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Human CD8$^+$ and CD4$^+$ T Cell Memory to Lymphocytic Choriomeningitis Virus Infection$^\nabla$

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Although cellular immunity to acute lymphocytic choriomeningitis virus (LCMV) infection has been well characterized in experimental studies in mice, the T cell response to this virus in humans is incompletely understood. Thus, we analyzed the breadths, magnitudes, and differentiation phenotypes of memory LCMV-specific CD8$^+$ and CD4$^+$ T cells in three human donors displaying a variety of disease outcomes after accidental needle stick injury or exposure to LCMV. Although only a small cohort of donors was analyzed at a single time point postinfection, several interesting observations were made. First, we were able to detect LCMV-specific CD8$^+$ and CD4$^+$ T cell responses directly *ex vivo* at 4 to 8 years after exposure, demonstrating the longevity of T cell memory in humans. Second, unlike in murine models of LCMV infection, we found that the breadths of memory CD8$^+$ and CD4$^+$ T cell responses were not significantly different from one another. Third, it seemed that the overall CD8$^+$ T cell response was augmented with increasing severity of disease, while the LCMV-specific CD4$^+$ T cell response magnitude was highly variable between the three different donors. Next, we found that LCMV-specific CD8$^+$ T cells in the three donors analyzed seemed to undergo an effector memory differentiation program distinct from that of CD4$^+$ T cells. Finally, the levels of expression of memory, costimulatory, and inhibitory receptors on CD8$^+$ and CD4$^+$ T cell subsets, in some instances, correlated with disease outcome. These data demonstrate for the first time LCMV-specific CD8$^+$ and CD4$^+$ T cells in infected humans and begin to provide new insights into memory T cell responses following an acute virus infection.

Lymphocytic choriomeningitis virus (LCMV), a single-stranded RNA virus and archetypical member of the family *Arenaviridae*, is a human pathogen maintained in a rodent host reservoir. Infection outcomes can range from subclinical disease to aseptic meningitis, encephalitis, life-threatening infections in immunosuppressed individuals, and severe congenital defects (5, 6, 9, 19). Despite the pathogenic nature of LCMV, there are no U.S. Food and Drug Administration-approved vaccines available. Moreover, antiviral therapy is limited to the use of the guanosine analogue ribavirin, which can lead to adverse side effects such as thrombocytosis, severe anemia, and birth defects (18, 37). Animal models of LCMV infection have clearly demonstrated that virus-specific CD8$^+$ and CD4$^+$ T cells play a pivotal role in viral clearance and protection (17, 20, 26, 27, 58, 59, 61). However, to date, human T cell responses to LCMV have not been characterized in detail.

We have begun to understand human LCMV-specific T cell responses by recent work with HLA transgenic mice, identifying HLA-restricted CD8$^+$ and CD4$^+$ T cell epitopes (10, 31, 32). Immunization of mice with HLA-restricted CD8$^+$ T cell epitopes is sufficient to confer protection against subsequent infection with LCMV in HLA transgenic mice (10). However, the degree of overlap between T cell epitopes identified in HLA transgenic mouse models and humans is incomplete (30, 55). Thus, whether the epitopes defined in the HLA transgenic model are useful to characterize human responses remains to be ascertained experimentally.

Human virus-specific CD8$^+$ T cell responses have been extensively characterized in the context of persistent virus infections, including infections with cytomegalovirus (CMV), Epstein-Barr virus (EBV), hepatitis C virus (HCV), and human immunodeficiency virus type 1 (HIV-1). By examining the expression profiles of surface markers such as CD45RA, CCR7, CD62L, CD28, and CD27, human CD8$^+$ T cells from different persistent virus infections could be categorized into phenotypically distinct memory populations (2, 12, 49). More recently, there have been several investigations examining the differentiation phenotypes of antiviral CD8$^+$ T cells responding to live-attenuated viral vaccines, such as those for smallpox virus (Dryvax) and yellow fever virus (YFV)-17D (1, 13, 38). These

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studies have shown that memory $CD8^+$ T cells generated after an acute infection with live-attenuated vaccines exhibit phenotypic and functional characteristics that are distinct from those described for human $CD8^+$ T cells specific for persistent viruses (1, 38). Importantly, effector memory $CD45RA^+$ $CCR7^-$ $CD8^+$ T cells from persistent infections have been defined as terminally differentiated, while after acute infections, CD45RA-expressing $CD8^+$ T cells exhibit a robust proliferative capacity. Although a great deal of knowledge characterizing Dryvax- and YFD-17D-specific $CD8^+$ T cells has been gained, results from these studies are difficult to generalize to other acute virus infections because of the attenuation state of the virus inoculum and a lack of data in regard to virus-specific $CD4^+$ T cells. Other acute virus infections have also been studied, such as hantavirus and influenza A virus (23, 56); however, these are not ideal experimental systems because exposure to virus and virus reencounter cannot be controlled.

In the present study, we report the identification of LCMV-specific $CD8^+$ and $CD4^+$ T cell epitopes restricted by various HLA molecules. In the case of HLA molecules previously investigated in HLA transgenic mouse models, we report an excellent overlap between epitopes recognized in humans and HLA transgenic mice. Utilizing these T cell epitopes, we characterized the breadths, magnitudes, and differentiation phenotypes of the memory $CD8^+$ and $CD4^+$ T cell responses generated after acute LCMV infection in a small cohort of human donors who displayed a range of disease outcomes.

MATERIALS AND METHODS

Ethics statement. Research conducted for this study was performed in accordance with approvals from the Institutional Review Board at the La Jolla Institute for Allergy and Immunology. All participants provided written informed consent prior to participation in the study.

Study subjects and PBMC samples. Healthy volunteers (>18 years of age) with a history of accidental needle stick injury or exposure to LCMV were recruited to the study following informed consent. Seropositivity was confirmed by measuring the serum IgM and IgG antibody titers for LCMV through immunofluorescence assay (Focus Diagnostics, Inc., Cypress, CA). Peripheral blood mononuclear cells (PBMC) were then isolated from heparinized blood or leukapheresis by gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), suspended in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO), and cryopreserved in liquid nitrogen. Donor PBMC were typed for HLA-A, -B, and -DR by high-resolution PCR (Blood Systems Laboratories, Tempe, AZ). Approval for all procedures was obtained from the Institutional Review Board (FWA 00000032). Donor AD108 was exposed to LCMV Armstrong clone 53b in 2003, and blood draws were taken in both 2008 and 2009. Donors AD123 and AD124 were exposed to LCMV clone 13 in 2001 and 2002, respectively, and both provided blood donations in 2009.

Bioinformatic analyses. Candidate epitopes were identified by a consensus epitope prediction approach essentially as described previously (41). For major histocompatibility complex (MHC) class I-restricted peptides, three prediction methods (artificial neural network [ANN], average relative binding [ARB], and the stabilized matrix method [SMM]), as described in reference 62, were applied to select peptides derived from the glycoprotein (GPC) (NCBI accession number P09991) and the nucleoprotein (NP) (NCBI accession number P09992) sequences of LCMV strain Armstrong clone 53b. Nine-mer peptides were ranked according to the their predicted ability to bind to the donor's specific HLA-A and -B molecules. The median rank in the three predictions was used to select the top 3% of class I peptides, which comprised 30 peptides/allele. For HLA-DR-restricted peptides, 15-mer peptides overlapping by 10 residues that covered the entire GPC and NP sequences were ranked using the same median rank consensus approach between ARB, SMM-align, and PROPRED predictions (60). Due to the lower confidence in MHC class II prediction performance, we chose the top 30% of class II peptides, resulting in 60 peptides/DR allele.

