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Vaccination with synthetic constructs expressing cytomegalovirus immunogens is highly T cell immunogenic in mice

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Abbreviations: CMV, human cytomegalovirus; E-DNA, enhanced DNA; EP, in vivo electroporation; Nabs, neutralizing Abs; gC, high molecular weight complexes

There is no licensed vaccine or cure for human cytomegalovirus (CMV), a ubiquitous β-herpesvirus infecting 60–95% of adults worldwide. Infection can cause congenital abnormalities, result in severe disease in immunocompromised patients, and is a major impediment during successful organ transplantation. In addition, it has been associated with numerous inflammatory diseases and cancers, as well as being implicated in the development of essential hypertension, a major risk factor for heart disease. To date, limited data regarding the identification of immunogenic viral targets has frustrated CMV vaccine development. Based upon promising clinical data suggesting an important role for T cells in protecting against disease in the transplantation setting, we designed a novel panel of highly-optimized synthetic vaccines encoding major CMV proteins and evaluated their immune potential in murine studies. Vaccination induced robust CD8+ and CD4+ T cells of great epitopic breadth as extensively analyzed using a novel modified T cell assay described herein. Together with improved levels of CMV-specific T cells as driven by a vaccine, further immune evaluation of each target is warranted. The present model provides an important tool for guiding future immunization strategies against CMV.

Human cytomegalovirus (CMV), also known as human herpesvirus 5, is a ubiquitous betaherpesvirus that has been the subject of roughly 50 y of clinical research.[1] Infection is lifelong and there is no licensed vaccine or cure.[2-5] It is highly seroprevalent in the human population (> 95% in developing countries) and has been estimated to infect 50–80% of adults over the age of 40 in the US alone (www.cdc.gov/CMV/index.html). Overt infection occurring during primary infection or reactivation can lead to a number of clinical diseases.[2,6] As a member of the 'TORCH' infections, it can lead to numerous congenital abnormalities including death,[7] and has been associated with cerebral palsy[8] and medulloblastoma.[9] It can also result in severe disease and death in immunocompromised and in HIV-infected individuals,[10,11] as well as being a major impediment during successful organ transplantation.[2] Haematopoietic and solid organ transplant recipients are most at risk for disease ranging from acute and chronic graft rejection, accelerated coronary artery disease after heart transplantation, and new onset diabetes mellitus.[12] Recently, a therapeutic CMV DNA vaccine encoding the gB and pp65 proteins, and formulated with poloxamer CRL1005 and benzalkonium chloride was shown to be immunogenic in a phase I trial,[13] then later demonstrated a significant reduction in occurrence and duration of episodes of CMV viremia for patients undergoing hemopoietic stem-cell transplantation in a recent phase 2, placebo-controlled trial.[14] Despite promising clinical data, the lack of an effective vaccine requires in some cases that antiviral treatments during transplantation be used which have demonstrated low clinical efficacy, can be highly toxic, can lead to invasive bacterial and fungal infection, are not suitable for long-term use, and contribute to the development of CMV drug resistance.[15-17] Development of an effective CMV vaccine would he highly desirable and may allow the reduction and restriction of antiviral treatments.

While it was widely held that latent or asymptomatic infection was virtually benign in healthy individuals, low-grade "micro-infection" with CMV can only be detected by highly sensitive assays[3,18] and has been implicated as a causative factor rather than an epiphenomenon in certain cancers, inflammatory, and hypertensive and pulmonary diseases.[3,9,18-20] As such, CMV has been directly implicated in essential hypertension,[3] which is a prevalent

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risk factor for a variety of cardiovascular diseases including stroke, coronary heart disease and renal and heart failure, affecting > 1 billion adults worldwide and costing the US an estimated $444 billion in 2010 (www.cdc.gov/chronicdisease/resources/publications/aag/dhdsp.htm).[21] This new evidence supports previous studies that have demonstrated pulmonary shedding of virus in asymptomatic patients[22] and has implicated CMV infection in atherosclerosis, coronary heart disease and cardiac transplant arteriopathy.[20,23-25] Furthermore, CMV genome and proteins are present in certain malignant tumors including colon cancer,[19] malignant glioblastoma,[26] medulloblastoma,[9] EBV-negative Hodgkins lymphoma,[27] prostatic carcinoma,[28] and colon and breast cancer,[29] in which virus-mediated "oncomodulation" may or may not have played a role in the cancer itself, but certainly in altering the progression of disease. CMV microinfection has also been associated with inflammatory bowel disease (90% of patients had active infection in bowel)[30] and can be detected in inflamed tissues in patients with rheumatoid arthritis, Sjögren's syndrome, dermato- and polymyositis, psoriasis, Wegener's granulomatosis, ulcerative colitis, and Crohn's disease.[18] The US Institute of Medicine and the US National Vaccine Program Office have ranked it with the highest priority in terms of health care dollars saved and improvement in quality adjusted life years.[31] An effective CMV vaccine could prove highly valuable, reducing congenital diseases and long-term sequelae, improving longevity of transplant patients and the transplants, as well as saving billions in annual healthcare costs and disability adjusted loss years in the developing world.[21]

The lack of a licensed vaccine for CMV has been complicated by the task of vaccine target selection due to the relative complexity of the CMV genome and its numerous glycoproteins associated with cell tropism and entry.[32] However, promising clinical[14,33,34] and preclinical[35-37] data suggest that an effective vaccine will induce both cellular and humoral immune responses. Recently, a purified recombinant CMV gB protein plus MF59 adjuvant (a sequalene-in-water emulsion) vaccine was immunogenic in phase I trials,[38-40] and in a phase II trial, demonstrated a 50% protection against virus transmission to women of child-bearing age.[34] While the protection rate achieved by this vaccine was much lower than desired for women at risk for CMV during pregnancy, it is the first to demonstrate protective efficacy of the gB immunogen in the clinic. Futher, CTLs and NAbs have been shown to aid in control and reduction of disease[2,14,41-44] and are critically dependent on adequate CD4 T cell help.[37,42,45] NAbs are also likely crucial for providing sterilizing immunity against viral transmission.[33,34,46,47]

To help in the identification and development of target CMV immunogens, we assembled a novel panel of CMV Ags including mainly glycoproteins, as well as matrix and charerones.[32,48] Each Ag gene was genetically-optimized and consensus-engineered,[49-51] and then administered as enhanced DNA (E-DNA) vaccines for immune evaluation in preclinical "proof-of-concept" rodent studies. Using a novel T cell assay described herein, it was determined that constructs elicited robust and highly-diverse T cell responses. Responses were constituted by both CD8+ and CD4+ T cells, both of which are considered critical in providing protection against post-transplant occurrence of CMV disease.[37,42,45] In addition, a majority of murine T cell epitopes identified herein also contained HLA that have previously been reported to contribute to the suppression of viremia and amelioration of overt disease.[2,14,41-44] These data suggest studies investigating the potential for generating vaccine-induced humoral immunity as well as protection in preclinical challenge models are warranted.

