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Genome-Wide Analysis Reveals a Highly Diverse CD8 T Cell Response to Murine Cytomegalovirus[1]

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Human CMV establishes a lifelong latent infection in the majority of people worldwide. Although most infections are asymptomatic, immunocompetent hosts devote an extraordinary amount of immune resources to virus control. To increase our understanding of CMV immunobiology in an animal model, we used a genomic approach to comprehensively map the C57BL/6 CD8 T cell response to murine CMV (MCMV). Responses to 27 viral proteins were detectable directly ex vivo, the most diverse CD8 T cell response yet described within an individual animal. Twenty-four peptide epitopes were mapped from 18 Ags, which together account for most of the MCMV-specific response. Most Ags were from genes expressed at early times, after viral genes that interfere with Ag presentation are expressed, consistent with the hypothesis that the CD8 T cell response to MCMV is largely driven by cross-presented Ag. Titration of peptide epitopes in a direct ex vivo intracellular cytokine staining assay revealed a wide range of functional avidities, with no obvious correlation between functional avidity and the strength of the response. The immunodominance hierarchy varied only slightly between mice and between experiments. However, *H-2*^{b}-expressing mice with different genetic backgrounds responded preferentially to different epitopes, indicating that non-MHC-encoded factors contribute to immunodominance in the CD8 T cell response to MCMV. ***The Journal of Immunology,*** **2006, 176: 3760–3766.**

Human CMV (HCMV)[3] is a β-herpesvirus that establishes a lifelong latent infection in the majority of people worldwide. It is a major cause of congenital malformations and of opportunistic infection in the immunocompromised. Furthermore, HCMV has a unique immunobiology in asymptomatic carriers. HCMV elicits an extremely large CD8 T cell response that continues to expand throughout life (1). In the elderly, HCMV-specific T cells dominate the repertoire to such an extent that they may impair the ability to respond to other Ags (2, 3). Understanding the causes and consequences of the immune system's unique preoccupation with HCMV requires an experimental model.

MCMV is a natural pathogen of the laboratory mouse (*Mus musculis*). Closely paralleling HCMV epidemiology, MCMV establishes lifelong latent infection in the majority of wild mice and causes similar opportunistic infection upon immunosuppression. It also elicits an extremely large CD8 T cell response that continues to increase throughout life (4–6). Thus, although HCMV and MCMV diverged with their hosts ~70 million years ago at the time of the mammalian radiation, coevolution of virus and host has achieved a tight conservation of the characteristic CMV immunobiology.

To make best use of the immunologically powerful C57BL/6 mouse model, we undertook a comprehensive definition of *H-2*^{b}-restricted CD8 T cell epitopes from MCMV. We used a genomic approach that avoided heavy reliance on epitope prediction algorithms. In this study, we describe 24 *H-2*^{b}-restricted MCMV epitopes from 18 different viral proteins to which responses can be detected directly ex vivo during acute infection. Together, these account for the majority of MCMV-specific T cells during the acute response and comprise a much broader response in a single animal than has been described for any other infection. In addition to establishing a basis for the investigation of CMV immunobiology in C57BL/6 mice, this endeavor has generated a rare, comprehensive definition of an immunodominance hierarchy to a large DNA virus in its natural host.

Materials and Methods

Mouse and virus strains

C57BL/6, 129/SvJ, BALB/c, BALB.B (C.B10-*H2*^{b}/LilMcdJ), and B6.C-*H2d*/bByJ mice were purchased from The Jackson Laboratory. C57BL/6 *H-2*^{dxb} mice were the F_1 generation of C57BL/6 mice bred to B6.C-*H2d*/bByJ mice. BALB *H-2*^{dxb} mice were the F_1 offspring of BALB/c mice bred to BALB.B mice. $K^{b+/+}D^{b-/-}$ and $K^{b-/-}D^{b+/+}$ mice were a gift from D. Raulet (University of California, Berkeley, CA). Mice aged 6–12 wk were infected i.p. with 2×10^6 PFU MCMV strain MW97.01 (7). MW97.01 is derived from a bacterial artificial chromosome of Smith strain MCMV. All mice were housed at Oregon Health & Science University, and all studies were approved by the Institutional Biosafety Committee and Institutional Animal Care and Use Committee.

Cloning of MCMV open reading frames (ORFs) and ORF fragments

The MCMV genome contains 170 predicted ORFs (8), of which 10 have been cloned as described previously (9–11). The remaining 160 ORFs were amplified by PCR in the presence of 8% DMSO, using MCMV MW97.01 DNA as a template. Gel-purified products were then cloned into pcDNA3.1/V5-His-TOPO (Invitrogen Life Technologies) using the TOPO cloning kit. All ORFs were sequenced from the 5′ end to confirm correct

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[3] Abbreviations used in this paper: HCMV, human CMV; MCMV, murine CMV; E, early; IE, immediate E; L, late; VIPR, viral gene that interferes with Ag presentation; ORF, open reading frame; ICS, intracellular cytokine staining.

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orientation and reading frame. Approximately one-third of the genes were sequenced from the 5′ end only, another one-third from both the 5′ and the 3′ end, and the last one-third were sequenced completely. A number of ORF sequences differed from the published sequence, which may be due to differences in MCMV strains used. Eighteen ORFs were subcloned into three to five smaller ORF fragments such that each fragment encoded an N-terminal Met start codon and overlapped the neighboring fragments by at least 11 aa.

