lines

MC57G cells are methylcholanthrene-induced fibrosarcoma cells of C57BL/6 origin.

Mice

C57BL/6, ST-NP, DEE (19), and Deleter (20) mice were bred at the Institute of Laboratory Animal Science (University of Zurich, Zurich, Switzerland). ST-NP mice were purchased directly from the Institute of Laboratory Animal Science (University of Zurich, Zurich, Switzerland) and were maintained by the animal facility of the Biomedical Research Institute (BRI) with C57BL/6 origin.

Viruses, infection of mice, and measurement of virus titers

LCMV-WE was originally obtained from Dr. F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) (21) and was propagated on L929 cells at a low moi. Mice were infected i.v. in the tail vein with the indicated amount of LCMV-WE in a final volume of 200 nl.

Vaccinia virus (VV), strain WR, was originally obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD). Recombinant VV (rVV) carrying the LCMV glycoprotein (VV-G2) was obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD). rVV expressing LCMV-derived gp33–41 or NP396–404 as a minigene were described previously (12). For priming of L338–346-specific CTL, mice were injected i.v. with 106 bone marrow-derived DC 14 days before were restimulated with irradiated and peptide-loaded (10−7 M) cells and constitutively express Cre recombinase. All mice had a pure C57BL/6 background and were kept under specific pathogen-free conditions. Mice were at least 6 wk old at the beginning of the experiments, groups were age- and sex-matched and experiments were performed in compliance with Swiss federal and cantonal laws on animal protection.

Experiments were performed in compliance with Swiss federal and cantonal laws on animal protection.

Immunohistochemistry

Organs were removed and snap-frozen in liquid nitrogen. Five-millimeter-thick cryosections were fixed in acetone for 10 min and subsequently incubated with rat-anti-LCMV NP (VLA; Ref. 24). Goat-anti-rat Ig (Caltag Laboratories) in 5% normal mouse serum was used as a secondary reagent and alkaline phosphatase-labeled donkey-anti-goat Ig (Jackson ImmunoResearch Laboratories) in 5% normal mouse serum as tertiary reagent. The substrate for the red color reaction was naphthol AS-Biphosphate (New Fuchsin). Endogenous alkaline phosphatase activity was quenched by levamisole. Sections were counterstained with hemalum.

RNA isolation and PCR for the detection of LCMV-NP

Total RNA from organs or blood of ST-NP × Deleter, ST-NP, or control C57BL/6 mice was isolated using TRIzol (Invitrogen Life Technologies). One-tenth of total RNA was freed from contaminating DNA (Turbo DNAfree; Ambion) according to the manufacturer's instructions. Successful cDNA synthesis was performed using random hexamers and Superscript II Reverse Transcriptase (18064-014; Invitrogen Life Technologies). PCR (40 cycles) was performed using a 10X PCR Master Mix (100 mM Tris, 500 mM KCl, 20 mM MgCl2, pH 9.2) with NP-specific primers: NP1713 forward (5′-GGG GTG CCT TCC GGT AAG C-3′) and NP2337 reverse (5′-GTC AGT GGT GGG GAG AGC AT-3′).

Measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin

AST, ALT, and bilirubin were determined in serum by standard clinical chemistry as described (25).

Generation of bone marrow-derived DC

Bone marrow-derived DC were generated from C57BL/6 mice as previously described (23).

Intracellular cytokine staining (ICS) for IFN-γ

ICS was performed as described (26). In some experiments, ICS was performed on restimulated cultures to increase the sensitivity and reliability of the usually very low CD4+ T cell responses. Splenocytes were restimulated for 5–6 days with bone marrow-derived DC loaded with 10−7 M of the relevant peptide in a splenocyte:DC ratio of 40.

In one experiment, VV-infected macrophages were used as sources of Ag. Macrophages were collected from the peritoneal cavity 3 days after i.p. injection of 1 ml of thioglycolate (30 g/l; Difco) and were infected with VV-WR, VV-G2, or VV-NY4 in vitro at a moi of 3 in 500 µl of IMDM for 60 min at 37°C. A total of 9.5 ml of IMDM were added and cells were rotated at 37°C for additional 5 h before centrifugation and resuspension in fresh IMDM plus 10% FCS plus 100 U/ml penicillin/streptomycin. Approximately 105 responder splenocytes were incubated with 3×105 infected macrophages per well to perform ICS as described above.

