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Immunodominance of an Antiviral Cytotoxic T Cell Response Is Shaped by the Kinetics of Viral Protein Expression

Hans Christian Probst,* Kathrin Tschannen,* Awen Gallimore,†† Marianne Martinic,* Michael Basler,†† Tilman Dumrese,* Emma Jones,† and Maries F. van den Broek‡‡

Lymphocytic choriomeningitis virus (LCMV) infection induces a protective CTL response consisting of gp- and nucleoprotein (NP)-specific CTL. We find that a small load of LCMV led to immunodominance of NP-CTL, whereas a large viral load resulted in dominance of gp-CTL. This is the first study describing that immunodominance is not fixed after infection with a given pathogen, but varies with the viral load instead. We assumed higher Ag sensitivity for NP-CTL, which would explain their preferential priming at low viral load, as well as their overstimulation resulting in selective exhaustion at high viral load. The higher Ag sensitivity of NP-CTL was due to faster kinetics of NP-epitope presentation. Thus, we uncover a novel factor that impinges upon immunodominance and is related to the kinetics of virus protein expression. We propose that CTL against early viral proteins swiftly interfere with virus replication, resulting in efficient protection. If these “early” CTL fail in immediate virus control, they are activated in the face of higher viral load compared with “late” CTL and are therefore prone to be exhausted. Thus, the observed absence of early CTL in persistent infections might not be the cause, but rather the consequence of viral persistence. The Journal of Immunology, 2003, 171: 5415–5422.

The CD8⁺ CTL recognize peptides presented by MHC class I molecules on the surface of APC (1). These peptides are predominantly generated from endogenous proteins in a proteasome- and TAP-dependent fashion (2–4). Viral peptides will therefore be presented on infected cells by MHC class I molecules, and as a result, virus-specific CTL are important effectors to control virus replication and spread (5–9).

Infection of C57BL/6 (H-2b) mice with lymphocytic choriomeningitis virus (LCMV) induces a strong and protective CTL response that is dominated by four epitopes: gp-derived gp33–41/Db, gp34–41/Kd, and gp276–286/Dd and nucleoprotein (NP)-derived NP396–404/Dd. The response in BALB/c (H-2b) mice is strongly dominated by NP118–126/Ld, but also responses to gp283–292/Kd are detectable (10–17). After infection with LCMV-Armstrong (Arm), the CTL response in C57BL/6 mice is dominated by NP396-CTL (15, 17), whereas infection of mice with faster replicating strains such as LCMV-WE, LCMV-Docile (Doc), or LCMV-Arm Clone13 or -t1b (18) results in immunodominance of gp33-CTL (10, 18, 19).

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4 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; Arm, Armstrong; DC, dendritic cell; Doc, Docile; m.o.i., multiplicity of infection; VV, vaccinia virus; ICS, intracellular staining; CTLp, precursor CTL.
Materials and Methods

Mice

C57BL/6 (H-2b) and BALB/c (H-2d) mice were bred in the Institut für Labortierkunde, University of Zurich (Zurich, Switzerland). 318 TCR-transgenic mice expressing the P14 TCR that is specific for LCMV gp33–41/D10 are on a C57BL/6 background (29). CB6F1 (H-2b/d) were obtained from Charles River Breeding Laboratories (Hanover, Germany). DIETER mice have a pure C57BL/6 background and expression of gp33–41 and NP396–404 as a transgene can be induced in their dendritic cells (DC) (30). All mice were kept under specific pathogen-free conditions and were at least 6 wk old at the beginning of the experiments. Animal experiments were performed in compliance with Swiss national and cantonal laws (Kantonales Veterinäramt Zürich) on animal protection.

Viruses

LCMV-WE was originally obtained from Dr. F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) (31)]. LCMV-Arm was obtained from M. Oldstone (The Scripps Clinic and Research Foundation, La Jolla, CA) (32) and LCMV-Doc was a variant isolated from a LCMV-WE carrier mouse and was obtained from C. Pfau (Department of Biology, Rensselaer Polytechnic Institute, Washington, DC) (33). LCMV was propagated on L929 cells at a low multiplicity of infection (m.o.i.). Vaccinia virus (VV), strain WR, was originally obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD). rVV carrying one of the two immunodominant CTL epitopes in the context of H-2d were generated by cloning synthetic oligonucleotides encoding for the peptide sequence (5′-CATG-xxx-xxx-xxx-xxx-3′, where xxx represents the peptide coding sequence and the underlined the added start codon) into the NcoI/BglII-digested transfer vector pScl11.3OR2 (34). The minigene sequences were: gp33 = AAAGCTGTGTACAATTTCGC CACCTGT (MKAVYNFATC) and NP396/H11005 = TTTCAACCACAAAAT. Recombinant viruses were selected by 5-bromo-2′-deoxyuridine and recombinant plasmids were identified by β-galactosidase activity as described elsewhere (35). rVV were plaque purified three times on BSC40 cells. As a control, VV expressing the vesicular stomatitis virus gp (VVc) was used. All VV were propagated at a low m.o.i. on BSC40 cells.