Results

Construction of CMV immunogen panel. We developed a novel panel of CMV immunogens comprised of mainly surface-associated proteins, as well as matrix and chaperones. Due to the complexity of the glycoproteins that may be involved in viral entry,[32,52] we selected a panel of traditional and novel neutralization-sensitive candidates for screening as vaccine targets for cellular immunity.[48] Structural glycoproteins typically grouping in high molecular weight complexes (gC) included gB (ORF UL55), gH (ORF UL75), gL (ORF UL115), gM (ORF UL100), gN (ORF UL73), and gO (ORF UL74).[46,47] The gB (gC-I) is an integral membrane protein that homodimerizes to form the Type 1 membrane protein, is essential for both in vivo and in vitro replication, is implicated in virus attachment, is required for entry and cell fusion, can be detected on the surface of both infected cells and virions,[53] represents a major target for NAbs,[46,54-56] and has been a predominant core component of CMV vaccine platforms.[35] Glycoproteins gM and gN (gC-II) heterodimerize to form the CMV "viral infectivity complex" required for virion assembly and egress and have been shown to elicit binding Abs during infection.[57] As a novel component for an CMV vaccine, glycoproteins gH, gL, and gO (gC-III) can heterotrimerize to form the gCIII fusion complex. During infection, gH is a potent immunogen as the target of NAbs that likely function by blocking a CMV post-attachment event such as membrane fusion or virus penetration.[58] However, it is known that co-expression of gL and gH is important for macromolecule expression since as gL serves as a chaperone for gH.[48] In addition, the chaperone proteins encoded by ORFs UL128-UL131A (UL128, UL130 and UL131A) were chosen since they facilitate gO in the formation of the gCIII complex, can incorporate into gH/gL or gH/gL/gO which alters viral tropism from fibroblasts to epithelial and endothelial cells,[46,56,59] and may serve as T cell targets. Expression of gO is not required for the production of infectious virus in vitro, but deficient mutant viruses are growth impaired.[60] While co-expression of gH and gL along with gB constitute the core fusion machinery, both are necessary and sufficient for fusion.[32] Lastly, the lower matrix protein pp65 (ORF UL83) was included in this study since it has been reported to elicit strong T cell epitopes and is a major component of current vaccine strategies.[35]

CMV immunogen consensus-engineering. To maximize the potential for broadly-reactive immunity, we generated amino acid consensus sequences from CMV clinical strains, and excluded those from potentially-divergent, highly-passaged lab-adapted strains,[49-51,61,62] a strategy shown previously to enhance protection against divergent strains of influenza and HIV.[63,64] Phylogenetic

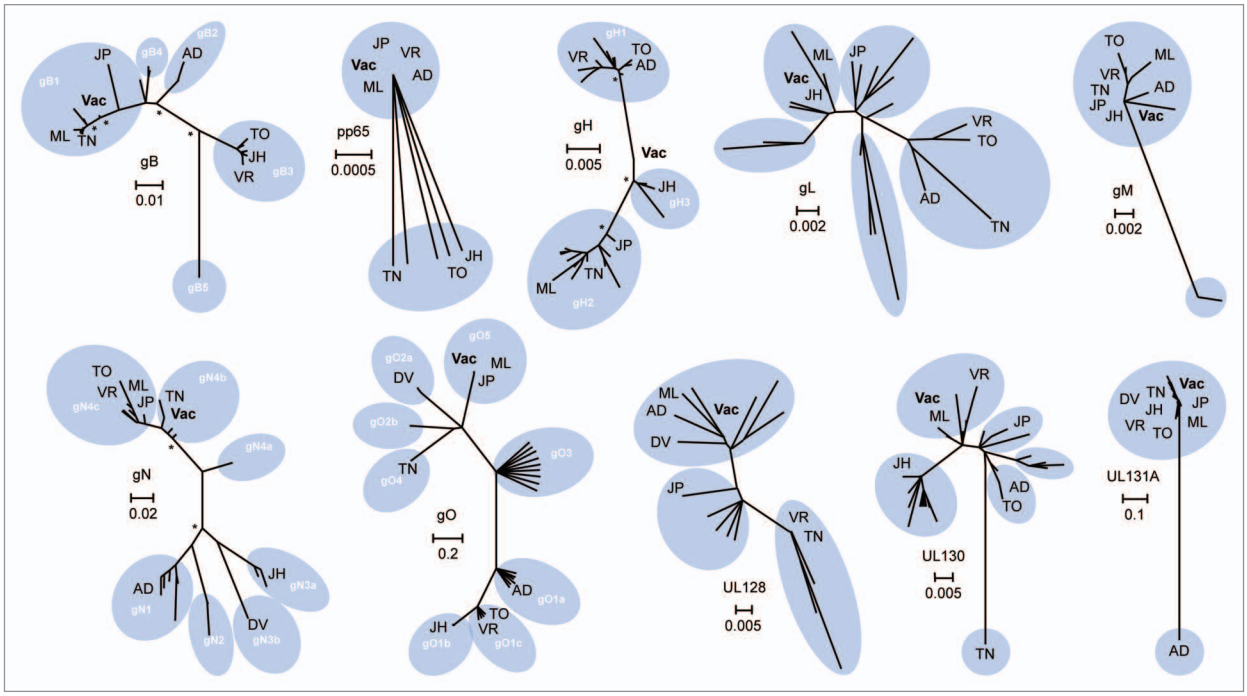

Figure 1. Phylogenetic analysis and consensus engineering of CMV immunogens. Phylogenetic trees of selected CMV immunogens are shown. The significance of the unrooted phylogenetic trees was verified by bootstrap analysis and significant support values (≥ 80%; 1,000 bootstrap replicates) are indicated by asterisks at major nodes. Previously reported genotypes are illustrated (white) and reference strains are indicated; AD, AD169; DV, Davis; JH, JHC; JP, JP; ML, Merlin; TO, Toledo; TN, Towne; VR, VR1814. CMV vaccine immunogens as derived by alignment of clinical strain sequence data are displayed (Vac) and scale bars signify distance of amino acids per site.

diversity for each CMV immunogen was determined using published sequences and results are displayed in **Figure 1**. Analysis of the gB confirmed the presence of four main variants (gB1-gB4)[65] and one nonprototypic variant (gB5).[66-69] Since the gB protein is relatively conserved among clinical and low-passage strains (~86% identical), we chose the consensus of these sequences. The vaccine sequence (Vac) was phylogenetically closest to the gB1 genotype which has been found to account for the majority of highly symptomatic individuals in the clinic.[70-72]

Components of the CMV gCIII fusion complex, gH, gL and gO were developed herein as novel candidate immunogens. Phylogenetic analysis of gH confirmed the presence of two main genotypes in addition to a possible third group including the newly reported JHC strain that was isolated from a bone marrow transplant patient.[73] Analysis confirmed a low level of variation among the gHs (~93% identical) which may explain why anti-gH MAbs appear broadly reactive.[58,74] Due to this high level of conservation, the DNA vaccine consensus immunogen fell between gH1 and gH2, and was closest to the putative third gH group (gH3) including with the JHC clinical isolate. Phylogenetic analysis of the gL protein, while similarly highly conserved (~91%), was less distinctly grouped. Upon removal of amino acid sequences of gLs from strains extensively passaged, the resultant DNA consensus immunogen fell closest to the JHC and Merlin clinical isolates. The gO, which is highly glycosylated, is highly variable at the 5' end[50] contributing to high polymorphism (~55% divergence). Thus, we chose the consensus sequence of the gO5 genotype group for our target immunogen since it has been described to be genetically linked with the gN4c genotype, the largest gN4 variant group and the most seroprevalent.[75] Identity within the gO5 subgroup was ~99% and thus, the consensus Ag was phylogenetically grouped within this subgroup that also included the Merlin and JP clinical isolates.

Recently, it was shown that UL128, UL130 and UL131A can form a pentameric complex with gH and gL, instead of the classically defined association of gH/gL/gO constituting the gCIII fusion complex.[76-79] Furthermore, that this complex has been described to elicit potent nAbs.[58,80] Due to the relatively high level of amino acid conservation upon removal of highly-passaged and lab-adapted strains (~87% for UL128, ~86% for UL130, and ~73% for UL131A), we used consensus sequences for each candidate vaccine immunogens. The UL128 vaccine sequence was phylogenetically grouped with the Merlin and Davis isolates, as well as the AD169 strain. However, both of the UL130 and UL131A sequences were phylogenetically distant from the Towne and AD169 lab strains, respectively, which have lost their ability to infect endothelial cells, epithelial cells, and leukocytes due to deletions or mutations of these genes.[79,81]

CMV gM and gN heterodimerize in the ER by both covalent disulfide bonding and noncovalent interaction to form the viral infectivity complex. While the gM is highly conserved among the CMV (~95%), the gN is extremely variable (~45%). Due to this relatively high identity among the gM, consensus of all clinical sequences determined our candidate vaccine immunogen. Conversely, due to the highly modified nature of gN, characterized by almost exclusive O-linked sugars, consensus of the gN4