Staining with tetrameric MHC class I-epitope complexes

Tetramer complexes containing biotinylated H-2Dd or H-2Kd, β2-microglobulin, the relevant peptide and streptavidin-PE were generated as described (27) and staining was performed as described (26).

Peptide-specific CTL lines

Splenocytes from mice that were primed with rVV33, rVV396 (12), or with L338-loaded bone marrow-derived DC 14 days before were restimulated with irradiated and peptide-loaded (10−7 M), thioglycolate-elicited macrophages (1 ml of thioglycolate i.p. at day −3) at a ratio of 20:1 in the presence of 25 U/ml recombinant mouse IL-2. Cultures were restimulated every week as described above at a ratio of 5:1 (12).

Kinetics of Ag presentation by cells from LCMV-infected mice

C57BL/6 mice were infected i.v. with 4×107 PFU of LCMV-WE. The high inoculum size was chosen to ensure maximal numbers of infected cells in the spleen. At defined time points after infection, spleens were homogenized and used as APC to stimulate IFN-γ production of CTL lines essentially as described (12). A total of 6×105 CTL of single specificity
were incubated in 96-well round-bottom plates at 37°C with 10⁶ splenocytes from infected or naive C57BL/6 mice or with 10⁶ naive C57BL/6 splenocytes with titrated amounts of the relevant antigenic peptide in 200 ml of medium containing 5 mg/ml brefeldin A to freeze their condition of Ag presentation. After 6 h, cultures were processed as described previously above under ICS.

**Statistical analysis**

Statistical analysis was done with the Student t test (distribution as indicated) and Excel software.

**Results**

**Generation and characterization of ST-NP mice**

ST-NP mice were generated by injection of the linearized transgenic construct (Fig. 1a) in the pronucleus of fertilized C57BL/6 oocytes according to standard protocols. To test whether NP expression was inducible in a ubiquitous fashion, ST-NP mice were bred to Deleter mice that ubiquitously and constitutively express Cre recombinase (20), which results in removal of the loxP-flanked STOP cassette and which should lead to ubiquitous expression of the transgenic NP. Organs and blood of ST-NP × Deleter mice were analyzed for NP expression by RT-PCR (Fig. 1b) and by immunohistology (Fig. 1c) and expression of NP was detected in all organs tested. Adequate processing and presentation of transgenic NP should result in NP-specific T cell tolerance in ST-NP × Deleter mice. To test this, we infected ST-NP × Deleter mice with LCMV strain WE (LCMV-WE) and analyzed the CD8⁺ and CD4⁺ T cell response to LCMV-glycoprotein (gp33–41/H-2Db, gp276–286/H-2Db and gp13–23/H-2I-Ab) and -NP (NP205–212/H-2Kb, NP289–406/H-2Db and NP309–328/H-2I-Ab) derived epitopes 8 and 14 days after infection by staining of blood cells with MHC class I-peptide tetramers (Fig. 1d) and intracellular staining of splenocytes for IFN-γ (Fig. 1e) after short in vitro restimulation with the relevant peptide (ICS). We found that both CD8⁺ and CD4⁺ T cells from ST-NP × Deleter mice were fully unresponsive to NP-derived epitopes, whereas the response to glycoprotein-derived epitopes was comparable to those of infected control ST-NP mice. Thus, transgenic NP is processed and presented in a way that specific T cells recognize it.

Absence of NP-specific RNA or protein as well as absence of NP-specific T cell tolerance upon LCMV infection of ST-NP single-positive littermates (or Deleter mice; data not shown) confirmed that the STOP cassette is tight.

