Thymic Selection and Adaptability of Cytotoxic T Lymphocyte Responses in Transgenic Mice Expressing a Viral Protein in the Thymus

By Matthias G. von Herrath,* Janel Dockter,* Michael Nerenberg,* Jean E. Gairin,*‡ and Michael B. A. Oldstone*

*From the *Department of Neuropharmacology, Division of Virology, The Scripps Research Institute, La Jolla, California 92037; and ‡Laboratoire de Pharmacologie et Toxocologie Fondamentales, Centre National de La Recherche Scientifique, 31400 Toulouse, France*

Summary

Upon primary challenge with lymphocytic choriomeningitis virus (LCMV), H-2^d (BALB/cByJ) mice mount a cytotoxic T lymphocyte (CTL) response to a single immunodominant domain of the viral nucleoprotein (NP) but no detectable response to the viral glycoprotein (GP). To manipulate this CTL response, the viral NP gene was expressed in the thymus and peripheral T lymphocytes using the murine Thy1.2 promoter. As a result, such Thy1.2-NP (H-2^d) transgenic (tg) mice deleted their high-affinity anti-LCMV-NP CTL, but generated equal numbers of lower-affinity NP CTL. Further, they made an alternative anti-LCMV-GP CTL response that is not normally found in non-tg mice indicating a hierarchial control of the CTL response. Unlike the H-2^d mice, H-2^b (C57Bl/6J) mice normally mount a CTL response to both LCMV-GP and -NP. When the LCMV-NP was expressed using the Thy1.2 promoter in these H-2^b mice, the LCMV-NP–specific CTL response was completely aborted and no CTL to new, alternative viral epitopes were generated. Dilutions of H-2^b or H-2^d NP peptides indicated that 3–4 logs less H-2^b NP peptide was required to sensitize syngeneic target cells for CTL-specific lysis, suggesting that the differing affinities of H-2^b and H-2^d major histocompatibility complex molecules for their peptides likely account for the total removal of NP CTL in the H-2^b mice but only partial removal in H-2^d mice made to express thymic NP. Thymic grafting experiments done with thymi from newborn Thy1.2-NP tg mice show that selection processes studied in this model are of central (thymic) origin and are not caused by Thy1.2-positive LCMV-NP–expressing T lymphocytes in the periphery.

The thymus plays a key role in the maturation and selection process of T lymphocytes. Undifferentiated $CD4^+/CD8^+$ doubly positive precursor cells mature into $CD4^+$ and $CD8^+$ single positive cells (1). They acquire restriction for self-MHC through a process of positive selection, which is thought to involve selection of those cells having TCRs with low but not zero affinity for complexes of self-peptide plus class II or I MHC molecules expressed on thymic epithelium (2, 3). In contrast, thymocytes with high affinity for self-MHC (plus peptide) are deleted in a process termed negative selection (3–9). Similarly, expression of viral antigens in the thymus leads to deletion of virus-specific T cells during thymocyte maturation. Although this process is beneficial in removing anti-self (potential autoimmune) T cells, it can also be harmful by providing a mechanism for viruses to persist over the life span of a host and cause chronic progressive disease (10–12). Furthermore, T cells with lower affinity can escape the negative selection process and emerge into the periphery, where they can cause autoimmune disease (13).

Virus-specific CTL are exquisitely specific in that they recognize a precise viral peptide of 8–12 amino acids (aa)[1] in length presented by a unique MHC molecule. In H-2^d mice, >98% of the primary CTL response to lymphocytic choriomeningitis virus (LCMV), whose genome encodes four proteins, focuses on only one viral determinant, the nucleoprotein (NP) aa 118–127. No detectable response to the viral glycoprotein (GP) is noted. H-2^b mice, in contrast, make LCMV CTL to both, viral NP (aa 396–404) and GP (aa 33–41 and aa 276–286). We adapted this well-characterized situation to study the plasticity of the anti-LCMV CTL response (14–17). By using the Thy1.2 promoter (18, 19), we developed transgenic mice (Thy1.2-NP tg mice) in which the viral NP gene was expressed in the thymus. We then asked the following four questions. First, are all anti-LCMV-NP CTL deleted in

[1] *Abbreviations used in this paper:* aa, amino acid; ARM, Armstrong; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; tg, transgenic, vv, vaccinia virus.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/11/1901/10 $2.00
Volume 180 November 1994 1901–1910

the thymus, or are only high-affinity NP CTL removed with low-affinity NP CTL still able to pass through the thymus and populate peripheral lymphoid organs? Second, are CTL to new, alternative epitopes now generated? Third, if so, is the altered or newly generated anti-LCMV CTL response sufficient to effectively control acute viral infection and prevent persistent infection? Fourth, can the same or a similar phenomenon be observed among several MHC haplotypes? The answers to these questions form the body of this report.

Materials and Methods

Generation of Tg Mice. A vector utilizing the murine Thy1.2 gene was designed to direct expression of viral protein to the thymus (19, and see Fig. 1). The Thy1.2 promoter directs higher expression of the tg RNA to the thymus than peripheral T cells or fibroblasts (19). The complete cDNA for the LCMV-NP coding regions was assembled from overlapping cDNA clones derived from the small (S)-RNA segment of LCMV-Armstrong (ARM) (16). Correct orientation and sequence were confirmed by the Maxim Gilbert method (20). After cloning and amplification, the Thy1.2-LCMV-NP cassette was isolated, purified on a high-resolution sucrose gradient, and used to generate tg mice as described (18). C57Bl/6J (H-2^b) × BALB/cByJ (H-2^d) (b × d) mice were used as a source of oocytes, and injected eggs were implanted in pseudopregnant CD1 females. Founder mice demonstrating integrated copies of the transgene were crossed to b × d for one generation to confirm transmission of the transgene. One line, Thy1.2-LCMV-NP 6-6, which had stably integrated and passed the LCMV-NP transgene, was selected for detailed study. To obtain Thy1.2-NP mice with a H-2^d– or H-2^b–restricted CTL response, b × d mice were bred to the fifth generation with BALB/cByJ or C57Bl/6 mice.

Biochemical Studies. Mice carrying the transgene were identified by hybridization of DNA extracted from tail biopsies using an LCMV-NP–specific probe (20, 21). RNA to be analyzed was extracted from their PBL and organs (thymus, brain, liver, and muscle) with the guanidinium-isothiocyanate method (20). Before PCR analysis of LCMV-NP RNA, RNA samples were treated with RNase-free RQ1 DNase to eliminate contaminating DNA. The RT-PCR was carried out as directed by the manufacturer (Perkin Elmer Cetus, Norwalk, CT) and PCR was run for 40 cycles. LCMV-NP–specific primers were: 5′ CAG TTA TAG GTG CTC TTC CGC 3′ and 5′ AGA TCT GGG AGC CTT GCT TTG 3′.

