The consequences of human lymphocytic choriomeningitis virus (LCMV) infection can be severe, including aseptic meningitis in immunocompetent individuals, hydrocephalus or chorioretinitis in fetal infection, or a highly lethal outcome in immunosuppressed individuals. In murine models of LCMV infection, CD8+ T cells play a primary role in providing protective immunity, and there is evidence that cellular immunity may also be important in related arenavirus infections in humans. For this reason, we sought to identify HLA-A2 supertype-restricted epitopes from the LCMV proteome and evaluate them as vaccine determinants in HLA-A2 transgenic mice. We identified four HLA-A*0201-restricted peptides—nucleoprotein NP169-177, glycoprotein precursor GPC10-16, GPC147-155, and zinc-binding protein Z49-58—that displayed high-affinity binding (≤275 nM) to HLA-A*0201, induced CD8+ T-cell responses of high functional avidity in HLA-A*0201 transgenic mice, and were naturally processed from native LCMV antigens in HLA-restricted human antigen presenting cells. One of the epitopes (GPC447-455), after peptide immunization of HLA-A*0201 mice, induced CD8+ T cells capable of killing peptide-pulsed HLA-A*0201-restricted target cells in vivo and protected mice against lethal intracranial challenge with LCMV.

Lymphocytic choriomeningitis virus (LCMV), the prototypic member of the family Arenaviridae, is a rodent-borne pathogen of humans. Infection outcome can range from subclinical infection to debilitating febrile disease with central nervous system involvement (10, 11, 35). Previous studies have demonstrated that LCMV is a significant etiologic agent of aseptic meningitis, accounting for ca. 8 to 9% of diagnosed cases (1, 32). Vertical transmission of LCMV from mother to fetus can result in severe teratogenic consequences, including hydrocephalus, chorioretinitis, or microcephaly (4, 5, 7, 29, 31). Currently there are no licensed vaccines available for LCMV. Detection of the virus in urine, feces, and/or saliva (14). Maintenance of the virus in the rodent population is thought to occur primarily through vertical transmission from infected dams to their pups (14). LCMV can also be introduced into hamster breeding colonies where a similar pattern of asymptomatic maintenance has been observed (24–26, 34). Transmission to humans likely occurs through direct contact with infected rodents or by exposure to excreta or infectious aerosols (10, 14). In the United States, ~5% of adults are seropositive for LCMV-specific antibodies (15). Most infections are presumed to occur from exposure to wild mice, but pet mice and hamsters, as well as experimentally infected rodents utilized in research settings, are also sources of exposure (19, 23). Finally, a recent LCMV outbreak occurred as a result of solid-organ transplantation from LCMV-infected donors to immunosuppressed recipients. This route of transmission led to an extremely high mortality rate, with seven of eight recipients succumbing to infection (21).

Experimental infection of mice with LCMV leads to several different outcomes that are dependent on the age of the mouse, the strain of the virus utilized, and the route of viral inoculation. Infection of immunocompetent mice by intraperitoneal (i.p.) inoculation with LCMV strain Armstrong results in a largely asymptomatic infection that is resolved 8 to 10 days postinfection. The cell-mediated immune response provides protective immunity in this setting; specifically, perforin-mediated lysis of infected cells by CD8+ T cells (28, 47). Gamma interferon (IFN-γ) production is important as well, considering that IFN-γ knockout mice cannot clear LCMV (3). Although passive antibody therapy can provide protection against infection (2, 50), the humoral response plays an insignificant role in resolution of a primary LCMV infection (13). In contrast to i.p. delivery of LCMV, intracranial (i.c.) inoculation of immunocompetent mice results in a lethal choriomeningitis. These

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animals, which succumb 6 to 9 days postinfection, die as a result of an immunopathologic CD8+ T-cell response directed against infected cells in the choroid plexus and the meninges (11). The murine i.c. model is highly relevant to human aseptic meningitis (resulting from LCMV infection) because disease in both settings is thought to occur by the same mechanism (10).