**Virus control is severely compromised in NP-tolerant mice**

We have shown previously that NP-derived CTL epitopes are presented 10–12 h before glycoprotein-derived epitopes on the surface of LCMV-infected cells (12) and we hypothesized that a delay in the induction of LCMV-specific CTL might have consequences for virus control. To address this question, we infected NP-tolerant ST-NP × Deleter and control mice with 10⁶ PFU LCMV-WE and measured viremia in the blood and virus load in the spleen by a focus-forming assay (24). Control mice had undetectable levels of virus by day 12 after infection (spleen) and were not viremic in contrast with ST-NP × Deleter mice that still had virus in blood and spleen by day 42 (Fig. 2a). Thus, we conclude that absence of early NP-specific CTL allows LCMV to replicate to higher levels resulting in severely delayed clearance. A parameter that correlates with the coexistence of Ag and CTL is LCMV-induced hepatitis (25), which manifests itself by increased levels of ALT, AST, and bilirubin in the serum. We found that all three parameters were more elevated and over a prolonged time period in NP-tolerant mice when compared with control mice (Fig. 2, b–d). This is in line with higher levels of LCMV in the face of LCMV-specific CTL as we observed in ST-NP × Deleter mice.

The higher load of LCMV in the liver was confirmed by immunohistology using VL-4 Abs to detect LCMV-derived NP (data not shown).

If early CTL indeed are important in rapid control of LCMV, we expected to see differences in early virus titers in infected mice, even if the inoculum size was low. Therefore, we infected NP-tolerant mice with 10⁵, 10⁴, or 10³ PFU LCMV-WE and measured virus titers in the spleen after 6 days. We found that virus titers in NP-tolerant mice were ~2- to 3-fold higher than those of control mice, independent of the inoculum size (data not shown).

**Absence of early NP-specific CTL promotes exhaustion of glycoprotein-specific CTL**

The final consequence of a high load and persistence of a noncytopathic virus is the gradual disappearance of virus-specific CTL to protect the host from lethal immunopathology, a process termed CTL exhaustion (13). We described previously that NP-specific CTL are more prone to exhaustion than glycoprotein-specific CTL, i.e., the virus load required to exhaust NP-specific CTL is lower than that to exhaust glycoprotein-specific CTL (12). Our hypothesis was that NP-specific CTL, being the first to recognize and control virus-infected cells, might encounter higher viral loads than glycoprotein-specific CTL as the latter are triggered 10–12 h after NP-specific CTL have begun eliminating infected cells. NP-tolerant ST-NP × Deleter mice allowed us to test whether the exhaustion of glycoprotein-specific CTL is achieved by nonexhausting loads of virus if NP-specific CTL are lacking. The size of the glycoprotein- and NP-specific CTL response was monitored by ICS at days 12, 20 (data not shown), and 42 after infection with 10⁶ PFU LCMV-WE (Fig. 2e). Whereas the CTL response to gp33–41 and gp276–286 remained at a high level (around 10% of CD8⁺ T cells for each epitope) until day 42 in control mice, the responses were already lower in NP-tolerant mice at day 20 (data not shown) and decreased to virtual absence by day 42 (<2.5% of CD8⁺ T cells for each epitope) (Fig. 2e). Therefore, absence of early NP-specific CTL makes the host more susceptible to virus persistence accompanied by progressive exhaustion of glycoprotein-specific CTL.

**Absence of NP-specific CD8⁺ T cells results in an enhanced clonal burst size of glycoprotein-specific CD8⁺ T cells early after infection with low amounts of LCMV**

The immunodominance of NP-specific CTL, which is invariably seen after infection with relatively low amounts of LCMV, was explained by the fact that NP-specific CTL are the first to interact with the virus and therefore had a better chance to expand than the later glycoprotein-specific CTL (12). If this were so, one would expect a larger clonal burst size of glycoprotein-specific CTL in absence of NP-specific CTL. To test this, we infected ST-NP × Deleter and control mice with a limiting amount (10 PFU) of LCMV-WE and analyzed the glycoprotein- (gp33–41/H-2Db, gp276–286/H-2Db) (Fig. 3a) and L-specific (L338–346/H-2Db) (Fig. 3b) CTL response in the spleen after 4, 5, 6, and 7 days by IFN-γ-ICS. As a control, NP-specific CTL (NP205–212/H-2Db, NP289–406/H-2Db) were analyzed (Fig. 3c). We had to measure these early responses by ICS and could not use tetramers because at these early time points the mice still carried virus, which resulted in TCR signaling and subsequent TCR down-regulation. As the interaction between the TCR and peptide/MHC I notoriously is a low-affinity interaction, down-regulation of TCR surface density as a result of recent antigenic stimulation is known to result in serious underestimation of frequencies if measured by tetramers. A few glycoprotein (the sum of gp33–41 and gp276–286 responses is shown) and
more L-specific CTL (L338–346) were detectable in ST-NP × Deleter mice as early as day 5 after infection after which they expanded to ∼13 and 1.2% of CD8+ T cells by day 6; in control mice, however, detection of glycoprotein-specific CTL was only possible from day 6 on and the size of the response was reduced by about one-third of that in ST-NP × Deleter mice. However, the number

