Impaired Anti-Viral T Cell Responses Due to Expression of the LY49A Inhibitory Receptor[1]

Allan J. Zajac,[2]* Russell E. Vance,† Werner Held,[3†] David J. D. Sourdive,[4]* John D. Altman,* David H. Raulet,† and Rafi Ahmed[5]*

Inhibitory receptors specific for alleles of MHC class I proteins play an important role in determining the reactivity and specificity of NK cells. To determine whether these receptors are also able to regulate T cell functions, we have studied anti-viral immune responses in mice transgenic for a class I-specific inhibitory receptor, Ly49A. Although nontransgenic mice express Ly49A primarily on NK cells and some T cells, the Ly49A transgenic mice express Ly49A on all lymphocytes, including T cells. We have assessed the activation, expansion, cytokine production, and cytotoxic activity of CD8 T cells in both transgenic and nontransgenic mice following infection with lymphocytic choriomeningitis virus. As expected, nontransgenic mice made a potent virus-specific CD8 T cell response following virus infection. However, as measured in cytolysis assays and by cytokine production, virus-specific CD8 T cell activity was reduced in Ly49A transgenic mice. This inhibition was largely, but not always exclusively, dependent upon the presence, either in vivo or in vitro, of the Ly49A ligand, H-2D^d. Strikingly Ly49A transgenic mice have reduced capacity to control infection with the virulent lymphocytic choriomeningitis virus variant clone 13. Overall, these studies demonstrate that expression of killer inhibitory receptors can modulate anti-viral T cell responses in vivo and in vitro. ***The Journal of Immunology,* 1999, 163: 5526–5534.**

In recent years, considerable advances have been made in our understanding of the molecular basis of NK cell recognition. Unlike T cells, NK cells typically lyse target cells that fail to express particular MHC class I complexes (1, 2). Several receptors that govern this recognition process have been defined and characterized (3). In humans, Ig superfamily members including the Ig killer inhibitory receptor and the leukocyte inhibitory receptor/Ig-like transcript families have been identified as receptors for classical class I molecules (class Ia molecules), but mouse counterparts of these receptors have not been reported. Instead, recognition of class Ia molecules in the mouse is accomplished by the lectin-like Ly49 receptors (4). Most of these class Ia-specific receptors discriminate allelic class Ia variants. A second lectin-like receptor family, the CD94/NKG2 receptors, has been identified in both humans and mice. CD94/NKG2 receptors recognize a class Ib molecule, HLA-E in humans (5–7) and Qa-1 in mice (8), that presents a peptide derived from the signal sequence of many class Ia molecules. The interaction of these various inhibitory receptors with cognate class I molecules on target cells results in the inhibition of NK cell lysis and cytokine release. Therefore, target cells that lack corresponding class I MHC molecules are rendered susceptible to attack by NK cells (1, 2). A rationale for such a system is thought to be provided by the observation that viruses and tumors often down-regulate self MHC class I in their efforts to evade recognition by T cells (9, 10).

Although inhibitory class I-specific receptors play an important role in determining the specificity and reactivity of NK cells, their role in regulating T cell activity is not well defined. Studies have demonstrated the existence of T cell subsets that express "NK" inhibitory receptors representing most of the defined receptor families. Ig killer inhibitory receptors have been shown to be functionally expressed by significant numbers of CD8 T cells (11–14). More recent studies demonstrated expression of CD94/NKG2 receptors by human T cells subsets including CD8 T cells (15). Interestingly, class I-specific inhibitory receptors have been shown to be expressed by HIV-specific T cells (16) and by T cells specific for tumor cell Ags (17). Also, an appreciable fraction of murine T cells have been shown to express the Ly49 receptors (Ref. 18 and M. Coles, C. McMahon, and D. H. Raulet, manuscript in preparation). Many of these Ly49$^+$ T cells are memory phenotype CD8$^+$ T cells, and the fraction of these Ly49$^+$ memory CD8$^+$ T cells increases dramatically with age (M. Coles, C. McMahon and D. H. Raulet, manuscript in preparation). Engagement of class I-specific inhibitory receptors has been shown to inhibit T cell functions in vitro (13, 18, 19), but evidence for a role for inhibitory receptors in regulating T cell responses in vivo is lacking. It will be interesting to determine whether inhibitory class I-specific receptors function broadly to regulate the responses of both NK and T cells.

Recently, mice transgenic for the Ly49A inhibitory receptor have been generated (19). In these mice, Ly49A is expressed by all NK cells, CD4 and CD8 T cells, and at variable levels by B cells. Because Ly49A is expressed by T cells in these transgenic mice,

*Emory Vaccine Center, Emory University, Atlanta, GA 30322; and †Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720

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[2] Current address: Department of Microbiology, University of Alabama, Birmingham, AL 35294.

[3] Current address: Ludwig Institute for Cancer Research, Lausanne Branch, Ch. Des Boveresses 155, 1066 Epalinges, Switzerland.

[4] Current address: Centre d'Etudes du Bouchet, Le Bouchet B.P. No. 3, 91710 Vert-le Petit, France.

[5] Address correspondence and reprint requests to Dr. Rafi Ahmed, Emory Vaccine Center, Emory University School of Medicine, G211 Rollins Research Building, 1510 Clifton Road, Atlanta, GA 30322. E-mail address: ra@microbio.emory.edu

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they provide a valuable tool to evaluate the effects of Ly49 expression on T cell functions. Ly49A has been shown to interact strongly with H-2D^d and H-2D^k (20), with some indirect evidence suggesting a weak interaction with H-2^b class I molecules (21).

Acute infection of mice with lymphocytic choriomeningitis virus (LCMV)[5] elicits massive expansion and activation of CD8 T cells (22–29). These CD8 T cells mediate potent virus-specific cytotoxicity and also produce anti-viral cytokines such as IFN-γ (27–29). The elaboration of LCMV-specific CD8 CTL responses is necessary for viral clearance (24, 30–33). Given the pronounced T cell response to LCMV, we have chosen to use this system to determine whether expression of Ly49 receptors by T cells can modulate anti-viral CD8 T cell activity.

Materials and Methods

Mice and virus

The generation of Ly49A transgenic mice and their backcrossing to B6 (H-2^b) and B10.D2 (H-2^d) genetic backgrounds has been previously described (19). These transgenic mice develop normally but express Ly49A on all CD4 and CD8 T cells and at variable levels on B cells. Ly49A transgenic and nontransgenic littermates were used for experiments involving LCMV infection. C57BL6/J (H-2^b) and B10.D2/nSnJ (H-2^d) mice were supplied by The Jackson Laboratory (Bar Habor, ME) and used as a source of feeder cells for IFN-γ enzyme-linked immunospot assays. Mice were housed in American Association for the Accreditation of Laboratory Animal Care accredited facilities at Emory University and at the University of California at Berkeley.