Peptides. Peptides were synthesized as crude material by Pepscan Systems (Lelystad, The Netherlands). Candidate epitopes were resynthesized by A and A Labs (San Diego, CA) and purified to 95% or greater homogeneity by reverse-phase high-pressure liquid chromatography (HPLC). The IEDB submission identification number for the MHC class I and II epitopes is 1000386.

MHC-peptide binding assay. Quantitative assays to measure the binding affinity of peptides to purified HLA class I and II molecules were based on the inhibition of binding of a radiolabeled standard peptide and were performed essentially as described elsewhere (11, 51, 52). Briefly, after a 2-day incubation, binding of the radiolabeled peptide to the corresponding MHC class I or II molecule was determined by capturing MHC/peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-One, Monroe, NC) coated with the W6/32 antibody (anti-HLA class I) or L243 antibody (anti-DRA) and measuring bound cpm using the Topcount microscintillation counter (Packard Instrument). The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC_{50}) was then calculated. Peptides were typically tested at 6 different concentrations covering a 100,000-fold dose range and in 3 or more independent assays. Under the conditions utilized, where [label] < [MHC] and $IC_{50} \geq$ [MHC], the measured IC_{50}s are reasonable approximations of the dissociation constant (K_D) values.

***Ex vivo* IFN-γ ELISPOT assay.** Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays were performed as previously described (43). In brief, 2×10^5 PBMC were incubated in the presence of peptide pools (10 μg/ml/peptide) corresponding to the donor's haplotype. Peptide pools yielding positive responses were deconvoluted, and individual peptides were tested at 10 μg/ml. To ensure MHC class I restriction when using MHC class I predicted binding peptides, $CD8^+$ T cells were purified by positive selection using anti-CD8 magnetic beads (Miltenyi Biotec, Auburn, CA). To ensure class II restriction when using MHC class II predicted binding peptides, $CD8^+$ T cells were depleted from the PBMC with anti-CD8 beads (Miltenyi Biotec). After 20 h of incubation at 37°C, plates were developed, and responses were calculated as described previously (43). Criteria for positivity were ≥20 net spot-forming cells (SFC)/10^6 cells, a stimulation index of ≥2.0, and a P value of ≤0.05 using a Student's t test in at least 2 experiments.

Tetramer staining and magnetic bead enrichment. MHC class I tetramers conjugated using allophycocyanin (APC)-labeled streptavidin (A*0201, B*0702, B*1501, and B*4402) were provided by the NIH Tetramer Core Facility (Atlanta, GA). MHC class II tetramers conjugated using phycoerythrin (PE)-labeled streptavidin (DRB1*1101 and DRB1*0701) were provided by the Tetramer Core Laboratory at the Benaroya Research Institute at Virginia Mason (Seattle, WA). PBMC ($\sim 50 \times 10^6$) were incubated in 0.5 ml phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (pH 8.0) (magnetically activated cell sorting [MACS] buffer) with a 1:100 dilution of class I tetramer for 30 min at 4°C or a 1:50 dilution of class II tetramer for 1 h at 37°C. The exception was the HLA-A*0201 GPC_{10-18} tetramer, which was incubated with PBMC at a 1:100 dilution for 1 h at 37°C. Tetramer-specific T cell populations were positively enriched by incubating cells with 50 μl of anti-APC or 50 μl of anti-PE microbeads (Miltenyi Biotech) for 20 min at 4°C. After washing, cells were resuspended in 5 ml MACS buffer and passed through a magnetized LS column (Miltenyi Biotech). The column was washed three times with 3 ml of MACS buffer, and after removal from the magnetic field, cells were collected with 5 ml of MACS buffer. The enriched tetramer-specific $CD8^+$ or $CD4^+$ T cells were resuspended in 0.1 ml MACS buffer and stained for cell surface antigens CD8-peridinin chlorophyll protein (PerCP) Cy5.5/PB (RPA-T8; eBioscience), CD27-fluorescein isothiocyanate (FITC) (M-T271; BD Biosciences), CD28-PE/APC (CD28.2; BD), CD62L-FITC (prey56; eBioscience), CD45RA-PECy7 (hl100; eBioscience), CD127-PE (RDR5; eBioscience), CCR5-FITC (2D7/CCR5; BD), CCR7-PE/APC (3D12; eBioscience), LAG3-FITC (R&D Systems), and PD1-PE/APC (MIH4; BD) and for CD4-PerCP Cy5.5/PB (OKT4 eBioscience; RPA-T4 BD), CD14-PerCP Cy5.5 (HCD14; BioLegend), and CD19-PerCP Cy5.5 (SJ25C1; BD) to exclude non-T cell-containing populations that bound tetramer. Fluorescence minus one (FMO) staining controls were used to set quadrant gates. Samples were acquired on an LSR II flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo software.

RESULTS

Breadth and magnitude of LCMV-specific memory $CD8^+$ T cell responses in a small cohort of individuals with resolved acute LCMV infection. We examined responses in three subjects who displayed various degrees of symptoms after accidental needle stick injury or exposure with two different strains of LCMV (Table 1). Subject AD108 was infected with the Arm-

TABLE 1. Characteristics of LCMV-seropositive donors

Donor	Sex	HLA class I	HLA class II (DRB1)	LCMV strain	Yr of infection	Route of infection	Symptoms and duration
AD108	Male	A*2402 A*3201 B*0702 B*1501	*1101 *1401	Armstrong clone 53b	2003	Unknown	None
AD123	Male	A*0201 B*1401 B*4402	*0701 *0801	Clone 13	2001	Leg needle stick	Flu-like illness and bacterial infection, 60 days
AD124	Male	A*0201 A*2402 B*4801 B*5401	*0803 *0901	Clone 13	2002	Eye	Aseptic meningitis, 4 to 5 days

strong clone 53b strain of LCMV, while subjects AD123 and AD124 were infected with the clone 13 strain. All three subjects displayed LCMV-specific serum IgG antibodies but were negative for LCMV-specific serum IgM (data not shown). The specificity of the serology assay was confirmed by testing sera from a total of 30 subjects with no history of LCMV exposure. All 30 subjects were negative for both LCMV-specific IgG and IgM antibodies. AD108 was clinically unaware of his infection, thus allowing us to study an asymptomatic acute LCMV infection. AD123 and AD124 were both symptomatic, and infection resulted in either a flu-like illness or aseptic meningitis, respectively. To investigate the breadth and magnitude of LCMV-specific memory $CD8^+$ and $CD4^+$ T cell responses following an acute LCMV infection, peptides derived from the GPC and NP predicted to bind to the donors' specific haplotypes and an HLA-A*0201-restricted epitope, Z_{49-58}, previously identified in HLA transgenic animals, were synthesized. PBMC isolated from the three donors were stimulated with the candidate peptides, and positive peptides were identified on the basis of their ability to stimulate IFN-γ production from $CD8^+$ T cells in an ELISPOT assay.