**FIGURE 1.** Generation and characterization of ST-NP and (ST-NP × Deleter) mice. *a*, Transgenic construct. *b*, Organs and blood from ST-NP × Deleter mice, single-positive littermates (ST-NP), and C57BL/6 wild-type mice were processed for RNA isolation and expression of NP was determined by RT-PCR. *c*, Organs from ST-NP × Deleter mice, single-positive littermates, and C57BL/6 control mice were processed for immunohistology and were stained with an Ab against LCMV-NP (VL4). The red precipitate visualizes LCMV-NP. ST-NP × Deleter mice are tolerant to LCMV NP-derived MHC class I and class II epitopes: ST-NP × Deleter mice and single-positive littermates were infected with 200 PFU LCMV-WE and glycoprotein- and NP-specific CD8+ and CD4+ T cell responses were measured by (*d*) tetramer staining in the blood and (*e*) IFN-γ-ICS on splenocytes (*d*) 8 and (*e*) 14 days later. Spontaneous IFN-γ-production in *e* was found in 0.1–1.5% of T cells. Four mice were used per group. *d* and *e*, One experiment of three is shown; *, p < 0.003; **, p < 0.006; and ***, p < 0.015 (n = 4, one-tailed distribution, paired). In *b* and *c*, analysis of one representative mouse is shown.
of glycoprotein-specific CTL was equalized again by day 7 in both groups of mice (Fig. 3a). The slightly reduced size of the L-specific response at day 5 after infection in ST-NP × Deleter was observed until day 7 after infection (Fig. 3b). The frequency of naive CTL precursors is so low that it precludes detection (<0.01% of CD8+ T cells), but immediately upon LCMV infection these precursors expand massively with an estimated doubling time of 6–8 h. The fact that glycoprotein-specific CTL are detectable, i.e., exceed the detection limit, in NP-tolerant mice 1 day before they can be detected in control mice suggests that more glycoprotein-specific CD8+ T cells are activated by virus-infected APCs if early NP-specific CTL (NP205-212, NP396-404) are lacking.

Kinetics of presentation of NP-, glycoprotein-, and L-derived epitopes in vivo

Very recently, LCMV L-protein-derived CTL epitopes were identified (Ref. 11; A. Bergthaler and D. Pinschewer, unpublished results), which made it necessary to investigate with
which kinetics the presentation of L-derived epitopes occurred as previously investigated for glycoprotein- and NP-derived epitopes (12). We infected C57BL/6 mice with 4 × 10^7 PFU LCMV-WE and used their splenocytes at different time points after infection as APC for gp33–41-, NP396–404-, and L338–346-specific CTL lines in the presence of brefeldin A to freeze the APC state of Ag presentation. After incubation for 5 h, the production of IFN-γ was measured by ICS.

We found that the gp33–41 epitope was detected by specific CTL after 12 h with a maximum at 16 h and beyond; the L338–346 epitope was detected by specific CTL with even slightly slower kinetics compared with the gp33–41 epitope. In contrast, the NP396–404 epitope was already at its maximum after 8 h (Fig. 4).

Thus, both L- and glycoprotein-derived epitopes are detected on infected cells considerably later than NP-derived epitopes. Stimulation of specific CTL with splenocytes loaded with titrated amounts of the individual peptides demonstrated that the CTL were monospecific and showed no reactivity to other LCMV-derived epitopes and that the Ag sensitivity of the three CTL lines was similar (data not shown). From these data, we conclude that not only glycoprotein-CTL, but also L-CTL encounter their nominal peptide on infected cells with delayed kinetics compared with NP-CTL. Obviously, our data only give information about the kinetics of detection of LCMV-derived peptides on MHC class I molecules on the surface of infected cells by CD8+ T cells and not about the kinetics by which individual viral proteins are produced in infected cells. The kinetics of detection, however, is what matters in the context of antiviral protection.

