Chronic Lymphocytic Choriomeningitis Virus Infection Actively Down-Regulates CD4$^+$ T Cell Responses Directed against a Broad Range of Epitopes[1]

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Activation of CD4$^+$ T cells helps establish and sustain CD8$^+$ T cell responses and is required for the effective clearance of acute infection. CD4-deficient mice are unable to control persistent infection and CD4$^+$ T cells are usually defective in chronic and persistent infections. We investigated the question of how persistent infection impacted pre-existing lymphocytic choriomeningitis virus (LCMV)-specific CD4$^+$ T cell responses. We identified class II-restricted epitopes from the entire set of open reading frames from LCMV Armstrong in BALB/c mice (H-2^d) acutely infected with LCMV Armstrong. Of nine epitopes identified, six were restricted by I-A^d, one by I-E^d and two were dually restricted by both I-A^d and I-E^d molecules. Additional experiments revealed that CD4$^+$ T cell responses specific for these epitopes were not generated following infection with the immunosuppressive clone 13 strain of LCMV. Most importantly, in peptide-immunized mice, established CD4$^+$ T cell responses to these LCMV CD4 epitopes as well as nonviral, OVA-specific responses were actively suppressed following infection with LCMV clone 13 and were undetectable within 12 days after infection, suggesting an active inhibition of established helper responses. To address this dysfunction, we performed transfer experiments using both the Smarta and OT-II systems. OT-II cells were not detected after clone 13 infection, indicating physical deletion, while Smarta cells proliferated but were unable to produce IFN-γ, suggesting impairment of the production of this cytokine. Thus, multiple mechanisms may be involved in the impairment of helper responses in the setting of early persistent infection. *The Journal of Immunology,* **2007, 179: 1058–1067.**

Immune responses resulting in viral clearance have been associated with the activation and expansion of virus-specific CD4$^+$ T cells. This effect is mediated by direct effector mechanisms and through the priming and maintenance of CTL responses which act to clear viral infection (1–7). However, certain viruses, such as HIV, lymphocytic choriomeningitis virus (LCMV)[3] and hepatitis C virus, have developed mechanisms to establish persistent infection. Despite potent responses early in the course of infection, CTL responses are often ineffective at clearing these viral infections (8–12). Several different mechanisms have been shown to contribute to the failure of T cell responses to contain viral replication in the course of persistent infections, including clonal exhaustion, overexpression of programmed death (PD)-1 and the rapid appearance of viral mutations resulting in escape variants (8, 13–20). Lack of effective CD4$^+$ T cell help early in infection, during the priming stage of the CD8$^+$ T cell response, may also contribute to the ultimate failure of the CTL response to clear infection (6, 20). Thus, accurate measurement of CD4$^+$ T cell responses early during infection is critical to determining how these responses promote development of effective CD8$^+$ T cell responses and more in general to understand how chronic viral infections are established.

To address the role of CD4$^+$ T cells in viral infection, we used the LCMV model (21–23). The LCMV genome consists of two ssRNA segments, the 3.4-kb small (S) and 7.2-kb large (L) segment. The L segment encodes the viral polymerase (L) and zinc-binding protein (Z), while the S segment encodes the nucleoprotein (NP) and glycoprotein precursor (GP), which is posttranslationally cleaved to yield a signal peptide (SP) and the two mature envelope glycoproteins, GP1 and GP2 (24, 25).

In adult immunocompetent mice, by selection of the appropriate LCMV strain, either an acute, self-limited infection (e.g., LCMV Armstrong) or a persistent infection (e.g., LCMV clone 13) is obtained (26). LCMV clone 13 was derived from the spleen of a mouse persistently infected from birth with LCMV Armstrong (27), and these isolates differ at only two amino acid positions, GP 260 in the virus glycoprotein GP1 and amino acid L 1079 (28–31). Sequence analysis has shown that both of these changes are associated with the persistent infection phenotype (28, 29, 32, 33). Preferential infection of particular cell subsets early in the course

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Received for publication January 16, 2007. Accepted for publication May 7, 2007.

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[1] This work was supported by 5S06GM059833-07 (to B.R.M.), HHSN266200400023C (to A.S. and M.J.B.), AI50840 (to M.J.B.), and National Institutes of Health Grant AI-065359 "Pacific Southwest Center For Biodefense and Emerging Infectious Diseases" (to J.B. and M.J.B.) and LLS 3248-05 (to E.J.). S.C. is a Cancer Research Institute Investigator.

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; PD, programmed death; S, small; L, large; Z, zinc-binding protein; NP, nucleoprotein; GP, glycoprotein precursor; α-DG, α-dystroglycan; ICCS, intracellular cytokine staining; WT, wild type; SFC, spot-forming cell.

of infection therefore defines the outcome of infection. In particular, viral strains expressing the F for L mutation in position 260 of GP1, such as clone 13, bind the cellular receptor α-dystroglycan (α-DG) with high affinity and result in chronic infection (34–36). $CD11^+$ dendritic cells are preferentially infected by high-affinity α-DG binders, thereby hindering the proper development of an adaptive immune response against the virus (34). By contrast, viral strains that bind α-DG with low affinity are associated with acute self-resolving infection (34–36).

It is well established that $CD8^+$ T cell responses play a primary role in successfully clearing acute phase LCMV infection (37), but these responses are altered and/or undetectable in the setting of persistent infection (38, 39). $CD8^+$ T cell responses to LCMV have been characterized primarily by measuring responses directed against numerous well-established MHC class I-restricted CTL epitopes in ELISPOT, CD8 tetramer, and intracellular cytokine staining (ICCS) assays (20, 40). In contrast, $CD4^+$ T cell responses against LCMV are not as well characterized. To date, only two LCMV-specific I-A^b-restricted $CD4^+$ T cell epitopes, GP_{61-80} and $NP_{309-328}$, have been described (41). Indeed the transgenic Smarta mouse, solely expressing T cells specific for LCMV GP_{61-80}, has aided in understanding the role of the $CD4^+$ T cell response in LCMV infection (39, 42, 43). During acute infection with LCMV Armstrong, potent $CD4^+$ and $CD8^+$ T cell responses develop that are associated with viral clearance (6, 8, 17, 44). By contrast, it has recently been shown that $CD4^+$ T cell responses are dysfunctional (based on their cytokine profile) during the early days following persistent infection, and this dysfunction may contribute to the ineffective CTL responses and persistent viral replication observed (17, 20, 39).

Molecular studies to evaluate the contribution of $CD4^+$ T cell responses to the overall immune response against LCMV are hampered by the paucity of defined epitopes. Although two I-A^b-restricted $CD4^+$ T cell epitopes have been identified, it is possible that other $CD4^+$ T cell responses against LCMV exist in the H-2^b haplotype which may play a role in controlling acute LCMV infection, but these epitopes have yet to be defined (45). Furthermore, epitopes have not been defined for other haplotypes. A more thorough understanding of the complexity of the $CD4^+$ T cell responses is important to study viral clearance and/or chronicity and may provide insights into how to develop interventions to prevent or resolve persistent infections.

In this study, we have identified a broad repertoire of nine new $CD4^+$ T cell epitopes in the response of BALB/c (H-2^d) mice against the Armstrong strain of LCMV infection. T cells specific for these nine epitopes were absent following LCMV clone 13 infection. Finally, experiments that involved peptide immunization or transfer of previously activated transgenic T cells followed by LCMV clone 13 infection showed that infection either rapidly eliminated $CD4^+$ T cell responses or impaired their ability to produce cytokines.

Materials and Methods

Peptide synthesis

Fifteen-mer peptides overlapping by 10 aa for the entire proteome of LCMV Armstrong strain (GP, National Center for Biotechnology Information (NCBI) accession number AAX49341; NP, NCBI accession number AAX49342; L, NCBI accession number AAX49344; Z: NCBI accession number AAX49343), GP_{61-80} and the $OVA_{323-336}$ peptide (46) were synthesized (Pepscan). The resultant 664 LCMV peptides for the entire proteome were divided into 83 pools, 8 peptides/pool for initial screening purposes. Specific peptides were subsequently tested individually at a concentration of 10 μg/ml.