Virologic and Immunologic Studies. Virus stocks consisted of LCMV-ARM (clone 53b), Pichinde virus, and vaccinia virus (vv) LCMV GP and NP recombinants that expressed LCMV-GP aa 1-398 and LCMV-NP aa 1-558 (16, 21). Virus was plaque purified three times on Vero cells and virus stock prepared by a single passage on BHK-21 cells. Stocks of recombinant vv were prepared by infection of 143 TK^- cells in media containing bromodeoxyuridine (22). MC57 (H-2^b) and BALB/Cl7 (H-2^d) cells used as CTL targets were grown as reported (21). K2A cells stably transfected with L^d were generated as described (15).

To determine CTL activity toward infected target cells, splenic CTL were obtained from mice 7 d after i.p. inoculation of 2 × 10^5 PFU of LCMV (21). E/T ratios used were 100:1, 50:1, 25:1, 12:1, and 6:1 for splenic CTL, and 10:1, 5:1, 2:1, and 1:1 for CTL clones. Target cells (MC57/H-2^b, BalbC17/H-2^d, or K2A/L^d) were infected with either LCMV or Pichinde virus for 1 h at 37°C (multiplicity of infection [MOI of 1]) or vv/LCMV recombinants for 1 h (MOI of 3). 48 (LCMV/Pichinde virus) of 12 h (vv) after infection, cells were labeled with ^{51}Cr (21). Incubation of virus-infected or -uninfected ^{51}CR-labeled targets with splenic CTL or CTL clones (21) lasted for 4.5–5 h, after which the amount of chromium released was measured (21). LU were defined as the reciprocal of the number of effector cells required for 25% lysis of 10^4 targets determined by correlating several E/T ratios with the respective ^{51}Cr-release values. Killing of peptide-coated uninfected target cells by CTL followed our reported procedures (23).

To determine CTL activity after secondary stimulation, spleen cells harvested from mice 30–120 d after primary inoculation with 10^5 PFU LCMV i.p. were incubated with MHC-matched, irradiated, LCMV-infected macrophages in the presence of T cell growth factor and irradiated syngeneic spleen feeder cells (21). For precursor frequency analysis, spleen cells were harvested on day 7 after primary LCMV infection. These cells were diluted serially and cultivated in 96-well flat bottom plates in the presence of T cell growth factor and syngeneic irradiated LCMV-infected (10^3 PFU/ml) spleen cells (10^5/well). After 9 d, each well was assayed for CTL lysis (described above) on target cells that were uninfected or infected with LCMV or vv and that expressed NP or GP. The fraction of positive cultures (lysis > 11%) was determined for each dilution.

The phenotype of surface molecules expressed on lymphocytes was tested by incubating cells with mAbs to murine CD4 (rat mAb YTS 191.1.1), CD8 (rat mAb YTS 169.4.2), or Thy1.2 for 30 min followed by incubation with mouse anti–rat Ig FITC reagent (21) and quantitation or sorting by FACS® (Becton Dickinson & Co., Mountain View, CA) (21). LCMV-specific antibody was determined by a solid-phase ELISA (21, 24, 25). Antibodies to LCMV-NP in the sera of tg mice were defined using [^{35}S]methionine-labeled LCMV-infected BHK cells, immune precipitation, and SDS-PAGE (21).

Thymic Transplantations. To isolate and observe the function of the thymus in our tg mice, we anesthetized SCID mice with ketamine and Metophane (Pitman-Moore, Mundelein, IL), and thymi excised from 1-d-old newborn tg or non-tg pups were implanted under their renal capsules using sterile conditions. Thymi were allowed to develop for 6 wk and then experimentally assayed for their ability to generate LCMV-specific CTL activity.

Immunohistochemical Studies. Pancreata and spleens were removed and quick-frozen in O.C.T. compound, after which 6–10 μm sections were cut, mounted on sialin-coated superfrost plus glass slides (Fisher Scientific, Pittsburgh, PA), fixed, and stained as reported (13). Primary antibodies were rat anti–mouse L3T4, rat anti–mouse Ly2 and Ly3 (Pharmingen, Sorrento Valley, CA), and secondary antibody was biotinylated goat anti–rat IgG (Boehringer Mannheim, La Jolla, CA). Hematoxylin-eosin stains were carried out on paraffin-mounted tissue fixed with Bouin's fixative.

Results

LCMV-NP RNA Is Expressed in Thymus, Spleen, Brain, and PBL of Thy1.2-NP tg Mice. When tested by PCR, LCMV-NP RNA (289-bp fragment) was expressed in the spleen, thymus, Thy1.2-positive PBL (FACS®-sorted twice) and brain of Thy1.2-NP tg mice (Fig. 2). However, no LCMV-NP RNA was found in Thy1.2-negative PBL or in pancreas, kidney, muscle, or liver from Thy1.2-NP tg mice. Despite several attempts using immunohistochemical staining techniques on tissues, we were unable to detect LCMV-NP protein in the thymus. As reported elsewhere (21), this is due to the inability of the antibody to detect low expression of NP trans-

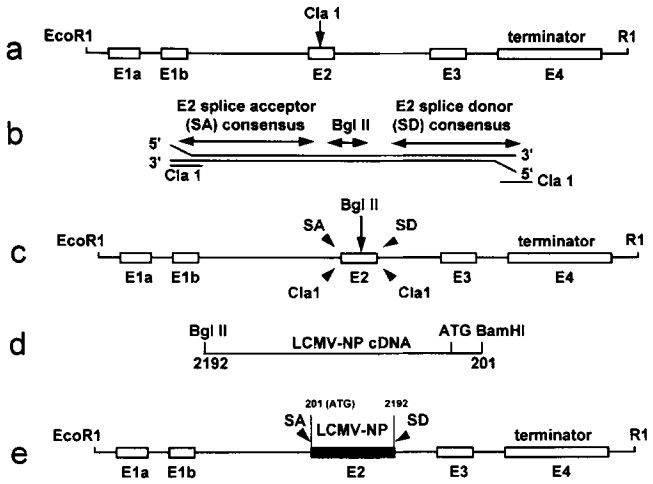

Figure 1. Construction of the cassette for generation of Thy1.2 NP tg mice. Within the murine Thy1.2 gene (*a*), exon 2 (*E2*) was replaced by a synthetic exon 2. Two 54-mer oligos (*b*) were synthesized, reconstructing the splice donor (*SD*) and splice acceptor (*SA*) sites and creating a unique BglII cloning site. The synthetic exon was inserted in place of the original E2 at the Cla1 site between exons E1b and E3 (*c*). The BglII site was now used to insert the full-length LCMV-NP cDNA (*d*) into the synthetic exon 2 of the Thy1.2 gene. The cloning vector used for these constructs was pUC13. The final cassette used for generation of tg mice was the EcoRI fragment containing all exons of the Thy1.2 gene and the LCMV cDNA, as shown in *e*.

gene protein. Such low levels of NP protein can be detected by MHC-restricted, NP-specific CTL (21).