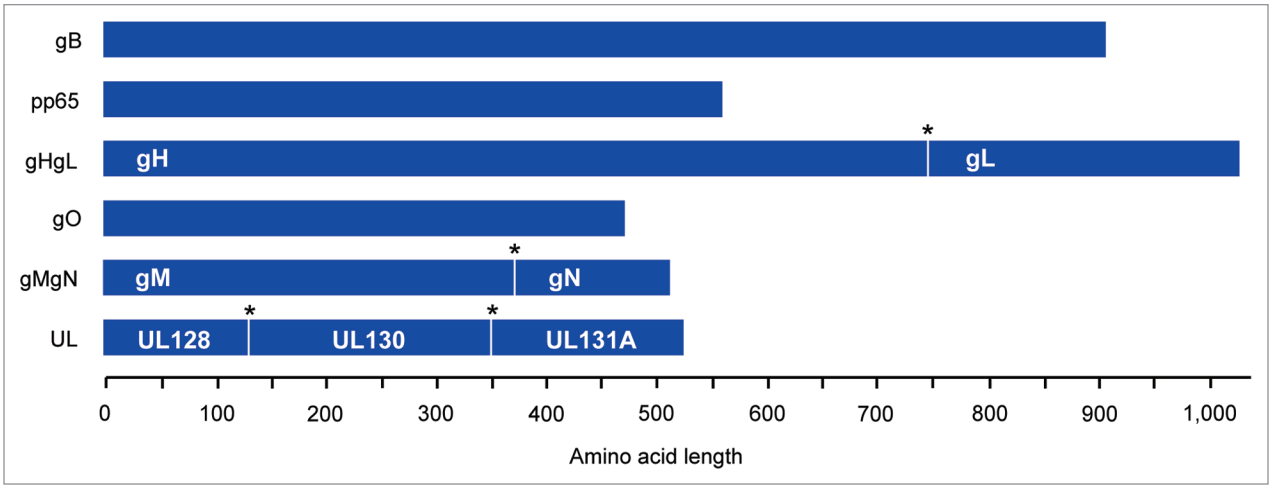

Figure 2. Construction of consensus CMV immunogens and structurally-relevant poly-proteins. Cartoon of consensus CMV immunogens. Poly-proteins are displayed and express multiple structurally-relevant proteins (labeled in white) that are separated by a furin cleavage site (*) for host cell post-translational modification. Amino acid length is defined. All proteins were subsequently genetically-optimized for expression in humans, commercially synthesized, and then subcloned into a modified pVAX mammalian expression vector.

subtype was used as the vaccine immunogen since this subgroup was reported to be the most prevalent of all clinical isolates in North America, Europe, China, and Australia.[75]

CMV pp65 was studied due to its current use in recent vaccine strategies as a T cell target based upon its elicitation of a dominant cellular immune response.[82,83] This protein is highly conserved among the CMV and was ~97% identical when not accounting for the 3' truncation associated with many published sequences. Thus, a synthetic, optimized, and consensus of the pp65 protein was developed as a target vaccine Ag that was phylogenetically similar to the JP, VR1814, Merlin and AD169 strains.

DNA vaccine construction. To potentially enhance the induction of NAbs upon vaccination, an additional strategy was employed consisting of the co-inclusion of multiple proteins of structural significance within the same vaccine construct (**Fig. 2**). Candidate viral glycoproteins requiring heterologous interaction for the construction of multimeric functional virion surface complexes were encoded in combination within the same DNA vaccine plasmid. Multiple protein-expressing plasmids gHgL, gMgN, and UL encoded ubiquitous endoproteolytic furin cleavage sites between immunogens to facilitate post-translational cleavage and modification. In this way, co-expression of structurally and functionally relevant proteins may hypothetically facilitate the formation of macromolecular complexes that express clinically- and virologically-relevant B cell epitopic determinants. This may be particularly critical in cases where coexpression is required for productive expression; gH requires coexpression of gL for intracellular transport and terminal carbohydrate modifications, and similarly, gL remains localized in the ER when expressed in the absence of gH.[48] Thus, plasmids were constructed for optimizing nascent coexpression of relevant proteins. In total, six amino acid target sequences were constructed and included the consensus-engineered gB, pp65, gHgL, gO, gMgN and UL (including the UL128-131A). Target sequences were genetically optimized at the nucleic acid level which included codon and RNA optimization, among others, for maximal protein expression. Transgenes were then synthesized and subcloned into modified pVAX1 mammalian expression vectors yielding the following plasmid vaccines: pHCMV-gB, pHCMV-pp65, pHCMV-gHgL, pHCMV-gO, pHCMV-gMgN, pHCMV-UL.

E-DNA vaccination was highly T cell immunogenic. Vaccines were evaluated for T cell immunogenicity in preclinical "proof-of-concept" rodent studies. Since it has been established that the diversity, or breadth, of the T cell response may be important for conferring protection,[84-86] we aimed to evaluate the comprehensive epitopic T cell response as induced by each vaccine construct. We developed a modified IFNγ ELISPOT assay that identified and measured subdominant and immunodominant CMV T cell epitopes. This was achieved by incubating samples with individual peptides, as opposed to whole or matrix peptide pools, to increase assay resolution; the traditional practice of pooling peptides for the sake of sample preservation, such as the use of matrix array pools, results in a reduction of assay sensitivity since total functional responses in pools containing multiple epitope-displaying peptides will effectively lower assay resolution, i.e., "drown-out" those of lower magnitude. Mice (n = 5/group) were vaccinated twice with in vivo electroporation (EP) delivery of the indicated CMV vaccines and IFNγ responses were measured 8 d later. E-DNA vaccination with each construct induced robust and broad IFNγ responses that recognized a diversity of T-cell epitopes (**Fig. 3 and Table 1**). All positive epitope-comprising peptides were subsequently confirmed and further characterized by FACS (data not shown). This modified approach was vaccine-specific and extremely sensitive since little IFNγ production was observed in control wells by stimulation with an irrelevant h-Clip peptide (Control).

Results from the modified ELISPOT assay showed that vaccination induced measurable T cell epitopes in all animals; gB induced 10 total epitopes, pp65-17, gH-28, gL-7, gO-5,