Virus and mice

Plaque-purified LCMV clone 13 ($LCMV_{cl13}$) clones were propagated in BHK-21 cells and tested for biological activity in vitro and in vivo (47). A second passage stock of subclone SC9 ($LCMV_{cl13\text{-}sc9}$) was used for all $LCMV_{cl13}$ experiments shown. $LCMV_{cl13\text{-}sc9}$ was fully sequenced and mutations GP^{F260L} and L^{K1079Q} were confirmed. Additional changes in $LCMV_{cl13\text{-}sc9}$ were L^{S108T}, L^{V177I}, L^{T1513K}, L^{H1665N}, GP^{R177A}, and GP^{A313E} (S. Crotty, unpublished data).

BALB/c (H-2^d), C57BL/6 (H-2^b), B6.SJL.P*tpcr*^{a} (B6/CD45.1), and B6.PL.*Thy1*^{a}/CyJ (B6/CD90.1) mice were purchased from The Jackson Laboratory. OT-II mice crossed to a CD90.1 background were provided by Dr. K. Sugie (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and Smarta-B6/CD45.1 mice were bred in-house. All studies were conducted at the California State University-San Marcos or La Jolla Institute for Allergy and Immunology, in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care and according to Institutional Animal Care and Use Committee-approved animal protocols.

Cell transfers and infections

BALB/c (H-2^d) mice were infected either with 2×10^5 PFU of LCMV Armstrong i.p. or $2–4 \times 10^6$ PFU of LCMV clone 13 i.v. To establish chronic infections, adult mice received $2–4 \times 10^6$ PFU of $LCMV_{cl13}$ by i.v. inoculation via retro-orbital injection (0.2 ml). Spleens were harvested between 4 and 12 after infection.

For the transfer model, OT-II $CD90.1^+$ and Smarta $CD45.1^+CD4^+$ T cells were cultured with 10 μg/ml cognate peptide in the presence of irradiated splenocytes. After 3 days, activated $CD4^+$ T cells were collected, washed, and rested for 1 wk in the presence of 20 ng/ml rIL-2. Rested cells were CFSE labeled and 2×10^6 CFSE-labeled OT-II $CD90.1^+$ T cells were transferred into B6/SJL (CD45.1/90.2) hosts along with 10^6 CFSE-labeled nontransgenic CD4 B6 wild-type (WT; CD45.2/90.2) T cells that served as an internal control. Two $\times 10^6$ CFSE-labeled Smarta $CD45.1^+$ T cells were transferred into B6 WT mice along with 10^6 CFSE-labeled CD4 $CD90.1^+$ control T cells. One day later, mice were infected i.v. with $2–4 \times 10^6$ PFU of LCMV clone 13 or left untreated. Relative frequencies of transgenic $CD4^+$ T cells in relation to the CFSE-labeled control population were determined in spleens by flow cytometric analysis between 4 and 12 days after infection using Abs to CD4, CD45.1, CD45.2, CD90.1, and CD90.2 (eBioscience).

ELISPOT assay

The IFN-γ ELISPOT assays were performed as previously described (48). Briefly, mouse $CD4^+$ T cells were isolated from the spleens of LCMV-infected mice with anti-$CD4^+$ magnetic beads (Miltenyi Biotec). One to 2×10^5 purified $CD4^+$ T cells were cultured with $1–2 \times 10^5$ syngeneic splenocytes from uninfected mice and peptides (either peptide pools or individual peptides, tested at 10 μg/ml) in flat-bottom 96-well nitrocellulose plates (Immobilon-P membrane; Millipore), which had been precoated with 2 μg/ml anti-mouse IFN-γ mAb (Mabtech). After 20 h, plates were washed with PBS/0.5% Tween 20 and then incubated with 1 μg/ml biotinylated anti-mouse IFN-γ mAb (Mabtech) for 3 h at 37°C. After additional washes with PBS/0.5% Tween, spots were developed by incubation with Vectastain ABC peroxidase (Vector Laboratories), then 3-amino-9-ethylcarbazole solution (Sigma-Aldrich), and counted by computer-assisted image analysis (Zeiss KS ELISPOT Reader).

Experimental values were expressed as the mean net spots per million $CD4^+$ cells ± SD for each peptide pool or individual peptide. For the initial screening of the 83 pools, responses against each pool were considered positive if 1) the number of spot-forming cells (SFCs) per 10^6 $CD4^+$ T cells exceeded the absolute value of the mean negative control wells (effectors plus APCs without peptide) plus 2 SDs; 2) the value exceeded 100 SFCs/10^6 $CD4^+$ cells; and 3) these conditions were met in at least two replicate independent experiments. Positive pools were deconvoluted into their eight individual components and tested again to determine which individual peptides were responsible for the pooled IFN-γ response. Responses against individual peptides were considered positive if they exceeded the threshold of the mean negative control wells (effectors plus APCs without peptide) by at least 2 SDs and exceeded a threshold of 200 SFCs/10^6 $CD4^+$ cells.

Intracellular cytokine assay for IFN-γ

Splenocytes from LCMV Armstrong- and clone 13-infected mice were cultured in 96-well U-bottom plates (2×10^6 cells/well) in complete RPMI (RPMI 1640 with 5% FBS, 20 mM HEPES, 2 mM L-glutamine, 1 U/ml penicillin G, and 100 μg/ml streptomycin) at 37°C in 5% CO_2 for 6 h, in the presence of the indicated peptides (at final concentration of 10 μg/ml).

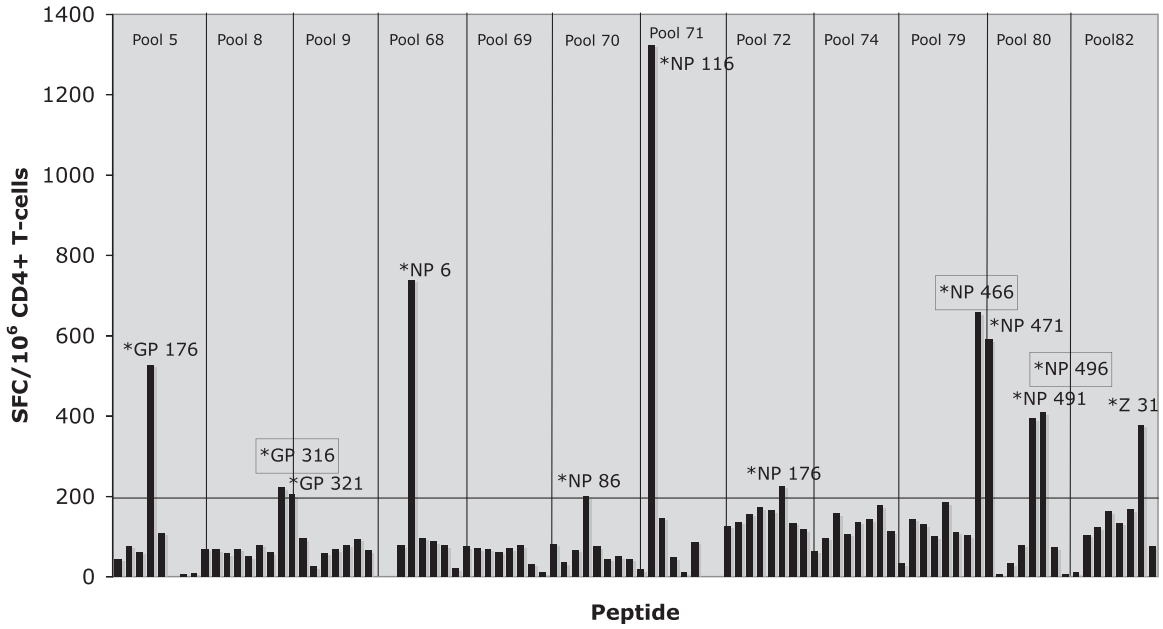

FIGURE 1. Identification of LCMV-derived CD4$^+$ epitopes by ELISPOT. BALB/c (H-2^d) mice were infected i.p. with 2×10^5 PFU of LCMV Armstrong. Eight days postinfection, CD4$^+$ T cells were purified and tested against each of the eight individual peptides contained in each positive pool at a concentration of 10 μg/ml per peptide. Responses against individual peptides were considered positive if they exceeded the threshold of the mean negative control wells (effectors plus APCs without peptide) plus 2 SDs and exceeded a threshold of 200 SFCs/10^6 CD4$^+$ cells. Each positive peptide is labeled by its proteomic location and an asterisk.