Dendritic cells

Bone marrow-derived DC were generated from the femora and tibiae of DIETER mice as previously described (36). Mice were primed by i.v. injection of 5 × 10⁷ DC.

Cell lines

MC57G are methylcholanthrene-induced fibrosarcoma cells of C57BL/6 origin. EL-4 are dimethylbenzanthracene-induced thymoma cells of C57BL/6 origin. P815 are mastocytoma cells from BALB/c origin. BSC40 cells are a subclone of the green African monkey kidney cell line BSC-1. NP118–126L-Kb-specific and gp34–41/Kc-specific T cell hybridomas carrying the loz2 reporter construct were a kind gift from M. Groettrup (University of Constance, Constance, Germany). The NP118 hybridoma recognizes NP118–126 (RPQAGVMYM) plus H-2Ld, the gp34 hybridoma gp34–41 (AVYNFATC) plus H-2Kb (37, 38). Peptide-specific CTL lines

Splenocytes from LCMV-WE memory mice (injected with 100 PFU LCMV-WE at least 2 mo before) were restimulated with irradiated and peptide-loaded (10⁻⁷ M), thiglycolate-elicited macrophages (1 ml of thiglycolate l.p. at day −3) at a ratio of 20:1 in the presence of 25 U/ml IL-2. Cultures were restimulated every week as described above at a ratio of 5:1. After three rounds of restimulation, CTL were found to be of single specificity. BALB/c memory CTL were restimulated with NP118–126 (RPQAGVMYM, L⁰) or with gp283–292 (GYCLTKWMIL, K⁰) and C57BL/6 memory CTL were restimulated with gp33–41 (KAVYNFATC, D⁰), gp276–286 (SGVENPgcYCL, D⁰), or with NP396–404 (FFQPQNGQPF, D⁰). CTL assays

To measure direct ex vivo LCMV-specific CTL responses, splenocytes were tested for cytolytic activity toward peptide-loaded (10⁻⁷ M) or un-loaded ⁵¹Cr-labeled EL-4 or P815 target cells in a 5-h assay. The CTL activity of LCMV-specific memory CTL was measured 5 days after in vitro restimulation of splenocytes with peptide-loaded (10⁻⁷ M), irradiated, synthetic thiglycolate macrophages (responder/stimulator = 20:1) in the presence of 25 U/ml IL-2. Standard dilutions of cultures were tested for cytolytic activity on peptide-loaded ⁵¹Cr-labeled EL-4 or P815 cells in a 5-h assay.

Staining with tetrameric MHC class I-peptide complexes

Tetrameric complexes containing biotinylated H-2Db or H-2Lb, β₂-microglobulin, the relevant peptide, and extravidin-PE were generated as described previously (39, 40). Approximately 5 × 10⁶ cells were stained with 0.5–1 µg of tetramer in 25 µl of FACS buffer (PB; PBS plus 2% FCS plus 0.02% NaN₃) at 20°C for 15 min. One microliter of anti-CD8α-FITC (clone 53–6.7) was added and staining was continued for 30 min at 4°C. Cells were washed three times, fixed (FACS Lysis Solution; BD Biosciences, Mountain View, CA) and were analyzed by flow cytometry (FACScan; BD Biosciences) using CellQuest software. For tetramer dissociation assays, cells were stained with tetramer as above, anti-CD8α-FITC, anti-CD45R0-biotin, and anti-I-Ab-biotin, for 30 min at 4°C, were washed once, were stained with streptavidin-CyChrome for 20 min at 4°C (all BD Pharmingen, San Diego, CA), and were washed three times. Cells were suspended in 0.5 ml of FACS buffer containing propidium iodide and 150 µg/ml mouse monoclonal IgG2a anti-β₂-m (T21–460, obtained from G. Hammerling, Deutsches Krebsforschungszentrum, Heidelberg, Germany) to prevent rebinding of dissociated tetramer at t = 0. Samples were incubated on ice for the indicated times and tetramer staining was analyzed on CD8⁺, CD4⁺, B220⁺, I-Ab⁺, and propidium iodide-ve cells. We measured the rate of decay using flow cytometry and we obtained linear decay plots of the natural logarithm of the normalized fluorescence vs time, indicating that tetramer dissociation was occurring stochastically and that the resulting tetramer staining half-lives should be proportional to the half-lives of the respective TCR-peptide-MHC complexes (41).