CTL Response to LCMV-NP Is Aborted in H-2^b Thy1.2-NP tg Mice. The LCMV-specific CTL response was measured in H-2^b Thy1.2-NP tg mice and their non-tg littermates 7 d after challenge with 10^5 PFU of LCMV i.p. As expected (21) and as shown in Table 1, CTL from non-tg C57BL/6 (H-2^b) and non-tg C57Bl/6 × BALB/c (b × d) mice killed both LCMV-GP- and -NP-expressing MHC-matched target cells, whereas CTL from BALB (H-2^d) mice killed only LCMV-NP-expressing syngeneic targets, but not those expressing LCMV-GP. In contrast, CTL from H-2^b Thy1.2-NP tg mice failed to kill LCMV-NP-expressing syngeneic targets, but did lyse those expressing LCMV-GP. Thus, the LCMV-NP-specific CTL response was specifically aborted in H-2^b Thy1.2-NP tg mice.

CTL Response to LCMV-NP in H-2^d Thy1.2-NP tg Mice Is Markedly Reduced but Not Aborted. In non-tg H-2^d mice, the primary CTL response was to viral NP with no response to GP (Table 1). As seen in Fig. 3 (*A*), unlike H-2^b mice, H-2^d Thy1.2-NP mice still generated a CTL response to LCMV-NP but of considerably lower activity than that of their age- and sex-matched non-tg littermates. This reduction of the CTL response was specific for LCMV, because tg and non-tg H-2^d mice made equivalent CTL responses to both Pichinde and vaccinia viruses (Table 2). Hence, thymic expression of the LCMV-NP transgene dampens the anti-LCMV-NP CTL response specifically, but not CTL responses against other viruses.

We then assessed the numbers and characteristics of these CTL with lower activity. As shown in Fig. 4 *B*, 10-fold-less antibody to CD8 was required for a 50% reduction of lysis by NP-specific CTL from Thy1.2-NP tg mice than from non-tg controls. Fig. 4 *A* documents that 10-fold-greater amounts of the immunodominant NP peptide (RPQASGVYMG) (50% end point lysis) was required to coat target cells for lysis by LCMV-NP-specific CTL from the tg than from the non-tg mice. Finally, Fig. 3 *B* shows that the number of CTL precursors for low-activity CTL in Thy1.2-NP tg mice virtually equaled those for high-activity CTL in their non-tg littermates. These data show that for H-2^d Thy1.2-NP tg mice, the reduced activity of CTL results from their lower affinity for LCMV-infected target cells and not from an overall reduction in numbers of CTL.

Less LMCV-NP Peptide Is Required to Sensitize H-2^b than

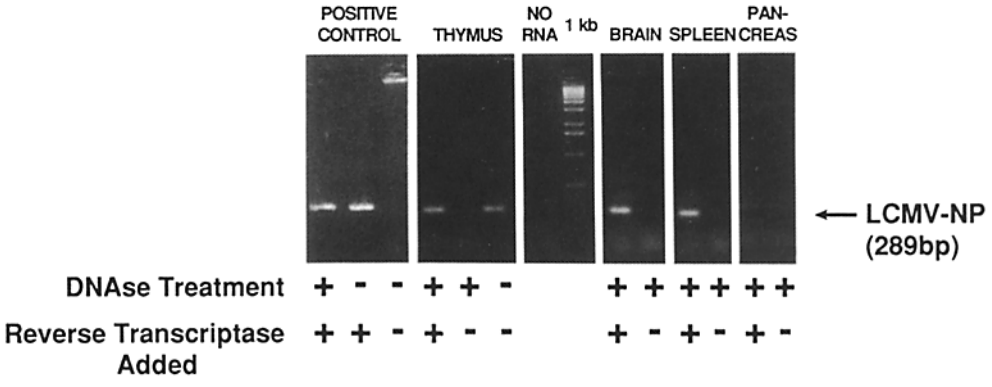

Figure 2. Detection of LCMV-NP RNA in thymus, spleen, brain, and Thy1.2-positive PBL but not in pancreas or Thy1.2-negative lymphocytes of Thy1.2-NP tg mice. RNA samples were obtained from organs of Thy1.2-NP tg mice. Splenic RNA from a mouse persistently infected with LCMV and containing viral RNA sequences in its spleen served as a positive control. All samples were treated with RNase-free RQ1 DNase before starting the reverse transcriptase reaction to remove traces of contaminating DNA. Negative control lanes without addition of reverse transcriptase do not show any LCMV-NP-specific product. Treatment of RNA with RQ1 DNase does not affect the amount of PCR product (first two lanes with control RNA on the left side of the graph).

Table 1. *Thy 1.2 NP (H-2^b) Tg Mice Fail to Generate LCMV-specific CTL to Viral NP but Generate CTL to Viral GP*

Day 7 primary splenic CTL from:	H-2 type	Percent specific ^{51}Cr released from targets infected with: H-2^b ARM	H-2^b vvNP	H-2^b vvGP	H-2^d ARM	H-2^d vvNP	H-2^d vvGP
Non-tg mice:							
C57/B16	b	46 ± 8	18 ± 3	24 ± 2	0	2 ± 1	2 ± 2
BALB/c	d	0	0	1 ± 1	64 ± 9	30 ± 7	6 ± 2
C57/B16 × BALB	b × d	54 ± 7	25 ± 3	31 ± 5	61 ± 10	36 ± 8	2 ± 2
Thy 1.2 NP tg mice:							
No. 38	b	24	2	17	7	4	5
No. 40	b	40	5	22	4	2	5
No. 41	b	43	2	23	6	1	5

6–8-wk-old Thy1.2 NP mice and non-tg littermates received 10^5 PFU i.p. of LCMV-ARM (clone 53b). 7 d later, lymphocytes were harvested from spleens and assayed for MHC-restricted LCMV-specific CTL activity. Effectors used at a ratio of 50:1 are shown. ^{51}Cr-labeled target cells were H-2^b (MC57) or H-2^d (BALB/Cl7) fibroblasts uninfected or infected with LCMV-ARM or vaccinia recombinants expressing LCMV-NP (vvNP) or LCMV-GP (vvGP). Variance was <10% in three separate experiments. Mean value ± 1 SD is shown for groups of 20 non-tg mice. The three tg mice represent equivalent data for >10 mice studied.