Brefeldin A (5 μg/ml; Sigma-Aldrich) was present throughout the incubation. Cells were harvested, washed with PBS containing 2% FBS, and stored on ice. Cells were stained with Abs to CD4 (BALB/c model) or a combination of CD4 with CD45.1or CD90.1 (transfer model), washed, and fixed in PBS containing 2% formaldehyde. Following fixation, the cells were permeabilized and stained for cytokines according to the manufacturer's directions (BD Pharmingen). Samples were resuspended in PBS containing 2% formaldehyde and data were acquired on a FACScan flow cytometer (BD Biosciences; 10^5–10^6 events acquired per sample). Data were analyzed with CellQuest software (BD Biosciences). For each T cell population, the peptide-specific responses to peptide stimulation were calculated by subtracting the percentage of cells that scored positive for IFN-γ production in the absence of peptide.

I-A^d and I-E^d peptide-binding assays

H-2^d class II MHC was purified and peptide- binding assays were performed essentially as previously described (49). Briefly, the mouse B cell lymphomas LB27.4 and A20 were used as sources of murine I-A^d and I-E^d molecules. MHC molecules were purified by affinity chromatography using the anti-I-A^d mAb MKD6 and the anti-I-E^d mAb 14.4.4. Quantitative peptide-binding assays were based on the inhibition of binding of radiolabeled probe peptide ROIV (artificial ligand; sequence YAHAAHAAHAAHAA HAA) to purified I-A^d molecules, or peptide 1398.01 (HIV vpu 30; sequence YRKILRQRKIDRLID) to purified I-E^d molecules. Assays were performed at pH 7.0 (I-A^d) or pH 4.5 (I-E^d) in PBS containing 0.15% pluronic F-68 and in the presence of a mixture of protease inhibitors (49). MHC binding of the radiolabeled peptide was determined by capturing MHC-peptide complexes on the respective Ab-coated Lumitrac 600 plates (Greiner Bio-one) and measuring bound cpm using the TopCount (Packard Instrument) microscintillation counter. The average IC_{50} nM ROIV and 1398.01 for the corresponding class II molecule was 7.5 and 120, respectively.

MHC restriction assays

Splenocytes derived from LCMV Armstrong-infected mice (see above) were stimulated for 6 days in vitro with each of the peptides. Specifically, 30×10^6 splenocytes were incubated with 10 μg/ml peptide in 10 ml of RPMI 1640 with 10% FBS. After 6 days, 1×10^5 splenocytes/well were used as effector cells and incubated with either RT2.10 C3 (I-E^d) or RT2.3 B2 (I-A^d) mouse fibroblast cell lines (provided by Andrea J. D'Sant, University of Rochester, Rochester, NY) at 10^5 cells/well and each specific peptide at a concentration of 10 μg/ml in an ELISPOT assay. A peptide was considered to be presented by either I-E^d or I-A^d if it elicited a positive ELISPOT response in at least two experiments and exceeded a threshold 10-fold magnitude compared with responses generated by the other MHC-restricted target cell line.

Immunizations

Five peptides were used for immunization studies: NP_{6-20}, $GP_{176-180}$, Z_{31-45}, and $OVA_{323-336}$. Groups of four BALB/c mice at 6 wk of age (see above) were immunized s.c. with 70 μg of each peptide in PBS/10% DMSO emulsified in CFA as previously described (50). Two weeks following immunization, a set of mice was sacrificed (day 0) or infected with 2×10^6 PFU of LCMV clone 13 i.v., or 2×10^5 PFU of LCMV Armstrong i.p., and then sacrificed either 4, 8, or 12 days after infection. Mice were immunized but not infected and sacrificed at the same time points. Cells were harvested, purified, and then tested in ELISPOT assays as described above.

Results

Identification of LCMV-derived CD4$^+$ T cell epitopes

A set of 664 overlapping peptides spanning the entire LCMV Armstrong proteome was subdivided into 83 pools of 8 peptides each, grouped in sequential order. To identify CD4$^+$ T cell epitopes, BALB/c mice were infected with 2×10^5 PFU of LCMV Armstrong via i.p. inoculation, and 8 days later splenic CD4$^+$ T cells were isolated and stimulated in vitro with each peptide pool. CD4$^+$ T cell reactivity against each pool was determined in standard IFN-γ ELISPOT assays. A total of 12 positive pools derived from NP, GP, or Z proteins were identified (data not shown).

To define specific epitopes, positive pools were decoded, and each of the eight individual peptides contained within a positive pool was individually tested. Fig. 1 shows the responses observed against individual peptides from each of the 12 positive pools. Twelve antigenic peptides were identified (each of the peptides identified as antigenic is marked by an asterisk). The peptides from two of the pools did not induce IFN-γ spot formation that exceeded the threshold for positivity (responses greater than the mean negative control wells, plus 2 SDs, and >200 SFCs/10^6 CD4$^+$ cells) and three positive peptides were contiguous with an