Intracellular cytokine staining (ICS) for IFN-γ

One million splenocytes were incubated in 200 µl of IMDM plus 10% FCS and antibiotics at 6 h at 37°C with 10⁻⁶ M of the specific peptide or with medium alone as a negative control. To enhance intracellular accumulation of IFN-γ, brefeldin A was added at a final concentration of 5 µg/ml for the whole duration of the culture. For staining, cells were put at 4°C, washed with FACS buffer (see above), and stained with anti-CD8α-FITC (BD Pharmingen) for 30 min at 4°C. Cells were washed twice with PB and fixed with 100 µl of 4% paraformaldehyde in PBS for 5 min at 4°C. Two milliliters of permeabilization buffer (PB: FB plus 0.1% w/v saponin) were added and cells were incubated for 10 min at 4°C. Cells were spun down and stained intracellularly with anti-mouse-IFN-γ-FITC (clone AN18; BD Pharmingen) in PB for ≥60 min at 4°C. After washing three times with PB, cells were resuspended in FB and analyzed by flow cytometry as described above.

Kinetics of Ag presentation by LCMV-infected CB6F1 macrophages

Thiglycolate-elicited macrophages were isolated and infected with LCMV-Arm or with LCMV-WE at a m.o.i. of 2 and were further incubated at 37°C for the indicated times. Infected macrophages were harvested (t = 0) and brefeldin A was added to a final concentration of 5 µg/ml to freeze cells in their state of Ag presentation. The degree of infection was measured by intracellular staining for LCMV NP using FITC-labeled monoclonal rat-anti-LCMV NP (VLA42), followed by FACS analysis. Macrophages of the same batch were used as targets in a chromium release assay and as stimulators in ICS using peptide-specific CTL lines as effectors/responders. For these experiments, part of the macrophages were labeled with ⁵¹Cr (in the presence of brefeldin A) and were incubated with titrated numbers of peptide-specific CTL in a 5-h Cr release assay in the presence of brefeldin A. Another part of the macrophages was used as stimulators of IFN-γ production in a 6-h ICS in the presence of brefeldin A (3 × 10⁴ macrophages plus 3 × 10⁶ tetramer-positive CTL) as described above. In the chromium release assay as well as in ICS, macrophages loaded with titrated amounts of each of the five peptides (10⁻⁶–10⁻¹² M) were included as a sensitivity control for the individual CTL lines.

Kinetics of Ag presentation by cells from LCMV-infected mice

CB6F1 mice were infected i.v. with 2 × 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 APCs. Five × 10⁷ T cell hybridoma cells were incubated overnight in 96-well round-bottom plates at 37°C with 1.5 × 10⁶ splenocytes from infected CB6F1 mice or with 1.5 × 10⁶ naive CB6F1 splenocytes with titrated amounts of the relevant antigenic peptide in 200 µl of medium containing 5 µg/ml brefeldin A to freeze their condition of Ag

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presentation. After incubation, cultures were processed as described previously (43). Briefly, cells were washed with PBS and were subsequently fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min at 4°C. The plates were washed with PBS and overlaid with 50 μl of staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-d-galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂ in PBS). T cell hybridomas that were stimulated through the TCR were fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min at 4°C. The plates were washed with PBS and overlaid with 50 μl of staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-d-galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂ in PBS). T cell hybridomas that were stimulated through the TCR can be visualized by blue staining, resulting from a lacZ reporter gene under control of the IL-2 promoter/enhancer (37). Triplicate cultures were microscopically examined, and the number of blue cells per well was counted after overnight incubation at 37°C.

Measurement of virus titers

LCMV titers were determined in the spleens and livers at indicated time points after infection. To determine LCMV titers, organs were removed, homogenized in MEM/2% FCS, and titrated by 10-fold dilution onto monolayers of MC57G cells in 24-well plates. LCMV was detected after 2 days of incubation at 37°C by an immunofocus assay using an LCMV NP-specific mAb (VL4), as previously described (42). The detection limit of this focus forming assay is 100 PFU/ml.

Results

Immunodominance is influenced by the LCMV inoculum and is not related to T cell intrinsic features such as TCR affinity or the capacity to expand