Mice were infected by i.p. injection with 2×10^5 PFU of LCMV (Armstrong) or by i.v. inoculation with 2×10^6 PFU of the macrophage-tropic LCMV isolate clone 13 (34, 35). Unless otherwise stated, responses were determined 8 days after infection with LCMV-Armstrong.

Peptide synthesis

Peptides corresponding to the LCMV-derived H-2L^d-restricted epitope NP118-126 (RPQASGVYM) and the H-2K^d-restricted GP283-291 epitope (GYCLTKWMI) were synthesized by F-moc chemistry using a Rainin Symphony peptide synthesizer (36). The H-2D^b-restricted epitopes GP33-41 (KAVYNFATM), GP276-286 (SGVENPGGYCL), and NP396-404 (FQPQNGQFI) were similarly produced (37). The GP33-41 peptide contains a carboxyl-terminal M residue rather than the naturally occurring C residue. This substitution enhances the binding affinity of the peptide for H-2D^b but does not alter its antigenicity (37).

Cytotoxicity assays

Standard ^{51}Cr release assays were performed to measure LCMV-specific T cell activity (24). Briefly, single-cell suspensions of splenocytes were prepared from mice at 8 days following infection with LCMV (Armstrong). Erythrocytes were removed by osmotic lysis using 0.83% NH_4Cl and cell preparations finally resuspended in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-ME, 100U/ml penicillin, and 100 μg/ml streptomycin (R10). The cytotoxic activity of these ex vivo effector cells was then determined.

Secondary effector cells were generated by restimulating splenocytes prepared from mice at 8 days postinfection. Splenocytes were seeded into replicate wells of a 24-well plate (10^7 cells/well), and the final volume was adjusted to 2 ml using R10 medium. Cultures were incubated for 5 days at 37°C in 6% CO_2 with a mixture (1 μg/ml each) of GP33-41, NP396-404, and GP276-286 peptides for H-2^b effectors and NP118-126 and GP283-291 peptides for H-2^d effectors. Secondary effector cells were washed twice before use in ^{51}Cr release assays.

BALB/clone 7 (H-2^d) or MC57 (H-2^b) fibroblast target cells were infected with LCMV clone 13 at a multiplicity of infection of 0.5 for 24 or 48 h, respectively. These target cells were radiolabeled by incubation with $Na_2{}^{51}CrO_4$ for 1 h at 37°C. Following washing, 2×10^4 labeled target cells were added to wells of 96-well flat-bottom plates. In certain assays, target cells were coated with peptides (0.1 μg/ml unless stated otherwise) rather than infected with LCMV. Splenocytes from LCMV-infected mice were then added to give the required E:T ratio in a final volume of 200 μL. Assays were performed in triplicate and allowed to proceed for 5–6 h before harvesting supernatants. Specific lysis was calculated using the formula: % specific lysis = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Spontaneous release is the number of radioactive counts released in the absence of effector cells, and total release is the number of counts released from target cells following treatment with 0.5% (v/v) Nonidet P-40.

IFN-γ enzyme-linked immunospot

Single-cell enzyme-linked immunospot assays to enumerate IFN-γ-producing cells were performed as previously described (28). Cultures were either left untreated or stimulated with peptide epitopes (0.1 μg/ml final concentration). Cultures were incubated for 40 h at 37°C in 6% CO_2. After this period, cells were removed and the plates processed as previously described.

Intracellular staining for IFN-γ

Responder cells (10^6) were cultured in 96-well flat-bottom plates in R10 medium supplemented with 50U/ml recombinant human IL-2. Cells were either left untreated or stimulated with LCMV-specific peptide epitopes. Brefeldin A (GolgiPlug; PharMingen, San Diego, CA) was added to all wells. Cultures were incubated for 5–6 h at 37°C in 6% CO_2 in an humidified incubator. After this period, cells were removed and stained with anti-CD8-PE for 30 min on ice. Intracellular staining was performed using the cytofix/cytoperm kit (PharMingen) in accordance with manufacturer's recommendations (28). Briefly, following cell-surface staining cells were washed and then treated with paraformaldehyde and saponin to fix and permeablize the cells. Intracellular staining was then done using anti-IFN-γ-FITC (XMG1.2) or anti-TNF-α-allophycocyanin (MP6-XT22) or with an irrelevant isotope-matched control Ab (R3-34). Stained cells were then washed and data acquired as described below.

MHC class I tetramers

The generation of LCMV-specific H-2D^b and L^d tetramers has been previously described (28, 38). Briefly, recombinant class I heavy chains were produced in *Escherichia coli* strain BL21(DE3). Monomeric complexes were refolded with human β_2-microglobulin and antigenic peptides (D^b heavy chains with GP33-41, NP396-404, and GP276-286 peptides and L^d heavy chains with NP118-126 peptide). Folded monomeric complexes were subjected to column chromatography using an S-300 column (Pharmacia, Piscataway, NJ). The purified monomers were then enzymatically biotinylated, using BirA enzyme, and further purified by ion exchange (Mono-Q column). Tetrameric complexes were assembled by the addition of allophycocyanin-conjugated streptavidin (Molecular Probes, Eugene, OR). Splenocyte preparations were costained using class I tetramers and anti-CD8 Abs and analyzed by flow cytometry, described below.

TCR Vβ usage

The diversity of TCR Vβ usage by CD8 T cells was analyzed by flow cytometry, as previously described (38). Splenocytes from naive mice were costained with anti-CD8 Abs and a panel of anti-TCR Vβ Abs. The TCR Vβ repertoire of Ag-specific CD8 T cells was determined by staining splenocytes from LCMV-Armstrong-infected mice with anti-CD8 Abs, L^d (NP118-126) tetramers, and a panel of anti-TCR Vβ Abs. Anti-TCR Vβ4, 5.1/5.2, 6, 7, 8.1/8.2, 9, 10, 13, and 14 Abs (clones KT4, MR9-4, RR4-7, TR310, MR5-2, MR10-2, B21.5, MR12-3, and 14.4, respectively) were all obtained from PharMingen. Anti-Vβ8.3 and Vβ12 Abs (clones CT-8C1 and CTVB12b, respectively) were purchased from Caltag (Burlingame, CA). Stained cells were analyzed by flow cytometry, described below.