Peptide predictions and synthesis

Candidate epitopes were predicted using SYFPEITHI and/or BIMAS software (⟨www.mpiib-berlin.mpg.de/MAPPP//binding.html⟩). All 8-, 9-, and 10-mer peptides were synthesized as crude peptides (65–95% pure by HPLC) by Genemed Synthesis or JPT and confirmed by mass spectrometry. Overlapping 15-mer peptides were synthesized by JPT at 50 nmol scale.

Stimulation of splenocytes with transfected cells or peptides

K41 cells, an *SV-40*-transformed *H-2^b* fibroblast cell line (gift from M. Michalek, University of Alberta, Edmonton, Canada) were plated at 4000 cells per well in 96-well flat-bottom plates. One day later, each well was transfected with 500 ng of plasmid DNA and 1.25 μl of FuGene 6 (Roche). Two days later, 8×10^5 splenocytes from infected mice were added per well in the presence of brefeldin A (GolgiPlug; BD Pharmingen) and incubated for 6–7 h at 37°C. Duplicate wells were combined into a single well in 96-well round-bottom plates for intracellular cytokine staining (ICS). For peptide stimulation, peptide was added to splenocytes for 6–7 h in the presence of brefeldin A at a concentration of ~10 μM for overlapping 15-mer peptides, 1 μg/ml for other peptides, or as indicated in the figure legend.

Surface and intracellular cytokine staining

Splenocytes were surface-stained with CD8α (53-6.7), CD11a (2D7), CD11c (HL3), CD44 (IM7), CD62L (MEL-14), Ly6C (AL-21), NKG2A (20d5), and/or CD43 (1B11). ICS for IFN-γ (XMG1.2) was performed with the Cytofix/Cytoperm kit (BD Pharmingen). Samples were acquired on a FACSCalibur (BD Pharmingen) with CellQuest software (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Results

Use of a comprehensive MCMV ORF library identifies 27 Ags recognized by CD8 T cells in acutely infected C57BL/6 mice

To comprehensively identify CD8 T cell Ags from MCMV, we cloned each of the 170 ORFs predicted from the genomic sequence of MCMV (8) into a mammalian expression plasmid. Initial screening of this library with a previously characterized T cell clone led to the identification of m141 ^{16}VIDAFSRL^{23} as a major MCMV epitope in C57BL/6 mice (data not shown). During this process, we realized that CD8 T cells taken directly from MCMV-infected mice could respond to transfected cells, which enabled us to screen the library directly ex vivo. The ORF library was transfected into *SV40*-transformed *H-2^b* fibroblasts (K41 cells) in a 96-well plate format and incubated with splenocytes from acutely infected C57BL/6 mice. ICS for IFN-γ was used to score for recognition. Twenty-seven ORFs elicited a CD8 T cell response that was more than five times above the background response to *lacZ* (Fig. 1 and Table I).

We selected 18 of these Ags for further study: 14 that elicited the strongest responses (*m141, M57, M38, m139, M102, M86, M33, m164, M78, M100, M31, M44, M45*, and *M77*), two that were recognized by other CD8 T cell clones (*M36* and *M97*, data not shown), one that is also an Ag in BALB/c mice (*m04*), and one that scored positive in preliminary screens of a partial library (*M112*). We determined the MHC restriction for these using CD8 T cells from MCMV-infected $K^{b+/+}D^{b-/-}$ and $K^{b-/-}D^{b+/+}$ mice. A combination of subcloning and computer-based epitope prediction using SYFPEITHI and BIMAS software (see *Materials and Methods*) was then used to identify peptide epitopes. Recognized ORFs were subcloned into overlapping fragments that were tested for T cell recognition. From the positive fragments, the top peptide candidates predicted by BIMAS and SYFPEITHI were then synthesized and tested. This methodology allowed us to identify 14 additional CD8 T cell epitopes (Table I).

Identification of peptides not predicted by computer algorithms

A combination of subcloning and peptide synthesis was used to identify major epitopes from *M57*, *M38*, and *M102* that were not predicted by the computer programs. *M57* elicited the second-strongest CD8 T cell response (Fig. 1), which was K^b-restricted (not shown), but the major computer-predicted epitopes were not recognized. Two rounds of subcloning narrowed the recognized region to residues 776–846, and a screen of 15-mer overlapping peptides mapped the determinant to residues 816–825 (Fig. 2*A*). All possible 10-, 9-, and 8-mer peptides from this region were