Since LCMV is a noncytopathic virus that replicates massively in its natural host, the mouse, simple titration of the inoculum does not cover a wide range of viral loads. To circumvent this problem and to be able to analyze immunodominance at gradually increasing viral loads, we took advantage of different viral isolates that are known to have different replication rates, such that LCMV-Arm < LCMV-WE < LCMV-Doc. We infected C57BL/6 mice with titrated (10-fold dilutions) amounts of different LCMV strains and measured virus titers 5 days later in the spleen. We then chose the inoculum size to analyze immunodominance such that we had a graded increase in viral load (see Fig. 1). We infected C57BL/6 mice with 10² or 10⁶ PFU of LCMV-Arm, with 10⁰, 10², or 10⁶ PFU of LCMV-WE, or with 10² or 10⁶ PFU of LCMV-Doc. The viral load was measured in spleen at days 6, 8, and 15 (Fig. 1A, upper panels) and in liver (comparable to spleen, data not shown) and was found to increase in the following order: 10² PFU LCMV-Arm < 10⁶ PFU LCMV-Arm < 10⁰ PFU LCMV-WE < 10⁶ PFU LCMV-WE < 10⁰ PFU LCMV-WE < 10⁶ PFU LCMV-Doc < 10⁰ PFU LCMV-Doc < 10⁶ PFU LCMV-Doc. Virus was not detectable anymore at day 50 in any of the mice, except for mice infected with 10⁶ PFU LCMV-Doc (50,000 PFU/spleen), which is in accordance with previously published findings (20). Tetrramer staining 15 days after infection (Fig. 1A, middle panels) showed clear immunodominance of NP396-CTL at low viral load, which gradually decreased with increasing viral load. This resulted in immunodominance of gp33-CTL at higher viral loads. Functional analysis of the CTL by ICS ex vivo at day 15 after infection made the observed difference even more clear (Fig. 1A, lower panels): At low viral load, NP396-CTL were immunodominant, whereas they were functionally undetectable (although physically present to some extent) after infection with 10⁶ PFU LCMV-WE. In contrast, the number of gp33-CTL increased at increasing viral load. High-dose (10⁶ PFU) LCMV-Doc resulted in such high amounts of rapidly spreading virus that also gp-specific CTL were functionally and physically exhausted (20, 40, 44), ultimately resulting in lifelong virus persistence. The apparent higher sensitivity to exhaustion of NP-CTL might be explained by a lower naive precursor CTL (CTL₀) frequency of NP-CTL compared with gp-CTL; however, this possibility cannot account for the preferential priming of NP-CTL at low virus load. The viral loads after infection with 10⁶ PFU LCMV-Arm and 1 PFU LCMV-WE were apparently similar (Fig. 1A, upper panels); however, the immunodominance profile was found to be different (Fig. 1A, middle and lower panels). This is possibly attributable to the fact that the resolution of the plaque assay is not sufficient to detect small, but probably biologically relevant differences in viral load immediately after infection. Moreover, analysis of the gp- and NP-CTL response after infection of CB6F₁ mice with 10² PFU LCMV-Arm and 10² or 10⁶ PFU LCMV-WE demonstrated that the NP396- and the NP118-specific response behaved similarly (i.e., going down upon increasing virus load) and opposite to the gp33-specific response (i.e., increasing upon higher virus load) (Fig. 1B). Because the NP118 response is a very strong one, selective exhaustion of NP-CTL at high virus load is unlikely to

![FIGURE 1. NP396-specific CTL are preferentially primed under conditions of low viral load and are gradually exhausted as viral load increases. A, C57BL/6 mice were infected with 10⁰, 10², or 10⁶ PFU of LCMV-Arm, LCMV-WE, or LCMV-Doc. Upper panels, Viral titers were measured in the spleen at indicated time points after infection. Middle panels, The physical presence of gp33- and NP396-CTL in the spleen was measured at day 15 after infection by tetramers. Lower panels, The functional presence of gp33- and NP396-CTL in the spleen was measured at day 15 after infection by ICS. The values are the mean of three mice; one representative experiment of four is shown. B, CB6F₁ mice were infected with 10² PFU of LCMV-Arm and 10² or 10⁶ PFU of LCMV-WE. Left panel, The physical presence of gp33-, NP396-, and NP118-CTL in blood was measured at day 29 after infection by tetramers. Right panel, The functional presence in the spleen was measured at day 29 after infection by ICS. The values represent the mean of two mice; one experiment of two is shown. gp33; NP396; NP118.](http://www.jimmunol.org/Downloadedfrom/fig1b.png)
result from low CTL\textsubscript{b} frequencies. The preferential priming of NP-CTL at low viral load and their exhaustion upon increasing Ag levels suggests that NP-CTL have somehow a higher sensitivity to Ag than gp-CTL. We considered the possibility that CTL bearing TCR with high affinity were preferentially activated at low virus load and were more likely to be overstimulated and exhausted (18, 20) at higher virus load. As a consequence, we had to assume that NP396-CTL generally have a TCR of higher affinity compared with gp33-CTL. The most reliable manner to determine TCR affinities is probably by BioCore; however, this method cannot be applied on polyclonal T cell populations. Therefore, we used tetramer dissociation on ex vivo-prepared CTL as a measure for the TCR affinities of polyclonal, but mono-specific CTL populations after infection with LCMV (41). We always included 318 TCR-transgenic T cells that are specific for gp33/D\textsuperscript{b} (29) as a reproducibility control. We found comparable tetramer dissociation kinetics of gp33-CTL and of NP396-CTL after infection with LCMV (Fig. 2, A and B), suggesting no apparent difference in TCR affinity between the two specificities. In addition, we found no difference in TCR affinities between infection with LCMV-WE and -Arm (Fig. 2, A and B). Also, by titrating peptides in ICS for IFN-\gamma on targets in a CTL assay, we did not find substantial differences between gp33-41 and NP396-404 (data not shown) provided that in these particular experiments we used the optimized D\textsuperscript{b} binder KAVYNFATM, with an affinity for D\textsuperscript{b} comparable to NP396-404, instead of the natural peptide KAVYNFATC. In addition, when we used the natural peptide (KAVYNFATC) and corrected for lower affinity to D\textsuperscript{b} (12), we also did not see differences in peptide titrations. The interaction of the TCR with H-2D\textsuperscript{d}/gp33-41 is not affected if the naturally occurring cysteine on position 9 is replaced by a methionine (45). Thus, the change of immunodominance after infection with different amounts of virus cannot be explained by differences in TCR affinity of polyclonal NP396- and gp33-CTL populations.