**FIGURE 3.** Absence of NP-specific CD8+ T cells results in an early enhanced clonal burst size of glycoprotein-specific CD8+ T cells. ST-NP × Deleter (■) and control mice (□) were infected with 10 PFU LCMV-WE and (a) the glycoprotein- (gp33–41, gp276–284), (b) L- (L338–346), and (c) NP- (NP205–212, NP396–404) specific CD8+ T cell response was determined in the spleen by IFN-γ-ICS. Data represent the mean of three mice and are presented as the pooled percentage of IFN-γ+ cells after gating on CD8+ cells for glycoprotein-, L-, and NP-derived epitopes. The experiment was repeated three times; *, p < 0.041 (two-tailed distribution, paired, n = 3); **, p < 0.018; ***, p < 0.031; and ****, p < 0.006 (one-tailed distribution, paired, n = 3).

To exclude that absence of a substantial part of the LCMV-specific CTL response rather than absence of early NP-specific CTL caused the severely compromised virus clearance in NP-tolerant mice, we compared viral clearance in NP-tolerant mice with that in glycoprotein-tolerant (DEE; Ref. 19) mice. To confirm NP- and glycoprotein-tolerance in ST-NP Deleter mice and in DEE mice, respectively, we infected mice with 200 PFU LCMV-WE and analyzed the CD8+ T cell response to the entire LCMV-glycoprotein and -NP 8 days after infection. Splenocytes were stimulated for 5 h in vitro in the presence of brefeldin A with C57BL/6 macrophages that were previously infected with wild-type vaccinia virus (VV-WR) or with VV expressing LCMV-glycoprotein (VV-G2) or LCMV-NP (VV-YN4). Subsequently, we stained splenocytes for surface CD8 and intracellular IFN-γ. CD8+ cells from ST-NP × Deleter mice were fully unresponsive to VV-YN4-infected macrophages whereas DEE mice lacked the glycoprotein response.
Deleter, DEE, and control mice infected with 10^6 PFU LCMV-WE that in control C57BL/6 mice (data not shown). The splenocytes were isolated at different time points after infection and were used as APC to stimulate IFN-γ production by gp33–41-, NP396–404-, and L338–346-specific CTL lines in the presence of 10^7 PFU LCMV-WE. Their splenocytes were isolated at different time after infection and were used as APC to stimulate IFN-γ production by these CTL lines in the presence of LCMV glycoprotein-derived peptides (data not shown). These data were confirmed by using NP- or L338–346-specific CTL lines in the presence of brefeldin A to freeze the status of Ag presentation of the infected splenocytes. Dashed line represents the percentage IFN-γ-producing CTL after incubation with splenocytes from uninfected mice.

(Fig. 5), thus demonstrating tolerance of CD8^+ T cells from ST-NP × Deleter mice to NP and of CD8^+ T cells from DEE mice to glycoprotein. Importantly, the use of rVV as Ag allowed analysis of the response to the total of all glycoprotein- and NP-derived epitopes, as it even included possible epitopes that were not previously described. These data were confirmed by using NP- or glycoprotein-derived peptides (data not shown) and the response to L338–346 in ST-NP × Deleter and DEE mice was comparable to that in control C57BL/6 mice (data not shown).

Measurement of virus titers in the blood and spleen of ST-NP × Deleter, DEE, and control mice infected with 10^7 PFU LCMV-WE revealed that absence of the glycoprotein response had only a small impact on viral clearance, whereas absence of the NP response resulted in severely impaired virus control (Table I). In line with high virus titers, NP-tolerant mice had high serum levels of AST and ALT (Fig. 2, b–d) at least until day 20 after infection and had detectable serum bilirubin at day 12. In contrast, the serum levels of AST and ALT in glycoprotein-tolerant mice were slightly elevated at day 12 after infection and bilirubin was undetectable (data not shown).

These results make it unlikely that absence of any large part of the LCMV-specific repertoire tips the balance in favor of the virus and thus supports our conclusion that absence of early NP-specific CTL allows LCMV to replicate to higher levels resulting in severely impaired clearance.