Flow cytometry

Splenocytes were prepared and stained in PBS, 2% (w/v) BSA, and 0.2% (w/v) NaN_3. Abs used included anti-CD8 (53-6.7), anti-CD44 (IM7), and anti-LFA-1 (2D7). All Abs were purchased from PharMingen. Cell were fixed in PBS and 2% (w/v) paraformaldehyde, and at least 10,000 events were acquired using either a FACScan or FACScaliber flow cytometer (Beckon Dickinson, San Jose, CA). Dead cells were excluded on the basis of forward and side light scatter. Data was analyzed using the computer program CellQuest (Beckon Dickinson).

Plaque assays

Viral titers were determined by plaque assay using Vero cell monolayers. After incubation for 4 days at 37°C in 6% CO_2, plaques were visualized by overnight staining with neutral red (34).

[5] Abbrevation used in this paper: LCMV, lymphocytic choriomeningitis virus.

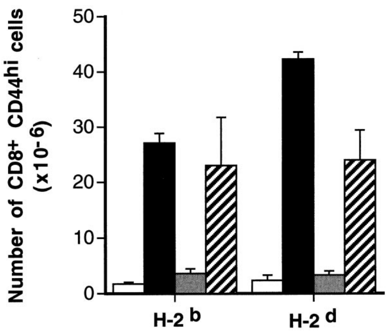

FIGURE 1. CD8 T cell activation following LCMV infection. Mice were either left untreated or infected with LCMV-Armstrong for 8 days. The number of activated (CD44^{high}) CD8 T cells in the spleens of both H-2^b and H-2^d mice was determined using flow cytometry. Cells were prepared from uninfected nontransgenic mice (□), LCMV-infected nontransgenic mice (■), uninfected Ly49A transgenic mice (▤), and LCMV-infected Ly49A transgenic mice (▨). Columns show the mean number of CD8$^+$ CD44^{high} cells per spleen ± SD. Three to five mice were analyzed per group.

Results

CD8 T cell expansion and activation

Acute infection of adult mice with LCMV (Armstrong) results in a massive activation and expansion of virus-specific CD8 T cells, which peaks at 8 days postinfection (22–29). To determine whether transgenic expression of Ly49A inhibits anti-viral T cell responses in vivo, we evaluated the absolute number and activation phenotype of splenic T cells in nontransgenic and Ly49A transgenic mice following LCMV infection. We used transgenic mice (or nontransgenic littermates) of two genetic backgrounds: B10.D2 (H-2^d) transgenics express a strong Ly49A ligand (i.e., D^d), whereas B6 (H-2^b) transgenics express only a weak, if any, Ly49A ligand (39). The number of spleen cells at 8 days after LCMV infection was determined, and two-color flow cytometric analysis was used to assess expression of the activation markers CD44 and LFA-1 by CD8 T cells (40, 41). Fig. 1 shows that LCMV infection resulted in an elevated number of activated (i.e., CD44^{high}) CD8 T cells in the spleens of all of the mice examined. However, the number of activated CD8 T cells was ~2-fold lower in H-2^d mice that expressed the Ly49A transgene, compared with nontransgenic H-2^d mice. In contrast, there were similar numbers of activated CD8 splenic T cells ($\sim 2.5 \times 10^7$) in transgenic and nontransgenic H-2^b mice. Similar results were obtained when LFA-1 expression by CD8 T cells was examined (data not shown). These data indicate that Ly49A expression inhibits virus-induced T cell expansion in H-2^d mice, which express a known Ly49A ligand, H-2D^d. However, in H-2^b mice, transgenic expression of Ly49A did not impair overall CD8 T cell activation following LCMV infection.

Anti-viral CTL activity in H-2^d mice

Normal mice elaborate a highly potent virus-specific CD8 CTL response following infection with LCMV. We investigated whether transgenic expression of Ly49A would be capable of inhibiting the generation of such a vigorous anti-viral CTL response. Direct ex vivo CTL activity was assessed using splenic effector cells prepared from either nontransgenic or Ly49A transgenic H-2^d mice at 8 days after LCMV (Armstrong) infection. As expected, effector cells from nontransgenic mice exhibited potent cytotoxicity against LCMV-infected target cells (22–28). Strikingly, the ex vivo CTL activity of effector cells from H-2^d Ly49A transgenic mice was 3- to 5-fold lower (Fig. 2*A*). This reduced cytotoxicity

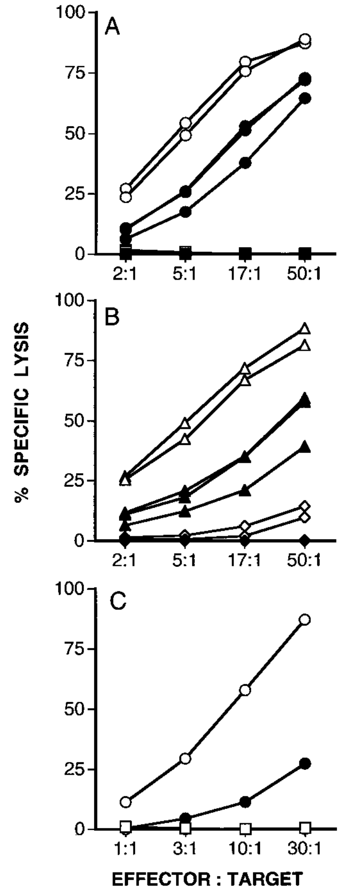

FIGURE 2. Virus-specific CTL activity in H-2^d mice. *A* and *B*, Splenocytes were prepared from two individual H-2^d nontransgenic (open symbols) and three individual Ly49A transgenic (closed symbols) mice 8 days after infection with LCMV. These effector cells were then tested directly ex vivo for their capacity to kill target cells. *A*, The specific lysis of noninfected (squares) and infected (circles) BALB/clone7 target cells is shown. *B*, The lysis of BALB/clone7 target cells coated with either NP118-126 (triangles) or GP283-291 (diamonds) peptides is shown. *C*, Splenocytes were prepared from nontransgenic (open symbols) and Ly49A transgenic mice (filled symbols) at 8 days postinfection. These cells were then restimulated in vitro for 5 days and tested for their ability to kill noninfected (squares) and LCMV infected (circles) BALB/clone7 target cells.

was also apparent if target cells were coated with either the immunodominant H-2L^d-restricted NP118-126 peptide epitope or the subdominant H-2K^d-restricted GP283-291 epitope (Fig. 2*B*). Furthermore, markedly impaired virus-specific CTL activity was also evident when the CTL activity of in vitro-stimulated secondary effector cells was assessed (Fig. 2*C*). The reduced CTL activity in Ly49A transgenic H-2^d mice is consistent with the lower number of activated spleen cells present in these mice. Taken together, these data show that in H-2^d mice Ly49A expression inhibits CD8 T cell activation and the elaboration of effector functions. In separate experiments, we sensitized ^{51}Cr-labeled BALB/clone 7 target cells with concentrations of NP118-126 peptide ranging from 1 μg/ml to $10^{-10}\mu$g/ml. With an E:T ratio of 50:1, nontransgenic effectors attained 50% of maximal lysis with between 10^{-7} and 10^{-8} μg/ml of peptide, whereas transgenic effectors attained 50% of maximal lysis with $\sim 10^{-6}$ μg/ml of peptide (data not shown).