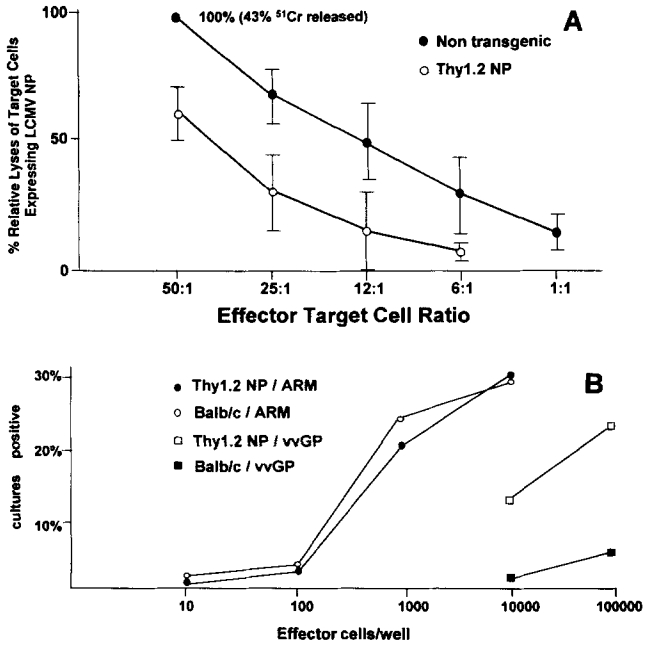

Figure 3. (*A*) Analysis of LCMV-NP–specific CTL generated in the spleens of non-tg H-2^d control (●) or Thy1.2-NP H-2^d tg (○) mice. Bar represents 2 SE for each time point. Targets were ^{51}Cr-labeled BALB/Cl7 (H-2^d) fibroblasts infected with vv/LCMV NP recombinant. H-2 restriction occurred, as killing of similarly infected H-2^b targets did not cause ^{51}Cr release. Four to five mice were used per group. See Materials and Methods section for CTL assay. (*B*) Numbers of CTL precursors in Thy1.2-NP tg mice and non-tg littermates. Analysis was performed as described in Materials and Methods. Positive cultures showed a specific lysis of > 11%. Activity of CTL from Thy1.2-NP tg mice on LCMV-infected targets (●) and targets expressing LCMV-GP (□). BALB/c mice LCMV targets (○) and targets expressing LCMV-GP (■). Average lysis found for BALB/c cultures was 22 ± 4%, for Thy1.2-NP cultures 11 ± 2% on LCMV targets and 23 ± 4% on GP-expressing targets.

H-2^d Targets for Lysis by CTL. The optimal LCMV-NP peptide for recognition by CTL of H-2^b mice is FQPQNGQFI (NP aa 396-404) and for H-2^d mice is RPQASGVYMG (NP aa 118-127). As shown in Fig. 5, at 10^{-13} M peptide FQPQNGQFI still sensitized H-2^b targets (Gairin, J.E., A. Tishon, and M.B.A. Oldstone, manuscript submitted for publication) for lysis by D^b-restricted anti-LCMV CTL. In contrast, 10^{-9} M peptide (or 4 log more) was required to sensitize H-2^d targets for L^d-restricted anti-LCMV CTL lysis.

The anti-LCMV Immune Response of Thy1.2-NP tg Mice Is Equivalent to that of Non-tg Littermates. Next, we questioned whether Thy1.2-NP tg mice cleared an acute LCMV infection as well as had their non-tg littermates. As Table 3 shows, by 3 wk after infection, the virus was completely cleared from serum, brain, lung, and liver as well as from PBL, spleen, central nervous system, and muscle (data not shown) in both groups of mice. By contrast, mice depleted of CD8 cells genetically (β_2-microglobulin knockout mice) or immunochemically (mice given antibody to CD8) failed to clear virus from sera or tissues (Table 3).

Non-tg mice inoculated with as little as 10 PFU of LCMV intracerebrally (i.c.) died of acute leptomeningitis within 10 d. Death resulted from the activity of LCMV-specific NP CTL in H-2^d mice as shown by depletion of CD8 cells and recon-

Table 2. *LCMV-NP (H-2^2) Tg Mice Make Normal CTL Responses to Pichinde and Vaccinia Virus but a Reduced Response to LCMV*

Virus innoculated	Day 7 primary spleen CTL obtained from	H-2 type	LU of virus-infected cells	
			H-2d	H-2b
Pichinde	BALB/c	d	32 ± −4	0
Pichinde	C57B1/6	b	0.2	22 ± −2
Pichinde	Thy1.2 NP	d	39 ± −2	0.3
Vaccinia	BALB/c	d	23 ± −4	0.1
Vaccinia	C57B1/6	b	0	17 ± −1
Vaccinia	Thy1.2 NP	d	33 ± −5	0.3
LCMV	BALB/c	d	25 ± −8	0
LCMV	C57B1/6	b	0.2	22 ± −4
LCMV	Thy1.2 NP	d	4 ± −2	0

6–8-wk-old Thy1.2 NP tg mice (bred to F_5 on Balb/H-2^d background) or non-tg mice were inoculated with Pichinde, vaccinia, or LCM virus. ^{51}Cr-labeled target cells were H-2^b (MC57) and H-2^d (BALB/Cl7) fibroblasts uninfected or infected with Pichinde, vaccinia, or LCM viruses. Procedure for determination of LU from ^{51}Cr release is given in the Materials and Methods. Numbers represent mean values ± 1 SE from four mice per group.

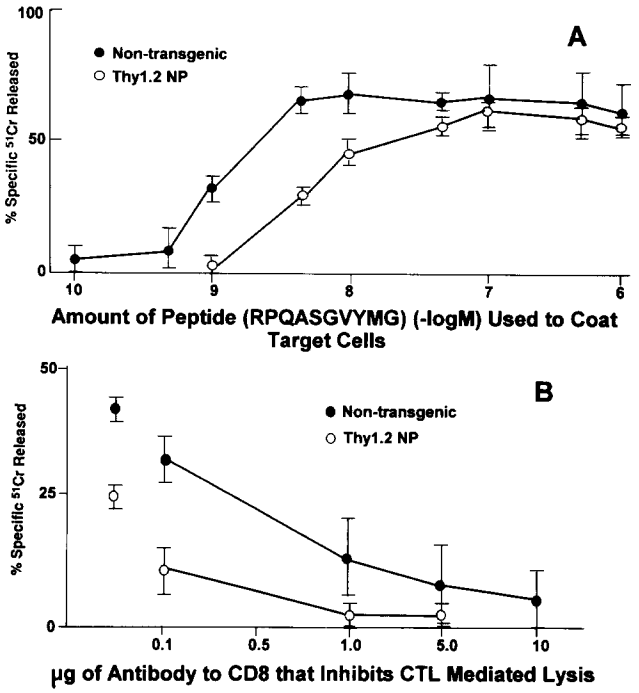

Figure 4. (*A*) Thy1.2-NP H-2^d tg mice (O) generate LCMV-NP–specific CTL with less affinity than NP-specific CTL made by age- and sex-matched non-tg controls (●). The NP sequence for which H-2^d CTL are restricted is RPQASGVYMG. Differing concentrations of this peptide were used to coat ^{51}Cr-labeled H-2^d BALB/C17 fibroblasts, and the mixture was incubated with CTL and E/T ratios of 50:1 and 25:1 with data from 50:1 shown. Coating of H-2^b ^{51}Cr-labeled targets with this peptide did not result in cell lysis. Bar indicates 2 SE for four to five mice used per each group. See Materials and Methods for ^{51}Cr-release assay. (*B*) Less antibody to CD8 (monoclonal GK1.5) is required to inactivate CD8$^+$ LCMV NP-specific CTL obtained from Thy1.2-NP H-2^d mice (O), than from age- and sex-matched non-tg controls (●). Bar indicates 2 SE. Four to five mice were used per group. mAb to CD8 at different dilutions was incubated with the CD8$^+$ effector cells. E/T ratios used were 50:1 and 25:1 with data from 50:1 shown.

stitution with L^d-restricted CTL clones that recognize NP aa 118–127 (26). After i.c. inoculation of 50 PFU LCMV into Thy1.2-NP mice (H-2^d or H-2^b), non-tg H-2^b or H-2^d controls, all mice (8–10 per group) died from leptomeningitis 7 –10 d after challenge. Mice depleted of CD8 cells did not die but became persistently infected.