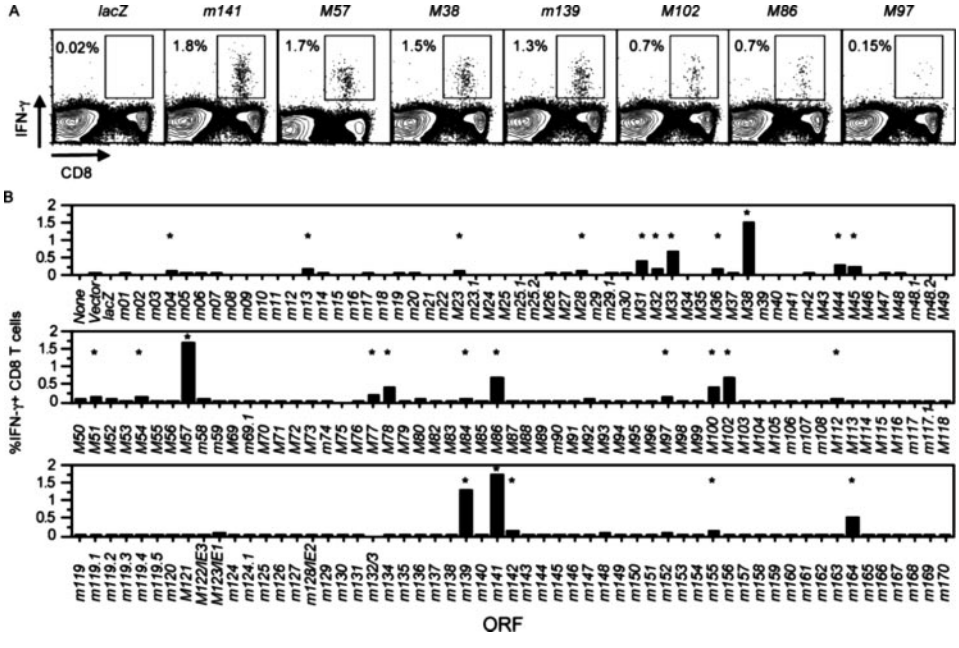


FIGURE 1. Use of the MCMV ORF library to identify CD8 T cell Ags directly ex vivo. Splenocytes from three MCMV-infected C57BL/6 mice (day 7) were pooled, incubated with K41 cells transfected with the indicated ORFs in the presence of brefeldin A, then stained for surface CD8 and intracellular IFN-γ. *A*, FACS plots showing the response to *lacZ* (background) and representative positive ORFs. *B*, Percentage of CD8 T cells responding to each ORF. The asterisk denotes ORFs to which the response was greater than five times above background. Results are representative of three similar experiments.

Table I. *Identified epitopes and Ags from the acute CD8 T cell response to MCMV in C57BL/6 mice*[a]

D^b-Restricted Epitopes

ID Rank	ORF	Amino Acids	Peptide Sequence	EC_{50} (log_{10} M)	T cell Clone	SYFPEITHI Rank	SYFPEITHI Score	BIMAS 9-Mer Rank	BIMAS 9-Mer Score	BIMAS Revised Rank	BIMAS Revised Score	HCMV Homolog	Family and/or Proposed Function
1	*M45*	985–993	HGIR**N**ASF**I**	−7.0	3,55	1	28	1	780	1	762	*UL45* (RRL)	Ribonucleotide reductase homolog
7	*M86*	1062–1070	SQNI**N**TVE**M**	−10.5		T2	26	T8	220	2	1125	*UL86* (MCP)	Major capsid protein
8	*M33*	47–55	GGPM**N**FVV**L**	−8.3		1	27	1	936	3	302	*UL33* (GCR)	Spliced GCR
9	*M112*	171–179	AAVQSATS**M**	−6.3		2	22	5	10	4	22	*UL112* (e1)	
10	*M44*	130–138	ACVH**N**QDI**I**	−8.2		T1	25	1	660	1	1003	*UL44* (DPAP)	DNA binding protein
14	*m164*	267–275	WAVN**N**QAIV	−8.7		5	20	4	39	2	108		Putative membrane glycoprotein
15	*M77*	474–482	GCVK**N**FEF**M**	−9.0		1	27	1	864	1	1024	*UL77*	Pyruvoyl decarboxylase homolog
16	*m04*	1–9	MSLVCRLV**L**	−5.5		T13	16	8	9	2	72		MHC I immune evasion
18	*M36*	213–221	GTVI**N**LTSV	−9.4		2	23	4	36	11	17	*UL36* (GF2)	US22 family homolog
ND	*M102*	436–445[a]				>19	<17	>15	<9	>64	<1	*UL102 (HP)*	Helicase-primase component

K^b-Restricted Epitopes

ID Rank	ORF	Amino Acids	Peptide Sequence	EC_{50} (log_{10} M)	T cell Clone	SYFPEITHI Rank	SYFPEITHI Score	BIMAS 8-Mer Rank	BIMAS 8-Mer Score	BIMAS 9-Mer Rank	BIMAS 9-Mer Score	HCMV Homolog	Family and/or Proposed Function
2	*m139*	419–426	TV**Y**G**F**CL**L**	−8.7		1	26	1	50			*US22* (GF2)	US22 family homolog
3	*M57*	816–824	SCLE**F**WQR**V**	−8.7	5	NP	NP			T248	0.2	*UL57* (MDBP)	Major DNA binding protein
4	*m141*	16–23	VIDA**F**SR**L**	−9.0	11	T1	22	T17	3			*US24* (GF2)	US22 family homolog
5	*M38*	316–323	SSPPMFR**V**	−8.0		76	9	T61	0.3			*UL38*	
6	*M78*	8–15	VD**Y**S**Y**PE**V**	−10.3		2	22	T7	10			*UL78*	GCR homolog
11	*M100*	72–79	RIID**F**DN**M**	−7.0		T3	21	1	19			*UL100* (gM)	Glycoprotein M
12	*m164*	283–290	GTTD**F**LW**M**	−5.5		T4	17	3	12				Putative membrane glycoprotein
13	*M97*	210–217	IISP**F**PG**L**	−5.7	96	T2	22	4	13			*UL97* (PK)	Phosphotransferase
17	*M38*	38–45	ST**Y**T**F**VRT	−5.7		T4	17	5	7			*UL38*	
ND	*M102*	446–455	SIVDLR**F**AV**L**	−8.2		NP	NP	NP	NP	NP	NP	*UL102 (HP)*	Helicase-primase component
ND	*M31*	297–305	VAPD**F**GVR**M**	−8.7		NP	NP			T7	3.6	*UL31*	
ND	*M102*	486–500[a]				>144	<8	>96	<0.3	>10	<4	*UL102 (HP)*	Helicase-primase component
ND	*M31*	276–285[a]				>84	<9	>68	<0.4	>80	<0.3	*UL31*	
ND	*M31*	341–355[a]				>96	<8	>124	<0.1	>127	<0.1	*UL31*	