In total, nine unique $CD8^+$ T cell epitopes were defined from the three individuals. $CD8^+$ T cells specific for the GPC, NP, and Z proteins were detected, indicating that all three viral proteins are immunogenic in humans (Table 2 and Fig. 1). As a specificity control, $CD8^+$ T cells isolated from three LCMV-seronegative individuals who expressed the same HLA class I molecules as our donor subjects (representative data are shown in Fig. 1) were also examined. Because the defined epitopes were restricted by 6 different HLA class I alleles, a total of 18 HLA-matched, LCMV seronegative PBMC (3 donors/allele) were tested to ensure that the epitope-specific responses were truly LCMV specific. None of the epitopes were recognized by $CD8^+$ T cells isolated from the seronegative donors, thus confirming the specificity of the defined epitopes.

Since three or four $CD8^+$ T cell epitopes were identified in each individual, disease severity and/or the infecting virus strain did not appear to affect the overall breadth of the memory $CD8^+$ T cell response. However, the magnitude of the total $CD8^+$ T cell response seemed to correlate with increased clinical severity, with the lowest responses detected in AD108 (total of 228 net SFC/10^6 cells) and significantly higher responses observed in AD123 and AD124 (totals of 734 and 863 net SFC/10^6 cells, respectively) ($P < 0.05$).

As shown in Table 2, the HLA restriction of the newly defined LCMV-specific $CD8^+$ T cell populations, inferred by the bioinformatic predictions, was further supported by experiments measuring epitope binding to purified HLA molecules *in vitro*. In 7 out of 10 cases, the epitope bound the relevant HLA molecule with high affinity (1 to 100 nM range), and in the 3 remaining cases it did so with weak to intermediate affinity (100 to 10,000 nM range). Two $CD8^+$ T cell epitopes ($GPC_{447-455}$ and $NP_{246-254}$) were recognized in more than one donor and were restricted by distinct MHC class I types. An overlapping

TABLE 2. Properties of LCMV-derived HLA class I-restricted $CD8^+$ T cell epitopes

Donor	Epitope[a]	Peptide sequence	ELISPOT assay result (avg no. of SFC/10^6 $CD8^+$ T cells)[b]	Predicted HLA restriction	Measured binding affinity (nM)[c]	Restriction (tetramer staining)[d]
AD108	GPC_{38-46}	FATCGIFAL	63	B*0702	6,230	ND
	$GPC_{447-455}$	YLVSIFLHL	46	B*1501	6,920	ND
	$NP_{246-254}$	AAVKAGAAL	46	B*0702	9.8	B*0702
	$NP_{414-422}$	KQFKQDSKY	73	B*1501	13	B*1501
AD123	$GPC_{447-455}$	YLVSIFLHL	172	A*0201	35	A*0201
	NP_{45-53}	SEVSNVQRI	84	B*4402	12	B*4402
	NP_{69-77}	SLNQTVHSL	348	A*0201	53	A*0201
	Z_{49-58}	YLCRHCLNLL	130	A*0201	3.9	A*0201
AD124	GPC_{10-18}	ALPHIIDEV	413	A*0201	57	A*0201
	GPC_{11-19}	LPHIIDEVI	151	B*5401	1,400	ND
	$NP_{246-254}$	AAVKAGAAL	299	B*4802	ND	ND

[a] Peptide position within LCMV strain Armstrong clone 53b GPC, NP, or Z protein.
[b] Number of net SFC per 10^6 purified $CD8^+$ T cells. Data are averaged from two independent experiments.
[c] Peptides were tested for binding to their predicted HLA class I allele. ND, binding was not determined because the purified HLA was not available.
[d] Restriction was determined by HLA class I tetramer staining. ND, tetramer staining was not performed.

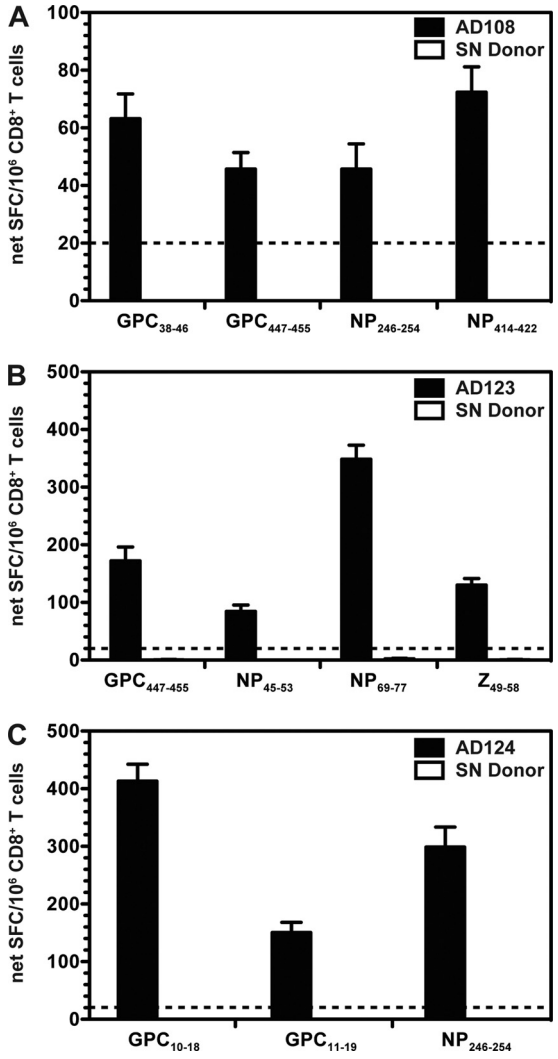

FIG. 1. Direct *ex vivo* detection of LCMV-specific memory $CD8^+$ T cell responses. Purified $CD8^+$ T cells from either the LCMV-seropositive donors (black bars) or a representative HLA-matched, LCMV-seronegative donor (white bars) were incubated with 10 μg/ml peptide, after which the number of IFN-γ-producing cells was enumerated in an ELISPOT assay. Shown are the average net SFC/10^6 $CD8^+$ T cells for AD108 (A), AD123 (B), and AD124 (C) in response to the defined epitopes from at least two independent experiments. The dashed lines indicate the threshold for peptide positivity (≥20 net SFC/10^6 cells). For each individual experiment, samples were tested in triplicate. Error bars indicate standard errors of the means (SEM).

epitope sequence was also defined in AD124, with GPC_{10-18} binding to HLA-A*0201 and GPC_{11-19} to HLA-B*5401.