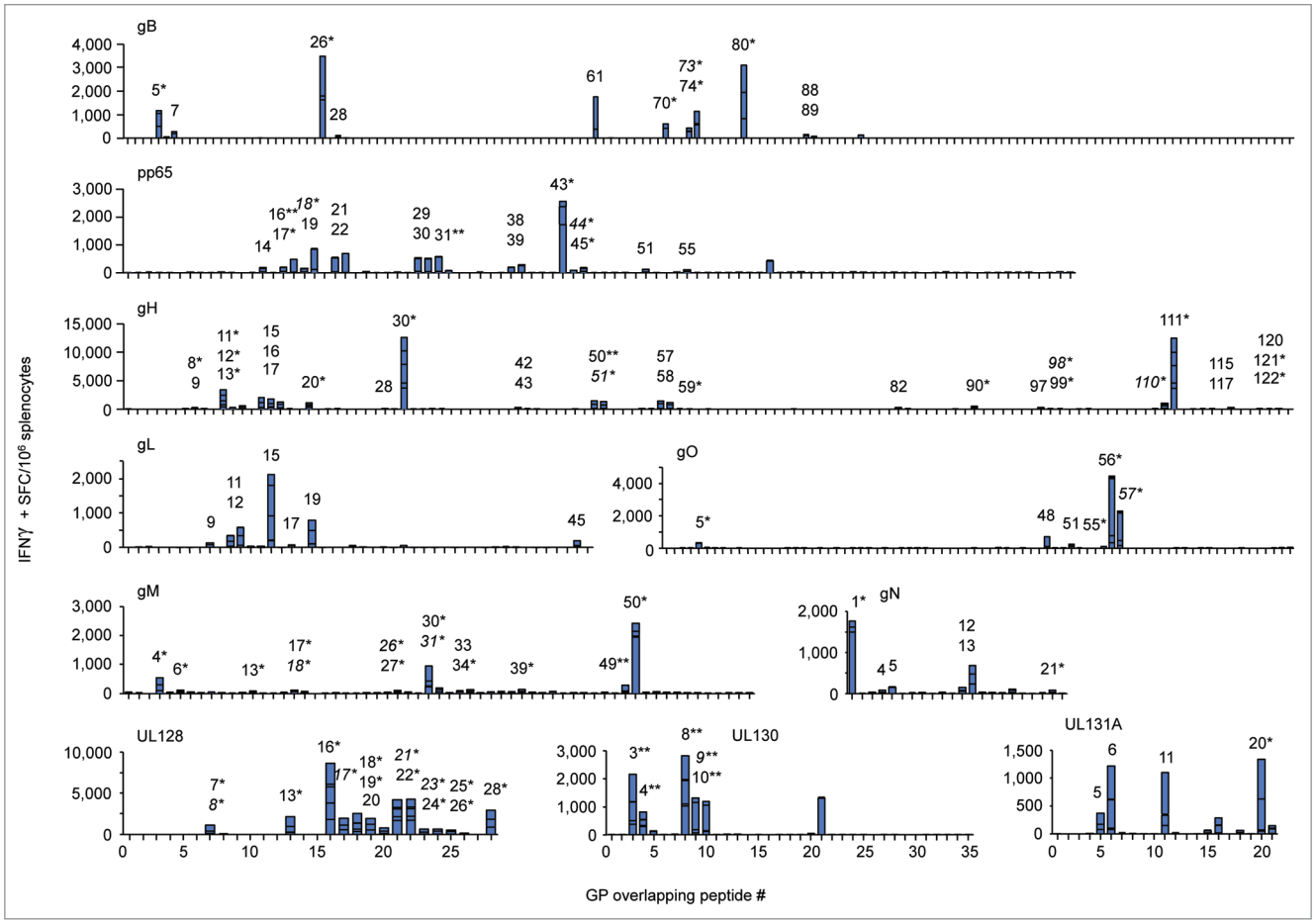

Figure 3. E-DNA vaccination was highly T cell immunogenic. Mice (n = 5/group) were immunized twice with EP delivery of the indicated CMV vaccine plasmid and IFNγ responses were measured by modified IFNγ ELISPOT. Splenocytes were incubated in the presence of individual peptides spanning each consensus CMV immunogen and results are shown in stacked bar graphs (summarized in **Table 1**). Peptides eliciting CD4-restriced IFNγ responses, CD8-restricted (*), and dual CD4/CD8-restricted responses (**), as determinded by FACS analysis, are individually numbered and displayed. Putative shared epitopes for contiguous positive peptide responses are italicized.

gM-12, gN-6, UL128-11, UL130-9, and UL131A - 4 (**Table 1**). When enumerated for each multi-protein expressing construct, pHCMV-gH induced a total of 35 epitopes, pHCMV-gMgN - 18, and pHCMV-UL - 24. These data also account for possible shared/partial T cell epitopes which were investigated for all instances of contiguous peptide responses as identified by modified ELISPOT assay (data not shown). Importantly, diverse epitope hierarchies were consistent and reproducible, and were comprised by both activated CD4+ and CD8+ T cells in all cases except for the gL immunogen which elicited CD4+ epitopes. Indeed, 6 of the 10 immunogens elicited T cell responses that were dominated by CMV-specific CD8+ T cells; approximately 81% of the average total gB-specific response was CD8-restricted, as well as at least 77% of gH, 86% of gO, at least 92% of gM, 60% of gN, and 98% of the UL128 response. Furthermore, vaccination with pHCMV-gHgL, pHCMV-gMgN, or pHCMV-UL generated CD8-dominant responses that constituted 67%, 80%, and 68% of the total average T cell response, respectively. Conversely, the CD4-restricted response was dominant after vaccination with pp65 (at least 50% was CD4+), gL (100%), and UL131A (74%). It was difficult to determine the composition of the T cell response for the UL130 immunogen since all 5 peptides contained dual CD4+/CD8+ epitopes. In most cases, the phenotypic composition of the vaccine-induced T cell response was not directly related to frequency of respective T cell epitopes; while gB vaccination elicited 5 of 10 (50%) epitopes that were CD4-restricted, they accounted for only 19% of the total average response. Similarly, after vaccination with gH or gN, 15 of 18 (54%) and 4 of 6 (67%) of CD4 T cell epitopes induced only 23% and 40% of the total average response, respectively. These data are likely explained by establishment of diverse epitope hierarchies constituted by an array of immunodominant and subdominant epitopes as driven by vaccination with the highly-optimized CMV constructs.

Each immunogen elicited at least 1 immunodominant epitope which was loosely defined as generating an IFNγ response approximately 2-fold over the highest subdominant response within that immunogen; single immunodominant epitopes were detected after vaccination with pp65 [$pp65_{235–267}$; CD8+ (peptide #43)], gL [$gL_{85–99}$; CD4+ (#15)], gO [$gO_{331–345}$; CD8+ (#56)],

Table 1. Identification and characterization of CMV vaccine-induced H-2^b T cell epitopes

									Best con. % rank (H-2^b)		
					ELISPOT		FACS	CD8+		CD4+	
								(≤ 0.6)		(≤ 28)	
Enhaced Plasmid Vaccine	**CMV Ag**	**Pep #**	**Position**	**Sequence**	**AVE**	**±SEM**	**T cell Restr.**	**D^b**	**K^b**	**I-A^b**	**Previously defined CMV epitopes (Blast - 90%; Allele (Ref))**
pHCMV-gB	**gB**	5	25-39	SSSTRGTSATHSHHS	388	140	8+			14.5	
		7	37-51	HHSSHTTSAAHSRSG	37	35	4+			18.4	
		26	**151-165**	RRSYAYIHTTYLLGS	**1,105**	**472**	**8+**	0.1	0.2	13.5	HLA-A*24:02[94]
		28	163-177	LGSNTEYVAPPMWEI	30	18	4+			4	
		61	361-375	AEDSYHFSSAKMTAT	577	430	4+		0.1	1.2	
		70	415-429	KYGNVSVFETTGGLV	183	89	8+		0.4		HLA-DR7[95]
		*73**	*433-447*	*QGIKQKSLVELERLA*	*95*	*73*	*8+*				
		74	439-453	SLVELERLANRSSLN	360	146	8+				
		80	**475-489**	SVHNLVYAQLQFTYD	**1,045**	**169**	**8+**		0.2		
		88	523-537	INPSAILSAIYNKPI	53	31	4+			20.7	HLA-A*24:02[94]
		89	529-543	LSAIYNKPIAARFMG	18	13	4+	0.3		2.5	HLA-A*24:02[94]
					3,798	1,544					
pHCMV-	**pp65**	14	79-93	HTYFTGSEVENVSVN	130	74	4+			11.4	HLA-A69[96]
		16	91-105	SVNVHNPTGRSICPS	106	46	8+/4+				HLA-A33[97]
		17	97-111	PTGRSICPSQEPMSI	161	127	8+	0.6			TMTL
		*18**	*103-117*	*CPSQEPMSIYVYALP*	*39*	*28*	*8+*		*0.3*		*TMTL*
		19	109-123	MSIYVYALPLKMLNI	427	196	4+	0.3	0.1	0.4	TMTL
		21	121-135	LNIPSINVHHYPSAA	192	132	4+			16.9	TMTL
		22	127-141	NVHHYPSAAERKHRH	277	178	4+			15.2	TMTL
		29	169-183	TRQQNQWKEPDVYYT	192	115	4+				TMTL
		30	175-189	WKEPDVYYTSAFVFP	216	117	4+		0.6	23.1	TMTL
		31	181-195	YYTSAFVFPTKDVAL	338	161	8+/4+	0.6	0.5	4.8	TMTL
		38	223-237	YVKVYLESFCEDVPS	87	50	4+				TMTL
		39	229-243	ESFCEDVPSGKLFMH	117	62	4+				TMTL
		43	**253-267**	DLTMTRNPQPFMRPH	**994**	**468**	**8+**	0.5			TMTL
		*44**	*259-273*	*NPQPFMRPHERNGFT*	*190*	*177*	*8+*				*TMTL*
		45	265-279	RPHERNGFTVLCPKN	150	97	8+				TMTL
		51	301-315	HFGLLCPKSIPGLSI	45	22	4+			27	TMTL
		55	325-339	QIFLEVQAIRETVEL	34	8	4+				TMTL
					3,466	1,853					
pHCMV-	**gH**	8	43-57	LNTYGRPIRFLRENT	38	33	8+				HLA-A*24:02[94]; HLA-II [98]
		9	49-63	PIRFLRENTTQCTYN	26	15	4+				HLA-II[98]
		11	61-75	TYNSSLRNSTVVREN	776	141	8+	0.1		24.5	HLA-A3[99]
		12	67-81	RNSTVVRENAISFNF	72	46	8+				HLA-A3[99]
		13	73-87	RENAISFNFFQSYNQ	94	28	8+	0.1	0.1		
		15	85-99	YNQYYVFHMPRCLFA	559	231	4+			3.5	HLA-B7[99]; HLA-A*24:02 [94]
		16	91-105	FHMPRCLFAGPLAEQ	419	199	4+			6.9	HLA-B7[99]; HLA-A*24:02 [94]