Considering the importance of CD8+ T cells in providing protection against LCMV infection, it is important that sensitive reagents to measure this response in the context of human infection or in response to vaccine candidates are developed. The identification of HLA-restricted epitopes from LCMV is required to develop tetrameric staining reagents and diagnostic assays. Such epitopes would also serve to determine the quality of immune responses, define correlates of protection and immunopathology, and ultimately guide the selection of candidate vaccines (39). The goal of the present study was to identify HLA-restricted epitopes from LCMV and evaluate whether they could provide protection against viral challenge. Accordingly, we screened the LCMV proteome for CD8+ T-cell epitopes using an HLA motif algorithm to predict potential epitopes corresponding to the HLA-A2 supertype family, which is represented in ~50% of the worldwide population, irrespective of ethnicity. We were able to identify four peptides (NP69-77, GPC447-455, and Z49-58) that were immunogenic after peptide immunization or LCMV infection of HLA-A*0201 transgenic mice and were endogenously processed from native LCMV antigens expressed in HLA-A*0201-expressing human antigen-presenting cells (APC). Peptide immunization of HLA-A*0201 mice with GPC447-455 induced CD8+ T cells that were able to kill peptide-pulsed targets in vivo and protect against lethal i.c. challenge with LCMV.

MATERIALS AND METHODS

Bioinformatic analyses. The LCMV open reading frames (ORFs) utilized in the present study were strain Armstrong 53b NP (NCBI accession number AAA46257), GPC (NCBI accession number NP_694851), L (NCBI accession number NP_694850), and Z (NCBI accession number AAA46299). Candidate HLA-A2 supertype epitopes were identified by using a previously described algorithm (12). Candidate peptides from each ORF (NP, n = 24; GPC, n = 21; L, n = 27; and Z, n = 1) were selected on the basis of both predicted binding affinity to HLA-A*0201 and amino acid sequence conservancy among all unique LCMV isolates for which amino acid sequences have been reported.

Peptides. Peptides (≥90% pure) were obtained from Genemed Synthesis, Inc. (South San Francisco, CA). Hepatitis B virus (HBV) ENV 378 (LPLIFCFLWV) was used as an irrelevant, HLA-A*0201-restricted peptide.

MHC peptide binding assays. Major histocompatibility complex (MHC) molecules were purified, and binding assays performed as previously described (41, 42). Briefly, 1 to 10 nM concentrations of radiolabeled peptide were coincubated with 1 μM to 1 nM concentrations of purified MHC in the presence of 1 to 3 μM human β2-microglobulin. After 2 days, the binding of the radiolabeled peptide to the corresponding MHC class I molecule was determined by capturing MHC-peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-One, Longwood, FL) coated with the W6/32 antibody and measuring the bound counts per minute using a TopCount microscintillation counter (Packard Instrument Co.).

Mice. HLA-A*0201/KR (referred to as HLA-A*0201 here) transgenic mice were bred at Pharmexa-Epimmune and The Scripps Research Institute. These mice represent the F1 generation resulting from a cross between HLA-A*0201 and amino acid sequence conservancy among all unique LCMV isolates for which amino acid sequences have been reported.

Oratory Animal Care and were done according to Institutional Animal Care and Use Committee-approved animal protocols.

Cells. JA2.1 cells (human Jurkat cells that express the HLA-A*0201/KR chimeric gene) (46) and LPS blasts (prepared as previously described [40]) were grown as previously described (40). CV-1 cells (CCL-70) and BSC-40 cells (CRL-2761), both from American Type Culture Collection were grown as di-

Virus. LCMV infections were conducted with strain Armstrong 53b. This virus was expanded in BHK-21 cells, and titers were determined on Vero E6 cells. vNP and vGPC (which express LCMV Armstrong 53b NP or GPC, respectively) are recombinant vaccinia virus (rVV) constructs that were generated on the Western Reserve (WR) background as previously described (49). rVV-Z and rVV-L (which express LCMV Armstrong 53b Z or L, respectively) were generated on the WR background according to the guidelines established by Blasco and Moss (8). Briefly, the LCMV Z and L genes were subcloned into the pRB21 transfer vector. CV-1 cells were infected with VV strain vRB12 (multiplicity of infection of 2) and transfected with 10 μg of transfer vector containing either Z or L. Viruses that underwent homologous recombination with the transfer vector were selected on the basis of their ability to form plaques. Z or L expression was confirmed for each recombinant virus via Western blot (data not shown). vRB12 and pRB21 were provided by B. Moss.