**Discussion**

Control of noncytopathic viruses is typically dependent on CTL that effectively eliminate infected cells after they recognized virus-derived peptides in the context of MHC class I molecules on the surface of infected cells. Obviously, the faster CTL can recognize virus-derived peptides become activated and start lysing infected cells, the more efficient virus control will be. We have previously shown in H-2^b and H-2^d mice that epitopes derived from different LCMV-encoded proteins are presented such that NP-derived epitopes are seen by specific CD8^+ T cells from LCMV-infected ST-NP mice (data not shown). These results make it unlikely that absence of any large part of the LCMV-specific repertoire tips the balance in favor of the virus and thus supports our conclusion that absence of early NP-specific CTL allows LCMV to replicate to higher levels resulting in severely impaired clearance.
Table I. Lack of the early NP-specific CTL response results in heavily impaired virus clearance, whereas lack of the late glycoprotein-specific CTL response has less impact

<table>
<thead>
<tr>
<th>LCMV Titer (log_{10} PFU/ml Blood or One-Half Spleen)</th>
<th>Day 9 p.i.</th>
<th>Day 12 p.i.</th>
<th>Day 20 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-NP × Deleter blood</td>
<td>4.94 ± 0.68</td>
<td>4.68 ± 0.42</td>
<td>4.55 ± 0.60</td>
</tr>
<tr>
<td>DEE</td>
<td>1.60 ± 0.55</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ST-NP × Deleter spleen</td>
<td>nd</td>
<td>6.09 ± 0.10</td>
<td>nd</td>
</tr>
<tr>
<td>DEE</td>
<td>nd</td>
<td>3.15 ± 0.05</td>
<td>nd</td>
</tr>
<tr>
<td>Control</td>
<td>nd</td>
<td>1.35 ± 0.49</td>
<td>nd</td>
</tr>
</tbody>
</table>

*ST-NP × Deleter (NP-tolerant), DEE (glycoprotein-tolerant), and control mice were infected i.v. with 10^6 PFU LCMV-WE. Virus titers were determined in the blood and spleen by a focus-forming assay on days 9, 12, and 20 after infection. The mean ± SD is shown, three to four mice were used per group; nd, not done.

To exclude that impaired virus control in NP-tolerant mice is due to different virus replication in cells that express transgenic LCMV-NP, we infected macrophages from ST-NP × Deleter mice and control C57BL/6 mice in vitro with LCMV-WE ( moi of 0.01, 0.1, or 1) and measured virus progeny in the supernatant 24, 48, or 72 h later. We found that LCMV replicated similarly in ST-NP × Deleter and in wild-type macrophages (data not shown). It has been suggested that ubiquitous and constitutive endogenous expression of LCMV-NP interferes with IFN-γ production (28, 29), which is crucial for immediate virus control (30). IFN-γ levels were measured in the sera 12, 24, and 48 h after high-dose LCMV infection, and no difference between NP-tolerant and control mice was found (data not shown), excluding impaired IFN-γ production as crucial factor for the observed difference in viral clearance between control and ST-NP × Deleter mice.

It is possible that thymic or peripheral expression of NP induces the development of NP-specific T regulatory cells. We did, however, not address this issue experimentally. The fact that we have clear evidence for physical absence of NP-specific CD8^+ T cells in ST-NP × Deleter mice supports central deletion rather than suppression mediated by (NP-specific) T regulatory cells.

We show that absence of the early NP-specific CTL allowed a larger clonal burst size of the glycoprotein-specific CTL response illustrating that early CTL efficiently compete with late CTL, at least under conditions of limited Ag. Transfer of glycoprotein-specific TCR-transgenic CD8^+ cells (TCR318) revealed that a higher percentage of the transferred cells became activated (as measured by T cell activation markers CD69, CD25, CD44, and CD62L) in NP-tolerant mice as compared with control mice (data not shown), also suggesting that more glycoprotein-specific CTL can encounter LCMV-infected APC if NP-specific CTL are absent. Competition by early CTL presumably acts through elimination of infected cells thereby reducing the available Ag for late CTL.