Table 1. Identification and characterization of CMV vaccine-induced H-2^b T cell epitopes (continued)

								Best con. % rank (H-2^b)			
					ELISPOT		FACS	CD8+ (≤ 0.6)		CD4+ (≤ 28)	
Enhaced Plasmid Vaccine	**CMV Ag**	**Pep #**	**Position**	**Sequence**	**AVE**	**±SEM**	**T cell Restr.**	**D^b**	**K^b**	**I-A^b**	**Previously defined CMV epitopes (Blast - 90%; Allele (Ref))**
		17	97-111	LFAGPLAEQFLNQVD	281	139	4+			25.5	HLA-B7[99]
		20	115-129	TLERYQQRLNTYALV	153	36	8+	0.6			HLA-A3[99]
		28	163-177	SIPHVWMPPQTTPHG	20	5	4+			1.2	
		30	**175-189**	PHGWKESHTTSGLHR	**2,942**	**81**	**8+**			25	
		42	247-261	MLLIFGHLPRVLFKA	78	58	4+	0.6	0.3	27.6	HLA-B7/A2/A3[99]
		43	253-267	HLPRVLFKAPYQRDN	24	9	4+			26.8	HLA-B7[99]
		50	295-309	DPDFLDAALDFNYLD	331	187	8+/4+		0.5		HLA-II[98]
		*51**	*301-315*	*AALDFNYLDLSALLR*	*307*	*181*	*8+*		*0.5*	*16.1*	*HLA-A*24:02*[94]
		57	337-351	RTVEMAFAYALALFA	340	190	4+		0.4	1.6	HLA-II[98]
		58	343-357	FAYALALFAAARQEE	265	157	4+	0.4		5.2	
		59	349-363	LFAAARQEEAGAEVS	27	17	8+			12.9	
		82	487-501	EIFIVETGLCSLAEL	64	31	4+				
		90	535-549	RLTRLFPDATVPATV	81	32	8+			6.5	
		97	577-591	ESFSALTVSEHVSYV	51	21	4+			15.9	HLA-A3[99]
		*98**	*583-597*	*TVSEHVSYVVTNQYL*	*10*	*5*	*8+*				*HLA-A3[99]; HLA-A*24:02* [94]
		99	589-603	SYVVTNQYLIKGISY	17	4	8+	0.1			
		*110**	*655-669*	*LLEYDDTQGVINIMY*	*191*	*83*	*8+*				
		111	**661-675**	TQGVINIMYMHDSDD	**2,864**	**136**	**8+**	0.4			HLA-A*24:02[94]
		115	685-699	EVVVSSPRTHYLMLL	22	14	4+			13.1	HLA-B7[99]
		117	697-711	MLLKNGTVLEVTDVV	58	23	4+	0.4			HLA-A2[99]
		120	715-729	TDSRLLMMSVYALSA	14	4	4+				
		121	721-735	MMSVYALSAIIGIYL	32	16	8+			7.4	HLA-A3[99]
		122	727-741	LSAIIGIYLLYRMLK	13	9	8+	0.5	0.2		HLA-A3[99]
					9,661	1,858					
	gL	9	49-63	ELTRRCLLGEVFQGD	25	15	4+				
		11	61-75	QGDKYESWLRPLVNV	76	41	4+			17.4	HLA-A*24:02[94]
		12	67-81	SWLRPLVNVTGRDGP	128	64	4+				HLA-A*24:02[94]
		15	**85-99**	LIRYRPVTPEAANSV	**483**	**220**	**4+**			0.1	
		17	97-111	NSVLLDEAFLDTLAL	16	11	4+				
		19	109-123	LALLYNNPDQLRALL	186	87	4+			19.8	
		45	265-279	PAHSRYGPQAVDAR	41	24	4+			14.2	
					955	462					
pHCMV-gO	**gO**	5	25-39	LLSLINCNVLVNSKG	65	47	8+	0.1			
		48	283-297	PYLSYTTSTAFNVTT	101	65	4+			2.4	
		51	301-315	YSATAAVTRVATSTT	43	8	4+			12.9	
		55	325-339	KSIMATQLRDLATWV	14	10	8+				
		56	**331-345**	QLRDLATWVYTTLRY	**784**	**317**	**8+**		0.2		
		*57**	*337-351*	*TWVYTTLRYRNEPFC*	*394*	*156*	*8+*				
					1,006	447					

Table 1. Identification and characterization of CMV vaccine-induced H-2^b T cell epitopes (continued)

								Best con. % rank (H-2^b)			
					ELISPOT		FACS	CD8+		CD4+	
								(≤ 0.6)		(≤ 28)	
Enhaced Plasmid Vaccine	**CMV Ag**	**Pep #**	**Position**	**Sequence**	**AVE**	**±SEM**	**T cell Restr.**	**D^b**	**K^b**	**I-A^b**	**Previously defined CMV epitopes (Blast - 90%; Allele (Ref))**
pHCMV-	**gM**	4	19-33	VFMVLTFVNVSVHLV	153	39	8+		0.2	22.2	
		6	31-45	HLVLSNFPHLGYPCV	31	7	8+	0.5	0.4	21.7	
		13	73-87	DSVQLVCYAVFMQLV	22	8	8+				
		17	97-111	VCWIKISMRKDKGMS	23	11	8+	0.3			
		*18**	*103-117*	*SMRKDKGMSLNQSTR*	*15*	*3*	*8+*				
		*26**	*151-165*	*SMIAFMAAVHFFCLT*	*14*	*4*	*8+*		*0.3*	*26.2*	
		27	157-171	AAVHFFCLTIFNVSM	21	10	8+	0.1			
		30	175-189	YRSYKRSLFFFSRLH	258	93	8+	0.1	0.1		
		*31**	*181-195*	*SLFFFSRLHPKLKGT*	*57*	*8*	*8+*	*0.1*	*0.1*	*24.4*	
		33	193-207	KGTVQFRTLIVNLVE	14	8	4+		0.3	17.7	
		34	199-213	RTLIVNLVEVALGFN	28	12	8+	0.4			
		39	229-243	FFVRTGHMVLAVFVV	32	15	8+				
		49	289-303	TFLSNEYRTGISWSF	83	38	8+/4+				
		50	**295-309**	YRTGISWSFGMLFFI	**627**	**441**	**8+**	0.1	0.1		
					1,293	681					
	gN	1	**1-15**	MEWNTLVLGLLVLSV	**472**	**343**	**8+**				
		4	19-33	SNNTSTASTPSPSSS	33	11	4+			2.7	
		5	25-39	ASTPSPSSSTHTSTT	67	36	4+			16	
		12	67-81	STTHDPNVMRPHAHN	46	14	4+			25	
		13	73-87	NVMRPHAHNDFYKAH	182	48	4+				
		21	121-135	RHCCFQNFTATTTKG	24	10	8+			8.6	
					824	462					
pHCMV-UL	**UL128**	7	37-51	NHPPERCYDFKMCNR	172	107	8+				
		*8**	*43-57*	*CYDFKMCNRFTVALR*	*12*	*5*	*8+*	*0.1*			
		13	73-87	IRGIVTTMTHSLTRQ	350	199	8+				
		16	**91-105**	NKLTSCNYNPLYLEA	**1,650**	**230**	**8+**	0.2	0.2		
		*17**	*97-111*	*NYNPLYLEADGRIRC*	*454*	*58*	*8+*				
		18	103-117	LEADGRIRCGKVNDK	443	163	8+				
		19	109-123	IRCGKVNDKAQYLLG	303	133	8+				
		20	115-129	NDKAQYLLGAAGSVP	100	52	4+			9.7	
		*21**	*121-135*	*LLGAAGSVPYRWINL*	*731*	*208*	*8+*		*0.2*	*24.4*	
		22	127-141	SVPYRWINLEYDKIT	739	202	8+		0.2		
		*23**	*133-147*	*INLEYDKITRIVGLD*	*65*	*36*	*8+*				
		24	139-153	KITRIVGLDQYLESV	89	50	8+				
		25	145-159	GLDQYLESVKKHKRL	56	32	8+				
		26	151-165	ESVKKHKRLDVCRAK	11	4	8+				
		28	163-171	RAKMGYMLQ	498	206	8+				
					4,412	1,379					