Unmapped Ags

ORF	HCMV Homolog	Family and/or Proposed Function
m13		Glycoprotein family m02
M23	*UL23* (GF2)	US22 family homolog
M28	*UL28*	
M32	*UL32* (pp150)	Tegument protein of HCMV
M51	*UL51*	
M54	*UL54* (DNA pol)	DNA polymerase Δ subtype
M84	*UL84*	Early nuclear nonstructural protein
m142		
m155		

[a] ND, Immunodominance rank not determined. Peptide sequence: (bold) preferred anchor residues; (underline) preferred auxiliary anchor residues; EC_{50}, peptide concentration yielding half maximal response by ex vivo ICS; T cell clone, antigens recognized by clones described in our previous publications; SYFPEITHI and BIMAS rank and score, rank among all possible peptides within the ORF; T, a tie; NP, not predicted (SYFPETIHI ranks only 8-mer peptides for K^b and 9-mer peptides for D^b); a, epitopes for which the minimal peptide is not known. For these, the highest ranked peptides that have been excluded are indicated.

synthesized and tested. The 9-mer ^{816}SCLEFWQRV^{824} was recognized at the highest dilution ($10^{-8.7}$M) and, hence, is most likely the naturally processed epitope.

Subcloning indicated that *M38* encodes two K^b-restricted epitopes. One, ^{38}STYTFVRT^{45}, was predicted by both BIMAS and SYFPEITHI, but the more dominant second epitope was not predicted by either software program. Subcloning mapped the determinant to aa 316–324. A series of 8-, 9-, and 10-mer peptides covering residues 316–325 was synthesized and tested (Fig. 2*B*). Residue 316 was clearly required as the N terminus, but surprisingly, no major difference in titration was seen between peptides terminating at V323 (P8), P329 (P9), or V325 (P10). We have not yet confirmed the identity of the naturally processed epitope but tentatively conclude that it is likely to be the 8-mer SSPPMFRV, with the P5 methionine occupying the pocket normally occupied by a phenylalanine residue.

A similar approach identified a 10-mer peptide, ^{446}SIVDLRFAVL^{455}, as a K^b-restricted epitope from M102 (Fig. 2*C*). This was especially striking, given that the last eight residues of this peptide (VDLRFAVL) fit the K^b motif well, yet the 8-mer was not recognized. Finally, subcloning has identified five more peptide regions from M31 and M102 that contain epitopes, from which we have not yet mapped four of the minimal determinants. The highest ranked predicted epitopes from M31 and M102 have been excluded for these four determinants (Table I), indicating that these epitopes are also poorly predicted by the programs.

Eighteen identified peptide epitopes account for the majority of the MCMV-specific CD8 T cell response in C57BL/6 mice

Incubation of splenocytes from acutely infected mice with 18 identified epitopes yields a characteristic immunodominance hierarchy (Fig. 3*A*). The sum of the responses to individual epitopes was 51% of all CD8 T cells. We wanted to determine the percentage of the total CD8 T cell response to MCMV that could be accounted for by these epitopes. Cells that newly express activation markers after MCMV infection are likely to be specific for MCMV, because adoptively transferred TCR transgenic CD8 T cells (OT-I) of irrelevant Ag specificity showed little bystander activation following MCMV infection (12). Therefore, to estimate the total response to MCMV, we assessed the expression of seven activation markers on CD8 T cells as a surrogate for Ag specificity (Fig. 3, *B* and *D*). When corrections were made for expression of the activation marker by naive T cells (false positives) and for Ag-specific T cells that failed to express each marker (false negatives)