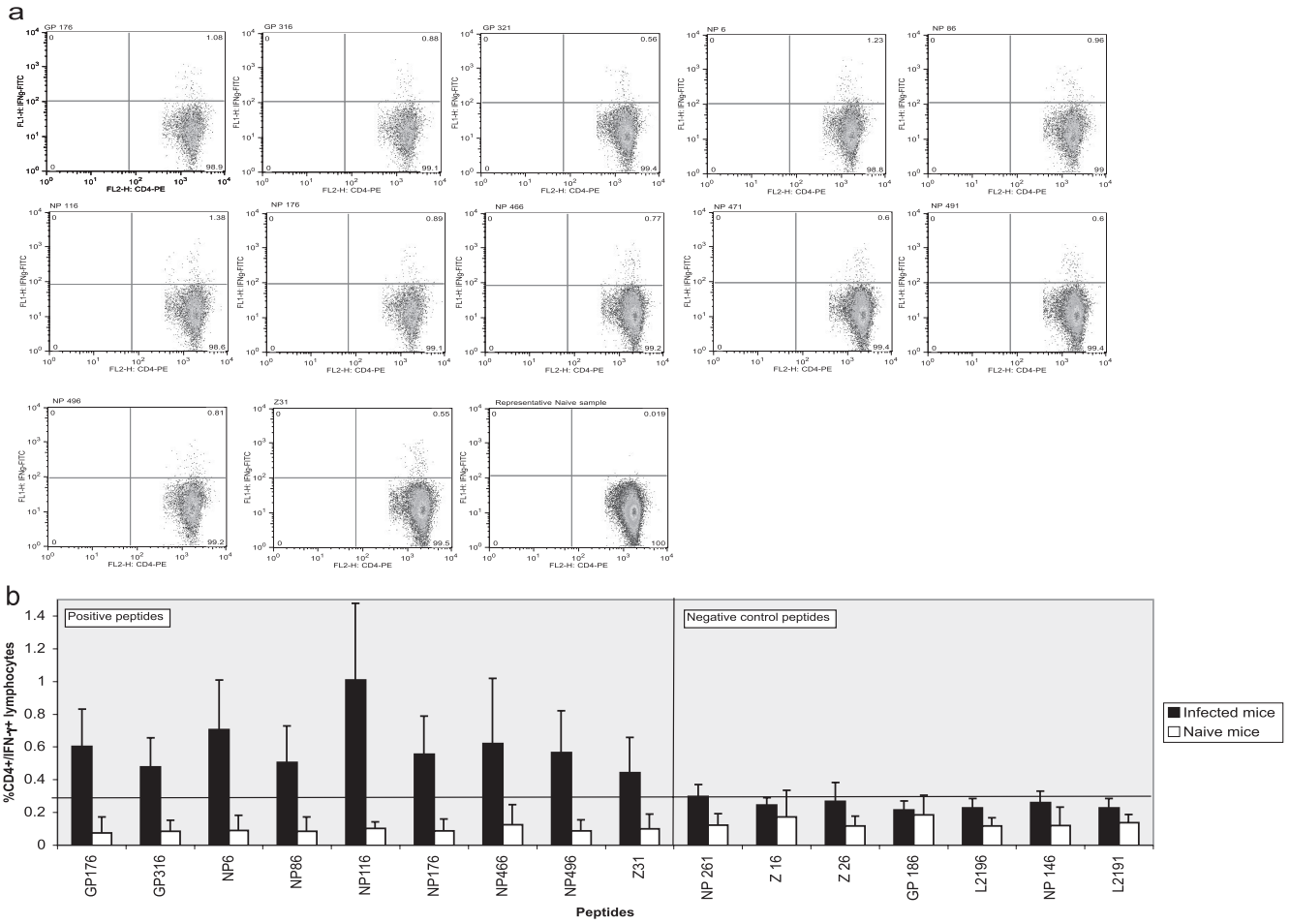

FIGURE 2. $CD4^+$ IFN-γ production as measured by ICCS. BALB/c ($H\text{-}2^d$) mice were infected i.p. with 2×10^5 PFU of LCMV Armstrong. Eight days after infection, splenocytes were purified and tested in ICCS assays measuring IFN-γ production against nine peptides identified as positive in the ELISPOT assays ($GP_{176-190}$, $GP_{316-330}$, NP_{6-20}, NP_{86-100}, $NP_{116-130}$, $NP_{176-190}$, $NP_{466-480}$, $NP_{496-510}$, and Z_{31-45}) as well as eight control peptides that were scored as negative in the ELISPOT assays ($NP_{261-275}$, Z_{16-30}, Z_{26-40}, $GP_{186-200}$, $L_{2196-2210}$, $NP_{146-160}$, and $L_{2191-2205}$. *a*, Representative responses against each of the following peptides: $GP_{176-190}$, $GP_{316-330}$, NP_{6-20}, NP_{86-100}, $NP_{116-130}$, $NP_{176-190}$, $NP_{466-480}$, $NP_{496-510}$, and Z_{31-45} and a peptide tested with a naive (uninfected) is shown. *b*, The mean responses against each peptide is shown. The threshold value to consider an IFN-γ response against a given peptide as positive was 0.3%.

overlapping, more strongly reactive peptide. Thus, nine unique, previously unidentified epitopes were discovered. The most vigorous response was observed with peptide $NP_{116-130}$ from pool 71, followed by NP_{6-120} from pool 68, and by an epitopic region encompassing two overlapping, consecutive peptides ($NP_{466-480}$ and $NP_{471-485}$). Additional responses were detected against $GP_{176-190}$, $GP_{316-330/321-335}$, NP_{86-100}, $NP_{176-190}$, $NP_{491-505/496-510}$, and Z_{31-45} peptides.

To verify that the IFN-γ responses observed in the ELISPOT assays were not due to contaminating $CD8^+$ T cells, we performed ICCS experiments. Mice were infected with LCMV and 8 days later splenocytes were isolated and incubated with each of the peptides and then stained for intracellular IFN-γ and $CD4^+$ and $CD8^+$ surface staining. Nine peptides identified as positive in the ELISPOT assays, as described above ($GP_{176-190}$, $GP_{316-330}$, NP_{6-20}, NP_{86-100}, $NP_{116-130}$, $NP_{176-190}$, $NP_{466-480}$, $NP_{496-510}$, and Z_{31-45}), as well as eight control peptides ($NP_{261-275}$, Z_{16-30}, Z_{26-40}, $GP_{186-200}$, $L_{2196-2210}$, $NP_{146-160}$, and $L_{2191-2205}$) which scored as negative in the ELISPOT assays were tested. In Fig. 2*a*, representative responses against each of the nine positive peptides and a naive control are depicted. In Fig. 2*b*, the mean responses against each of the peptides are shown.

The percentage of $CD4^+$ cells producing IFN-γ in response to each individual peptide was measured from LCMV Armstrong-infected and uninfected control mice. The percentage of $CD4^+IFN\text{-}\gamma^+$ releasing cells from naive mice was $0.12 \pm 0.06\%$ and, accordingly, we defined a value of 0.3% (mean $\pm$ 3 SD) as a threshold value to consider IFN-γ responses against a given peptide as positive.

As expected, the eight control peptides were negative according to these criteria. In contrast, nine of nine of the epitopes identified in the ELISPOT assay scored positive for IFN-γ production by gated $CD4^+$ cells in the ICCS assay. The percentage of IFN-γ^+CD4^+ cells ranged from 0.45–1% against the peptide panel. The strongest response observed was against $NP_{116-130}$, followed by NP_{6-20}, $NP_{466-480}$, $GP_{176-190}$, $NP_{496-510}$, $NP_{176-190}$, NP_{86-100}, $GP_{316-330}$, and Z_{31-45}. With the exception of Z_{31-45}, the hierarchy of the magnitude of responses corresponded to those observed in the ELISPOT assay (Fig. 1). In eight of the nine cases, the newly identified epitopes were not associated with IFN-γ staining from $CD8^+$ T cells tested in parallel (data not shown). The exception was represented by $NP_{116-130}$, which contains a previously identified MHC class I-restricted epitope, $NP_{118-126}$ (41). Indeed, the $NP_{116-130}$ peptide elicited a $CD8^+$ T cell response with 22.8% of the $CD8^+$ cells