Cytokine production by anti-viral T cells in H-2^d mice

To further investigate the ability of Ly49A to inhibit CD8 T cells functions, IFN-γ and TNF-α production by anti-viral T cells was

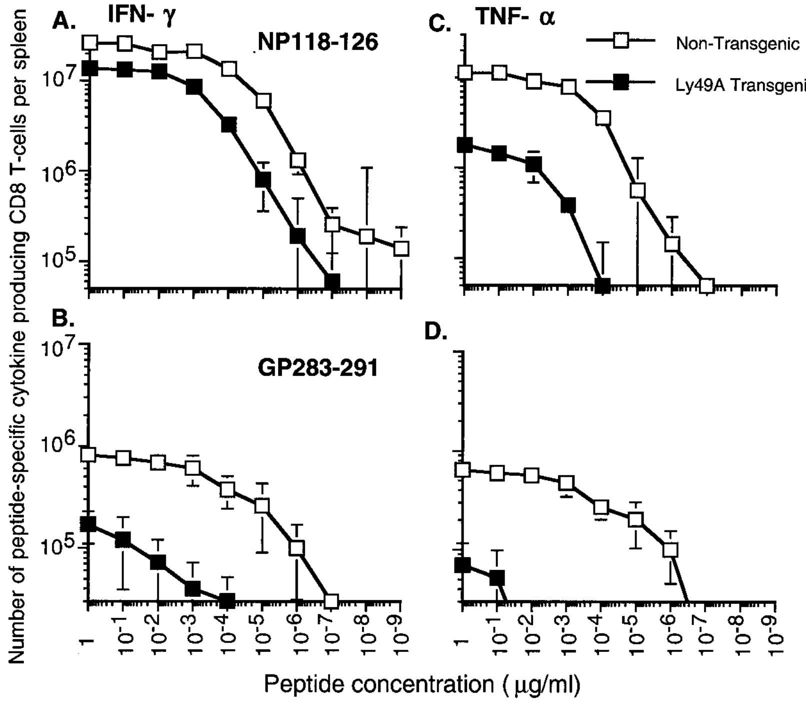

FIGURE 3. Virus-specific cytokine production in H-2^d mice. Splenocytes were prepared from LCMV infected nontransgenic (open squares) and Ly49A transgenic (filled squares) mice at 8 days postinfection. IFN-γ (*A* and *B*) and TNF-α (*C* and *D*) production by CD8 T cells was determined using intracellular cytokine staining following 5 h stimulation with various concentrations of peptide (*A* and *C*, NP118-126; *B* and *D*, GP283-291). Each point represents the number of splenic CD8 T cells that produce IFN-γ or TNF-α in response to the given dose of peptide. Error bars show SDs.

examined following stimulation with graded doses of antigenic peptides. Fig. 3 shows intracellular cytokine staining data obtained using effector cells from either nontransgenic or Ly49A transgenic H-2^d mice following LCMV infection. Strikingly, ~50% of splenic CD8 T cells isolated from nontransgenic H-2^d mice produced IFN-γ in response to the immunodominant NP118-126 epitope (Fig. 3*A*). In Ly49A transgenic mice, the number of IFN-γ-producing CD8 T cells was consistently lower. Based on multiple determinations, there was an overall 2.5-fold reduction in the absolute number of NP118-126-specific IFN-γ-producing CD8 T cells in the transgenic H-2^d mice (Fig. 3). The number of TNF-α-producing NP118-126-specific CD8 T cells was also reduced in Ly49A transgenic H-2^d mice (Fig. 3*C*). As expected, the addition of the subdominant GP283-291 epitope stimulated IFN-γ and TNF-α production by a smaller number of CD8 T cells from nontransgenic mice (Fig. 3, *B* and *D*; Refs. 28, 29, and 36). However, even lower numbers of cytokine-producing cells were apparent after stimulation of Ly49A transgenic effector cells (Fig. 3, *B* and *D*). Following stimulation with NP118-126 peptide, both nontransgenic and Ly49A transgenic effector cells exhibited similarly shaped dose response curves, which reached plateau at a peptide concentration of 10^{-2} μg/ml. By comparison with nontransgenic mice, the GP283-291-specific response of Ly49A transgenic mice was somewhat more rapidly extinguished as the peptide concentration was lowered (Fig. 3). Unstimulated cells did not produce significant amounts of either IFN-γ or TNF-α (data not shown). In addition, isotype-matched control Abs stained <0.2% of CD8 T cells, even after stimulation with the dominant NP118-126 epitope (data not shown). These data provide independent evidence that Ly49A expression impairs CD8 T cell responses in vivo in H-2^d mice.

Anti-viral CTL and cytokine production in H-2^b mice

To investigate the effect of Ly49A expression on anti-viral effector activity in H-2^b mice, standard ^{51}Cr release assays were performed. At 8 days postinfection, direct ex vivo CTL activity was measured, and the CTL activity of effector cells following in vitro restimulation was also determined. The ability of effector cells to kill untreated, virus-infected, and peptide-coated MC57 cells was assessed. In addition, because H-$2D^d$ is known to interact strongly with Ly49A (20), CTL activity was also measured using peptide-pulsed RMA (H-2^b) and H-$2D^d$-transfected RMA cells (RMA-D^d).