When antibody to LCMV and LCMV-NP was measured by ELISA and Western blot (21) in tg and non-tg H-2^d mice 3 wk after i.p. challenge with 10^5 PFU of LCMV, titers were equivalent in both groups. Similarly, the overall number of CD4 and CD8 cells in peripheral blood and spleens of the Thy1.2-NP mice was equivalent to that in non-tg controls (four mice/group) as determined by FACS® analysis at 3, 7, 14, and 30 d after LCMV infection (data not shown).

Thy1.2-NP H-2^d Mice Generate New CTL to an Alternative LCMV-GP Epitope. CTL generated by H-2^d Thy1.2-NP mice displayed roughly threefold less specific activity toward LCMV (whole virus) and LCMV-NP than did non-tg age-

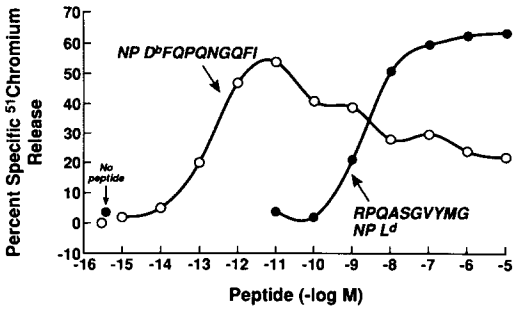

Figure 5. Amount of H-2^b restricted NP peptide FQPQNGQFI (aa 396–404) and H-2^d–restricted NP peptide RPQASGVYMG (aa 118–127) required to coat appropriate H-2^d or H-2^b targets for lysis. See legend to Fig. 3 *A*.

Table 3. *Acute LCMV Infection Is Cleared by H-2^d Thy1.2 NP Tg Mice as Efficiently as by Non-Tg Mice*

Host (10 mice/group)	PFU/ml serum			PFU/g tissue day 21		
	Day 7	Day 14	Day 21	Brain	Liver	Lung
Non-tg immunocompetent	7×10^3	0	0	0	0	0
Non-tg CD8 depleted	$1 \times 10^{4.5}$	1×10^5	$1 \times 10^{5.2}$	$1 \times 10^{4.5}$	$1 \times 10^{4.6}$	ND
Tg Thy1.2 NP	7×10^3	0	0	0	0	0

Clearance of LCMV in Thy1.2 NP mice. Mice were infected with 2×10^5 PFU i.p. LCMV and viral titers (PFU/ml or PFU/g) assessed 7, 14, or 21 d later in serum and selected tissues (brain, lung, and liver) by plaque assay (see Materials and Methods).

and sex-matched controls (Fig. 3 *A*). Nevertheless, Thy1.2-NP tg mice cleared virus with the same kinetics and as efficiently as non-tg littermates. Further, both groups showed similar kinetics and outcome of CTL-mediated lethal choriomeningitis after i.c. inoculation of virus. To further evaluate this issue, we measured the virus-specific CTL response over the course of infection. Table 4 shows that for as long as 60–120 d after infection, Thy1.2-NP H-2^d mice continued to generate low-activity LCMV-NP–specific CTL. However, unlike the non-tg controls, the tg mice produced a new anti-LCMV-GP CTL response (Table 4). Precursor frequencies were, however, 50–100-fold lower than those found for low-activity NP CTL (Fig. 3 *B*). Therefore, GP-specific CTL activity could be measured only after secondary in vitro amplification of CTL. Using several vv/LCMV GP minigene recombinants (14, 15, 17, 22, 23), the H-2^d–restricted epitope was mapped between aa 1 and 171 of LCMV-GP.

Lymphocytes Developing from the Thymus of H-2^d Thy1.2-NP tg Mice Exhibit lower CTL Activity than those from non-tg Littermates. Final studies evaluated the development of lymphocytes from thymi obtained from Thy1.2-NP H-2^d tg mice and transplanted under the renal capsules of H-2^d

Table 4. *Generation of a Novel CTL Response to LCMV-GP in H-2^d Thy1.2-NP Tg Mice*

CTL cultures tested				Percent specific ^{51}Cr released from targets infected with:						
				H-2^d			H-2^b			Ld
Secondary CTL obtained from:	H-2 type	Wk after LCMV inoculation	E/T ratio	ARM	vvGP	vvNP	ARM	vvGP	vvNP	vvGP
Non-tg	d	8	5:1	81	6	71	4	1	0	1
Thy1.2 NP	d	4	10:1	48	25	35	3	2	1	ND
tg			5:1	38	20	23	ND	ND	ND	ND
			2:1	27	17	ND	ND	ND	ND	ND
		6	5:1	47	22	22	2	1	0	14
		10	10:1	60	24	25	5	3	2	6
			5:1	46	19	19	ND	ND	ND	ND
			2:1	32	15	0	ND	ND	ND	ND
Non-tg	b	8	5:1	3	1	0	51	36	16	ND
Thy1.2 NP	b	6	5:1	2	1	0	48	24	2	ND
tg		10	10:1	1	3	4	56	34	1	ND
			5:1	1	2	1	54	28	2	ND

6–8-wk-old Thy1.2-NP H-2^d tg and non-tg mice were inoculated with $1–2 \times 10^5$ PFU LCMV i.p. At 4–10 wk after inoculation, spleens were harvested and placed in culture with syngeneic, irradiated, and LCMV-infected macrophages (see Materials and Methods). After 2 wks in culture, lymphocytes were harvested and tested in a 5–6-h ^{51}Cr-release assay against syngeneic or allogeneic target cells that were uninfected or infected with LCMV or vaccinia recombinants expressing LCMV-NP or LCMV-GP. An L^d-expressing target cell line was also used (K2A fibroblasts). Numbers represent the mean of triplicate samples with variance <10%. Similar results were obtained in three other experiments (three mice per group).