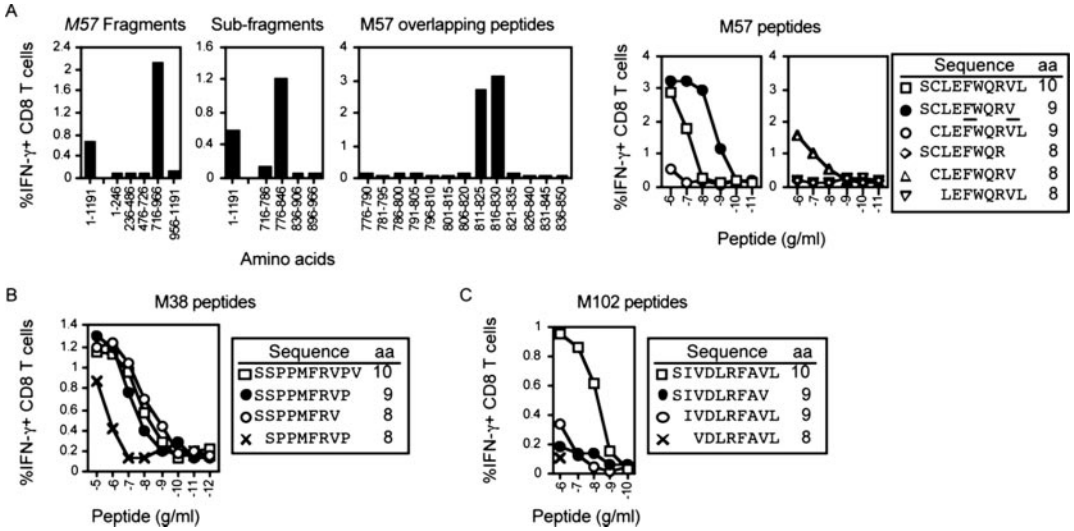


FIGURE 2. Identification of epitopes not predicted by computer algorithms. *A*, M57 fragments and overlapping peptides were tested by ex vivo ICS to map the epitope to aa 816–825. The 8-, 9-, and 10-mer peptides covering this region were titrated and tested. A similar procedure was followed for M38 (*B*) and M102 (*C*). The final peptide titrations for each are shown. Results are representative of two or more similar experiments.

(Fig. 3*C*; see the figure legend for details), we calculated that between 49 and 60% of CD8 T cells were specific for MCMV. The results were remarkably consistent between different activation markers (Fig. 3*D*). The ICS assay may overestimate the percentage of T cells responding to peptide during the acute response to infection, when most effector T cells are highly susceptible to apoptosis, because there is selective loss during the in vitro incubation period of activated CD8 T cells that are not rescued by cognate Ag. Thus, the true sum of responses to the individual epitopes in Fig. 3*A* is likely to be somewhat <51%. However, we can conclude that the identified epitopes account for the majority of the MCMV-specific CD8 T cell response.

Immunodominance cannot be predicted by functional avidity

T cell functional avidity, or the concentration of peptide required to elicit half-maximal IFN-γ production, depends both on the affinity of peptide for MHC and the TCR affinity for the peptide-MHC complex. To assess the relationship between functional avidity and rank in the immunodominance hierarchy, we quantified the frequency of T cells that could produce IFN-γ over a range of peptide concentrations (Fig. 4*A* and Table I). The peptide concentration yielding half-maximal stimulation (EC_{50}) varied markedly between different peptides, from $10^{-5.5}$ to $10^{-10.5}$. However, there was no obvious correlation between the EC_{50} and each epitope's rank in the immunodominance hierarchy (Fig. 4*B*).

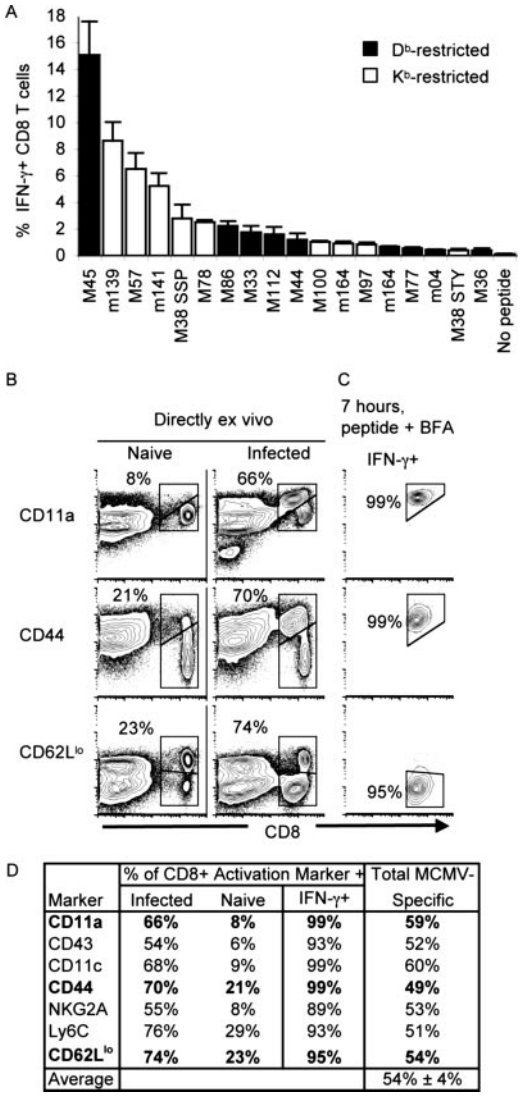
Immunodominance is affected by genes encoded outside the MHC

To assess the influence of genes outside the MHC on the immunodominance hierarchy among CD8 T cell epitopes, we infected two strains of mice, 129/SvJ and BALB.B, which share the *H-2*^{b} haplotype with C57BL/6 mice but differ in their non-MHC genes. The CD8 T cell response to seven individual epitopes was assessed in each strain (Fig. 5*A*); and four further D^b-restricted and three further K^b-restricted epitopes were assessed as pooled peptides. Although mice of each strain responded to all epitopes tested, the hierarchy differed significantly between strains. For example, whereas the response to M45 was always greater than the response to m139 in C57BL/6 mice, the reverse was true in 129/SvJ mice. We also noted that the total size of the measured CD8 T cell response was highest in C57BL/6 and lowest in BALB.B mice.