LCMV-specific memory $CD4^+$ T cell responses in same three individuals with resolved acute infection. $CD4^+$ T cells targeted both the GPC and NP, with three unique epitopes derived from the GPC and nine from the NP (Table 3 and Fig. 2). A single epitope ($NP_{521-535}$), originally defined in HLA-DRB1*0101 transgenic mice (33), was also recognized by AD123 (Table 4). Epitope-specific responses for AD108 and AD123 were not detectable in HLA-matched, LCMV-seronegative donors (representative data are given in Fig. 2). The overall breadth of the LCMV-specific memory $CD4^+$ T cell response did not appear to correlate with disease severity and/or the infecting virus strain. Unlike the case for the LCMV-specific $CD8^+$ T cell response, the magnitude of the total $CD4^+$ T cell response was highly variable between the three different donors examined. The total net SFC/10^6 $CD4^+$ T cells for AD108, AD123, and AD124 were 121, 1,279, and 100, respectively.

The restricting alleles of the LCMV-specific $CD4^+$ T cell populations inferred by the predicted binding affinities were further supported by the measured binding affinities (Table 3). In 7 out of 14 cases, the epitope bound the relevant HLA molecule with high affinity (l to 100 nM range), and in the 7 remaining cases it did so with weak to intermediate affinity (100 to 10,000 nM range). Two GPC-specific epitopes (GPC_{66-80} and GPC_{71-85}) were recognized in more than one donor and were restricted by different HLA-DRB1 molecules. GPC_{66-80} and GPC_{71-85} both bound to HLA-DRB1-*0701 in AD123 and to HLA-DRB1-*0901 in AD124.

HLA restriction of LCMV-specific T cell populations determined by MHC-peptide tetramer staining. To further characterize the dynamics of the LCMV-specific T cell response and the differentiation phenotypes of LCMV-specific $CD8^+$ and $CD4^+$ T cells, MHC-peptide tetramer reagents were prepared for selected epitopes with binding affinities to the predicted HLA allele of <100 nM (Tables 2 and 3). Because the frequencies of LCMV-specific $CD8^+$ and $CD4^+$ T cell populations detected by ELISPOT assay in the three individuals were relatively low, we employed tetramer staining followed by a magnetic bead enrichment technique (see Materials and Methods) to increase the sensitivity of detection (4, 45).

The tetramer staining experiments confirmed HLA restriction of seven $CD8^+$ and four $CD4^+$ T cell responses (representative data are given in Fig. 3). We were able to detect LCMV-specific T cell responses at frequencies 30- to 600-fold above background. For some $CD8^+$ and $CD4^+$ T cell populations, we were unable to confirm the HLA restriction inferred on the basis of the HLA predictions (Tables 2 and 3), because purified HLAs for binding assays and/or tetramer preparations were not available. Only a small number of background-level tetramer-positive cells were detected with the LCMV-specific tetramers in the control HLA-matched, LCMV-seronegative individuals (Fig. 3), thus confirming that tetramer specificity was derived from the LCMV peptide and HLA molecule combination.

Phenotypic characterization of the LCMV-specific memory $CD8^+$ T cells. Next, we assessed the differences between effector and central memory T cells using CD45RA, a transmembrane tyrosine phosphatase reexpressed on late-differentiated $CD8^+$ T cells (12), and CCR7, a chemokine receptor that controls homing to secondary lymphoid organs (49). As shown in Fig. 4, we found that LCMV-specific tetramer-positive $CD8^+$ T cells consisted predominantly of $CD45RA^+$ $CCR7^-$ effector memory cells in the three different donors analyzed. We also analyzed the expression of CD62L, an L-selectin lymph node homing molecule. LCMV-specific $CD8^+$ T cell subsets consisted mainly of $CD62L^{low}$ effector memory cells but also contained $CD62L^{high}$ central memory cells (Fig. 4), particularly in subjects AD108 and AD124. Interestingly, the majority of tetramer-positive $CD8^+$ T cells isolated from AD123 consistently displayed $CD45RA^{low}$ and $CD62L^{low}$ ex-

TABLE 3. Properties of LCMV-derived HLA class II-restricted $CD4^+$ T cell epitopes

Donor	Epitope[a]	Peptide sequence	ELISPOT assay result (avg no. of SFC/10^6 $CD4^+$ T cells)[b]	Predicted HLA restriction	Measured binding affinity (nM)[c]	Restriction (tetramer staining)[d]
AD108	NP_{86-100}	KNVLKVGRLSAEELM	121	*1101	23	*1101
AD123	GPC_{66-80}	DIYKGVYQFKSVEFD	239	*0701	16	*0701
	GPC_{71-85}	VYQFKSVEFDMSHLN	190	*0701	373	ND
	$GPC_{341-355}$	HLFKTTVNSLISDQL	71	*0701	2.0	ND
	$NP_{106-120}$	LEKLKAKIMRSERPQ	33	*0801	403	ND
	$NP_{236-250}$	NISGYNFSLGAAVKA	328	*0701	13	*0701
	$NP_{261-275}$	LESILIKPSNSEDLL	134	*0701	60	*0701
	$NP_{281-295}$	AKRKLNMFVSDQVGD	53	*0701	228	ND
	$NP_{311-325}$	EGWPYIACRTSIVGR	45	*0701	480	ND
	$NP_{356-370}$	VGLSYSQTMLLKDLM	26	*0701	3.8	ND
	$NP_{411-425}$	VDQKQFKQDSKYSHG	128	*0701	339	ND
	$NP_{521-535}$	MDCIIFESASKARLP	32	*0801	75	ND
AD124	GPC_{66-80}	DIYKGVYQFKSVEFD	62	*0901	791	ND
	GPC_{71-85}	VYQFKSVEFDMSHLN	38	*0901	1,200	ND

[a] Peptide position within LCMV strain Armstrong clone 53b GPC or NP.
[b] Number of net SFC per 10^6 purified $CD4^+$ T cells. Data are averaged from two independent experiments.
[c] Peptides were tested for binding to their predicted HLA class II allele.
[d] Restriction was determined by HLA class II tetramer staining. ND, tetramer staining was not determined.

pression compared with the other two donors (medians of 34% $CD45RA^+$ and 12% $CD62L^+$). In most instances, we found similar expression patterns of CD45RA, CCR7, and CD62L in both the tetramer-positive $CD8^+$ T cells and the total $CD8^+$ T cell population within the same donor.

To further determine the phenotypic nature of the LCMV-specific memory $CD8^+$ T cells, we examined the coexpression of CD127 (interleukin-7 receptor alpha [IL-7Rα]), which marks precursors destined to differentiate into long-lived memory cells (25), and the chemokine receptor CCR5, which is associated with activated memory cells (46). We found that the majority of tetramer-positive $CD8^+$ T cells reexpressed CD127 in subject AD108 (median of 69% $CD127^+$), whereas CD127 was expressed at progressively lower levels in AD123 and AD124 (medians of 22% and 10% $CD127^+$, respectively). Bimodal expression patterns were observed for CCR5 in all three donors.