Table 1. Identification and characterization of CMV vaccine-induced H-2^b T cell epitopes (continued)

								Best con. % rank (H-2^b)			
					ELISPOT		FACS	CD8+		CD4+	
								(≤ 0.6)		(≤ 28)	
Enhaced Plasmid Vaccine	CMV Ag	Pep #	Position	Sequence	AVE	±SEM	T cell Restr.	D^b	K^b	I-A^b	Previously defined CMV epitopes (Blast - 90%; Allele (Ref))
	UL130	3	13-27	LLLCAVWATPCLASP	332	146	8+/4+			4.6	
		4	19-33	WATPCLASPWSTLTA	104	33	8+/4+			22.9	
		8	**43-57**	KLTYSKPHDAATFYC	**465**	**169**	**8+/4+**			15.1	
		*9**	*49-63*	*PHDAATFYCPFLYPS*	*237*	*185*	*8+/4+*	*0.2*			
		10	55-69	FYCPFLYPSPPRSPL	222	179	8+/4+	0.6		0.6	
					1,360	712					
	UL131A	5	25-39	AEKNDYYRVPHYWDA	61	34	4+				
		6	31-45	YRVPHYWDACSRALP	223	130	4+			16.2	
		11	**61-75**	LNYHYDASHGLDNFD	**429**	**220**	**4+**			12.1	
		20	115-129	PHARSLEFSVRLFAN	255	145	8+		0.6		
					967	530					

Epitope-containing peptides were identified by IFNγ ELISPOT (≥ 10 SFC/10^6 splenocytes AND ≥ 80% animal response rate) and then confirmed/characterized by FACS for function (≥ 3–5 x 10^4 CD3+ cells were acquired) and phenotype (CD4 and/or CD8 expression by CD3+/CD44+/IFNγ+ cells). Predicted epitopes of peptides confirmed to be CD8-restricted are underlined (as determined by best consensus % rank). All previously-described epitopes (by 90% Blast) are referenced; no H-2^b epitopes reported herein, as well as any for UL128, UL130, UL131A, gM, gN and gO, have been previously described (IEDB; www.iedb.org). Contiguous peptides with putative shared and/or partial epitopes as confirmed by ELISPOT (data not shown) are italicized and indicated (*). Immunodominant T cell epitopes are boldface and total responses on average ± SEM are displayed and do not include putative shared and/or partial epitopic responses. TMTL, Too many to list.

gM [gM$_{295–309}$; CD8+ (#50)], gN [gN$_{1–15}$; CD8+ (#1)], UL128 [UL128$_{91–105}$; CD8+ (#16)], UL130 [UL130$_{43–57}$; CD8+/CD4+ (#8)], and UL131A [UL131A$_{61–75}$; CD4+ (#11)]. Two CD8-restricted immunodominant epitopes were detected following vaccination with either gB or gH that were comprised by peptides gB$_{151–165}$ (#26), gB$_{475–489}$ (#80), gH$_{175–189}$ (#30), and gH$_{661–675}$ (#111). When considering the multi-protein expression strategy employed by several of these vaccines, immunodominance among the T cell epitopes occurring within multiple immunogens may shift. While both the gH and the gL contain immunodominant epitopes, that from the gL becomes subdominant to the two within the gH since it induces responses that are 2-fold lower, and the immunodominance hierarchy shifts from a total of 3 to 2. This is also true for the pHCMV-UL in which the immunodominant epitope occurring within the UL128 is over 2-fold greater than those within the UL130 and the UL131A, and thus the total immunodominant epitopes elicited by vaccination of all three immunogens is 1 instead of 3. However, immunodominance does not change following vaccination with the pHCMV-gMgN since each of the single immunodominant epitopes remains approximately 2-fold higher than the greatest subdominant response between the two immunogens. Altogether, these data show that each of the plasmids was immune potent and drove CMV-specific T cell responses that were CD8-dominant and recognized a broad array of T cell epitopes.

Discussion

Based upon promising clinical[14,33,34] and preclinical[35-37] data suggesting that an effective CMV vaccine will induce cellular immune responses, a combination of traditional and surface glycoproteins, matrix and chaperones were selected for evaluation for T cell immunogenicity following delivery as E-DNA vaccines.[32,48] While 'first-generation' DNA vaccines were poorly immunogenic and elicited mainly CD4-dominant T cell responses, recent technological advancements have dramatically improved their immunogenicity in recent clinical trials.[87,88] Nucleic acid synthesis technologies have revolutionized the way genes can be developed via elimination of destabilizing RNA structures, implementation of species-specific codon-usage, modification of GC content and others, rendering these genes capable of strong in vivo expression when inserted into 'highly-optimized' plasmid DNA.[87] In response to polymorphism, likely attributed to the accumulation of spontaneous mutations and/or by inter- and intra-strain homologous recombination induced by selective pressure (**Fig. 1**),[50,51,89] immunity can be further directed toward multiple circulating strains by "consensus-engineering" of the amino acid sequence of the vaccine immunogen.[63,64] Furthermore, structurally-relevant immunogens can be incorporated into the same plasmid vaccines for the co-expression of virologically-relevant macromolecular

complexes (**Fig. 2**). Cellular uptake and subsequent Ag expression are substantially increased when highly-concentrated plasmid vaccine formulations are administered with EP, a technology that uses brief square-wave electric pulses within the vaccination site to drive plasmids into transiently permeabilized cells.[90] In theory, a cocktail of DNA plasmids could be assembled for directing a highly-specialized immune response against any number of variable Ags.

E-DNA vaccination with each CMV construct herein was highly T cell immunogenic in preclinical 'proof-of-concept' murine studies, generating robust and broad T cell responses as extensively analyzed by a novel modified ELISPOT assay developed herein (**Fig. 3** and **Table 1**). This may be critical since prevention of CMV infection and disease in the transplantation setting may require a greater cellular response than is needed to prevent congenital infection. While little quantitative data regarding the induction of CMV-specific T cell responses in mice as driven by a vaccine exist, vaccination herein with the pHCMV-pp65 generated a T cell response that was on average over 40% greater than a previous pp65 DNA vaccine administered three times with a poloxamer-based delivery system that induced ~2,000 IFNγ+ SFC/10^6 cells.[91] Importantly, responses from both CD8+ and CD4+ helper cells were observed which may be required for protection against post-transplant occurrence of CMV disease,[37,42,45] as well as driving diverse T cell hierarchies of which subdominant responses can make significant contributions to protection.[84-86] These data demonstrate that 'next-generation' DNA vaccine technologies are effective at inducing CD8+ T cell responses in contrast to prior strategies that induced mainly CD4-dominant responses. Additionally, a majority of epitopes identified for gB, pp65, gH, and gL also contained HLA that have previously been reported to contribute to the suppression of viremia and amelioration of overt disease (**Table 1**).[2,14,41-44] In summary, the elicitation, identification, and characterization of extensive T cell responses as driven by a vaccine herein provide an important tool for guiding CMV vaccine development. These data suggest further study of these constructs as vaccine components in strategies aiming to generate broad T cell responses, and investigation of their potential for generating Abs capable of neutralization and protection in preclinical challenge models is warranted.