Immunizations and viral challenges. To evaluate peptide immunogenicity, CD8+ T-cell avidity, endogenously processing of peptides from native LCMV antigens or in vivo CTL killing, HLA-A*0201 mice (8 to 14 weeks old) were inoculated subcutaneously at the base of the tail with a mixture of CD8+ T-cell peptides (50 μg of each peptide per mouse) and the helper T-cell peptide human lambda repressor 12 (YLEDDRLKAIYKKK), chicken ovalbumin 323-339 (GPFTKAYLQRLPPPRPPI) (46), and HIV core 128 (TPPVDHAAAH(E)2) (46) of each peptide per mouse) that had been emulsified 1:1 in incomplete Freund’s adjuvant (IFA). For i.p. challenge studies, mice (HLA-A*0201 or CB6F1) were immunized with peptide-IFA emulsions and challenged 14 days postimmunization via i.p. inoculation with 2 × 106 PFU of LCMV. At 4 days postchallenge, spleens were harvested for CD8+ T-cell purification and LCMV titer determination. To determine the virus titer, a portion of each spleen was homogenized, and 10-fold dilutions of these homogenates were plated on Vero E6 cells and overlaid with 1.4% agarose–Dulbecco modified Eagle medium. Cells were fixed, and plaques counted 4 days postinoculation. For lethal challenge studies, equal numbers of HLA-A*0201 mice were inoculated subcutaneously with peptide-IFA emulsions or peptide-CpG mixtures (CpG ODN 1826 [3.8 nmol per mouse] and a PADRE [AFFFFAAATLKAAA]-CD8+ T-cell epitope fusion peptide (50 nmol/mouse) as described by Daftarian et al. (17). Mice were boosted 11 days later and then 7 days after the boost challenged i.c. with 105 50% lethal doses (LD50) of LCMV. Mice were examined for 30 days after challenge.

Because the results after peptide-IFA and peptide-CpG immunization did not differ, they were reported as pooled data in Table 2.

ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (44). Briefly, 4 × 104 splenic CD8+ T cells were labeled with anti-CD8 bioconjugate (Miltenyi Biotec). Cells were cultured with 106 peptide-pulsed or rVV-infected JA2.1 target cells. Target cells were pulsed by incubating them with peptide for at least 2 h at room temperature, followed by three washes to remove free peptide. For rVV infections, JA2.1 cells were infected (multiplicity of infection) of 10) 18 h prior to the assay. Effector and target cells were incubated in flat-bottom 96-well nitrocellulose plates (Immobilon-P membrane; Millipore) precoated with 50 μl of anti-IFN-γ monoclonal antibody (Mabtech AN18; 10 μg/ml)/well. After 16 to 20 h, the plates were washed, and the wells were incubated with 100 μl of biotinylated anti-IFN-γ monoclonal antibody (Mabtech R4A6A2; 1 μg/ml) for 2 h. After additional washing, spots were developed by sequential incubation with Vectastain ABC peroxidase (Vector Laboratories) and 3-amin-9-ethylcarbazole solution (Sigma-Aldrich) and counted by computer-assisted image analysis (Zeiss KS ELISPOT reader).

Each assay was performed in three replicate wells, and the experimental values were expressed as the mean spots/106 CD8+ T cells ± the standard deviation for each peptide. The responses of CD8+ T cells derived from peptide immunized mice against JA2.1 cells pulsed with irrelevant peptide (HBV ENV 378) or infected with irrelevant VV constructs were measured to establish background values.

In vivo cytoxicity assay. Spleenocytes from HLA-A*0201 mice were labeled with 0.3 μM or 0.06 μM of CFSE (5-carboxy-fluorescein diacetate succinimidyl ester; Molecular Probes). CFSE− cells were pulsed with 1 μg/ml of GFP-C33-43, GPC447-455, NP69-77, or Z49-58, while CFSE+ cells were pulsed with 1 μg/ml of the irrelevant peptide HBV ENV 378. After extensive washing, equal numbers of CFSE− and CFSE+ cells were mixed and delivered (~8 × 106 cells

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protection against lethal lymphocytic choriomeningitis

Identification of candidate HLA-A*0201-restricted CD8+ T-cell epitopes. To identify candidate CD8+ T-cell epitopes from LCMV, the NP, GPC, L, and Z amino acid sequences of LCMV strain Armstrong 53b were screened for potential HLA-A2 supertype epitopes using bioinformatic algorithms as described in Materials and Methods (12). A total of 73 candidate peptide sequences (nonamers and decamers) were identified (NP, n = 24; GPC, n = 21; L, n = 27; and Z, n = 1). To determine whether each of these peptides would be recognized in the context of an LCMV infection, HLA-A*0201 transgenic mice were infected with 2 × 10^5 PFU of LCMV strain Armstrong 53b via i.p. inoculation. Ten days later, splenic CD8+ T cells were isolated and screened for reactivity to each candidate peptide in an ex vivo IFN-γ ELISPOT assay. Candidate peptides were considered immunogenic if they induced IFN-γ spot formation by CD8+ T cells in response to peptide-pulsed JA2.1 target cells that received increasing gradient doses of peptide. +, Statistically significant response; −, response not statistically significant.