Similar results were obtained using both primary ex vivo (Fig. 4, *A–C*) and secondary effector cells (Fig. 4, *D–E*). The general trends were: 1) both nontransgenic and transgenic H-2^b mice elaborated a potent virus-specific CTL response; however, the response of effector cells from Ly49A transgenic mice is marginally reduced in some cases; 2) these CTL can kill LCMV-infected MC57 cells (Fig. 4, *A* and *D*) and RMA cells (H-2^b) pulsed with LCMV-derived peptide epitopes (Fig. 4, *B* and *E*); and 3) peptide-pulsed RMA cells coexpressing H-$2D^d$ (RMA-D^d) were killed by CTL from H-2^b nontransgenic mice; however, CTL from H-2^b Ly49A transgenic mice failed to kill these target cells (Fig. 4, *C* and *F*). In separate experiments, we checked GP276-286-specific killing activity using peptide-pulsed MC57 target cells. When adjusted for background lysis, detected on nonpulsed targets, specific lysis by nontransgenic and transgenic H-2^b effectors was 11.8 and 6.1%, respectively, at an E:T ratio of 50:1 (data not shown).

These data make two important points. First, the activity of H-2^b CTL, expressing Ly49A, is inhibited if target cells coexpressing H-$2D^d$ are used. This shows that if the responding cells encounter target cells that express both a ligand for the TCR (a viral peptide/H-2^b complex) and a ligand for Ly49A (D^d), then CD8 T cell effector functions are strongly inhibited. Second, expression of Ly49A in H-2^b mice may have a slight effect upon the elaboration of virus-specific CTL in some cases.

The ability of effector cells from LCMV-infected H-2^b mice to synthesize IFN-γ and TNF-α was also determined. Fig. 5 shows the absolute number of GP33-41-, NP396-404-, and GP276-286-specific cytokine-producing cells in nontransgenic and Ly49A transgenic mice following stimulation with various doses of antigenic peptide. In comparison with nontransgenic H-2^b mice, the number of GP33-41- and NP396-404-specific cytokine-producing cells was slightly reduced in Ly49A transgenic mice. However, the

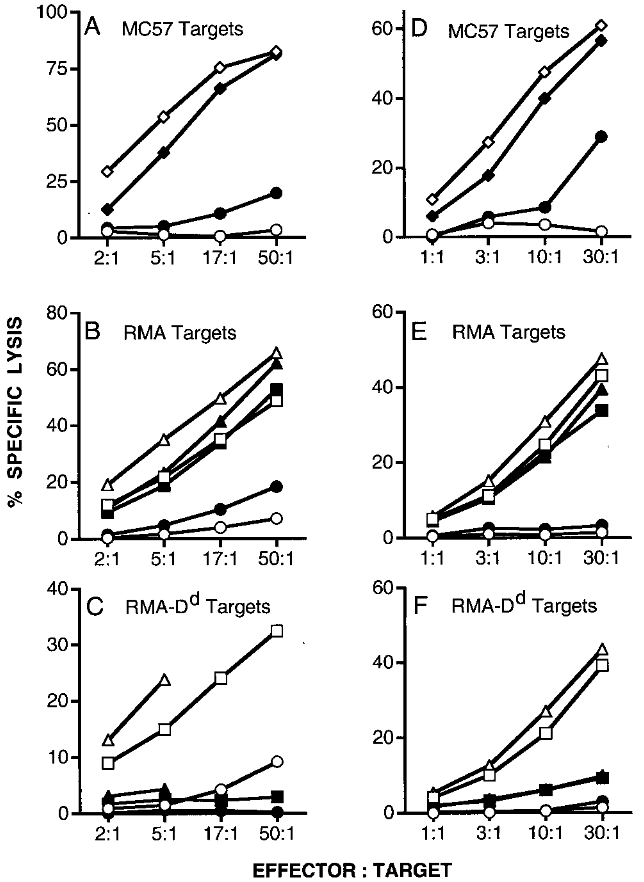

FIGURE 4. Virus-specific CTL in H-2^b mice. Splenocytes from LCMV-infected nontransgenic (open symbols) and Ly49A transgenic (closed symbols) H-2^b mice were tested for virus-specific CTL activity. *A–C*. Splenocytes were prepared from mice at 8 days after LCMV infection, and killing activity was determined directly ex vivo. *D–F*, Splenocytes were isolated from mice at 8 days postinfection and restimulated in vitro for 5 days; CTL activity was then determined. MC57 (*A* and *D*), RMA (*B* and *E*), and RMA-D^d (*C* and *F*) cells were used as targets. The target cells were either left untreated (circles), infected with LCMV (diamonds), or coated with either GP33-41 (triangles) or NP396-404 (squares) peptides. Representative data from one of three experiments are shown in *A*; a total of nine nontransgenic and eight Ly49A transgenic mice were analyzed. *B–F*, Representative data from one of two experiments are shown. In these panels, a total of five nontrangenic and five Ly49 A transgenic mice were analyzed.

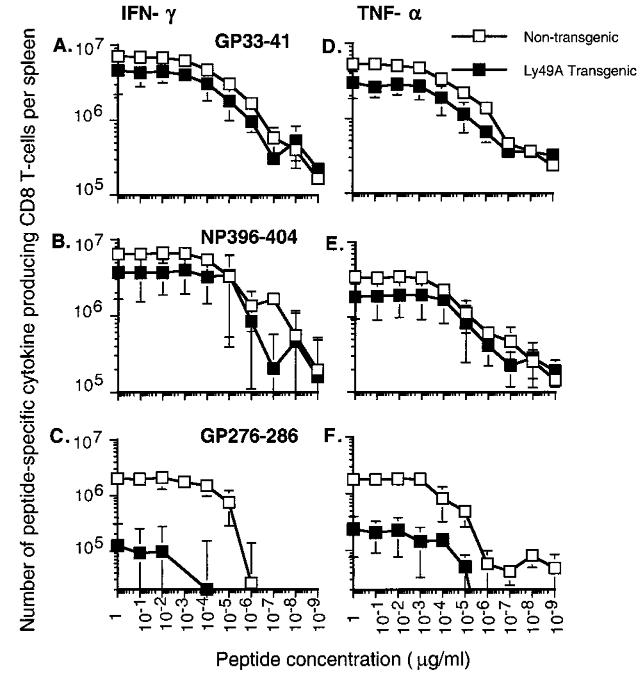

FIGURE 5. Virus-specific cytokine production in H-2^b mice. Splenocytes were prepared from LCMV-infected nontransgenic (open squares) and Ly49A transgenic (filled squares) mice at 8 days postinfection. IFN-γ (*A–C*) and TNF-α (*D* and *E*) production by CD8 T cells was determined using intracellular cytokine staining following 5 h stimulation with various concentrations of peptide (*A* and *D*, GP33-41; *B* and *E*, NP396-404; *C* and *F*, GP276-286). Each point represents the number of splenic CD8 T cells that produce IFN-γ or TNF-α in response to the given dose of peptide. Error bars show SDs.