TABLE 4. Summary of overlap between HLA class I- and II-restricted LCMV T cell epitope recognition in human and HLA transgenic mouse systems

Epitope	Sequence	Identification[a] in:		Reference(s)[b]
		Human system	Transgenic mouse system	
GPC_{10-18}	ALPHIIDEV	Y	Y	10
GPC_{11-19}	LPHIIDEVI	Y	NT	
GPC_{34-43}	AVYNFATCGI	NT	Y	10, 31
GPC_{38-46}	FATCGIFAL	Y	N	
$GPC_{112-120}$	FTNDSIISH	N	Y	31
GPC_{46-55}	LVSFLLLAGR	NT	Y	31
$GPC_{447-455}$	YLVSIFLHL	Y	Y	10
NP_{45-53}	SEVSNVQRI	Y	NT	
NP_{69-77}	SLNQTVHSL	Y	Y	10
$NP_{246-254}$	AAVKAGAAL	Y	N	
$NP_{414-422}$	KQFKQDSKY	Y	NT	
$NP_{521-535}$	MDCIIFESASKARLP	Y	Y	33
Z_{24-33}	TTYLGPLSCK	NT	Y	31
Z_{49-58}	YLCRHCLNLL	Y	Y	10, 31

[a] Y, epitope was identified; N, epitope was not identified. Class I epitope recognition in HLA transgenic mice was examined following inoculation of mice with the LCMV strain Armstrong (10) or recombinant VACV (rVACV) expressing LCMV GPC, NP, or Z (31). Class II epitope recognition was examined after priming mice with an rVACV expressing one of the LCMV proteins followed by boosting with LCMV-specific peptide pools (33). Epitope-specific T cell responses were measured by IFN-γ ELISPOT assays. NT, not tested because the epitope sequence was not predicted to bind to the donor's HLA molecules or because HLA transgenic mice were not available.
[b] Reference(s) for epitope identification in HLA transgenic mice.

Characterization of the LCMV-specific $CD8^+$ T cells in terms of expression of costimulation and inhibitory receptors. To assess the ability of the memory $CD8^+$ T cells to respond to costimulatory signals, we studied the expression of CD27 and CD28. These costimulatory receptors have also been used to phenotypically differentiate distinct memory $CD8^+$ T cell subsets following persistent virus infections (2). We found that tetramer-positive $CD8^+$ T cells from AD108 and AD123 showed uniformly high expression of CD27 and heterogeneous expression of CD28, while only ~30% of tetramer-positive $CD8^+$ T cells from AD124 expressed both CD27 and CD28.

We then examined the expression of two inhibitory receptors, programmed death 1 (PD1) and lymphocyte activation gene 3 (LAG3), that both negatively regulate T cell activation and proliferation when upregulated transiently in acute LCMV Armstrong infection and long term in clone 13 infection of mice (3, 7). Recently, it was shown that blockade of both PD1 and LAG3 improved $CD8^+$ T cell responses and reduced the viral load in LCMV clone 13-infected mice (7). We found that tetramer-positive $CD8^+$ T cells from all three donors did not express PD1, which is consistent with a resolved acute infection and clearance of virus. In contrast, we observed upregulated expression of LAG3 in the three donors (medians of 6% for AD108, 19% for AD123, and 4% for AD124). Interestingly, a high degree of variability was observed when examining the expression of LAG3 in other tetramer-positive $CD8^+$ T cell populations from AD123. Ten percent of HLA-A*0201

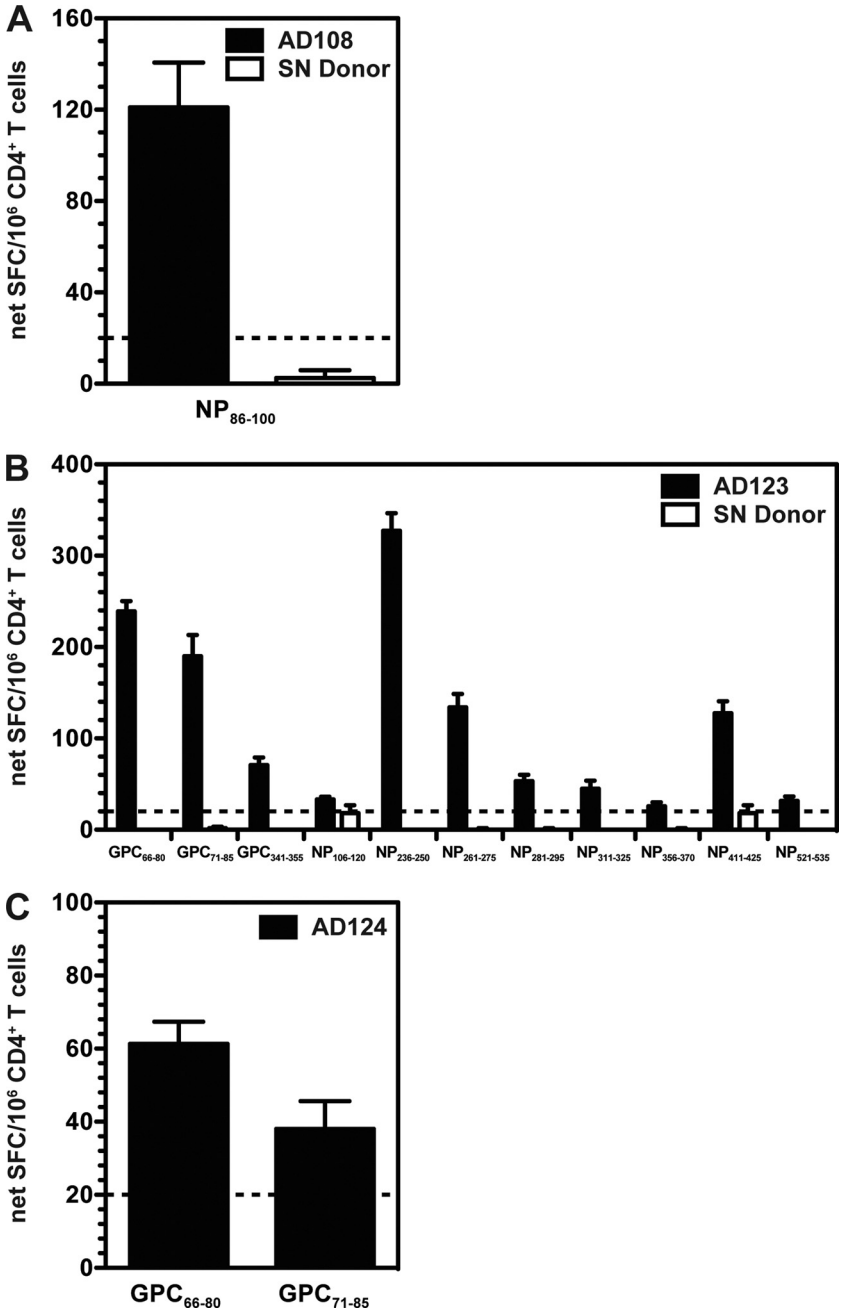

FIG. 2. Direct *ex vivo* detection of LCMV-specific memory $CD4^+$ T cell responses. PBMC enriched for $CD4^+$ T cells from either the LCMV-seropositive donors (black bars) or a representative HLA-matched, LCMV-seronegative donor (white bars) were incubated with 10 μg/ml peptide. The number of IFN-γ-producing cells was enumerated in an ELISPOT assay. Shown are the average net SFC/10^6 $CD4^+$ T cells for AD108 (A), AD123 (B), and AD124 (C) in response to the defined epitopes from at least two independent experiments. An HLA-matched, LCMV-seronegative donor for AD124 was not available. The dashed lines indicate the threshold for peptide positivity (≥20 net SFC/10^6 cells). For each individual experiment, samples were tested in triplicate. Error bars indicate SEM.