To distinguish between factors intrinsic to the responding CTL populations and LCMV-specific features, we primed mice with 5 \times 10\textsuperscript{5} bone marrow-derived DIETER DC transgenic for gp33-41 and NP396-404 (30) and challenged them 10 days later with 2 \times 10\textsuperscript{6} PFU rVV expressing gp33-41 or NP396-404 as a minigene (VV33, VV396) or with 100 PFU LCMV-WE. We found that gp33-CTL and NP396-CTL expanded to a similar extent after VV minigene challenge (Fig. 2C). In contrast, NP396-CTL expanded considerably stronger than gp33-CTL after LCMV challenge. This indicated that the difference in expansion seen in LCMV-challenged mice is not an intrinsic property of the individual CTL populations but rather depends on LCMV as such.

**LCMV-infected macrophages present LCMV NP-derived CTL epitopes with increased kinetics compared with LCMV gp-derived CTL epitopes**

Using infected cells in vitro, it has been shown that the LCMV NP is detectable at least 12 h before LCMV gp on the protein level (28). Thus, NP-derived epitopes can be presented before gp-derived epitopes, which may have important consequences for CTL induction: NP-CTL will be primed before gp-CTL and they will thus start controlling the virus before most gp-specific precursors had the chance to interact with their nominal peptide on LCMV-infected cells. Therefore, NP-CTL generally will be primed in the face of higher viral load than gp-CTL and can interfere with priming of gp-CTL at the same time. We investigated the existence of kinetic differences in the onset of presentation between gp- and NP-derived epitopes by infecting thioglycolate-elicited CB6F1 (H-2\textsuperscript{d}) macrophages with LCMV-Arm or -WE at a m.o.i. of 2, resulting in infection of 100\% of the macrophages as demonstrated by intracellular staining with an mAb against LCMV NP (VL4 (42)). Subsequently, their status of Ag presentation was frozen at different time points after infection by brefeldin A, and these macrophages were used as stimulators for IFN-\gamma production (ICS) by mono-specific CTL lines specific for three gp-derived (gp33-41/29, gp276-286/D\textsuperscript{d}, gp283-292/K\textsuperscript{d}) and two NP-derived (NP118-126/L\textsuperscript{d}, NP396-404/D\textsuperscript{d}) epitopes. We found that gp-derived epitopes were detectable by CTL after 7-8 h of infection and reached their maximum after 10 h, whereas NP-derived epitopes reached their maximum around 2 h after infection (Fig. 3). This was true for both LCMV-Arm (Fig. 3, left panels) and -WE (Fig. 3, middle panels), excluding the possibility that differences in immunodominance seen in vivo after infection with LCMV-Arm (NP396 immunodominant) or -WE (gp33 immunodominant) were due to differences in Ag processing or presentation between the two LCMV strains. Because differences in Ag sensitivity between the individual CTL lines would obscure our data, we included CB6F1 macrophages loaded with titrated amounts of peptide in the same experiment to check the Ag sensitivity of CTL lines. Half-maximal stimulation of IFN-\gamma production required 10\textsuperscript{-10} M gp33,
CB6F₁ macrophages with titrated amounts of peptide in the same experiment. For both hybridomas, maximal numbers of blue cells were found at $10^{-7}$ M peptide or higher and background numbers were found below $10^{-10}$ M peptide. This shows that the gp34/K<sup>α</sup>-specific and the NP11/L<sup>d</sup>-specific hybridoma were equally sensitive to Ag.

The kinetics of Ag presentation by in vivo-infected splenocytes were similar to those by in vitro-infected, thioglycolate-elicited macrophages (Fig. 4). gp34-specific T cells were activated by splenocytes that were infected for 12 h or more, and splenocyte infection for 20 h or more induced maximal numbers. In contrast, infection of splenocytes for 4 h or more resulted in the presentation of the NP118 epitope and maximal levels were reached after 12 h. The maximal numbers of activated hybridomas after incubation with infected splenocytes were ~50% of the maximal values observed after incubation with peptide in both cases. This may be explained by the fact that peptides were present throughout the overnight culture, thus avoiding the influence of peptide off-rates as is the case with infected splenocytes in the presence of brefeldin A.

Presentation by in vivo-infected splenocytes displayed a delay of ~2 h for both epitopes (NP and gp derived) compared with the in vitro-infected macrophages (Fig. 3). This may be explained by the lower peptide sensitivity of the hybridomas (~$10^{-10}$ M (64)) compared with the mono-specific CTL lines we used (Fig. 3) or by the fact that it takes LCMV slightly longer to infect cells in vivo than in vitro, where the cells were infected at a high m.o.i. and in very close contact with the virus.