In BALB/c mice, the acute CD8 T cell response is dominated by two epitopes: an L^d-restricted epitope from pp89/IE1 (encoded by *m123*) and a D^d-restricted epitope from m164 (13). In contrast, we identified no epitopes in C57BL/6 mice that are encoded by known immediate early genes. To compare the impact of non-MHC genes on the relative immunodominance of these Ags, we bred F_1 mice expressing both *H-2*^{b} and *H-2*^{d} haplotypes on either a BALB or C57BL/6 background. These mice were tested for their response to four *H-2*^{b}-restricted and four *H-2*^{d}-restricted epitopes. In mice carrying BALB background genes, the prototypical BALB/c H-2^d restricted epitopes dominated the response (Fig. 5*B*). In contrast, in mice carrying C57BL/6 background genes, responses to *H-2*^{b}-restricted epitopes were codominant with *H-2*^{d}-restricted epitopes. Again, the total size of the measured CD8 T cell response was much higher in mice of the C57BL/6 background. These results further confirm that non-MHC genes can have a marked impact on the immunodominance of CD8 T cell epitopes. They also indicate that the lack of IE Ags in C57BL/6 mice is probably due to a lack of peptides from these proteins that bind well to K^b and D^b, rather than a bias against IE Ags per se.

Discussion

The results presented here represent a comprehensive description of a CD8 T cell immunodominance hierarchy to a large DNA virus in its natural host. The phenomenon of immunodominance, or how the immune system chooses which epitopes from an infectious agent to respond to, remains poorly understood (14, 15). CD8 T cell responses often are remarkably focused on apparently few epitopes, even for complex organisms with large genomes. For example, the response to MCMV in BALB/c mice is thought to be dominated by two epitopes, pp89 and m164 (5). The response to HSV-1 in C57BL/6 mice may be even narrower. Some evidence suggests that a single epitope may account for 60–90% of the response (16), even though HSV-1 encodes >70 other proteins. Finally, a recent analysis of the CD8 T cell response to vaccinia virus in C57BL/6 mice identified only one major immunodominant epitope from 256 ORFs and only five epitopes overall (17). In contrast, the CD8 T cell response to MCMV in C57BL/6 mice described here is strikingly broad. Responses to 27 individual Ags were clearly detectable directly ex vivo (Fig. 1). Responses to 18



D

Marker	% of CD8+ Activation Marker +			Total MCMV-Specific
	Infected	Naive	IFN-γ+	
CD11a	**66%**	**8%**	**99%**	**59%**
CD43	54%	6%	93%	52%
CD11c	68%	9%	99%	60%
CD44	**70%**	**21%**	**99%**	**49%**
NKG2A	55%	8%	89%	53%
Ly6C	76%	29%	93%	51%
CD62Llo	**74%**	**23%**	**95%**	**54%**
Average				54% ± 4%

FIGURE 3. Eighteen identified epitopes account for the majority of the MCMV-specific CD8 T cell response. *A*, Definition of the acute immunodominance hierarchy. Splenocytes from acutely infected mice ($n = 4$) were incubated with the indicated peptides and analyzed by ICS. The graph shows the mean ± SD of individual mice. *B*, De novo expression of activation markers by CD8 T cells following MCMV infection. In the same experiment as *A*, uncultured CD8 T cells from naive ($n = 3$) or infected ($n = 4$) mice were surface stained for seven activation markers. Representative staining from naive and infected mice is shown for three markers. Numbers represent the percentage of CD8 T cells expressing the activation marker. *C*, Expression of activation markers on Ag-specific CD8 T cells. Splenocytes incubated with M45 peptide were costained for activation markers. For all markers, similar staining was obtained with CD8 T cells specific for two to five additional peptides (data not shown). *D*, Use of de novo expression of activation markers to estimate the total MCMV-specific CD8 T cell response. The percentage of CD8 T cells that expressed the activation marker as a result of MCMV infection was calculated by subtracting the percentage of CD8 T cells in naive mice that expressed the marker from the percentage of CD8 T cells in infected mice that expressed the marker. To estimate the percentage of MCMV-specific CD8 T cells, the result was corrected for false negatives by dividing by the percentage of IFN-γ^+ cells that expressed the marker. Results are representative of three similar experiments.