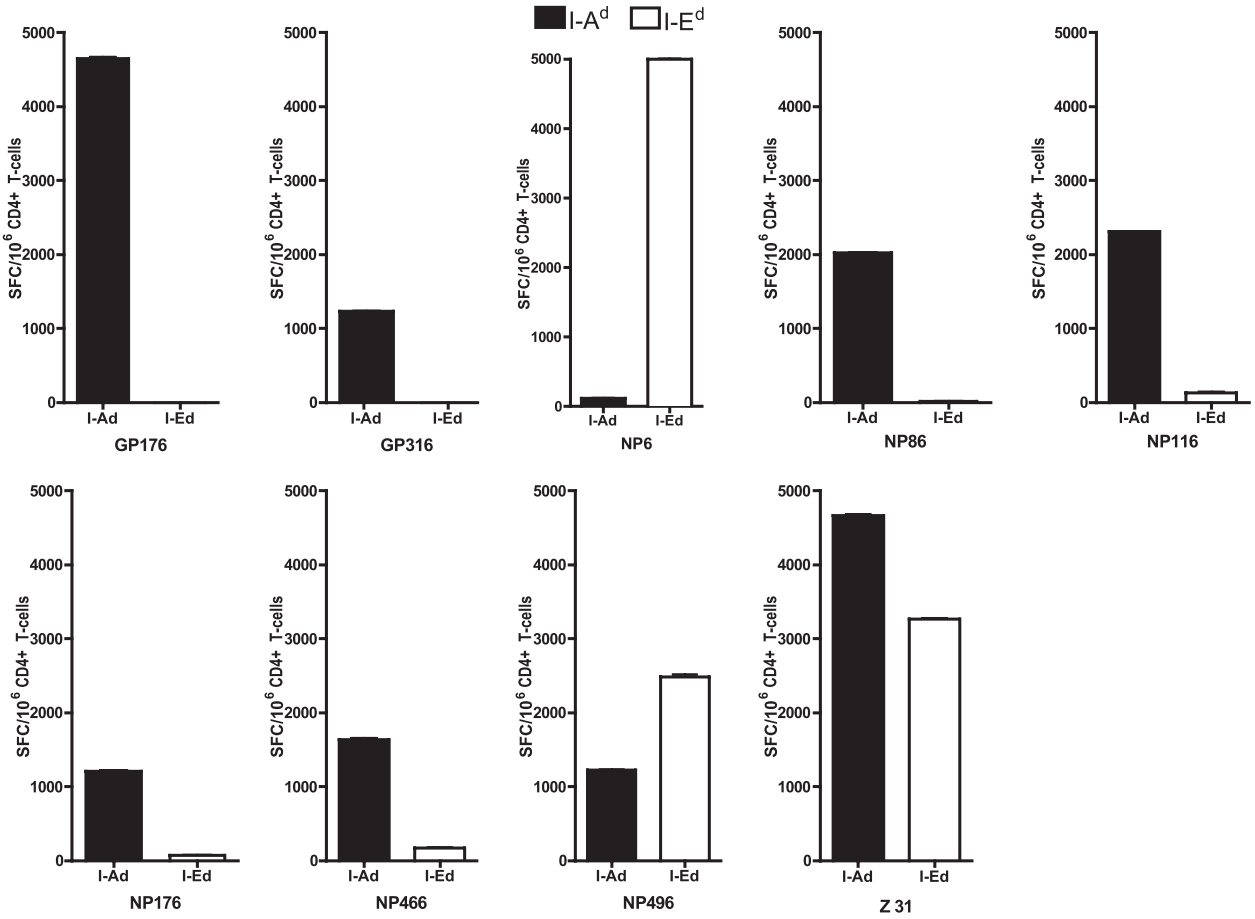

FIGURE 3. Identification of MHC molecule(s) presenting each new peptide. BALB/c (H-2^d) mice were infected i.p. with 2×10^5 PFU of LCMV Armstrong. Eight days after infection, splenocytes were purified and stimulated for 6 days in vitro with each of the peptides. After stimulation, 1×10^5 splenocytes/well were used as effector cells and incubated with either RT2.10 C3 (I-E^d) or RT2.8 B2 (I-A^d) cell lines at 1×10^5 cells/well and each specific peptide at a concentration of 10 μg/ml in an ELISPOT assay. A peptide was considered to be presented by either I-E^d or I-A^d if it elicited a positive ELISPOT response in at least two experiments and exceeded a threshold of 10-fold magnitude compared with response generated by the other MHC-restricted target cell line. A representative response against each peptide is shown here.

producing IFN-γ (data not shown), thus demonstrating that this region contains overlapping $CD4^+$ and $CD8^+$ T cell epitopes.

MHC restriction of the identified epitopes

To determine the MHC class II restriction for each of the identified epitopes, we conducted MHC:peptide-binding assays and Ag presentation experiments. For the Ag presentation experiments, we used the mouse fibroblasts RT2.3B2 and RT10.3CS which express either I-A^d or I-E^d, respectively. ELISPOT assays were performed using purified $CD4^+$ T cells from infected BALB/c mice and RT2.3B2 and RT10.3CS cell lines as APCs. Representative results from one of multiple replicate experiments are shown in Fig. 3. For seven of the nine epitopes, peptide presentation was restricted to a single MHC molecule. The majority of the epitopes ($GP_{176-190}$, $GP_{316-330}$, $NP_{116-130}$, NP_{86-100}, $NP_{176-190}$, and $NP_{466-480}$) were restricted by I-A^d. NP_{6-20} was restricted by the I-E^d molecule.

Table I. *$CD4^+$ T cell responses detected against LCMV-derived epitopes in LCMV Armstrong infection*

Protein/Position	Sequence	ELISPOT[a]	ICCS[b]	I-E^d Binding[c] (nM)	I-A^d Binding[c] (nM)	Restricting MHC Molecule[d]
$GP_{176-190}$	DAQSAQSQCRTFRGR	528	0.60	1666	4634	I-A^d
$GP_{316-330}$	CDMLRLIDYNKAALS	223	0.48	4,904	**27**	I-A^d
NP_{6-20}	EVKSFQWTQALRREL	736	0.71	1,506	2144	I-E^d
NP_{86-100}	KNVLKVGRLSAEELM	201	0.51	4,596	**19**	I-A^d
$NP_{116-130}$	SERPQASGVYMGNLT	1325	1.00	23,272	**20**	I-A^d
$NP_{176-190}$	PSLTMACMAKQSQTP	226	0.56	26,180	**41**	I-A^d
NP_{466-80}	SQNRKDIKLIDVEMT	658	0.62	36,081	**87**	I-A^d
$NP_{496-510}$	GWLCKMHTGIVRDKK	409	0.57	**95**	**974**	I-A^d and I-E^d
Z_{31-45}	SCKSCWQKFDSLVRC	378	0.44	**517**	**437**	I-A^d and I-E^d

[a] IFN-γ^+ SFC/10^6 $CD4^+$ T cells.
[b] Percent $CD4^+$IFN-γ^+ lymphocytes.
[c] Bold numbers are values <1,000 nM, the threshold of binding.
[d] As measured in APC assays.

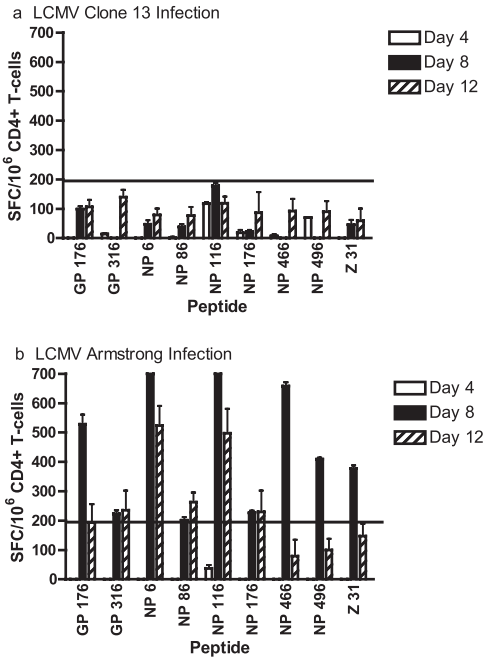

FIGURE 4. Profile of $CD4^+$ T cell responses against clone 13 infection. BALB/c ($H\text{-}2^d$) mice were infected with LCMV clone 13 (*a*) or LCMV Armstrong (*b*). Four, 8, and 12 days postinfection, splenocytes were purified and tested with peptides at a concentration of 10 μg/ml in ELISPOT assays. Responses were considered positive if they exceeded the threshold of the mean negative control wells (effectors plus APCs without peptide) plus 2 SDs and exceeded a threshold of 200 SFCs/10^6 $CD4^+$ cells.

$NP_{496-510}$ and Z_{31-45} were effectively presented by both cell lines, demonstrating that these peptides contained two overlapping epitopes restricted by $I\text{-}E^d$ and $I\text{-}A^d$.

MHC:peptide-binding assays performed on our panel of peptides confirmed the restriction results obtained above in seven of the nine cases. $H\text{-}2^d$ class II MHC was purified, and peptide-binding assays were performed for each peptide using both $I\text{-}E^d$ and $I\text{-}A^d$, essentially as previously described (49). The data are presented in Table I. $I\text{-}A^d$ bound $GP_{316-330}$, NP_{86-100}, $NP_{116-130}$, $NP_{176-190}$, and $NP_{466-480}$. Both molecules bound $NP_{496-510}$ and Z_{31-45}. Neither molecule bound $GP_{176-190}$ and NP_{6-20} below the 1000 nM threshold level. Table I summarizes the restriction specificities as well as the IFN-γ levels detected both by ELISPOT and ICCS.