Methods

Ethics Statement. Animal experimentation was conducted following UPenn IACUC and School of Medicine Animal Facility guidelines for housing and care of laboratory animals and performed in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of NIH.

E-DNA vaccine construction. Plasmid constructs encoded full-length CMV proteins: pHCMV-gB, pHCMV-pp65, pHCMV-gHgL, pHCMV-gO, pHCMV-gMgN, pHCMV-UL (UL128, UL130 and UL131A). Immunogens were consensus, as determined by alignment using Vector NTI® (Invitrogen), of publically available (GenBank) CMV clinical strains (passaged < 6 times in tissue culture), and resultant sequences are listed below. This strategy excluded sequences from highly-passaged lab-adapted strains that may diverge in sequence from clinical strains, thus maximizing the potential for broadly-reactive immunity. Phylogenetic analysis was performed by multiple-alignment with ClustalW using *MEGA* version 5 software. Commercial genetic optimization was proprietary and included codon and RNA optimization, among others, for species-specific protein expression in humans, and all genes were synthesized and subcloned into a modified pVAX1 mammalian expression vector (GeneArt or GenScript). Several plasmids (pHCMV-gHgL, pHCMV-gMgN and pHCMV-UL) encoded multiple immunogens separated by a furin cleavage site (RGRKRRS).

Consensus CMV immunogen sequences. *gB.* MES RIW CLV VCV NLC IVC LGA AVS SSS TRG TSA THS HHS SHT TSA AHS RSG SVS SQR VTS SEA VSH RAN ETI YNT TLK YGD VVG VNT TKY PYR VCS MAQ GTD LIR FER NIV CTS MKP INE DLD EGI MVV YKR NIV AHT FKV RVY QKV LTF RRS YAY IHT TYL LGS NTE YVA PPM WEI HHI NSH SQC YSS YSR VIA GTV FVA YHR DSY ENK TMQ LMP DDY SNT HST RYV TVK DQW HSR GST WLY RET CNL NCM VTI TTA RSK YPY HFF ATS TGD VVD ISP FYN GTN RNA SYF GEN ADK FFI FPN YTI VSD FGR PNS ALE THR LVA FLE RAD SVI SWD IQD EKN VTC QLT FEW ASE RTI RSE AED SYH FSS AKM TAT FLS KKQ EVN MSD SAL DCV RDE AIN KLQ QIF NTS YNQ TYE KYG NVS VFE TTG GLV VFW QGI KQK SLV ELE RLA NRS SLN LTH RTK RST DGN NTT HLS NME SVH NLV YAQ LQF TYD TLR GYI NRA LAQ IAE AWC VDQ RRT LEV FKE LSK INP SAI LSA IYN KPI AAR FMG DVL GLA SCV TIN QTS VKV LRD MNV KES PGR CYS RPV VIF NFA NSS YVQ YGQ LGE DNE ILL GNH RTE ECQ LPS LKI FIA GNS AYE YVD YLF KRM IDL SSI STV DSM IAL DID PLE NTD FRV LEL YSQ KEL RSS NVF DLE EIM REF NSY KQR VKY VED KVV DPL PPY LKG LDD LMS GLG AAG KAV GVA IGA VGG AVA SVV EGV ATF LKN PFG AFT IIL VAI AVV IIT YLI YTR QRR LCT QPL QNL FPY LVS ADG TTV TSG STK DTS LQA PPS YEE SVY NSG RKG PGP PSS DAS TAA PPY TNE QAY QML LAL ARL DAE QRA QQN GTD SLD GQT GTQ DKG QKP NLL DRL RHR KNG YRH LKD SDE EEN V.

pp65. MES RGR RCP EMI SVL GPI SGH VLK AVF SRG DTP VLP HET RLL QTG IHV RVS QPS LIL VSQ YTP DST PCH RGD NQL QVQ HTY FTG SEV ENV SVN VHN PTG RSI CPS QEP MSI YVY ALP LKM LNI PSI NVH HYP SAA ERK HRH LPV ADA VIH ASG KQM WQA RLT VSG LAW TRQ QNQ WKE PDV YYT SAF VFP TKD VAL RHV VCA HEL VCS MEN TRA TKM QVI GDQ YVK VYL ESF CED VPS GKL FMH VTL GSD VEE DLT MTR NPQ PFM RPH ERN GFT VLC PKN MII KPG KIS HIM LDV AFT SHE HFG LLC PKS IPG LSI SGN LLM NGQ QIF LEV QAI RET VEL RQY DPV AAL FFF DID LLL QRG PQY SHE PTF TSQ YRI QGK LEY RHT WDR HDE GAA QGD DDV WTS GSD SDE ELV TTE RKT PRV TGG GAM AGA STS AGR KRK SAS SAT ACT AGV MTR GRL KAE STV APE EDT DED SDN EIH NPA VFT WPP WQA GIL ARN LVP MVA TVQ GQN LKY QEF FWD AND IYR IFA ELE GVW QPA AQP KRR RHR QDA LPG PCI AST PKK HRG.

gH. MRP GLP SYL TVF AVY LLS HLP SQR YGA DAA SEA LDP HAF HLL LNT YGR PIR FLR ENT TQC TYN SSL RNS TVV REN AISF NFF QSY NQY YVF HMP RCL FAG PLA EQF LNQ VDL TET LER YQQ RLN TYA LVS KDL ASY RSF SQQ LKA QDS LGE QPT TVP PPI DLS IPH VWM PPQ TTP HGW KES HTT SGL HRP HFN QTC ILF DGH DLL FST VTP CLH QGF YLI DEL RYV KIT LTE DFF VVT VSI DDD TPM LLI FGH LPR VLF KAP YQR DNF ILR QTE KHE LLV LVK KDQ LNR HSY LKD PDF LDA ALD FNY LDL SAL LRN SFH RYA VDV LKS GRC QML DRR TVE MAF AYA LAL FAA ARQ EEA GAE VSV PRA LDR QAA LLQ IQE FMI TCL SQT PPR TTL LLY PTA VDL AKR ALW TPN QIT DIT SLV RLV YIL SKQ NQQ HLI PQW ALR QIA DFA LKL HKT HLA SFL SAF ARQ ELY LMG SLV HSM LVH TTE RRE IFI VET GLC SLA ELS HFT QLL AHP HHE YLS DLY TPC SSS GRR DHS LER LTR LFP DAT VPA TVP AAL SIL STM QPS TLE TFP DLF CLP LGE SFS ALT VSE HVS YVV TNQ YLI KGI SYP VST TVV GQS LII TQT DSQ TKC ELT RNM HTT HIS TAA LNI SLE NCA FCQ SAL LEY DDT QGV INI MYM HDS DDV LFA LDP YNE VVV SSP RTH YLM LLK NGT VLE VTD VVV DAT DSR LLM MSV YAL SAI IGI YLL YRM LKT C.

gL. MCR RPD CGF SFS PGP VIL LWC CLL LPI VSS AAV SVA PTA AEK VPA ECP ELT RRC LLG EVF QGD KYE SWL RPL VNV TGR DGP LSQ LIR YRP VTP EAA NSV LLD EAF LDT LAL LYN NPD QLR ALL TLL SSD TAP RWM TVM RGY SEC GDG SPA VYT CVD DLC RGY DLT RLS YGR SIF THE VLG FEL VPP SLF NVV VAI RNE ATR TNR AV RLP VST AAA PEG ITL FYG LYN AVK EFC LRH QLD PPL LRH LDK YYA GLP PEL KQT RVN LPA HSR YGP QAV DAR.