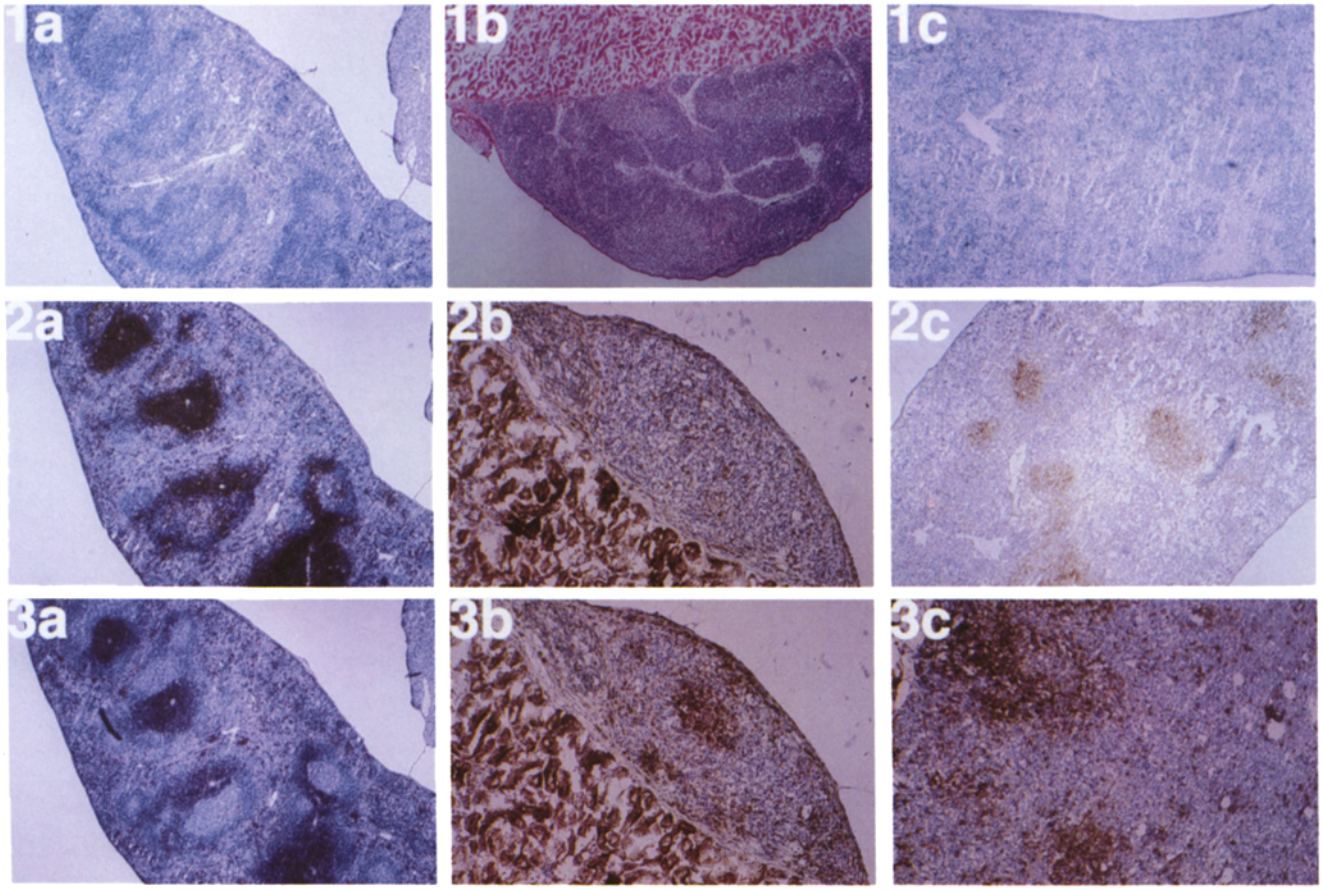

Figure 6. Immunohistochemical study to detect CD4 and CD8 bearing lymphocytes in normal spleen as compared to those in thymus and spleen 7 wk after renal capsule grafting of Thy1.2-NP (H-2^d) newborn thymi into SCID mice. Panels show the following tissue stained with: (*1a*) normal spleen, hematoxylin; (*2a*) normal spleen, CD4 stain and hematoxylin; (*3a*) normal spleen, CD8 stain, and hematoxylin; (*1b*) SCID kidney and capsule after grafting of Thy1.2-NP thymus, hematoxylin-eosin; (*2b*) SCID kidney and capsule after grafting of Thy1.2-NP thymus, CD4, and hematoxylin; (*3b*) SCID kidney and capsule after grafting of Thy1.2-NP thymus, CD8 stain, and hematoxylin; (*1c*) SCID spleen and hematoxylin; (*2c*) SCID spleen after grafting of Thy1.2-NP thymus, CD8 stain, and hematoxylin; and (*3c*) SCID thymus after grafting of Thy1.2-NP thymus, CD8 stain, and hematoxylin.

C.B.17/*scid/scid* (SCID) mice. 6–7 wk after transplantation of thymi or fat tissue (negative control) from Thy1.2-NP mice or of thymi from non-tg littermates (positive control), SCID mice received 10^5 PFU i.p. of LCMV, and 7 d later their spleens were removed and analyzed for anti-LCMV and anti-LCMV-NP CTL activity. Fig. 6 demonstrates (*a*) the localization of CD4 and CD8 lymphocytes in a normal spleen; (*b*) the thymic transplant under the renal capsule of SCID mice; and (*c*) CD4 and CD8 lymphocytes in the spleen and thymus of a SCID mouse after transplant. As seen in Fig. 6, transplanted thymic lymphoid cells migrated from under the renal capsule into lymphoid organs. Neither CD4 nor CD8 lymphocytes were detected in any SCID mouse without a thymic transplant, and kidneys of the SCID mice did not show GVHD. As shown in Table 5, CTL killing of LCMV- or LCMV-NP–expressing target cells was reduced two- to threefold in SCID recipients of the Thy1.2 NP H-2^d transplants compared to SCID mice receiving thymi from BALB/c mice or from non-tg littermates.

Discussion

Tg mice were used as a model to study the impact of a viral gene expressed in the thymus upon subsequent generation of the CTL response to the same virus. To develop this model, one viral gene of LCMV, the NP, was expressed in tg mice using the murine Thy1.2 promoter. This allowed us to determine whether negative selection of CTL was total or partial and to study whether new CTL responses to "unexpected" epitopes were generated once the NP CTL response had been disrupted. Our data show that in H-2^b tg mice, the CTL response to LCMV-NP is completely aborted. By contrast, in H-2^d tg mice the immunodominant NP response is diminished but not aborted. These findings are inversely associated with the affinity between MHC/viral (NP) peptide and CTL. 10,000-fold fewer H-2^b NP peptide molecules (dilution end point 10^{-13} M) were needed to sensitize H-2^b targets for CTL killing than H-2^d NP peptide (10^{-9} M). In addition, H-2^d tg mice generated a new CTL response to LCMV-GP, which mapped between aa 1 and 171.

Table 5. *CTL Activity Developed from Thymi of Thy1.2 NP Tg Mice Transplanted into SCID Mice*

Experimental group			CTL response		
			Percent specific ^{51}Cr-release from target cells infected with:		
			H-2^d		H-2^b
Thymus donor (H-2^d)	Thymus recipient (H-2^d)	No. of mice	LCMV-ARM	vvNP	LCMV-ARM
BALB/c	SCID	2	50 ± −8	28 ± −7	2 ± −1
Nonexpressor: Thy1.2 NP	SCID	4	45 ± −12	22 ± −5	3 ± −2
Expressor: Thy1.2 NP	SCID	6	20 ± −8	10 ± −9	2 ± −1
Fat tissue	SCID	4	2 ± −4	3 ± −1	4 ± −2

Thymi were obtained from Thy1.2 NP tg and non-tg mice within 24 h after birth and transplanted under the renal capsule of syngeneic SCID mice. 6–8-wk later, the recipients were inoculated with 10^5 PFU-LCMV i.p. and 7 d later their spleens were removed and tested in a 5–6-h ^{51}Cr-release assay for specific CTL activity against LCMV- and LCMV-NP–infected targets (see footnotes to Tables 1 and 2).