Characterization of $CD4^+$ T cell responses after clone 13 infection

To determine whether responses directed against these $CD4^+$ T cell epitopes could be detected in a persistent viral infection setting, we infected BALB/c mice with LCMV clone 13. Groups of four mice each were infected with either 4×10^6 PFU of LCMV clone 13 i.v. or 4×10^6 PFU of LCMV Armstrong i.v. Splenic $CD4^+$ T cells were purified 12 days postinfection, and IFN-γ production to each of the epitopes was measured in ELISPOT assays.

$CD4^+$ T cells responses against all nine epitopes were largely absent in the setting of early clone 13 infection (Fig. 4*a*). A weak activity against $NP_{116-130}$ was noted 8 days postinfection, with a magnitude of 178 SFC/10^6 $CD4^+$ T cells. As a control, responses to the epitopes were determined in splenocytes from mice infected with LCMV Armstrong (Fig. 4*b*). The response against all epitopes peaked 8 days postinfection in this setting and began to wane by 12 days postinfection. This is in contrast with the lack of a measurable response against clone 13, suggesting that clone 13 infection is associated with defective $CD4^+$ T cell activation against the LCMV Armstrong epitopes.

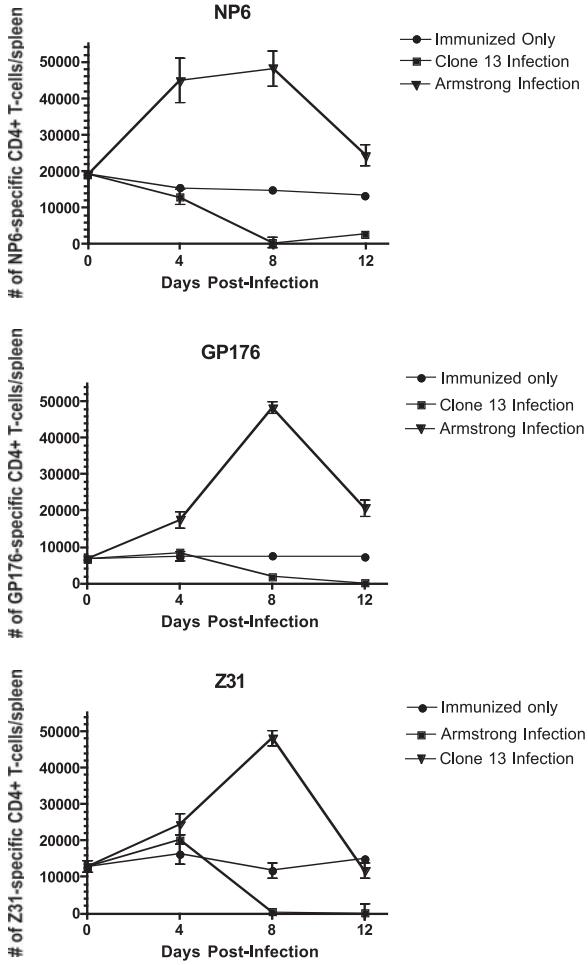

FIGURE 5. $CD4^+$ T cell responses generated by peptide immunization followed by LCMV clone 13 or Armstrong infection. Groups of four BALB/c mice were immunized s.c. with each peptide individually, NP_{6-20}, $GP_{176-180}$, and Z_{31-45}. Fourteen days following immunization (day 0), subsets of mice were infected with LCMV clone 13, LCMV Armstrong, or remained uninfected and then were sacrificed either 4, 8, or 12 days after the infection time point. Cells were harvested, purified, and then tested in ELISPOT assays against each individual epitope. Responses were considered positive if they exceeded the threshold of the mean negative control wells (effectors plus APCs without peptide) plus 2 SDs and exceeded a threshold of 200 SFCs/10^6 $CD4^+$ cells. Absolute numbers of Ag-specific $CD4^+$ T cells per spleen are shown.

Suppression of $CD4^+$ T cell responses to LCMV epitopes by clone 13 infection

The absence of detectable $CD4^+$ T cell responses after clone 13 infection led us to hypothesize that these responses may play a role in resolution of acute infection. To address this point, we performed immunizations with a subset of these epitopes, consisting of NP_{6-20}, $GP_{176-190}$, and Z_{31-45}. Following a previously described protocol (48), mice were immunized s.c. with each of the peptides. Two weeks after immunization (noted as day 0), mice either remained uninfected or were infected with 2×10^6 PFU of LCMV clone 13 i.v. or 2×10^5 PFU of LCMV Armstrong i.p. and then sacrificed either 4, 8, or 12 days after infection. At each time point, we assayed for reactivity against the immunizing peptide in the ELISPOT assay (Fig. 5).

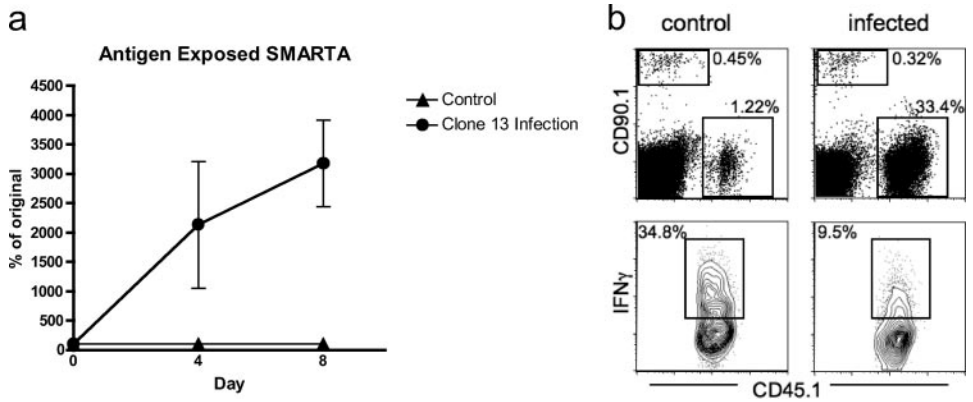

FIGURE 6. IFN-γ impairment of transferred Smarta-transgenic T cells upon LCMV clone 13 infection. *a*, Smarta CD45.1$^+$CD4$^+$ T cells were cultured with 10 μg/ml cognate peptide in the presence of irradiated splenocytes. After 3 days, activated CD4$^+$ T cells were collected, washed, and rested for one week in the presence of 20 ng/ml rIL-2. Rested cells were CFSE-labeled and 2×10^6 CFSE-labeled Smarta CD45.1$^+$ T cells were transferred into B6 WT mice along with 10^6 CFSE-labeled CD4 CD90.1$^+$ control T cells. One day later, mice were infected i.v. with 2×10^6 PFU of LCMV clone 13 or left untreated (control). Relative frequencies of transgenic CD4$^+$ T cells in relation to the CFSE-labeled control population were determined in spleens by flow cytometric analysis between 4 and 12 days after infection using Abs to CD4, CD45.1, CD45.2, CD90.1, and CD90.2. *b*, ICCS for IFN-γ in CD45.1$^+$ Smarta cells upon a brief stimulation with GP_{61-80} peptide in the presence of Brefeldin A. The FACS plots show representative data of one control and one infected animal ($n = 3$).

In all groups, CD4$^+$ T cell responses could be measured only against the immunizing peptide (not against any of the other epitopes; data not shown) 14 days after immunization (denoted as day 0). In the uninfected groups, a peptide-specific response was maintained throughout all the time points tested. After infection with LCMV Armstrong, the magnitude of the responses increased, peaking in each case at day 8 postinfection. By 12 days postinfection, the responses against the immunogen had subsided, but were still detectable.