number of GP276-286-specific cytokine-producing T cells was ~10-fold lower in Ly49A transgenic mice (Fig. 5, *C* and *F*). Similar overall trends were apparent for both IFN-γ- and TNF-α-producing cells, and both nontransgenic and Ly49A transgenic effectors exhibited similar dose responses to each individual epitope. In separate experiments, we also titrated ability of the peptide epitopes to sensitize ^{51}Cr-labeled MC57 target cells for lysis by both nontransgenic and transgenic effector cells. Target cells were coated with various concentrations of peptide ranging from 1 μg/ml to $10^{-10}\mu$g/ml. These peptide-coated targets cells were assayed for lysis by both nontransgenic and Ly49A transgenic effector cells, using an E:T ratio of 50:1. With both transgenic and nontransgenic effectors, 50% of maximal lysis was attained with between 10^{-5} and 10^{-6} μg/ml of GP33-41 peptide, and with between 10^{-6} and 10^{-7} μg/ml of NP396-404 peptide (data not shown).

Analysis using MHC class I tetramers

The results, described above, show the functional activity of the LCMV-specific CD8 T cell response in nontransgenic and Ly49A transgenic mice during acute infection. However, these assays do not necessarily reveal the total number of virus-specific CD8 T cells. Therefore, we used MHC class I tetramers complexed with LCMV epitopes to directly visualize anti-viral CD8 T cells in acutely infected nontransgenic and Ly49A transgenic mice. Following LCMV infection of nontransgenic and Ly49A transgenic H-2^b mice, there was a pronounced expansion of GP33-41- and NP396-404-specific CD8 T cells and a slightly lower GP276-286-specific response (Fig. 6, *A–C*, and Fig. 7, *A–C*). The number of LCMV-specific CD8 T cells in infected Ly49A transgenic H-2^b mice was somewhat lower than that in similarly infected nontransgenic mice, with the most marked difference in the GP276-286-specific response. Fig. 7, *A–C*, compares the absolute number of epitope-specific CD8 T cells, determined by tetramer staining, with number of cytokine-producing peptide-specific CD8 T cells in H-2^b mice. These data suggest that the slightly reduced GP33-41- and NP396-404-specific response in Ly49A transgenic mice is due to impaired expansion of Ag-specific CD8 T cells. However, the lower GP276-286 response results from both a reduction in the overall magnitude of the response and also diminished effector activity by these cells.

L^d (NP118-126) tetramers were used to analyze the responses of H-2^d mice (Figs. 6*D* and 7*D*). The magnitude of the NP118-126 response, visualized using L^d (NP118-126) tetramers, was similar in both nontrangenic and Ly49A transgenic mice. However, the capacity of these cells to produce IFN-γ and, more strikingly, TNF-α was reduced in acutely infected Ly49A transgenic mice.

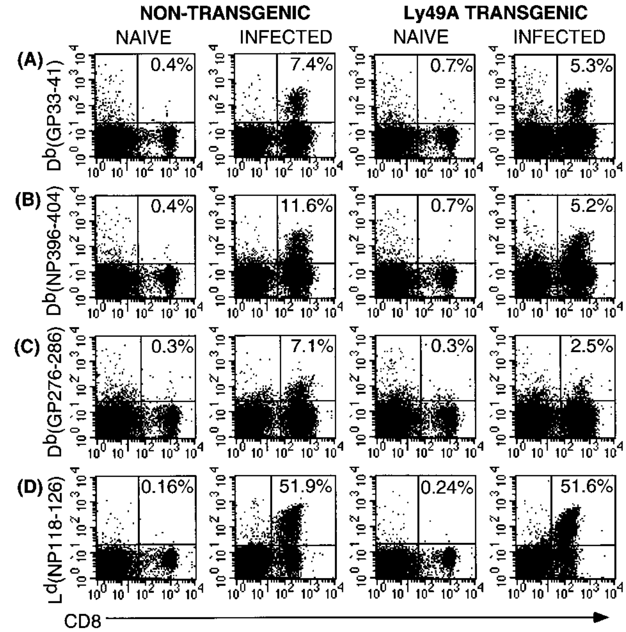

FIGURE 6. Analysis of anti-viral CD8 T cell responses using MHC class I tetramers. Splenocytes were prepared from naive or LCMV-infected (day 8 Armstrong) nontransgenic and Ly49A transgenic mice, as indicated. Splenocytes from H-2^b mice (*A–C*) were costained with anti-CD8-PE Abs, and the indicated H-2D^b-peptide tetrameric complex conjugated to allophycocyanin. *D,* Splenocytes from H-2^d mice were analyzed using anti-CD8-PE and H-2L^d (NP118-126) class I tetramers. The values indicate the percentage of CD8 T cells that costain with the appropriate MHC class I tetramer.

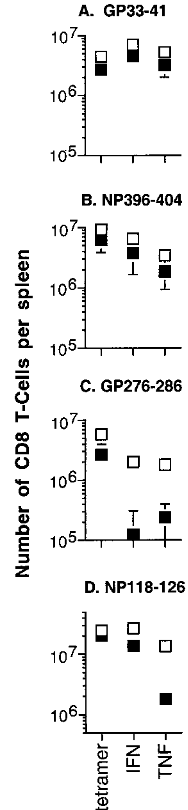

FIGURE 7. Comparison of tetramer analysis and cytokine production by virus-specific CD8 T cells. Non transgenic (open squares) and Ly49A transgenic (filled squares) mice were infected with LCMV-Armstrong and splenic responses analyzed 8 days later. H-2^b mice were analyzed for their GP33-41-, NP396-404-, and GP276-286-specific response (*A*, *B*, and *C*, respectively). *D,* NP118-126-specific responses in H-2^d mice. Each panel shows the number of CD8 T cells that costain with appropriate MHC class I tetramer, the number of CD8 T cells that produce IFN-γ, and the number of CD8 T cells that produce TNF-α. Cytokine production was determined by intracellular staining following stimulation with 1 μg/ml of antigenic peptide. All values are shown $\pm$ SD.

This reduced effector activity suggests that transgenic expression of Ly49A impairs the responsiveness of these cells (Fig. 7*D*).