gO. MGK KEM IMV KGI PKI MLL ISI TFL LLS LIN CNV LVN SKG TRR SWP YTV LSY RGK EIL KKQ KED ILK RLM STS SDG YRF LMY PSQ QKF HAI VIS MDK FPQ DYI LAG PIR NDS ITH MWF DFY STQ LRK PAK YVY SEY NHT AHK ITL RPP PCG TVP SMN CLS EML NVS KRN DTG EKG CGN FTT FNP MFF NVP RWN TKL YIG SNK VNV DSQ TIY FLG LTA LLL RYA QRN CTR SFY LVN AMS RNL FRV PKY ING TKL KNT MRK LKR KQA LVK EQP QKK NKK SQS TTT PYL SYT TST AFN VTT NVT YSA TAA VTR VAT STT GYR PDS NFM KSI MAT QLR DLA TWV YTT LRY RNE PFC KPD RNR TAV SEF MKN THV LIR NET PYT IYG TLD MSS LYY NET MSV ENE TAS DNN ETT PTS PST RFQ RTF IDP LWD YLD SLL FLD KIR NFS LQL PAY GNL TPP HER RAA NLS TLN SLW WWL QYP.

gM. MAP SHV DKV NTR TWS ASI VFM VLT FVN VSV HLV LSN FPH LGY PCV YYH VVD FER LNM SAY NVM HLH TPM LFL DSV QLV CYA VFM QLV FLA VTI YYL VCW IKI SMR KDK GMS LNQ STR DIS YMG DSL TAF LFI LSM DTF QLF TLT MSF RLP SMI AFM AAV HFF CLT IFN VSM VTQ YRS YKR SLF FFS RLH PKL KGT VQF RTL IVN LVE VAL GFN TTV VAM ALC YGF GNN FFV RTG HMV LAV FVV YAI ISI IYF LLI EAV FFQ YVK VQF GYH LGA FFG LCG LIY PIV QYD TFL SNE YRT GIS WSF GML FFI WAM FTT CRA VRY FRG RGS GSV KYQ ALA TAS GEE VAA LSH HDS LES RRL REE EDD DDD EDF EDA.

gN. MEW NTL VLG LLV LSV AAS SNN TST AST PSP SSS THT STT VKA TTT ATT STT TAT STT SST TST KPG STT HDP NVM RPH AHN DFY KAH CTS HMY ELS LSS FAA WWT MLN ALI LMG AFC IVL RHC CFQ NFT ATT TKG Y.

UL128. SPK DLT PFL TAL WLL LGH SRV PRV RAE ECC EFI NVN HPP ERC YDF KMC NRF TVA LRC PDG EVC YSP EKT AEI RGI VTT MTH SLT RQV VHN KLT SCN YNP LYL EAD GRI RCG KVN DKA QYL LGA AGS VPY RWI NLE YDK ITR IVG LDQ YLE SVK KHK RLD VCR AKM GYM LQ.

UL130. LRL LLR HHF HCL LLC AVW ATP CLA SPW STL TAN QNP SPP WSK LTY SKP HAD ATF YCP FLY PSP PRS PLQ FSG FQR VST GPE CRN ETL YLL YNR EGQ TLV ERS STW VKK VIW YLS GRN QTI LQR MPR TAS KPS DGN VQI SVE DAK IFG AHM VPK QTK LLR FVV NDG TRY QMC VMK LES WAH VFR DYS VSF QVR LTF TEA NNQ TYT FCT HPN LIV.

UL131A. MRL CRV WLS VCL CAV VLG QCQ RET AEK NDY YRV PHY WDA CSR ALP DQT RYK YVE QLV DLT LNY HYD ASH GLD NFD VLK RIN VTE VSL LIS DFR RQN RRG GTN KRT TFN AAG SLA PHA RSL EFS VRL FAN.

Immunization, mice, and challenge. Adult female C57BL/6 (H-2^b) mice 6 – 8 weeks of age were purchased from The Jackson Laboratory. Mice were immunized i.m. by needle injection of 45 μg plasmid DNA resuspended in water and immediately followed by EP at the same site, using a three-pronged Minimally Invasive Device inserted approximately 2 mm intramuscularly as previously described[92] using the CELLECTRA® adaptive constant current device (Inovio Pharmaceuticals, Inc.). Square-wave pulses were delivered through a triangular 3-electrode array consisting of 26-gauge solid stainless steel electrodes and two constant-current pulses of 0.1 Amps were delivered for 52 msec/pulse separated by a 1 sec delay. For T cell studies, spleens were harvested 8 days post-immunization.[93] Briefly, splenocytes were resuspended in RPMI 1640 medium (Mediatech Inc.) supplemented with 10% FBS, 1X Anti-Anti, and 1X β-ME (Invitrogen).

ELISPOT assays. Standard IFNγ ELISPOT assay has been described[93] and was modified herein for comprehensive analysis of T cell breadth. Identification and measurement of subdominant and immunodominant CMV T cell epitopes were assessed by stimulating splenocytes with individual peptides (15-mers overlapping by 9 amino acids; 2.5 μg/ml final) spanning each consensus CMV immunogen, as opposed to whole or matrix peptide pools. Peptides containing T cell epitopes were identified (≥10 AVE IFNγ+ spots AND ≥ 80% animal response rate; summarized in **Table 1**) and then later confirmed functionally and phenotypically by FACS. Possible shared/partial T cell epitopes were addressed for all instances of contiguous peptide responses as identified by modified ELISPOT assay. Here, cells were stimulated individually with each of the contiguous peptides, as well as in combination for comparison (data not shown), and were

defined as 'shared/partial' if the combined response was not greater than either of the two individual responses.

Flow cytometry. For splenocyte ICS, 1 x 10^6 cells/well were added to 96-well plates and stimulated for 5 h for stimulation with individual peptides (2.5 μg/ml final) for functional confirmation of all peptides identified by modified ELISPOT as well as phenotypic characterization (**Table 1**). Immunostaining of splenocytes and transfected 293Ts was performed in 96-well plates and cells were pre-stained with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen). Splenocytes were surface-stained for CD19 (V450; clone 1D3), CD4 (PE-Cy7; clone RM4-5), CD8α (APC-Cy7; clone 53-6.7) and CD44 (PE-Cy5; clone IM7) (BD Biosciences), washed three times in PBS + 1% FBS, permeabilized with BD Cytofix/Cytoperm™ kit, and then stained intracellularly with IFNγ (APC; clone XMG1.2) and CD3 (PE-cy5.5; clone 145-2C11) (eBioscience). All cells were fixed in 1% paraformaldehyde. LSRII flow cytometer (BD Biosciences) was used for acquisition and FlowJo (Tree Star) for data analysis. Activated T cells were gated by FACS: total lymphocytes, live (LD) CD3+ cells that were negative for CD19 and LIVE-DEAD (dump channel), singlets (excludes cell doublets), CD4+ and CD8+ cells, activated cells (CD44+), and peptide-specific IFNγ-producing T cells were gated.

Statistical analyses. Significance for unrooted phylogenetic trees was determined by maximum-likelihood method and verified by bootstrap analysis and significant support values (≥80%; 1,000 bootstrap replicates) were determined by *MEGA* version 5 software.

Disclosure of Potential Conflicts of Interest

D.B.W. notes that he and his laboratory has several commercial relationships with companies in the area of vaccines. These include him receiving consulting fees or stock ownership for Advisory/Review Board service, speaking support, or research support from commercial entities including Inovio Pharmaceuticals, Bristol-Myers Squibb, VGXI, Pfizer, Virxsys Co., Johnson & Johnson, Merck & Co., Sanofi Pasteur, Althea, Novo Nordisk, Statens Serum Institut, Aldevron, Novartis, Incyte, and possibly others. No writing assistance was utilized in the production of this manuscript.

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