After infection with LCMV clone 13, 4 days postinfection, T cell responses were observed against the immunizing peptide, with magnitudes between 8,334–20,000 Ag-specific CD4$^+$ T cells per spleen. These responses are similar in magnitude to those obtained in uninfected animals. The responses induced by peptide immunization were specific: no responses were observed against the other epitopes tested or in naive mice, nonpeptide-immunized mice (data not shown). These findings indicate that peptide immunization was effective in generating a CD4$^+$ epitope-specific T cell response.

By contrast, in the day 8 and 12 clone 13 postinfection time points, responses against the immunizing peptides were either dramatically reduced or undetectable. The failure to generate CD4$^+$ T cell responses in the setting of clone 13 infection is not due to defective APCs in the ELISPOT assay, because the infected CD4$^+$ T cells were purified and incubated with naive splenocytes serving as APCs. These results suggest that clone 13 infection actively down-regulates helper responses.

To address whether this dysfunction is caused by physical deletion of cytokine impairment, we performed transfer experiments using LCMV GP_{61-80}-specific Smarta- transgenic cells (39, 42, 43). Specifically, Smarta CD45.1$^+$CD4$^+$ T cells were cultured with 10 mg/ml cognate peptide in the presence of irradiated splenocytes. After 3 days, activated CD4 T cells were collected, washed, and rested for 1 wk in the presence of 20 ng/ml rIL-2. Rested cells were CFSE-labeled and 2×10^6 CFSE-labeled Smarta CD45.1$^+$ T cells were transferred into B6 WT mice along with 10^6 CFSE-labeled CD4 CD90.1$^+$ control T cells. One day later, mice were infected i.v. with 2×10^6 PFU of LCMV clone 13 or left untreated (control). Relative frequencies of transgenic CD4$^+$ T cells in relation to the CFSE-labeled control population were determined in spleens by flow cytometric analysis between 4 and 12 days after infection using Abs to CD4, CD45.1, CD45.2, CD90.1, and CD90.2. ICCS for IFN-γ was performed on infected and control samples after a brief stimulation with cognate peptide in the presence of Brefeldin A. In Fig. 6*a*, we illustrate that these transferred Smarta cells proliferated after clone 13 infection, reaching approximately a 30-fold expansion as compared with the levels of originally transferred cells. Control animals (uninfected) did not show any proliferation of the transferred cells. In Fig. 6*b*, intracellular cytokine profiles reveal that even though Smarta cells did not proliferate in the control animals, these transferred cells had the capacity to produce IFN-γ in sharp contrast to the profile of Smarta-transferred cells after clone 13 infection which had a reduced IFN-γ profile. These results indicate that if restimulated with cognate Ag, cells have the capacity to proliferate after clone 13 yet are impaired in their ability to produce IFN-γ.

Suppression of the CD4$^+$ T cell response to a non-LCMV epitope by clone 13 infection

To assess whether immunosuppression of CD4$^+$ T cell responses after clone 13 infection also affects epitopes not derived from LCMV, we performed an immunization experiment using a CD4 epitope from chicken OVA, followed by clone 13 infection. For this purpose, we used $OVA_{323-336}$, an I-A^b-restricted epitope which elicits a robust CD4$^+$ T cell response (46). Immune responses were assessed in either peptide-immunized animals tested either 4, 8, or 12 days after clone 13 infection (Fig. 7). Control animals that were not infected with LCMV clone 13 (peptide-immunized only) maintained an OVA-specific response throughout the different time points assessed, with the weakest response measured at 14,550 OVA-specific CD4$^+$ T cells per spleen 12 days after infection. However, the OVA-specific CD4$^+$ T cell responses rapidly declined in the LCMV clone 13-infected animals. Four days postinfection, the response was assessed at 4,720 OVA-specific CD4$^+$ T cells/spleen CD4$^+$ T cells. This response continued to decline, reaching 1,300 OVA-specific CD4$^+$ T cells per spleen when measured at 8 days postinfection and remained low at 12 days postinfection. This experiment highlights the conclusion that the immunosuppression caused by LCMV clone 13 infection is not specific to LCMV-derived epitopes but affects other established, non-LCMV-specific CD4$^+$ T cell responses.

We also performed transfer experiments using the $OVA_{323-339}$-specific OT-II-transgenic T cells to address whether nonspecific

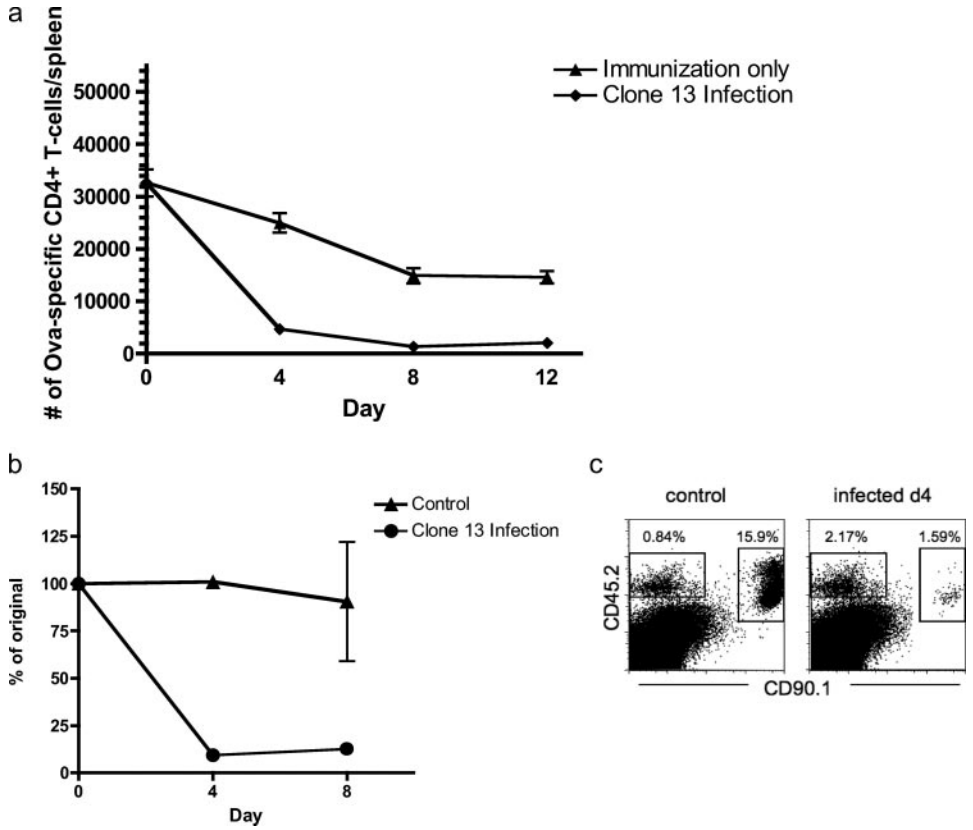

FIGURE 7. Deletion of OVA-specific $CD4^+$ T cell responses upon LCMV clone 13 infection. *a*, BALB/c mice were immunized s.c. with the $OVA_{323–336}$ peptide. Fourteen days after immunization (day 0), subsets of mice were infected with LCMV clone 13 or remained uninfected and then were sacrificed either 4, 8, or 12 days after the infection time point. Cells were harvested, purified, and then tested in ELISPOT assays against the $OVA_{323–336}$ peptide. Responses were considered positive if they exceeded the threshold of the mean negative control wells (effectors plus APCs without peptide) plus 2 SDs and exceeded a threshold of 200 SFCs/10^6 $CD4^+$ cells. Absolute numbers of Ag-specific $CD4^+$ T cells per spleen are shown. *b*, OT-II $CD90.1^+CD4^+$ T cells were cultured with 10 μg/ml cognate peptide in the presence of irradiated splenocytes. After 3 days, activated $CD4^+$ T cells were collected, washed, and rested for 1 wk in the presence of 20 ng/ml rIL-2. Rested cells were CFSE-labeled and 2×10^6 CFSE-labeled OT-II $CD90.1^+$ T cells were transferred into B6/SJL (CD45.1/90.2) hosts along with 10^6 CFSE-labeled nontransgenic CD4 B6 WT (CD45.2/90.2) T cells that served as an internal control. One day later, mice were infected i.v. with $2–4 \times 10^6$ PFU of LCMV clone 13 or left untreated. Relative frequencies of transgenic $CD4^+$ T cells in relation to the CFSE-labeled control population were determined in spleens by flow cytometric analysis between 4 and 12 days after infection using Abs to CD4, CD45.1, CD45.2, CD90.1, and CD90.2. *c*, Representative FACS plot of transferred OT-II and control cells in control and infected mice 4 days after infection with LCMV clone 13.