NP_{69-77}, 23% of HLA-A*0201 $GPC_{447-455}$, and 42% of HLA-B*4402 NP_{45-53} were positive for LAG3 (median of 19% $LAG3^+$) (data not shown).

Phenotypic characterization of the LCMV-specific memory $CD4^+$ T cells. Our understanding of $CD4^+$ T cell differentiation following acute virus infection in humans remains unclear. To begin to fill this knowledge gap, we applied the tetramer enrichment technique to phenotypically characterize LCMV-specific $CD4^+$ T cell populations isolated from AD108 and AD123. We were unable to phenotype $CD4^+$ T cells from AD124, as the MHC class II molecules DRB1*0803 and DRB1*0901 were not available for tetramer preparation. The expression of markers used to dissect the $CD8^+$ T cell populations was also used to characterize the LCMV-specific $CD4^+$ T cells.

Overall, we observed two key differences between the

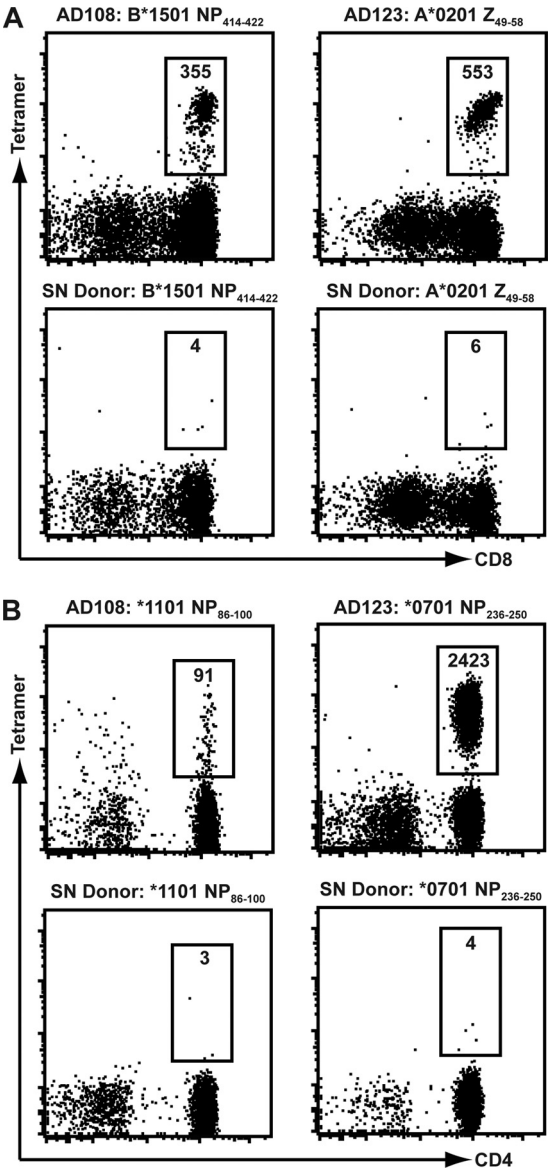

FIG. 3. HLA restriction of LCMV-specific $CD8^+$ and $CD4^+$ T cell responses using MHC tetramers. PBMC from AD108, AD123, or HLA-matched, seronegative individuals were stained with MHC class I tetramers (A) or class II tetramers (B), and tetramer-positive cells were isolated following magnetic bead enrichment. Plots are gated on either $CD3^+$ $CD8^+$ (A) or $CD3^+$ $CD4^+$ (B) lymphocytes, and the numbers indicate the total number of tetramer-positive cells isolated from each donor's PBMC.

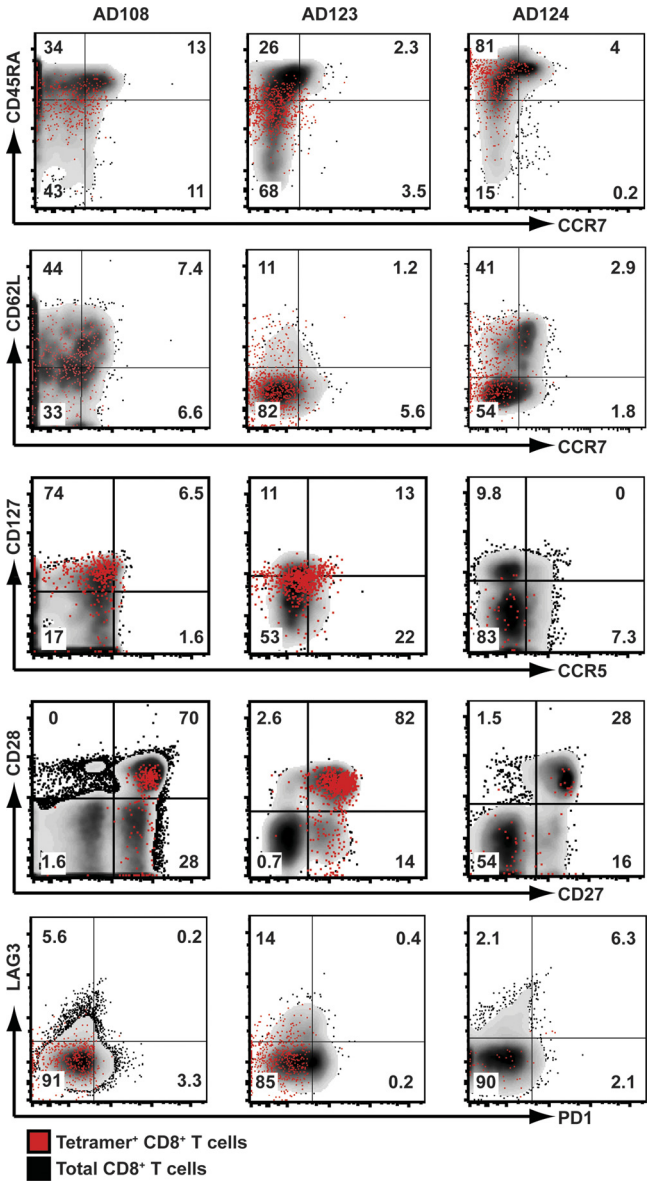

FIG. 4. Memory phenotype of LCMV-specific $CD8^+$ T cells. PBMC from donors AD108, AD123, and AD124 were stained with the MHC class I tetramers HLA-B*1501 $NP_{414-422}$, HLA-A*0201 Z_{49-58}, and HLA-A*0201 GPC_{10-18}, respectively. Tetramer-positive cells were enriched following incubation with magnetic beads. There was a 10-fold reduction in the number of tetramer-positive $CD8^+$ T cells isolated from AD124 when examining CD127, CCR5, CD27, CD28, LAG3, and PD1 expression, as fewer PBMC were initially used. Plots are gated on either total $CD3^+$ $CD8^+$ T cells (black background) or LCMV-specific $CD3^+$ $CD8^+$ T cells (red dots). The numbers represent the percentages of tetramer-positive $CD3^+$ $CD8^+$ T cells in the gate.