**Kinetic differences in the presentation of LCMV-derived CTL epitopes can be visualized in vivo**

To show the relevance of faster kinetics of presentation of NP-derived epitopes in vivo, we designed the following experiment:

**LCMV-infected splenocytes ex vivo present LCMV NP-derived CTL epitopes with increased kinetics compared with LCMV gp-derived CTL epitopes**

LCMV is known to infect a variety of cells in vivo, such as DC and macrophages (46), and, therefore, in vitro-infected, thioglycolate-elicited macrophages may not represent the physiologically relevant population presenting LCMV-derived epitopes in vivo. To analyze the kinetics of Ag presentation in vivo, we infected CB6F₁ mice with $2 \times 10^7$ PFU LCMV-WE and used their splenocytes at different time points after infection as APC for NP118–126/L<sup>d</sup>- or gp34–41/K<sup>α</sup>-specific T cell hybridomas (38, 43). To exclude the possibility that differences in Ag sensitivity between the two hybridomas would influence our result, we included uninfected CB6F₁ macrophages with titrated amounts of peptide in the same experiment. For both hybridomas, maximal numbers of blue cells were found at $10^{-7}$ M peptide or higher and background numbers were found below $10^{-10}$ M peptide. This shows that the gp34/K<sup>α</sup>-specific and the NP11/L<sup>d</sup>-specific hybridoma were equally sensitive to Ag.

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**LCMV-infected splenocytes ex vivo present LCMV NP-derived CTL epitopes with increased kinetics compared with LCMV gp-derived CTL epitopes**

LCMV is known to infect a variety of cells in vivo, such as DC and macrophages (46), and, therefore, in vitro-infected, thioglycolate-elicited macrophages may not represent the physiologically relevant population presenting LCMV-derived epitopes in vivo. To analyze the kinetics of Ag presentation in vivo, we infected CB6F₁ mice with $2 \times 10^7$ PFU LCMV-WE and used their splenocytes at different time points after infection as APC for NP118–126/L<sup>d</sup>- or gp34–41/K<sup>α</sup>-specific T cell hybridomas (38, 43). To exclude the possibility that differences in Ag sensitivity between the two hybridomas would influence our result, we included uninfected CB6F₁ macrophages with titrated amounts of peptide in the same experiment. For both hybridomas, maximal numbers of blue cells were found at $10^{-7}$ M peptide or higher and background numbers were found below $10^{-10}$ M peptide. This shows that the gp34/K<sup>α</sup>-specific and the NP11/L<sup>d</sup>-specific hybridoma were equally sensitive to Ag.

The kinetics of Ag presentation by in vivo-infected splenocytes were similar to those by in vitro-infected, thioglycolate-elicited macrophages (Fig. 4). gp34-specific T cells were activated by splenocytes that were infected for 12 h or more, and splenocyte infection for 20 h or more induced maximal numbers. In contrast, infection of splenocytes for 4 h or more resulted in the presentation of the NP118 epitope and maximal levels were reached after 12 h. The maximal numbers of activated hybridomas after incubation with infected splenocytes were ~50% of the maximal values observed after incubation with peptide in both cases. This may be explained by the fact that peptides were present throughout the overnight culture, thus avoiding the influence of peptide off-rates as is the case with infected splenocytes in the presence of brefeldin A.

Presentation by in vivo-infected splenocytes displayed a delay of ~2 h for both epitopes (NP and gp derived) compared with the in vitro-infected macrophages (Fig. 3). This may be explained by the lower peptide sensitivity of the hybridomas (~$10^{-10}$ M (64)) compared with the mono-specific CTL lines we used (Fig. 3) or by the fact that it takes LCMV slightly longer to infect cells in vivo than in vitro, where the cells were infected at a high m.o.i. and in very close contact with the virus.

**Kinetic differences in the presentation of LCMV-derived CTL epitopes can be visualized in vivo**

To show the relevance of faster kinetics of presentation of NP-derived epitopes in vivo, we designed the following experiment:
We reasoned that “early” memory CTL (e.g., NP-CTL) recognize infected cells and start to eliminate them before “late” CTL (e.g., gp-CTL) had the chance to interact with their Ag, and thus substantially interfere with the priming of the latter. As a consequence, early memory CTL (e.g., NP-CTL) should block priming of late CTL (e.g., gp-CTL) upon infection with LCMV, but not vice versa. Thus, we primed mice with VV33, VV396, or VVc and challenged them 30 days later with 100 PFU LCMV-WE to investigate whether we could prime gp-CTL in the face of a pre-existing NP-CTL memory response and vice versa. It is important to note that priming with VV33 or with VV396 protects mice against subsequent LCMV challenge, resulting in very low (undetectable) LCMV levels (data not shown). We measured the frequency of gp33- and NP396-CTL in the spleen by tetramer staining (Fig. 5) at day 9 after LCMV challenge and confirmed the data by a primary ex vivo CTL assay 9 days after LCMV infection (data not shown).