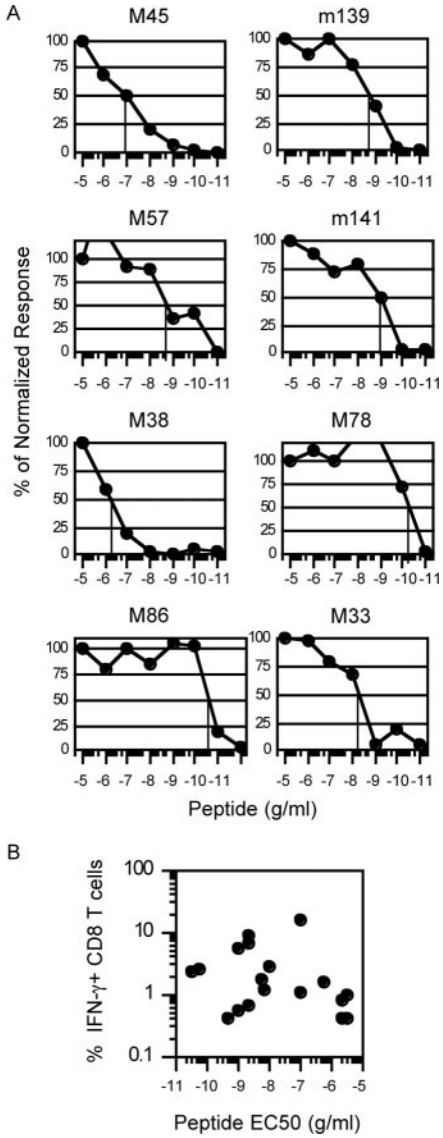


FIGURE 4. CD8 T cell functional avidity for peptide-MHC complexes does not predict the MCMV epitope immunodominance hierarchy. In the same experiment as Fig. 3, splenocytes from the four acutely infected mice were pooled and incubated with various concentrations of the 18 peptides shown in Fig. 3*A*. After a 7-h incubation in brefeldin A with peptide, cells were analyzed by ICS. The percentage of IFN-γ^+ CD8 T cells at each peptide concentration was normalized to the response at the highest peptide concentration, after subtracting background (no peptide) staining from all samples. *A*, The functional avidity curves for the first eight peptides in the immunodominance hierarchy from Fig. 3*A* are shown as representatives. *B*, The size of the CD8 T cell response, as determined in Fig. 3*A*, was plotted as a function of CD8 T cell functional avidity (EC_{50}) for all 18 peptides tested in Fig. 3*A*.

of these Ags have been mapped to peptide epitopes, to which responses are detected in MCMV-infected but not naive mice (Table I and data not shown). The D^b-restricted $M45_{985–993}$ epitope was clearly dominant but constituted <25% of the MCMV-specific response and was followed by five K^b-restricted subdominant epitopes (*m139*, *M57*, *m141*, *M38*, and *M78*) that each elicited a substantial CD8 T cell response.

We consider it unlikely that the broad response we have identified here is an exception to the more general rule of narrow immunodominance. A recent comprehensive analysis of the T cell response to HCMV in seropositive, chronically infected adults indicated that subjects mounted a CD8 T cell response against a median of 8 ORFs. However, the number of Ags recognized varied widely between individuals, ranging from 1 to 39 ORFs (18).

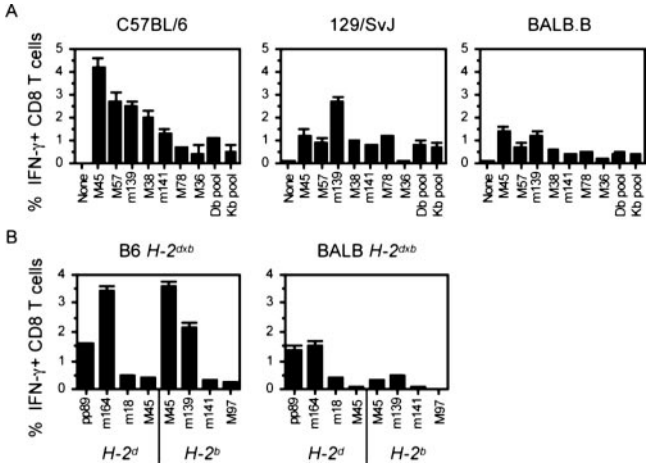


FIGURE 5. Genes not linked to the H-2 complex affect immunodominance. *A* and *B*, The indicated mouse strains were infected with MCMV for 7 days, and the CD8 T cell response to various peptides was determined by ICS. Peptides from *M33*, *M44*, *m164*, and *m04* were included in the D^b pool, and peptides from *M100*, *M97*, and *m164* were included in the K^b pool. Results are representative of two similar experiments.

When sensitive screening methodologies such as the one used here are applied to other systems, we suspect that similarly broad responses will be uncovered in many cases. However, the experience with HCMV (18), and with vaccinia (17) and HSV-1 (16) in C57BL/6 mice, suggests that in some instances even sensitive screening techniques will still reveal a highly focused response.