tetramer-positive $CD8^+$ and $CD4^+$ T cell populations analyzed. First, we found that tetramer-positive $CD4^+$ T cells consisted predominantly of $CD45RA^-$ $CCR7^-$ $CD62L^-$ effector memory cells (Fig. 5). Second, when examining CD27 and CD28 expression, we found that the tetramer-positive $CD4^+$ T cells uniformly expressed high levels of both costimulatory receptors. Expression of CD127 was divergent between the two donors analyzed. Greater than 70% of tetramer-positive $CD4^+$ T cells isolated from AD108 reexpressed high levels of CD127, whereas the majority of tetramer-positive $CD4^+$ T cells from AD123 expressed CD127 at only low to moderate levels (median of 16% $CD127^+$). Interestingly, similar to the case for LCMV-specific $CD8^+$ T cells from AD123, LAG3 expression was also upregulated on tetramer-positive $CD4^+$ T cells in this donor. This phenotype was seen on other tetramer-positive $CD4^+$ T cell populations isolated from AD123 (median of 27% $LAG3^+$) (data not shown). Both CCR5 and PD1 were not expressed on any of the $CD4^+$ T cell populations analyzed.

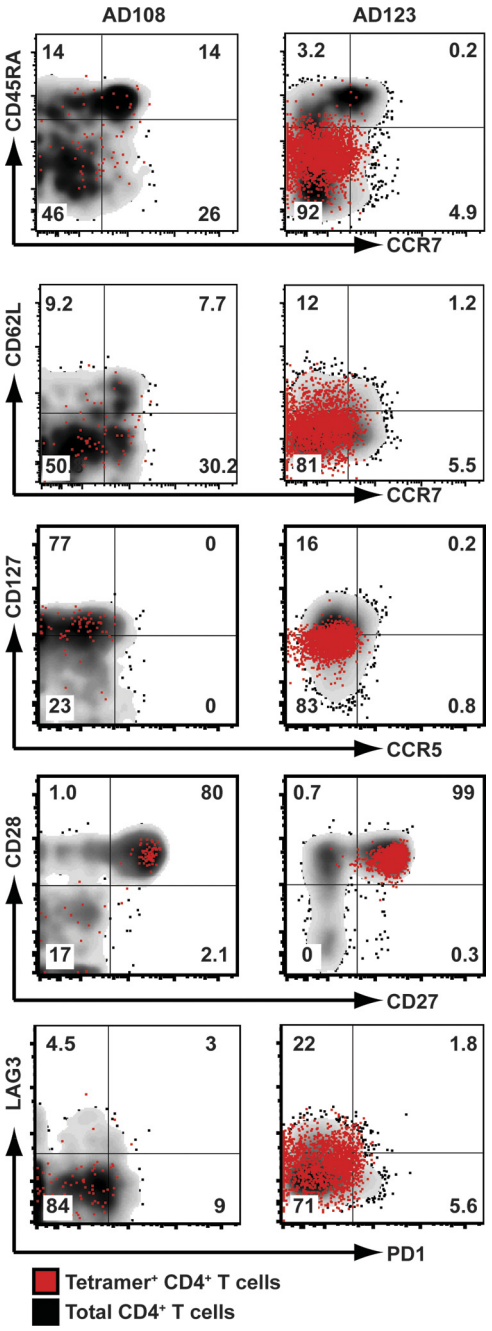

FIG. 5. Memory phenotype of LCMV-specific $CD4^+$ T cells. PBMC from donors AD108 and AD123 were stained with the MHC class II tetramers HLA-DRB1*1101 NP_{86-100} and HLA-DRB1*0701 $NP_{236-250}$, respectively. Tetramer-positive cells were enriched following incubation with magnetic beads. Plots are gated on either total $CD3^+$ $CD4^+$ T cells (black background) or LCMV-specific $CD3^+$ $CD4^+$ T cells (red dots). The numbers represent the percentages of tetramer-positive $CD3^+$ $CD4^+$ T cells in the gate.

DISCUSSION

The present study represents the first characterization of human memory T cell responses to LCMV in the setting of accidental needle stick injury or exposure with different strains of LCMV in three individuals who displayed distinct disease outcomes. Responses against a total of 9 unique $CD8^+$ and 12 $CD4^+$ T cell epitopes were detected directly *ex vivo* at 4 to 8 years after LCMV exposure. We were able to rapidly identify these T cell epitopes, as HLA binding predictions are increasingly more accurate and IFN-γ ELISPOT assays allow quick screening of a large number of peptides. Most importantly, the integration of bioinformatic approaches with cellular immune assays might lead to the development of optimized vaccines and drugs tailored to personalized prevention and treatment of virus infection. Thus, with the advent of epitope identification in humans, personalized medicine is becoming a reality.

Despite our findings, the small number of individuals available to us for study and the significant time elapsed from infection dictate that these evaluations should be interpreted with caution. The data trends and conclusions suggested in this study would be strengthened by increasing the number of subjects and/or by analysis of the same three subjects at multiple time points to enable documentation of differentiation changes of the defined T cell populations. Unfortunately, we have been constrained by the wide geographic distribution of our donors and thus are unable to obtain repeat PBMC donations from the same three subjects. Further studies with additional donors and time points postinfection would be required to more firmly establish the observations reported here. These are areas of future research.

Although the donor cohort size was small, we detected a high degree of overlap in the epitopes recognized in infected humans and in previous studies utilizing HLA transgenic mice. Of the 5 HLA-A*0201-restricted LCMV-specific $CD8^+$ T cell epitopes originally defined in HLA-A*0201 transgenic mice, 4 were also recognized by HLA-A*0201-positive donors in this study, thus demonstrating an 80% concordance rate between humans and mice. The facts that multiple HLA-A*0201-positive donors recognized the HLA-A*0201-restricted $CD8^+$ T cell epitopes and that immunization with these epitopes protected mice against LCMV challenge further validate their potential use as vaccine candidates. The high rate of concordance between the two systems underscores the importance of HLA transgenic mice as a suitable model for identifying human T cell epitopes from small RNA viruses, such as LCMV. HLA transgenic mice also serve as a preliminary probe for identifying T cell epitopes from viruses that infrequently infect humans and that could be viewed as potential bioterrorist threats.

In the three donors analyzed, we found that the breadths of the memory $CD8^+$ and $CD4^+$ T cell responses did not significantly differ from one another and were not influenced by disease severity and/or the infecting LCMV strain. In contrast, the overall magnitudes of memory $CD8^+$ and $CD4^+$ T cell responses varied between the different donors. Compared to the total $CD8^+$ T cell response magnitude, the total $CD4^+$ T cell response was similar in AD108, higher in AD123, and lower in AD124. These findings were interesting, as they differ from what was previously observed in murine models of LCMV infection and also in the case of other acute viral infections in humans. Thus, it appears that different viruses might induce qualitatively different T cell responses. In murine models of LCMV, studies have demonstrated that a broader repertoire of $CD8^+$ T cell responses exists after an acute LCMV infection (16, 32). Murine models have also demon-

strated major differences in the magnitudes of memory $CD8^+$ and $CD4^+$ T cell responses. Memory is stable in $CD8^+$ T cell responses, while the levels of memory $CD4^+$ T cells are 20-fold lower and gradually decline (15, 24). Our findings also differ from those for other acute viral infections in humans, including severe acute respiratory syndrome (SARS) coronavirus (35) and vaccinia virus (VACV) (38), where memory $CD8^+$ T cell responses are more frequent and greater in magnitude than $CD4^+$ T cell responses (10, 31).