RESULTS

Identification of immunogenic LCMV peptides after infection with LCMV. HLA-A*0201 mice (A), C57BL/6 mice (B), or BALB/c mice (C) were inoculated with 2 × 10^5 PFU of LCMV strain Armstrong 53b via i.p. inoculation. Splenic CD8+ T cells were isolated 10 days later and exposed to JA2.1 target cells that had been pulsed with 10^{-9} M of each of the listed peptides in an ex vivo IFN-γ T-cell proliferation assay. Peptides that induced significant IFN-γ spot formation compared to an irrelevant HLA-A*0201-restricted peptide are denoted by an asterisk; the dotted line indicates the maximum number of spots produced by CD8+ T cells exposed to the irrelevant peptide.

FIG. 1. Identification of immunogenic LCMV peptides after infection with LCMV. HLA-A*0201 mice (A), C57BL/6 mice (B), or BALB/c mice (C) were inoculated with 2 × 10^5 PFU of LCMV strain Armstrong 53b via i.p. inoculation. Splenic CD8+ T cells were isolated from HLA-A*0201 mice 11 to 14 days after infection with LCMV, the NP, GPC, L, and Z amino acid sequences of LCMV, the NP, GPC, L, and Z amino acid sequences of LCMV strain Armstrong 53b were screened for potential HLA-A2 supertype epitopes using bioinformatic algorithms as described in Materials and Methods (12). A total of 73 candidate peptide sequences (nonamers and decamers) were identified (NP, n = 24; GPC, n = 21; L, n = 27; and Z, n = 1). To determine which of these peptides would be recognized in the context of an LCMV infection, HLA-A*0201 transgenic mice were infected with 2 × 10^5 PFU of LCMV strain Armstrong 53b via i.p. inoculation. Ten days later, splenic CD8+ T cells were isolated and screened for reactivity to each candidate peptide in an ex vivo IFN-γ ELISPOT assay. Candidate peptides were considered immunogenic if they induced IFN-γ spot formation by CD8+ T cells in response to peptide-pulsed JA2.1 target cells that had been pulsed with 10^{-9} M of each of the listed peptides in an ex vivo IFN-γ T-cell proliferation assay. Peptides that induced significant IFN-γ spot formation compared to an irrelevant HLA-A*0201-restricted peptide are denoted by an asterisk; the dotted line indicates the maximum number of spots produced by CD8+ T cells exposed to the irrelevant peptide.
formation that was significant compared to an irrelevant HLA-A*0201-restricted peptide.

Using this approach, we identified 13 peptides that were immunogenic in HLA-A*0201 mice (Fig. 1A and Table 1). Immunogenic peptides were identified from each of the four ORFs: six from NP (NP69-77, NP101-109, NP235-244, NP236-244, NP240-248, and NP315-323), four from GPC (GPC10-18, GPC447-455, GPC116-125, and GPC447-455), two from L (L1189-1197 and L1734-1742), and one from Z (Z49-58). These results demonstrated the immunogenicity of the above-listed peptides but did not rule out the possibility that some of the peptides may be restricted by H-2b or H-2d alleles which, in addition to HLA-A*0201, are expressed in these HLA transgenic mice. To determine whether peptides were restricted to HLA-A*0201, H-2b, H-2d, or a combination of these backgrounds, we repeated the experiment utilizing either C57BL/6 (Fig. 1B) or BALB/c (Fig. 1C) mice. CD8 T cells obtained from C57BL/6 or BALB/c mice 10 days postinfection were exposed to each of the 13 immunogenic peptides identified in Fig. 1A. The majority of the peptides (8 of 13) were not reactive in C57BL/6 or BALB/c mice, confirming that they were HLA-A*0201 restricted (Table 1). We found three peptides (NP235-244, GPC10-18, and GPC116-125) that were reactive in C57BL/6 mice and two peptides (GPC10-18 and L1189-1197) that were reactive in BALB/c mice. One of the H-2b reactive peptides, GPC34-43, has previously been shown to be an immunodominant epitope restricted by H-2Kd (27). In the case of GPC116-125 (SIISHNFCNL), a nested epitope, GPC116-125 (ISHNFCNL), has been previously reported, indicating that the 10-mer identified in the present study is likely a less optimal derivation of the previously reported H-2b-restricted epitope (45). The remaining murine-restricted peptides have not been described previously.