In contrast, no additional responses were generated in H-2^b mice. CTL responses of both the H-2^b and H-2^d tg mice were effective and efficient in controlling LCMV infection.

The CTL response to LCMV of H-2^b and H-2^d mice maps to genes (NP or GP) encoded by the S-RNA (16, 27–32). The CTL response in H-2^b mice maps to both GP (aa 33–41 and aa 276–286) and NP (aa 396–404) proteins, whereas in H-2^d mice the CTL response maps only to a single immunodominant NP epitope, aa 118–127 (15, 28). Here we show that expression of NP in the thymus of H-2^b mice completely aborts the NP CTL response, but retains the CTL response to LCMV-GP. The complete removal (negative selection) of H-2^b–restricted CTL was directly associated with a high-affinity interaction of H-2^b NP CTL with MHC class I plus peptide. In CTL end point titrations (Fig. 5) this peptide was effective at a concentration of 10^{-13} M. In contrast, expression of the NP transgene in H-2^d mice resulted in partial negative selection of CTL to NP. High-affinity NP-reactive CTL were removed, but not lower-affinity CTL to the same epitope. In contrast to the H-2^b NP peptide, the H-2^d NP peptide requires a 10,000-fold higher concentration to sensitize syngeneic target cells for CTL lysis (Fig. 5).

Thymic and peripheral Thy1.2-positive LCMV-NP–expressing T cells cannot be lysed by LCMV-NP–specific CTL in vivo or in vitro. Evidence for this is derived from finding nonaltered numbers of CD4 and CD8 cells as determined by FACS® (Von Herrath, M.G., unpublished experiments) in Thy1.2 tg mice before and after infection with LCMV. Further, ^{51}Cr-labeled thymic cells or PBL from Thy1.2 tg mice cannot be lysed by LCMV-NP–specific CTL in vitro (data not shown). The reason(s) why these cells resist CTL-mediated lysis is not clear. However, these observations strongly suggest that the avidity threshold (peptide density?) between T cell and APC required for negative selection in the thymus is less than that required for recognition and/or triggering of activated CTL (4, 9). As shown by thymus transfer experiments (Table 5), this negative selection of high-affinity cells is due to events taking place in the thymus rather than effects of peripheral Thy1.2-positive T cells expressing the NP protein.

The repertoire of T cells is selected in the thymus when T lymphocytes come in contact with MHC class I and II molecules plus peptide (1, 3, 6, 33). We show here that MHC class I haplotypes, because of different peptide/MHC/TCR complex affinities, may vary in terms of the completeness of the negative selection process. This observation may explain, in part, the differing susceptibilities and incidences of autoimmune diseases among different MHC haplotypes (34, 35) as well as the presence of low-affinity T lymphocytes (13, 36) in certain autoimmune responses. Our experimental results in a virus infection model agree with those of other investigators (3–5, 7, 8), who have correlated quantitation of thymic selection with MHC class I peptide binding affinity in nonviral systems.

Our H-2^d tg Thy1.2-NP generated a new LCMV-GP–specific CTL response, perhaps to compensate for the removal of the high-affinity NP-CTL. Although the mechanism(s) by which this occurred is uncertain, this switch to an alternative L^d-restricted CTL response indicates its adaptability and probable hierarchial control. The H-2^d CTL re-

sponse generated in Thy1.2 NP tg mice (low-activity CTL to NP and new GP CTL) is able to control LCMV infection in vivo. The number of precursors for the low-affinity CTL to NP in Thy1.2 H-2^d tg mice equaled the numbers found for high-affinity NP CTL in non-tg littermates. However, these tg H-2^d mice had 50–100-fold fewer GP CTL than low-affinity NP-CTL. This probably explains why the GP CTL response was only uncovered after secondary stimulation but not as a primary CTL response. Indeed, in > 500 individual non-tg H-2^d mice, a primary GP-specific CTL response was not present after LCMV challenge (14, 21, and our unpublished observations). Possibly, the alternative L^d-restricted GP response exists but is suppressed by the immunodominant high-affinity NP response in non-tg mice. However, when the immunodominant H-2^d NP CTL response is diminished, hierarchial control of the NP epitope is broken, GP-specific CTL occur, and can be cloned by limiting dilution (von Herrath, M. G., H. Lewicki, and M. B. A. Oldstone, unpublished results). A hierarchy of factors that control MHC genes in the generation of antiviral CTL responses has been noted elsewhere (35, 37). For example, H-$2L^d$ is the sole MHC haplotype in normal BALB/c mice ($K^dI^dD^dL^d$) for generating a CTL response to influenza virus (35). However, in the absence of this L^d haplotype, the H-$2K^d$ serves to restrict the CTL response. Similar observations have been made with LCMV (38).

The work presented here shows the adaptability of an antiviral CTL response in two given MHC environments. In H-2^b mice, thymic expression of viral NP causes complete negative selection of high-avidity NP CTL but the anti-LCMV-GP CTL response remains unaffected. In H-2^d mice, NP CTL of lower avidity are not deleted, pass to the periphery, and can be activated. Additionally, a new CTL response to an alternative viral epitope on LCMV-GP is generated, which is restricted by the same MHC allele. In both scenarios, however, the altered CTL responses suffice to clear viral infection in vivo. These in vivo findings in mice undergoing infection with a natural viral pathogen suggest that negative selection is only complete if the affinity between TCR and MHC/peptide complex is high and support the recently proposed affinity model for thymic selection (7, 8). Finally, our findings with LCMV are likely to be pertinent in understanding the presence or absence of CTL responses, MHC control, and different autoimmune phenotypes observed in humans infected in utero or at birth with hepatitis B virus, HIV, or cytomegalovirus.

The authors thank Drs. Lindsay Whitton, Juan Carlos de la Torre, David Tough, Charles Surh, Mark Horwitz, and Jim Waters for helpful discussions and Gay Schilling and Jody Anderson for assistance with the manuscript.

This is Publication Number 7770-NP from the Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA. This manuscript is supported by U.S. Public Health grants AI-09484, NS-12428, AG-04342 and a Juvenile Diabetes Foundation Fellowship and Fellowship by the Deutsche Forschungsgemeinschaft to M. G. von Herrath.

Address correspondence to Dr. M. B. A. Oldstone, Department of Neuropharmacology, Division of Virology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.

Received for publication 12 May 1994 and in revised form 12 July 1994.