We also found that the total LCMV-specific $CD8^+$ T cell response was augmented with increasing severity of disease, while the total $CD4^+$ T cell response was highly variable between the different donors. This variability could be due to the small size of the donor cohort analyzed. These findings are in agreement with studies on other acute virus infections in humans, including Sin Nombre virus and dengue virus, which have demonstrated that elevated levels of virus-specific $CD8^+$ T cells correlate with heightened disease severity (29). Infection with Sin Nombre virus, a hantavirus that causes fulminant life-threatening disease, results in high frequencies of tetramer-specific $CD8^+$ T cells in patients with severe hantavirus pulmonary syndrome compared to those in patients with moderate disease (29, 39, 40). Similarly, higher frequencies of circulating dengue virus-specific $CD8^+$ T cells are seen in patients with acute secondary dengue virus infection (40). Thus, whether disease outcome correlates with the virus-specific T cell response seems to depend on the infecting virus, and the fine balance between protective immunity and immunopathology. However, in our case, we have been able to study only infections resolved some time previously, so it is difficult to determine the importance of these cells in the development of acute infection and pathogenesis.

To our knowledge, this is one of the first studies where the differentiation phenotypes of human LCMV-specific $CD8^+$ and $CD4^+$ T cells have been compared following an acute, live nonattenuated virus infection in the absence of antigenic reencounter (14, 28, 36, 50). When examining the differences between effector memory and central memory on the basis of CD45RA, CCR7, and CD62L expression patterns, we found that LCMV-specific $CD8^+$ T cells had a predominantly effector memory phenotype, with the majority of tetramer-positive $CD8^+$ T cells reexpressing CD45RA. This $CD45RA^+$ effector memory phenotype confirms what was previously observed in vaccinees acutely infected with either Dryvax or YFV-17D (1, 13, 38, 44) and expands these observations to LCMV infection. Interestingly, this general observation held true for the three different donors, despite dramatic differences in the severity of disease associated with the infection. Interestingly, we noted that in some instances MHC-peptide tetramer staining detected a 5-fold-greater number of LCMV-specific T cells than was demonstrated to produce IFN-γ (HLA-B*1501-restricted $NP_{414-422}$ and HLA-A*0201-restricted Z_{49-58}). It has been previously reported that in some cases there is a 1:1 correspondence between the number of antigen-specific T cells detected by IFN-γ ELISPOT and tetramer staining assays, while in other cases this correspondence is not observed. As noted by Murali-Krishna et al. (42), in BALB/c mice infected with LCMV, both IFN-γ ELISPOT and tetramer staining detected approximately 50% of $CD8^+$ T cells recognizing the immunodominant epitope $NP_{118-126}$. However, in LCMV-infected C57BL/6 mice, 33% of $CD8^+$ T cells recognizing the immunodominant H-$2D^b$-restricted $NP_{396-404}$ epitope were detected by IFN-γ ELISPOT, while only 23% of $CD8^+$ T cells bound the H-$2D^b$ $NP_{396-404}$ tetramer. In humans, there is also a correlation between the number of antigen-specific T cells detected by IFN-γ ELISPOT and tetramer staining assays; however, the ratio is usually not 1:1. In agreement with our data, in HIV-infected individuals, tetramer staining detected approximately a 5- to 10-fold-greater number of $CD8^+$ T cells recognizing HLA-A02-restricted Gag and Pol-specific epitopes than were demonstrated to produce IFN-γ (22, 54). A larger number of antigen-specific T cells could be detected by tetramer staining assays, because tetramer-positive T cells might be producing other cytokines or no cytokines at all. Another possibility is that a portion of tetramer-positive T cells are no longer able to proliferate and are undergoing apoptosis.

The availability of novel MHC class II tetramers allowed us for the first time to evaluate the memory phenotype of virus-specific $CD4^+$ T cells resulting from an acute viral infection in humans. Importantly, we demonstrated that LCMV-specific $CD4^+$ T cells had an effector memory phenotype different from that of $CD8^+$ T cells. The majority of tetramer-positive $CD4^+$ T cells lacked CD45RA expression. The lack of CD45RA expression has been suggested to reflect a possibly less mature or differentiated effector memory phenotype (48, 53). Thus, it seems that following acute LCMV infection in humans, $CD4^+$ T cells have a less mature memory phenotype than $CD8^+$ T cells. Considering the phenotypic differences between $CD8^+$ and $CD4^+$ T cell subsets unearthed in the present study, this suggests that a more thorough examination of virus-specific $CD4^+$ T cells is needed in future studies.

The majority of LCMV-specific $CD8^+$ and $CD4^+$ T cells expressed high levels of both CD28 and CD27, which is consistent with the early differentiation phenotype also seen in influenza virus-infected individuals and Dryvax and YFD-17D vaccinees (1, 23, 38). We expanded on these findings by demonstrating that the level of CD28 and CD27 expression appears to correlate with disease outcome. Specifically, we found that the tetramer-positive $CD8^+$ T cells from AD124 were predominantly $CD28^-$ $CD27^-$, suggesting a late differentiated phenotype also seen in CMV infections (2). Interestingly, early $CD28^+$ $CD27^+$ differentiated antiviral T cells have been associated with protective immunity (47), suggesting that a more severe disease outcome, as seen in AD124, might be related to the quality of the antiviral $CD8^+$ T cell response.

Finally, CD127 and LAG3 expression also correlated with clinical manifestation. CD127 is reexpressed on antiviral $CD8^+$ T cells in resolved infections (8, 25) but is not present at significant levels on CMV- and EBV-specific $CD8^+$ T cells, demonstrating that persistent viral antigen suppresses CD127 expression (21, 34, 57). In our study, CD127 was expressed at high levels on LCMV-specific $CD8^+$ and $CD4^+$ T cells in the asymptomatic AD108 donor, while CD127 expression rapidly declined with increased disease severity in AD123 and AD124. LAG3 expression was also upregulated on all tetramer-positive T cell subsets examined, especially $CD8^+$ and $CD4^+$ T cells in donor AD123. Similarly, the upregulation of CD127 on HBV-specific $CD8^+$ T cells correlated with a loss of PD1 expression (8). These results suggest a reciprocal relationship between CD127 and inhibitory receptor expression.

In conclusion, these data collectively demonstrate for the first time that memory $CD8^+$ and $CD4^+$ T cells can be identified in infected humans. Most notably, the rapid identification of T cell epitopes from infected humans suggests that personalized prevention and treatment of virus infection are possible. In addition, this study not only confirms previous findings with live attenuated viral vaccines but also begins to show that disease outcome correlates with both the magnitude of the memory $CD8^+$ T cell response and the phenotypes of both LCMV-specific $CD8^+$ and $CD4^+$ T cells. These data suggest that a closer examination of LCMV-specific $CD8^+$ and $CD4^+$ T cells with a larger donor cohort is needed in future studies.

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