TCR Vβ usage in H-2^d mice

We have previously reported that acute infection of H-2^d mice elicits a marked L^d-restricted NP118-126-specific response and that many of the responding CD8 T cells express TCR Vβ10 genes (38). We analyzed TCR Vβ usage by CD8 T cells in naive nontransgenic and Ly49A transgenic mice and also examined the pattern of Vβ usage by L^d-restricted NP118-126-specific T cells at the peak of the acute anti-viral response. Fig. 8*A* shows that the peripheral CD8 T cell repertoire is similar in both naive nontransgenic and Ly49A transgenic H-2^d mice. This pattern changes following LCMV infection. In our initial analysis, we observed that nontransgenic H-2^d mice contained substantial numbers of activated Vβ10$^+$ CD8 T cells ($7.08 \times 10^6 \pm 2.3 \times 10^6$; $n = 4$) and that this number was reduced in similarly infected Ly49A transgenic mice ($2.17 \times 10^6 \pm 7.12 \times 10^5$; $n = 5$). We further investigated this alteration in the NP118-126 CD8 T cell repertoire using costaining with tetramers and a panel of anti-TCR Vβ Abs. Fig. 8*B* shows that transgenic expression of Ly49A skews the repertoire of the responding T cells. Most significantly, the usually prominent Vβ10 response was substantially depressed in Ly49A transgenic mice, while in the same mice, Vβ13 expression by anti-viral CD8 T cells was substantially elevated.

Control of LCMV infection

By comparison with nontransgenic mice, CD8 T cell activity was substantially reduced in H-2^d Ly49A transgenic mice, and slightly reduced in H-2^b Ly49A transgenic mice. Despite this reduced activity, all strains of mice examined were able to control infection with the Armstrong isolate of LCMV. Thus, no viremia was detectable at 8 days post infection (data not shown). This finding indicates that although transgenic expression of Ly49A can inhibit T cell functions, the residual response is sufficient to eradicate acute LCMV (Armstrong) infection.

The clearance of the more virulent LCMV variant, clone 13, was investigated to provide a more stringent test of the in vivo effects of Ly49A expression on anti-viral T cell functions. Clone 13 is a macrophage-tropic strain of LCMV, which rapidly disseminates in vivo (34, 35, 42). Fig. 9 shows that viremia was greater in clone 13-infected Ly49A transgenic mice than in nontransgenic mice. Significantly, Ly49A transgene expression in both H-2^d and H-2^b mice resulted in elevated viral titers, by a factor of 10- to 100-fold. Because viral clearance in LCMV infections has been shown to depend largely on the activity of virus-specific CTLs (30–33), these observations support the notion that Ly49A can impair T cell activity in H-2^d mice and provide further evidence for an inhibitory effect of Ly49A in H-2^b mice.

Discussion

In this study, we have investigated the ability of Ly49 receptors to inhibit anti-viral CD8 T cell responses. The results demonstrate a

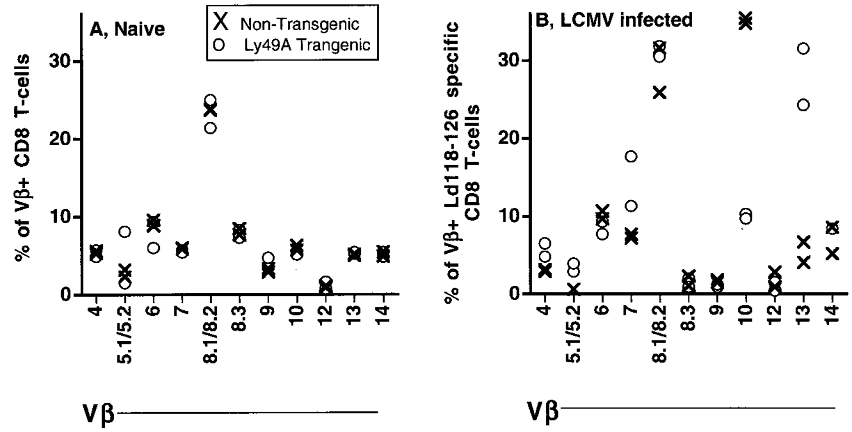

FIGURE 8. CD8 T cell receptor Vβ repertoire analysis in H-2[d] mice. The diversity of the splenic CD8 T cell Vβ repertoire was analyzed in naive or LCMV infected (day 8 Armstrong) nontransgenic (crosses) and Ly49A transgenic (circles) H-2[d] mice. *A,* Splenocytes from naive mice were costained with anti-CD8 and the indicated anti-TCRVβ Ab. *B,* The TCRVβ usage by L[d]-NP118-126-specific CD8 T cells is shown. Splenocytes, isolated from LCMV-Armstrong-infected mice, were costained with anti-CD8, L[d] (NP118-126) tetramers and the indicated anti-TCR Vβ Ab. Stained cells were analyzed by flow cytometry, and the results of two individual mice from each group are shown.

clearly impaired antiviral CD8 T cell response due to Ly49A transgene expression and a correspondingly impaired capacity of mice to clear a viral infection. The data suggest that inhibition of the response occurs at two levels: at the level of induction of activated CTL from naive precursor cells during the viral infection and at the level of the encounter between the activated CTLs and the target cells during the effector phase. Inhibition at the induction phase is suggested by the reduced number of CD44^{high} CD8 T cells present in the transgenic mice 8 days after primary infection and also by the reduced number of virus-specific CD8 T cells detected by tetramer staining. The reduced number of cytokine-producing CD8 T cells in the transgenic mice also suggests that Ly49A expression inhibits the expansion of cytokine-producing virus-specific T cells. Inhibition of effector cells is suggested by the results with CTLs from transgenic H-2[b] mice. The capacity of these CTL to lyse an infected H-2[b] target cell line was strongly inhibited when the target cell was transfected with D[d], while no such inhibition was observed with nontransgenic effector cells. Inhibition during the effector phase is also suggested by the finding that, in some cases, tetramer staining detected a large number of epitope-specific cells, most of which failed to produce cytokines following stimulation. These data indicate that activation of effector CD8 T cells by target cells is subject to inhibition through Ly49 receptors, as has already been demonstrated for NK cells (3, 4). Although a previous study demonstrated that the interaction of Ly49A on CD4 T cells with cognate class I molecules can inhibit alloantigen-induced T cell

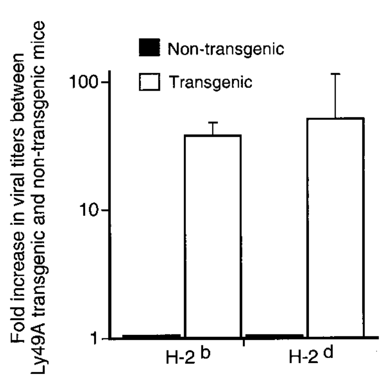

FIGURE 9. Control of virulent LCMV infection. Both nontransgenic (■) and Ly49A transgenic (□) H-2[b] and H-2[d] mice were infected with the virulent LCMV strain clone 13. Serum virus titers were measured by plaque assay at 8 days postinfection. For each haplotype of mice, the bars represent the fold increase in viral titers in Ly49A transgenic mice compared with nontransgenic mice. Three to five mice were analyzed per group, and the error bars represent the range of differences observed.

proliferation in vitro (19), the present work is the first to examine the effect of Ly49A signaling on the cytotoxic response to a natural murine pathogen in vivo.