LCMV L protein has recently been shown to induce a specific CTL response during infection (11). Analysis of the kinetics of viral L protein epitope presentation revealed that L-derived epitopes were detected on infected cells by specific CTL with similar kinetics to glycoprotein-derived epitopes, both of which are considerably delayed compared with NP-derived epitopes. These findings support the idea put forward in this work that early CTL are more efficient in virus control and that absence of those readily may result in a carrier status of hosts infected with large inocula of noncytopathic viruses. Although another product of the L-RNA segment, the small size (99 aa) zinc-binding protein (Z) might be an additional source of so far unknown CTL epitopes, predicted MHC class I-binding peptides spanning the Z protein did not induce IFN-γ-production by cells derived from LCMV-infected mice (11).

The transiently elevated virus titers after infection of glycoprotein-tolerant DEE mice show that absence of glycoprotein-specific CTL also has an impact on virus control, but a minor one compared with the impact of early NP-specific CD8^+ T cells.

Our finding that early CTL more efficiently control noncytopathic viruses is in line with different clinical observations. Vaccination of macaques with early SIV-derived proteins such as Rev and Tat induced better protection against SIV challenge than did vaccination with late proteins (31). Similarly, longitudinal analysis of HIV-specific CTL responses in infected individuals with high plasma levels of viral RNA showed that Rev- and Tat-specific CTL were the first to disappear (32–34), similar to what happens to early LCMV NP-specific CTL in the face of high LCMV burden. In addition, the development of CMV disease upon lung or heart transplantation in CMV-positive patients was found to correlate strongly with virtual absence of CTL responses against the immediate early-1 protein of CMV, whereas no correlation between disease and CTL responses to the late pp65 protein was apparent (35).

It has been proposed that efficient and fast CTL-mediated lysis of infected cells is beneficial for the prognosis of individuals infected with noncytopathic viruses that have the tendency to persist. Here, we actually provide the first strong in vivo evidence for this thesis, as we show that a relatively short delay of CTL-mediated elimination of cells infected with a noncytopathic virus has a dramatic impact not only on virus control itself but also on the sometimes life-threatening immunopathology that is often associated with infection with noncytopathic viruses. Based on our own findings and the correlations found in patients, we suggest that this principle has general applicability, which should be considered in the development or improvement of efficient vaccines. An efficient vaccine for protection against noncytopathic viruses should therefore include early proteins (or epitopes derived of those) to ensure maximal restriction of replication. In addition, however, it should contain late proteins (or epitopes derived of those) as well, as it is obvious that CTL responses against early proteins, although the most efficient, are also prone to exhaustion when virus levels in the host are high.

**Acknowledgments**

We thank T. Rülicke (Institute of Laboratory Animal Science, University Hospital Zurich) for pronuclear injection of the pβact-STOP-NP construct; A. Novotny and A. Fitsche (Clinical Pathology, University Hospital Zurich) for expert immunohistology; L. Bestmann (Institute of Clinical Chemistry, University Hospital Zurich) for AST, ALT, and bilirubin measurements; A. Matus (F. Miescher Institute Basel) for the pβact vector; and R. Kuehn (Cologne, Germany) for providing W. Beyer (Heinrich Pette Institute, Hamburg, Germany) for the GpTM Deleter (NP-tolerant), DEE (glycoprotein-tolerant), and control mice were infected i.v. with 10^6 PFU LCMV-WE. Virus titers were determined in the blood and spleen by a focus-forming assay on days 9, 12, and 20 after infection. The mean ± SD is shown, three to four mice were used per group; nd, not done.

**Disclosures**

The authors have no financial conflict of interest.

**References**


Kotturi, M. F., B. Peters, F. Buendia-Laysa, Jr., J. Sidney, C. Oseroff, J. Botten,

Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh,


Oehen, S. U., P. S. Ohashi, K. Burki, H. Hengartner, R. M. Zinkernagel, and

Thyagarajan, B., M. J. Guimaraes, A. C. Groth, and M. P. Calos. 2000. Mam-


Hany, M., S. Oehen, M. Schulz, H. Hengartner, M. Mackett, D. H. Bishop, H. Overton, and R. M. Zinkernagel. 1989. Anti-viral protection and prevention of lymphocytic choriomeningitis or of the local footpad swelling reaction in mice by immunization with vaccinia-recombinant virus expressing LCMV-WE nucleo-