After VV396 priming, pre-existing memory NP396-CTL were found to expand massively upon LCMV challenge (Fig. 5). gp33- CTL were not primed by LCMV in the presence of memory NP396-CTL (Fig. 5). This can be explained by different kinetics of Ag presentation: NP396-CTL could start to control LCMV-infected cells before gp33-CTL could efficiently interact with them, resulting in inhibition of gp33-CTL priming. In the reverse situation, VV33 priming results in enhanced expansion of gp33-CTL after LCMV challenge compared with VVc priming, thus demonstrating the increased number of gp33-specific precursors due to VV33 priming (Fig. 5). Interestingly, and in contrast with the situation described above, pre-existing memory gp33-CTL did not interfere with the priming of NP396-CTL (cf VVc and VV33 in Fig. 5). This shows that gp33-CTL, even although present as memory cells and at increased CTLp numbers, could not interfere with the priming of NP396-CTL. Because gp33 is presented with delayed kinetics compared with NP, gp33-CTL were too late to interfere with NP396-CTL priming. The ex vivo CTL assay performed on day 9 confirmed the tetramer data (data not shown). It is unlikely that differences in priming between VV33 and VV396 are responsible for the observed findings: Priming with VV33 or with VV396 resulted in a specific CTL response of similar size as determined by tetramer analysis (0.8–1.0% of the CD8^+ at day 10 after infection) and by limiting dilution analysis (1 in 10^4 splenocytes at day 7 after infection). The frequency of specific memory CTL was comparable for VV33- and VV396-infected mice over at least 5 wk as measured by protection against LCMV challenge (data not shown).

**Discussion**

LCMV infection induces a protective CTL response against both gp- and NP-derived epitopes. We describe here that inocula leading to a relatively low virus load (e.g., LCMV-Arm) resulted in immunodominance of NP-CTL, whereas increasing viral load (e.g., LCMV-WE or -Doc) coincided with decreasing NP-CTL, ultimately resulting in immunodominance of gp-CTL. We explained this finding by a higher sensitivity to Ag of NP-CTL compared with gp-CTL: NP-CTL thus would be preferentially activated by low levels of Ag and (partially) exhausted (18, 20, 44) upon increasing viral load. It has been suggested (46) that different strains of LCMV infect different cells in vivo; this would preclude comparison between strains as we do here. We infected C57BL/6 mice with 10^6 PFU of LCMV-Arm, -WE, or -Doc and stained splenocytes for subset markers and LCMV NP. We found that, independent of the strain used to infect, 5–8% of CD11c^+, 0.7–1.5% of CD11b^+ , and <0.5% of CD19^+ or TCRβ^+ cells stained positive for LCMV NP. This excludes that changes in immunodominance were due to different tropism of the individual strains. Comparison of the gp33- and the NP396-specific CTL response after infection with 1 or with 100 PFU of LCMV-WE demonstrates that the gp33-specific response increased, whereas the NP396 response decreased with higher viral load (Fig. 1). The data of Ou et al. (44) can be interpreted along the same line: They showed that infection of mice deficient for the IFN-α/β receptor (A129 mice) with 100 PFU of LCMV-WE or with 10^5 PFU of LCMV-Arm resulted in a substantially higher viral load and in selective exhaustion of NP396-CTL when compared with wild-type control mice. Thus, also without varying the virus strain/dose used for infection, but by choosing the conditions such that the virus replicates to higher titers, NP-CTL were found to gradually disappear. We considered that the higher Ag sensitivity of NP-CTL could be due to 1) higher TCR affinity of NP-CTL, 2) prolonged presentation of NP compared with gp by infected cells (47), or 3) different kinetics of presentation of NP vs gp epitopes (28). We found no difference in TCR affinity as measured by tetramer dissociation (41) between NP396- and gp33-CTL or by peptide titration in CTL assay and in ICS. The finding that in mice persistently infected with LCMV gp expression was not found after day 15, whereas NP could be detected in all mice even after 80 days, has been used to explain the relatively high sensitivity to overstimulation of NP-CTL (18). However, the prolonged presence of a stable protein, like NP, does not mean that CTL epitopes are generated continuously: It has been proposed by Yewdell et al. (48) that the majority of the MHC class I-binding peptides are derived from defective ribosomal products and thus heavily depend on protein neosynthesis. Two studies supported this hypothesis (49, 50), and we showed recently for LCMV NP that no CTL epitopes were generated from this long-lived protein, unless neo-synthesis took place (51). Therefore, the relevance of the observation that gp is not as persistent as NP in chronically infected mice to (over)stimulation of NP-CTL is not certain. We cannot formally exclude the possible contribution of cross-priming to the phenomenon we describe here. However, we do not expect cross-priming to be an important pathway for priming a CTL response against a pathogen that very efficiently infects DC.