Avidity of CD8 T-cell responses against LCMV peptides after peptide immunization. To determine the avidity of CD8 T-cell responses induced after peptide immunization, in vitro peptide titration experiments were performed next. For these experiments, HLA-A*0201 mice were immunized with a subset of the LCMV peptides identified in Fig. 1 (NP69-77, NP101-109, NP235-244, NP236-244, NP240-248, NP315-323, GPC10-18, GPC447-455, Z49-58, L1189-1197, or L1734-1742). CD8 T cells were isolated 11 to 14 days postimmunization and exposed to JA2.1 target cells that had been pulsed with gradient doses of the immunizing LCMV peptide in an ex vivo IFN-γ ELISPOT assay. An asterisk denotes the lowest concentration of peptide that yielded a significant response. Avidity data was used to determine the lowest concentration of peptide that yielded a significant response.
pulsed murine LPS blasts that had been derived from CB6F1 mice (these mice represent the F1 generation resulting from a cross between a BALB/c and a C57BL/6 mouse). In the context of murine MHC class I peptide presentation, GPC10-18 was not reactive, whereas L1189-1197 and NP235-244 were reactive to an endpoint of 5 \times 10^{-10} M and 2.5 \times 10^{-11} M of peptide, respectively (Fig. 2 and data not shown). Considering that the HLA-A\*0201-restricted endpoint reactivity of GPC10-18 and L1189-1197 was 10^{-9} M and that of NP235-244 was \geq 6.25 \times 10^{-11} M, the HLA-A\*0201 presentation resulted in significantly higher functional avidity compared to the murine MHC class I presentation.

Natural processing of immunogenic peptides from native antigens in HLA-restricted human APCs. Next, we sought to address whether the peptides could be endogenously processed from native viral antigen by human APCs. To express LCMV proteins within JA2.1 cells, rVV were designed to express full-length LCMV NP (vvNP), GPC (vvGPC), Z (vvZ), or L (vvL) (see Materials and Methods). To assess processing from intact LCMV antigens, HLA-A\*0201 mice were immunized with individual LCMV peptides (NP69-77, GPC10-18, GPC447-455, Z49-58, L1189-1197, or L1734-1742). Splenic CD8^+ T cells were isolated 11 to 14 days later and exposed to JA2.1 cells that had been pulsed with peptide (LCMV peptide or irrelevant peptide) or infected with rVV (irrelevant or relevant rVV) in an ex vivo IFN-\gamma ELISPOT assay. Peptides or rVV were considered immunogenic if they induced IFN-\gamma spot formation that was significant compared to JA2.1 target cells that had been pulsed with an irrelevant HLA-A\*0201-restricted peptide or infected with an irrelevant VV construct. Immunogenic responses are denoted by an asterisk.

Peptide immunization is protective against LCMV challenge in HLA-A\*0201 mice. We next addressed whether the identified epitopes could confer protection against LCMV challenge in HLA-A\*0201 mice. Figure 4A outlines the logistics of the challenge experiment. Groups of HLA-A\*0201 mice (n = 5 per group) were initially immunized with the following CD8^+
immunization with GPC33-41 led to a significant decrease in LCMV replication after challenge (Fig. 4B). As expected, prior animals (adjuvant alone) displayed evidence of high titer titration. Spleens were harvested from each mouse on day 4 post-immunization, mice were inoculated i.p. with 2 × 10^5 PFU of LCMV strain Armstrong 53b. On day 14 postimmunization, splenic CD8^+ T cells were isolated from mice that were immunized without an accompanying viral challenge (C) or immunized and challenged with LCMV (D). CD8^+ T cells were exposed to JA2.1 target cells that had been pulsed with 10^7 M of the immunizing peptide in an ex vivo IFN-γ ELISPOT assay. The fold increases in epitope-specific CD8^+ T-cell frequencies in challenged mice compared to unchallenged mice are indicated.