References

1. Sprent, J. 1990. Introduction: the thymus and T cell differentiation. *Seminars in Immunology.* 2:1.
2. Hogquist, K., S. Jameson, W. Heath, J. Howard, M. Bevan, and F. Carbone. 1994. T-cell receptor antagonist peptides induce positive selection. *Cell.* 76:17.
3. Allen, P. 1994. Peptides in positive and negative selection, a delicate balance. *Cell.* 76:593.
4. Pircher, H., U. Rohrer, D. Moskophidis, R. Zinkernagel, and H. Hengartner. 1991. Lower receptor avidity required for thymic clonal deletion than for effector T cell function. *Nature (Lond.).* 351:482.
5. Wallace, V., J. Penninger, and T. Mak. 1993. CD4, CD8 and tyrosine kinases in thymic seletion. *Curr. Opin. Immunol.* 5:235.
6. Sprent, J., E. Gao, and S. Webb. 1990. T cell reactivity to MHC molecules: immunity versus tolerance. *Science (Wash. DC).* 248:1357.
7. Ashton-Rickardt, P., A. Bandeira, J. Delaney, L. Van Kaer, H.-P. Pircher, R. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T-cell selection in the thymus. *Cell.* 76:651.
8. Sebzda, E., V. Wallace, J. Mayer, R. Yeung, T. Mak, and P. Ohashi. 1994. Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science (Wash. DC).* 263:1615.
9. Karjalainen, K. 1994. High sensitivity, low affinity paradox of T-cell receptor recognition. *Curr. Opin. Immunol.* 6:9.
10. Ahmed, R., A. Salmi, D. Butler, M. Chiller, and M.B.A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med.* 160:521.
11. de la Torre, J.C., and M.B.A. Oldstone. 1992. Selective disruption of growth hormone transcription machinery by viral infection. *Proc. Natl. Acad. Sci. USA.* 89:9939.

12. Jamieson, B., F. Somasundaram, and R. Ahmed. 1991. Abrogation of tolerance to a chronic viral infection. *J. Immunol.* 147:3521.
13. von Herrath, M., J. Dockter, and M.B.A. Oldstone. 1994. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. *Immunity.* 1:231.
14. Whitton, J.L. 1990. Lymphocytic choriomeningitis virus CTL. *Seminars in Virology.* 1:257.
15. Whitton, J.L., A. Tishon, H. Lewicki, J. Gebhard, T. Cook, M. Salvato, E. Joly, and M.B.A. Oldstone. 1989. Molecular analysis of a five-amino acid CTL epitope: an immunodominant region which induces non-reciprocal CTL cross-reactivity. *J. Virol.* 63:4303.
16. Southern, P.J., M.K. Singh, Y. Riviere, D. Jacoby, M. Buchmeier, and M.B.A. Oldstone. 1987. Molecular characterization of the genome S RNA segment of LCMV. *Virology.* 157:145.
17. Klavinskis, L., J.L. Whitton, and M.B.A. Oldstone. 1989. Monoclonal engineered vaccine which expresses an immunodominant T cell epitope induces CTL that confer protection from lethal virus infection. *J. Virol.* 63:4311.
18. Nerenberg, M., S. Hinrichs, R. Reynolds, G. Khoury, and G. Jay. 1987. The tat-gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. *Science (Wash. DC).* 237:1324.
19. Nerenberg, M., T. Minor, J. Price, D. Ernst, T. Shinohara, and H. Schwartz. 1991. Transgenic thymocytes are refractory to transformation by the HTLV I tax gene. *J. Virol.* 65:3349.
20. Maniatis, T., J. Sambrooke, and E.F. Fritsch. 1990. Molecular Cloning: A Laboratory Manual. Nancy Ford and Chris Nolan, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Oldstone, M.B.A., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell.* 65:319.
22. Klavinskis, L., J.L. Whitton, E. Joly, and M.B.A. Oldstone. 1990. Vaccination and protection from a lethal viral infection: identification, incorporation and use of a CTL glycoprotein epitope. *Virology.* 178:393.
23. Oldstone, M.B.A., J.L. Whitton, H. Lewicki, and A. Tishon. 1988. Fine dissection of a nine-amino acid glycoprotein epitope, a major determinant recognized by lymphocytic choriomeningitis virus–specific class I–restricted H-2D^b CTL. *J. Exp. Med.* 168:559.
24. Buchmeier, M., H. Lewicki, O. Tomori, and M.B.A. Oldstone. 1981. Monoclonal antibodies to LCMV: generation, characterization and cross-reactivity with other arenaviruses. *Virology.* 113:73.
25. Buchmeier, M., R. Welsh, F. Dutko, and M.B.A. Oldstone. 1980. The virology and immunobiology of LCMV infection. *Adv. Immunol.* 30:275.
26. Klavinskis, L., A. Tishon, and M.B.A. Oldstone. 1989. Efficiency and effectiveness of cloned virus-specific CTL *in vivo*. *J. Immunol.* 143:2013.
27. Dutko, F., and M.B.A. Oldstone. 1983. Genomic and biological variation among commonly used LCMV strains. *J. Gen. Virol.* 64:1689.
28. Whitton, J.L., P.J. Southern, and M.B.A. Oldstone. 1988. Analysis of the CTL responses to glycoprotein and nucleoprotein components of LCMV. *Virology.* 162:3211.
29. Ahmed, R., J.A. Byrne, and M.B.A. Oldstone. 1984. Virus specificity of cytotoxic T lymphocytes generated during acute lymphocytic choriomeningitis virus infection: role of the H-2 region in determining cross reactivity for different lymphocytic choriomeningitis strains. *J. Virol.* 51:34.
30. Gairin, J.E., and M.B.A. Oldstone. 1993. Virus and cytotoxic T-lymphocytes: crucial role of viral peptide secondary structure in MHC class I interactions. *J. Virol.* 67:2903.
31. Salvato, M.S., E. Shimomaye, K. Schweighofer, and M.B.A. Oldstone. 1989. Mapping genes of LCMV which determine CTL response. *UCLA Symp. Mol. Cell. Biol. New Ser.* 90:329.
32. Riviere, Y., and M.B.A. Oldstone. 1986. Genetic reassortants of lymphocytic choriomeningitis virus: unexpected disease and mechanism of pathogenesis. *J. Virol.* 59:363.
33. Takahama, Y., E. Shores, and A. Singer. 1992. Negative selection of precursor thymocytes before their differentiation into CD4$^+$, CD8$^+$ cells. *Science (Wash. DC).* 258:653.
34. Ohashi, P., S. Oehen, P. Aichele, H. Pircher, B. Odermatt, P. Herrara, Y. Higuchi, K. Buerki, H. Hengartner, and R.M. Zinkernagel. 1993. Induction of diabetes is influenced by the infectious virus and local expression of MHC class I and TNF-alpha. *J. Immunol.* 150:5185.
35. Doherty, P., W. Biddison, J.R. Bennink, and B. Knowles. 1978. CTL responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. *J. Exp. Med.* 148:534.
36. Heath, W., L. Kjer Nielsen, and M.W. Hoffman. 1992. Avidity for antigen can influence the helper dependency of CD8$^+$ lymphocytes. *J. Immunol.* 151:5993.
37. Zinkernagel, R.M., A. Athage, S. Cooper, G. Kreeb, B. Klein, B. Sefton, L. Flaherty, J. Stimpfling, D. Schreffler, and J. Klein. 1978. Ir genes in H-2 regulate generation of antiviral CTL. *J. Exp. Med.* 148:592.
38. Allan, J., and P. Doherty. 1985. Consequences of a single Ir gene defect for the pathogenesis of LCMV. *Immunogenetics.* 21:581.