In contrast, pathogens with smaller genome sizes might generally be expected to elicit a narrower response. Lymphocytic choriomeningitis virus encodes ~3,400 amino acids, compared with MCMV's ~68,000. Accordingly, the CD8 T cell response to lymphocytic choriomeningitis virus is focused on only three epitopes in BALB/c and six epitopes in C57BL/6 mice, even though >50% of CD8 T cells are virus specific (19). Similarly, for influenza virus (~4200 aa), seven epitopes have been identified for C57BL/6 mice (20)

What sort of viral proteins dominate the CD8 T cell response to CMVs? HCMV and MCMV genes are expressed in three temporal waves, early (E), immediate early (IE), and late (L). MCMV, and HCMV both encode viral genes that interfere with MHC class I Ag presentation (VIPRs) (21, 22), which are expressed during the E phase. If CD8 T cell priming occurs predominantly via infected APCs, IE Ags or structural virion proteins, both of which could be presented before VIPR expression, would be predicted to dominate the CD8 T cell response. Indeed, by screening for Ags that were presented by infected cells (where VIPRs were functioning), the first Ags identified for both HCMV and MCMV fell into these classes (23–25). However, the data presented here are in keeping with recent reports that cast this model into doubt. Previously, we reported no difference in the size of the CD8 T cell response to M45 in C57BL/6 mice infected with either wild-type MCMV or a mutant lacking the VIPR *m152* (12), even though *m152* profoundly impairs the presentation of this epitope both in vitro (12) and in vivo (26). Similarly, when an HCMV mutant lacking VIPRs was used to detect responses, it was found that CD8 T cells from HCMV-infected subjects recognized a wide variety of IE- and E-encoded Ags, even though T cells specific for E Ags could not recognize wild-type virus-infected cells (27). Recently, a genome-wide survey of HCMV CD8 T cell response using overlapping peptides also has detected a wide variety of IE, E, and some L Ags (18).

Although the transcriptional class of many MCMV ORFs has not been verified experimentally, based on homology with HCMV, it is clear that the vast majority of the 27 Ags identified in this study (Fig. 1*B*) are encoded by E genes. Interestingly, no IE Ags were identified. The lack of IE Ags in C57BL/6 mice is probably due to a lack of peptides from these proteins that bind well to K^b and D^b, rather than a bias against IE Ags per se, because $H\text{-}2^b \times H\text{-}2^d$ F1 mice on a C57BL/6 background responded equally well to the L^d-restricted IE1 Ag and D^b-restricted E Ag M45 (Fig. 5*B*). Among the E-encoded Ags, there was no discernable preference for class of protein: nuclear, cytosolic, and membrane proteins are all represented (Table I). Of note, only 12 of the 27 identified Ags were detected in MCMV virions (28), which suggests that processing of epitopes from virion proteins is not a major route of CD8 T cell priming. These results are consistent with the idea that most of the CD8 T cell response to MCMV is primed by cross-presented Ag (12), with a large percentage of viral proteins being candidates for recognition.

Once the parental protein Ag had been identified, we used epitope prediction programs to identify the minimal determinant (Table I). Our experience underscores both the utility and the limitations of these programs. Nine of 10 D^b-restricted epitopes were predicted by BIMAS and/or SYFPEITHI, but only 8 of 14 K^b-restricted epitopes were predicted. The reason that K^b-restricted epitopes were not predicted appears to be predominantly based on size; several of our epitopes were longer than the canonical K^b-binding 8-mer peptides. If the CD8 T cell response to MCMV is largely primed by cross-presented Ag, one intriguing possibility is that epitopes derived from cross-presented Ag may differ from those that are conventionally processed. For example, N-terminal trimming could be inefficient in the cross-presenting Ag processing compartment.

The basis for the immune system's choice of particular peptides to be immunodominant CD8 T cell epitopes also remains poorly understood. Peptide titration experiments (Fig. 4) revealed that the functional avidity of CD8 T cells for their cognate peptide-MHC was a poor predictor of immunodominance. It also was intriguing that non-MHC-encoded genes influenced both the size of the overall CD8 T cell response and the order of the immunodominance hierarchy (Fig. 5). The reason for these differences is not clear. One possibility is that the CD8 T cell response is influenced by the efficacy of NK cell control of MCMV during acute infection. NK cells from C57BL/6 mice express the activating NK cell receptor Ly49H, which can be activated by the MCMV protein m157 on infected cells, rendering this strain more resistant to MCMV than most other strains (29, 30). $CD8\alpha^+$ DCs are uniquely competent for cross-presentation (31) and CD8 T cell priming in various infections (32). Following MCMV infection, this subset is depleted from the spleens of $Ly49H^-$ mouse strains but is preserved in $Ly49^+$ strains (33). Thus, because BALB and 129/SvJ mice have much higher viral loads, higher levels of viral Ag, and fewer $CD8\alpha^+$ DCs after MCMV infection, it is perhaps not unexpected that the CD8 T cell immunodominance hierarchy would be different from that of C57BL/6 mice (Fig. 5). We are currently investigating the effect of $Ly49H^+$ NK cells on CD8 T cell priming. However, we note that the responses in BALB.B and 129/SvJ mice also differ from each other, indicating that genes other than *Ly49H* are most likely involved as well.

Historically, research interest in HCMV has focused on its pathogenesis in the immunocompromised, such as transplant and AIDS patients. Recent interest also has focused on the consequences of HCMV infection in apparently asymptomatic individuals. In particular, the massive size of the CMV-specific T cell response in asymptomatic carriers has only recently been appreciated. This response may contribute to immunopathology in vascular and other diseases, and it may come to dominate the T cell

repertoire in the elderly to such an extent that it compromises immunity to other agents (2). However, in human infection, these possibilities remain largely a matter for speculation. Our description of the CD8 T cell response to MCMV in C57BL/6 mice should facilitate the use of this powerful immunological model in understanding the immunobiology of HCMV carriage, in both health and disease.

Disclosures

The authors have no financial conflict of interest.

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