helper responses are impaired and/or deleted after clone 13 infection. Comparable to the Smarta cells, OT-II $CD90.1^+CD4^+$ T cells were stimulated with cognate peptide, expanded, and rested in the presence of 20 ng/ml rIL-2. Rested cells were CFSE-labeled and 2×10^6 CFSE-labeled OT-II $CD90.1^+$ T cells were transferred into B6/SJL (CD45.1/90.2) hosts along with 10^6 CFSE-labeled nontransgenic CD4 B6 WT (CD45.2/90.2) T cells that served as an internal control. One day later, mice were infected i.v. with 2×10^6 PFU of LCMV clone 13 or left untreated. Relative frequencies of transgenic $CD4^+$ T cells in relation to the CFSE-labeled control population were determined in spleens by flow cytometric analysis between 4 and 12 days after infection using Abs to CD4, CD45.1, CD45.2, CD90.1, and CD90.2. In contrast to the Smarta transfer experiments, the frequency of transferred OT-II T cells was greatly reduced by day 8 postinfection as compared with control animals, indicating physical deletion of the OT-II T cells (Fig. 7*b*).

Discussion

We have identified a set of nine LCMV-specific $CD4^+$ T cell epitopes recognized following acute infection with LCMV Armstrong of BALB/c mice. The nine $CD4^+$ T cells epitopes are derived from the NP, GP, and Z proteins. Eight of them are derived from the NP and GP proteins which are expressed early and abundantly in infected cells, providing the major components of LCMV virions. Consistent with their abundance, these proteins play a significant role in the immunodominance of cellular immune response against the virus (51). Our data provide the first evidence of $CD4^+$ T cell responses against the Z protein for LCMV or any other Arenavirus. This protein possesses a metal-binding RING finger domain and regulates viral transcription and translation (52, 53). Our data suggest that responses directed against this protein may play a role in cellular immunity during acute infection. By contrast, despite the large size of the L protein (2210 residues) (39, 54), we did not identify any $CD4^+$ T cell epitopes derived from this protein.

The strongest $CD4^+$ T cell response identified during acute infection was directed against a region that contains a nested $CD8^+$ T cell epitope. That the $NP_{116–130}$ peptide contains not only a $CD8^+$ T cell epitope, but also a $CD4^+$ T cell epitope was demonstrated in ICCS assays and is also consistent with its binding to $I\text{-}A^d$ in vitro. Previous research has described the $NP_{118–126}$ MHC class I-restricted response as the immunodominant CTL response in BALB/c mice (55, 56). Our studies identify this region as containing overlapping $CD4^+$ and $CD8^+$ T cell epitopes. It is tempting to speculate that the overlap of the two epitope types might be related to the immunodominance of this viral region.

The use of various LCMV strains and varying routes of infection results in alternate outcomes of infection. For instance, infection of adult mice with LCMV Armstrong results in viral clearance while the use of an immunosuppressive viral strain (e.g., clone 13) results in persistent infection (26, 27, 57). We were interested in evaluating the breadth and magnitude of the $CD4^+$ T cell response to LCMV in acute and persistent infections. It has previously been shown that during acute LCMV infection, functional $CD4^+$ T cell responses led to effective CTL responses and viral clearance (8, 39, 58). Interestingly, in the case of the related Lassa fever virus, seropositive individuals from endemic areas have strong memory $CD4^+$ T cells responses against the virus (59, 60). In the case of HIV infection, strong $CD4^+$ T cell responses are detected in individuals who are treated with antivirals early in the course of infection, suggesting that $CD4^+$ T cell responses can be maintained if viral replication is contained (5, 61, 62). Understanding the role of $CD4^+$ T cell responses in acute infection may provide insights into correlates of successful immune responses in contrast to persistent infections where immune responses fail to control viremia.

In this study, we showed that $CD4^+$ T cell responses elicited by prior immunization with peptide epitopes were not sustained following a LCMV clone 13 challenge that results in chronic infection, although responses to these epitopes were readily demonstrated following LCMV Armstrong acute infection. Varga and Welsh (63) showed that LCMV-specific $CD4^+$ T cells were detectable after clone 13 infection in C57BL/6 mice ($H\text{-}2^b$), but never reached the same level measured after Armstrong infection. Other groups (39, 47, 64) have shown that $CD4^+$ T cell responses quickly become nonfunctional in the setting of persistent LCMV infection as measured by reduced cytokine production, specifically IL-2 and TNF-α.

Our studies significantly extend these previous findings by showing that even though $CD4^+$ T cell responses can be elicited by peptide immunization, these responses are dramatically impaired at least at the level of IFN-γ secretion by day 12 after clone 13 infection. In addition, not only were LCMV-specific $CD4^+$ T cell responses affected, but also an OVA-specific response was suppressed as compared with uninfected controls. The mechanism by which these cells are affected include physical deletion in the case of transferred OVA-specific cells and cytokine production dysfunction of the transferred Smarta cells, despite their rapid proliferation after clone 13 infection. As a caveat to these data, it is possible that the OVA-specific cells are circulating or present in another organ not detected by our assays. Nevertheless, these data suggest that different mechanisms might regulate survival and functional activity, at least judged by IFN-γ production, of the transferred cells. It is interesting to note that Oxenius et al. (38) showed that transferred Smarta cells could survive for up to 6 wk in the setting of LCMV-docile infection, another persistent LCMV strain, but these Ag-specific cells could not proliferate by day 50 after adoptive transfer.

The role of cytokines in chronic LCMV infection has been highlighted by studies showing that blocking IL-10 results in restored function and eliminates persistent infection (65, 66). LCMV-specific $CD4^+$ T cells and dendritic cells expressed greater levels of IL-10 following LCMV clone 13 infection as compared with LCMV Armstrong infection, and inhibiting IL-10 production resulted in viral clearance (66). It is possible to speculate that particular LCMV-specific $CD4^+$ epitopes may be associated with varying IL-10 production, in turn potentially leading to effective immunocytotherapy strategies.

It has recently been shown that Ag-specific $CD4^+$ T cells express high levels of PD-1 and therefore undergo apoptosis in the setting of HIV and LCMV infections (13, 66). In HIV infection, these cells can be rescued with anti-PD-1 Abs resulting in restored function (13). A possible strategy to rescue $CD4^+$ T cells from apoptosis may involve the use of blocking PD-1 function concomitant with active immunization to engender strong Ag-specific $CD4^+$ T cells. This hypothesis may be tested using the LCMV model of chronic infection with the epitopes we have defined.

$CD4^+$ T cell responses have been shown to be instrumental in establishing and sustaining effective $CD8^+$ T cell responses in the setting of viral and bacterial infection (67–69). Our studies illustrate the complex nature of the $CD4^+$ T cell response against LCMV and suggest that chronic infection is linked to active downregulation of $CD4^+$ T cell responses. Developing feasible strategies to maintain effective $CD4^+$ T cell responses in the setting of persistent infection may prevent the establishment of chronic LCMV infection and may also be applicable to chronic infections caused by other viruses, such as hepatitis C virus and HIV, where $CD4^+$ T cell responses are also impaired.

Acknowledgments

We thank Andrea J. D'Sant for providing us mouse fibroblast cell lines to perform the restriction experiments. We also thank Howard Grey for critical review of this manuscript.

Disclosures

The authors have no financial conflict of interest.

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