Protection is HLA restricted. To verify that the protection observed after immunization with GPC447-455 or NP69-77 in Fig. 4 was HLA-A*0201 restricted, we repeated the immunization and challenge protocol in CB6F1 mice (Fig. 6A). These mice have the same genetic background as HLA-A*0201 mice but do not express HLA-A*0201. Although GPC33-41 immunization led to a significant titer reduction (> 99%, P = 0.0004), immunization with GPC447-455 or NP69-77 did not reduce virus titers in CB6F1 mice, confirming that the protection observed was HLA-A*0201 restricted (Fig. 6B). We also measured the frequency of epitope-specific CD8^+ T cells from these animals on day 4 postchallenge (Fig. 6C). Consistent with the observed protection from GPC33-41 immunization, we saw a significant number of GPC33-41-specific CD8^+ T cells compared to an irrelevant peptide. However, in the case of GPC447-455 or NP69-77-specific CD8^+ T cells on day 18 postimmunization (range, 41.67 ± 8.33 to 616.67 ± 60.09 mean spots per 10^6 CD8^+ T cells). However, peptide-immunized mice that were challenged on day 14 postimmunization with LCMV showed an expansion of CD8^+ T cells that were specific for the immunizing peptide 4 days after challenge (Fig. 5). This expansion was observed for each of the three peptides that led to significant titer reduction in Fig. 4. (GPC33-41, 7.4-fold; GPC447-455, 7.5-fold; and NP69-77, 112-fold). Interestingly, expansion of epitope-specific CD8^+ T cells after LCMV challenge was also observed for each of the remaining peptides, despite the fact that they did not provide protection.

Protection is epitope specific. To provide evidence of epitope-specific protection, we measured the frequency of epitope-specific CD8^+ T cells in HLA-A*0201 mice that were immunized with peptide (GPC33-41, GPC447-455, NP69-77, Z49-58 or GPC10-18) and then either challenged or not with LCMV. We wanted to determine whether an expansion of epitope-specific CD8^+ T cells after viral challenge would correlate with protection. The results of this experiment are displayed in Fig. 5. Peptide-immunized mice that were not challenged displayed significant numbers of epitope-specific CD8^+ T cells.
prior peptide immunization followed by challenge did not lead to expansion of peptide-specific CD8\(^+\) T cells in these HLA-A*0201-negative animals. This observation confirms the HLA-A*0201 restriction of these epitopes and discounts any contributing murine MHC class I presentation in this challenge model.

**Peptide Immunization protects HLA-A*0201 mice from lethal challenge with LCMV.** We next evaluated whether the reduction in virus titer observed after immunization with GPC33-41, GPC447-455, or NP69-77 (Fig. 4) correlated with protection against lethal LCMV challenge. To do so, HLA-A*0201 mice were immunized with GPC33-41, GPC447-455, NP69-77, or adjuvant alone (control) as described in Materials and Methods. Mice were boosted 11 days postimmunization and then challenged 7 days later with 10 LD\(_{50}\) of LCMV strain Armstrong 53b via i.c. inoculation. As a positive control, we also included LCMV-immune mice, which had been infected with 2\(^\times\)10\(^5\) PFU of LCMV via i.p. inoculation 30 prior to i.c. challenge with 10 LD\(_{50}\) of LCMV.

All deaths in peptide-immunized groups occurred 6 to 8 days postchallenge. The results of this experiment are listed in Table 2. Immunization with GPC33-41 and GPC447-455 led to significant (\(P = 0.049\)) protection, with three of six mice surviving in each group. The final peptide, NP69-77, which protected one of six animals, did not lead to significant protection. All deaths in peptide-immunized groups occurred 6 to 8 days postchallenge.

**Peptide immunization of HLA-A*0201 mice induces CD8\(^+\) T cells that kill in vivo.** As a final correlative measure to the protection observed in Fig. 4 and Table 2, we evaluated whether peptide immunization of HLA-A*0201 mice with GPC33-41 or any of the four HLA-A*0201-restricted epitopes would induce...
CD8+ T cells capable of killing peptide-pulsed target cells in vivo. Mice were immunized with peptide (GPC10-18, GPC33-41, GPC447-455, NP69-77, Z49-58, or adjuvant alone (control). Ten days later, CFSE-labeled target cells (CFSElo) were delivered via i.v. injection. The results of this experiment are displayed in Fig. 7. Each of the peptides examined was able to induce specific killing of peptide-pulsed targets. The magnitude of killing was greatest after immunization with GPC33-41 (range, 61.0 to 72.0%). Of the four HLA-A2-supertype-restricted epitopes from the LCMV proteome, the level of killing induced by each of the four peptides examined was able to bind at least three of the alleles with high affinity (defined as 500 nM or less) (40).