The transgene is expressed broadly in hemopoietic cells, but it appears likely that the inhibitory effects documented here were largely due to expression of Ly49A by CD8 CTL and their precursor cells. Cytolysis per se requires no cell type other than virus-specific CTLs, arguing against a role for any other transgene-expressing cell type in the process. The primary induction of LCMV-specific CTL is known to be largely unaffected by depletion of CD4 T cells, making it unlikely that the inhibition is an indirect effect mediated through helper T cells (30, 31, 42, 43). Furthermore, clearance of acute LCMV infection requires CD8 T cells, with little detectable role of CD4 T cells, B cells, or NK cells (24, 30–33, 42, 43). These considerations make it likely that the impaired clearance of the virus is due to transgene expression in CD8 T cells. Hence, Ly49A expression can inhibit viral Ag-specific T cells in a physiological setting.

In H-2[b] mice, which are thought to lack a physiological ligand for Ly49A, the overall expansion of CD44^{high} CD8 T cells was unaffected by the transgene, and the expansion of Ag-specific T cells was only marginally reduced. However, a somewhat surprising finding was that transgene expression inhibited some aspects of the response in H-2[b] mice. An effect on the number of cytokine-producing CD8 T cells was observed, though this was only marked in the case of the relatively weak GP276-286 epitope. It also appeared that the CTL response was slightly depressed in transgenic H-2[b] mice in some assays, though the effect was very weak. Significantly, however, the transgene clearly impaired the capacity of H-2[b] mice to control infection with the clone 13 variant of LCMV. It is likely that infection with this variant is a particularly sensitive assay for CD8 T cell function. There are least two possible explanations for the effect seen in H-2[b] mice. One possible explanation is that Ly49A functionally interacts with H-2[b] class I molecules weakly, resulting in inhibition of some but not all parameters of the response. While cell-cell adhesion assays have failed to detect binding of Ly49A to H-2[b] cells, there are hints that such an interaction may occur in vivo (44–46). For example, a small transgene-dependent alteration in the NK cell Ly49 receptor repertoire was observed in Ly49A transgenic H-2[b] mice when compared with transgenic class I-deficient mice (21). This observation suggests that there may be a weak interaction between Ly49A and H-2[b] class I molecules. An additional possibility is that Ly49 receptor expression can cause some degree of inhibition even without interacting with a class I ligand. A precedent for ligand-independent signaling of Ag receptors in lymphocytes has been described in the case of signaling by the pre-T cell receptor (47).

Our analysis of Vβ usage by CD8 T cells in H-2^d mice shows that although transgenic expression of Ly49A does not detectably affect the TCR repertoire of naive peripheral CD8 T cells it does affect the NP118-126-specific response following viral infection. We have previously shown that at the peak of the acute response over 80% of Vβ10$^+$ CD8$^+$ T cells are NP118-126 specific in H-2^d mice (38). The expression of Ly49A reduces this usually prominent anti-viral response. This is consistent with the notion that Ly49A expression can diminish the expansion of virus-specific CD8 T cells. However, the overall response of Ly49A transgenic H-2^d mice is not as drastically reduced, and this is most likely due to an increase in the proportion of Vβ13$^+$ NP118-126-specific CD8 T cells in the transgenic mice. One possibility is that expression of Ly49A preferentially inhibits the usually more dominant Vβ10 NP118-126 response but allows the emergence subdominant virus-specific T cells. Alternatively, expression of the transgene may alter thymic selection, such that elevated numbers of Vβ13$^+$ LCMV-specific clones emerge in transgenic but not nontransgenic mice.

It remains unclear why certain T cell responses and effector functions differ in their susceptibility to suppression by inhibitory receptors. Expression of inhibitory receptors severely impairs NK cell responses; however, the effects on T cell responses appears more variable. De Maria et al. (16) have demonstrated the presence of inhibitory receptor-expressing CD8 T cells during HIV infection. However, in this case, the reduction of cytolytic activity was not absolute. As shown in this study, the effects of Ly49A expression on CD8 T cell responses are diverse and include only subtle effects (for example, GP33-41 responses), impairment of effector activity (for example, NP118-126 responses), and reduced expansion and effector activity (for example, GP276-286 responses). An important parameter that may distinguish the effect of inhibitory receptors on CD8 T cell responses compared with NK cell responses is the nature of the signal delivered through the TCR. The elaboration of T cell responses, including proliferation/expansion, cytotoxicity, and cytokine production, is likely to be determined by the interplay between the strength of the activating signal from the TCR and the strength of the inhibitory signal delivered through the inhibitory receptor. This model would account for the differential effects of Ly49A expression on epitope-specific CD8 T cell responses. For example, in H-2^b mice GP33-41 and NP396-404 responses are only marginally impaired; however, the GP276-286 response is more dramatically reduced. This suggests that the GP276-286 epitope may act as a weak agonist, inducing less pronounced T cell activation, and, consequently, this response is more susceptible to inhibition by Ly49A.

The inhibition of the T cell response caused by the Ly49A transgene was incomplete, probably explaining why the mice were able to clear LCMV-Armstrong. Nevertheless, a clear effect of the transgene on viral clearance was observed with the LCMV variant, clone 13. Because this macrophage-tropic variant disseminates to many tissues rapidly and is not completely cleared in nontransgenic mice (34, 35, 42), we were able to assess whether transgene expression affects clone 13 viral loads. Under these conditions, we observed a 10- to 100-fold increase in viral titers in transgenic mice compared with normal mice. In light of the finding that a fraction of CD8 T cells in normal mice can express Ly49 receptors, it is tempting to speculate that these receptors play a role in regulating T cell responses to persistent Ags, such as chronically infecting viruses or autoantigens. The fact that Ly49 transgene expression has clear effects on the usually very potent response to LCMV suggests that the inhibitory Ly49A signals must themselves be very potent and could play a role in physiological scenarios. The expression and function of these receptors in T cells may represent a mechanism to modulate T cell activity after periods of chronic T cell activation or under specific conditions of antigenic exposure.

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