We analyzed the kinetics of presentation of NP- and of gp-derived epitopes by LCMV-WE- and -Arm-infected macrophages, because we argued that faster presentation of NP-derived epitopes could account for a better protective capacity on a per cell basis.
(19), for better priming at limited viral load, and for higher sensitivity to exhaustion at high viral load. We found that, independent of the LCMV strain used, both NP-derived epitopes were sufficiently presented to stimulate CTL as early as 2.5 h after infection, whereas it took at least 8 h before three different gp-derived epitopes were recognized. It is unlikely that different traveling times of particular MHC class I molecules from the endoplasmic reticulum to the cell surface account for the kinetic differences observed here, as H-2D^b is the restricting element of one NP-derived and two gp-derived peptides. To formally exclude the possibility that in vitro-infected, thioglycolate-elicited macrophages differed with respect to Ag presentation from those cells that are naturally infected by LCMV in vivo, we used splenocytes isolated from LCMV-infected mice at different time points after infection as APC to stimulate gp- or NP-specific T cell hybridomas (37, 38, 43). Using this approach, we found similar kinetic differences between the presentation of gp- and NP-derived epitopes. We used above readouts for Ag presentation rather than peptide elution from MHC class I molecules, because the latter method requires a large amount of cells (>5 x 10^6 per time point and virus) that is impossible to obtain from other sources than from cell lines, the latter being probably abnormal in Ag processing or presentation.

As a result of faster kinetics of NP presentation, NP-CTL encounter their nominal peptide on every infected cell before gp-CTL do and will therefore be activated earlier and probably in the face of higher viral loads. The consequences of delayed gp presentation were demonstrated in vivo: pre-existing memory NP-CTL inhibited priming of gp-CTL, whereas pre-existing memory gp-CTL did not interfere with NP-CTL priming. In an attempt to investigate consequences of kinetic differences in epitope presentation for in vivo priming in a direct manner, we analyzed the expansion kinetics of NP- and gp-specific CTL at different early time points after infection. To this end, we performed ICS at 3, 4, 5, 6, 7, 8, and 9 days after infection. We detected LCMV-specific CTL from day 5 onward; however, we did not find substantial differences in expansion kinetics of NP- vs gp-CTL (data not shown). Detection of specific CTL by tetramers was unreliable as long as virus was still present (approximately until day 7), probably due to TCR down-regulation. Kinetic differences of 8 h may not lead to detectable differences in expansion kinetics, because the time required for initial T cell activation and proliferation is much longer than 8 h, thus masking possible initial differences. Moreover, it may well be that possible differences in expansion kinetics could not be detected anymore by day 5 because most of the virus usually is eliminated by then and a substantial part of the NP-CTL may be exhausted before they could expand to numbers that allow detection. No comparative data on naive CTLp frequencies are currently available. Alternatively, small differences in the frequencies of CTLp could probably cover up differences in expansion kinetics.

Kinetic differences in epitope presentation may also explain the finding that a NP118-CTL escape mutant of LCMV, that still expressed the gp283 CTL epitope, was controlled slower than the wild-type virus in BALB/c mice, but equally fast in CB6F1 mice (that could use an additional NP-derived epitope, NP396) (13). It was found in the same study that the 118-CTL escape mutant efficiently primed subdominant gp283-CTL in BALB/c mice, but not in CB6F1 mice, whereas LCMV-WE did not prime gp283-CTL in either mouse strain: Absence of an early NP-specific response in BALB/c mice (but not in CB6F1 mice) results in slower virus control and allows the priming of otherwise subdominant and later gp-specific responses. Results obtained by mathematical analysis of the LCMV-specific CTL response in BALB/c mice (52) can also be explained by kinetic differences in epitope generation.

Based on our findings, we propose the general rule that CTL against early epitopes normally clear most of the virus and are at risk to be exhausted if the initial viral load is relatively high. Experiments showing that early NP-CTL are 100 times more effective in protecting mice against LCMV compared with late gp-CTL (Ref. 19 and M. F. van den Broek and H. C. Probst, unpublished data) fit this hypothesis. Data on specific CTL responses in other viral infections than LCMV seem to support the general applicability of our hypothesis. Vaccination of macaques with early simian immunodeficiency virus proteins (Rev, Tat) induced a protective immunity that was substantially more effective than vaccination with late simian immunodeficiency virus proteins (Pol, Gag) (53). Along the same line, longitudinal analysis of the HIV-specific CTL response in individuals with a high HIV load revealed that Rev- and Tat-specific CTL were the first to disappear (54–56). In analogy to what we described here for LCMV infection, namely, evidence for early activation of specific CTL followed by their gradual functional or even physical disappearance after they were unable to eliminate the Ag in due time can be found in a variety of infections, such as HIV (57, 58), hepatitis B (59–61), and hepatitis C (62) and also in tumor-bearing patients (63).

We propose that CTL against early viral proteins swiftly interfere with virus replication, resulting in efficient protection. If these early CTL fail in immediate virus control, they are activated in the face of higher viral load compared with late CTL and are therefore prone to be exhausted.

Thus, the observed absence of CTL in persistent infections might not be the cause, but rather the consequence of viral persistence.

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