**DISCUSSION**

The consequences of human LCMV infection can be severe, ranging from aseptic meningitis in immunocompetent individuals; hydrocephalus, chorioretinitis, or microcephaly in fetal infections; or a highly lethal outcome in immunosuppressed individuals (10, 11, 21, 35). The reservoir rodent has a worldwide distribution; thus, the risk of LCMV infection exists on a global scale. Experimental murine infection with LCMV represents one of the most carefully studied host-pathogen models. Our understanding of protective immunity in this model is clear: CD8+ T cells are both required and sufficient to provide protective immunity (11, 33). Furthermore, immunization of mice with CD8+ T-cell epitopes has been shown to provide protection against lethal LCMV disease (6, 37, 48). To date, HLA-restricted epitopes have not been identified or evaluated as vaccine determinants. In the present study, we have addressed this deficiency by identifying four novel HLA-A2-supertype-restricted epitopes from the LCMV proteome. Because the HLA-A2-supertype family is represented in ca. 50% of the general population, these epitopes should be broadly applicable as diagnostic reagents for evaluating CD8+ T-cell responses in the context of infection and/or vaccine trials. Furthermore, because one of the epitopes (GPC447-455) was protective against lethal disease in HLA-A*0201 transgenic mice, it is a candidate epitope for vaccine design.

Previously, we utilized a similar approach to identify HLA-A*0201-restricted epitopes from Lassa virus (LASV) (9). In that study we utilized motif-based algorithms to identify candidate peptides, in vitro MHC binding analysis to identify high-affinity (≤500 nM) binding peptides, peptide immunization of HLA transgenic mice to evaluate in vivo immunogenicity of high-affinity peptides and, finally, expression of native LASV antigens in HLA-restricted human APC to evaluate endogenous processing of peptides. Only 3 of the 18 immunogenic peptides identified in that study were processed correctly from native LASV antigens in human APC. Therefore, considerable effort was spent evaluating MHC binding and immunogenicity for peptides that ultimately were not validated as epitopes. One observation from this earlier work suggested that we could improve the efficiency with which we identify and validate HLA-restricted epitopes. When we infected HLA-A*0201 mice with an rVV expressing either LASV NP or GPC and then screened CD8+ T cells from these mice for reactivity against the original panel of predicted pep-

**TABLE 3.** LCMV epitopes bind to multiple HLA-A2 supertype alleles with high affinity

<table>
<thead>
<tr>
<th>LCMV peptide</th>
<th>Binding affinity (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPC447-455</td>
<td>52</td>
</tr>
<tr>
<td>NP69-77</td>
<td>142</td>
</tr>
<tr>
<td>Z49-58</td>
<td>17</td>
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* Peptide position within LCMV (strain Armstrong 53b) NP, GPC, or Z.
tides ($n = 83$), all three of the validated epitopes were reactive. This result suggested that we could have circumvented the majority of the MHC binding analysis and immunogenicity screening had we simply started by screening CD$^8^+$ T cells from infected mice for reactivity against predicted peptides. Therefore, that is the approach we applied in the present study. Using CD$^8^+$ T cells from LCMV-infected HLA-A*0201 mice, we were able to successfully screen 73 candidate peptides for immunogenicity within the constraints of a single assay. The alternative would have been to immunize ~15 groups of mice with pools of five nonoverlapping peptides in order to screen the entire set of candidate peptides for immunogenicity. Although the current approach potentially missed peptides that the HLA mice did not mount a detectable CD$^8^+$ T-cell response against after infection, it did successfully allow us to map new determinants with high efficiency. Therefore, we suggest that this more efficient approach be considered in future epitope identification studies.

The viral challenge studies revealed that not all of the epitopes identified afforded equal protection after immunization. In Fig. 4 we screened each of the HLA-A*0201-restricted epitopes for their ability to protect against viral replication. Despite the fact that immunization with each peptide led to similar levels of in vivo killing (Fig. 7) and induced IFN-γ-secreting CD$^8^+$ T cells that were similar in frequency and functional avidity (Fig. 2), there was considerable variation in the ability of these epitope-specific CD$^8^+$ T-cell populations to restrict viral replication; only immunization with GPC$_{447-455}$ or NP$_{69-77}$ was able to significantly reduce the virus titer. These observations indicate that measurements of IFN-γ secretion and in vivo killing of peptide-pulsed APC by CD$^8^+$ T cells after peptide immunization may not be sensitive indicators of the protective potential for a given peptide. Indeed, an investigation of protective versus nonprotective antimalaria CD$^8^+$ CTL clones found that whereas secreted cytokines such as IFN-γ and tumor necrosis factor alpha did not differ between clones, the expression of the adhesion molecules CD44 and VLA-4 was upregulated in protective clones (36). Another study found that a nonprotective CTL clone specific for cytomegalovirus phenotypically differed from protective clones by exhibiting low levels of tumor necrosis factor alpha secretion and CD8 expression but that IFN-γ expression did not differ among clones (20). Finally, the fact that uniform levels of in vivo killing were observed for protective and nonprotective epitopes may reflect that there are differences among these peptides with regard to antigen processing and presentation during LCMV infection.

In Fig. 4, we also evaluated GPC$_{33-41}$, an immunodominant, H-2$^d$-restricted peptide, for its ability to restrict viral replication after peptide immunization. We found that the levels of titer reduction observed after immunization with the protective HLA-A*0201 peptides was significantly less than that observed after immunization with GPC$_{33-41}$. These differences in titer reduction may be attributable to differences in MHC expression in the HLA-A*0201 mice (HLA-A*0201 versus H-2$^d$), but it could also be that the H-2$^d$-restricted epitope is an exceptionally good epitope, since its immunodominance during LCMV infection in H-2$^d$-restricted mice is well established.

The viral challenge studies also revealed that epitope-primed CD$^8^+$ T cells were able to expand in response to LCMV infection (see Fig. 5). Surprisingly, this observation applied both to protective and to nonprotective CD$^8^+$ T-cell responses. The two peptides (GPC$_{33-41}$ and GPC$_{447-455}$) that induced the highest level of protection displayed ~7-fold increases in effector cells after challenge compared to similarly immunized mice that were not challenged (Fig. 5). However, the range of expansion for the remaining nonprotective peptides varied considerably (5.9- to 825-fold). These outcomes, either modest or robust expansion after challenge, could be a result of epitope-specific CD$^8^+$ T cells that were able to proliferate in response to antigen presentation but not efficiently kill target cells (examples include GPC$_{10-18}$ [825-fold expansion] and NP$_{69-77}$ [112-fold expansion]) or, alternatively, were merely unable to proliferate efficiently in response to viral challenge (Z$_{49-58}$ [5.9-fold expansion]). In the case of GPC$_{10-18}$ it could also be that the initial epitope-specific CD$^8^+$ T-cell frequencies were too low at the time of challenge to effectively blunt the initial rounds of viral replication (37). In this scenario, these T-cell populations would in essence be overrun by early viral replication and then rapidly expand in direct proportion to the increasing viral antigen load in the infected host.

A subset of the epitopes (Z$_{49-58}$, HLA-A*0201 restricted; L$_{1189-1197}$, H-2$^d$ restricted) identified in the present study correspond to the Z and L proteins. To our knowledge this is the first report of MHC class I-restricted epitopes derived from these arenavirus ORF. Although peptide immunization of HLA-A*0201 mice with Z$_{49-58}$ induced a high avidity CD$^8^+$ T-cell response, this response did not effectively limit viral replication after viral challenge. This could be a result of differential levels of Z expression compared to GPC or NP during LCMV infection. However, the fact that Z$_{49-58}$-primed T cells were able to expand after challenge (5.9-fold compared to unchallenged mice) indicates that failure to protect was not due to a paucity of antigen to drive CD$^8^+$ T-cell expansion. Furthermore, the fact that mice mounted responses against the Z and L epitopes after a primary infection (see Fig. 1) demonstrated that the level of expression of these proteins was sufficient to induce a detectable CD$^8^+$ T-cell response. Indeed, a previous study demonstrated that splenocytes from LCMV-infected CBA/Ca or TG8.1 mice could effectively kill target cells that expressed LCMV L via infection with an rVV construct (18). Therefore, collectively these findings indicate that the Z and L ORF should be included in future epitope identification studies involving members of the arenavirus family.

The lethal challenge experiments demonstrated that the titer reduction observed after peptide immunization of HLA-A*0201 mice with GPC$_{447-455}$ (76%) or GPC$_{33-41}$ (97%) (see Fig. 4) correlated with significant protection against lethal disease (Table 2). Furthermore, peptide immunization did not lead to an exacerbation of disease as evidenced by the fact that none of the peptide immunized mice displayed increased kinetics to lethal endpoint compared to controls (data not shown). These findings represent the first demonstration of HLA-restricted protection against LCMV infection and correlate with our previous observations of HLA-restricted protection against rVV expressing LASV proteins (9). Therefore, these studies collectively demonstrate that HLA-restricted epitopes represent effective vaccine determinants for the pathogenic Old World arenaviruses.
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