A RANDOMIZED MULTICENTER TRIAL OF THE EFFECTS OF MELANOMA-ASSOCIATED HELPER PEPTIDES AND CYCLOPHOSPHAMIDE ON THE IMMUNOGENICITY OF A MULTIPEPTIDE MELANOMA VACCINE

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SUPPLEMENTAL TEXT AND DATA

Supplemental Methods:

12 melanoma peptides restricted by MHC Class I molecules (12MP), used in vaccines:

HLA-A1 restricted peptides:

DAEKSDICTDEY (Tyrosinase $_{240-251}$, with substitution of S for C at residue 244),

SSDVIPIGTY (Tyrosinase $_{146-156}$),

EADPTGHSY (MAGE-A1 $_{161-169}$),

EVDPIGHLY (MAGE-A3 $_{168-176}$);

HLA-A2 restricted peptides:

YMDGTMSQV (Tyrosinase $_{369-377D}$),

IMDQVPFSV (gp100 $_{209-217,}$ 209-2M),

YLEPGPVTA (gp100 $_{280-288}$),

GLYDGMEHL (MAGE-A10 $_{254-262}$);

HLA-A3 restricted peptides:

ALLAVGATK (gp100 $_{17-25}$),

LIYRRRLMK (gp100 $_{614-622}$),

SLFRAVITK (MAGE-A1 $_{96-104}$),

ASGPGGGAPR (NY-ESO-1_{53-62}).

6 melanoma helper peptides restricted by HLA-DR molecules (6MHP):

AQNILLSNAPLGPQFP (Tyrosinase $_{56\text{-}70}$, HLA-DR4),

FLLHHAFVDSIFEQWLQRHRP (Tyrosinase $_{386\text{-}406}$, HLA-DR15),

RNGYRALMDKSLHVGTQCALTRR (Melan-A/MART-$1_{51\text{-}73}$, HLA-DR4),

TSYVKVLHHMVKISG (MAGE-3 $_{281\text{-}295}$, HLA-DR11),

LLKYRAREPVTKAE (MAGE-1,2,3,6 $_{121\text{-}134}$, HLA-DR13) , and

WNRQLYPEWTEAQRLD (gp100 $_{44\text{-}59}$, HLA-DR4 & -DR1).

Peptide vaccine preparation: Peptides for the vaccines were synthesized and purified (>95%) under GMP conditions (Multiple Peptide Systems, now Polypeptide Group, San Diego, CA). The peptides were then solubilized, sterile-filtered, mixed, vialed and lyophilized under GMP conditions by Merck Biosciences AG Clinalfa (Läufelingen, Switzerland) in single-use vials tested for sterility, identity, purity, potency, general safety, pyrogenicity, and stability in accordance with Code of Federal Regulations (CFR) guidelines[35].

ELIspot assays. Briefly, 200,000 viable PBMC were plated per well, and pulsed with synthetic peptide (10 mcg/ml), in quadruplicate. Controls included irrelevant peptides, a mixture of viral peptides (CEF peptide pool), PMA-ionomycin and PHA. Assessment of immunologic response was based upon the following definitions:

N_{vax} = number T-cells responding to vaccine peptide; N_{neg} = number T-cells responding to maximum of two negative controls; $R_{vax} = N_{vax}/N_{neg}$. A patient was considered to have a T-cell response to vaccination (binary yes/no) only if all of the following criteria were met: (1) N_{vax} exceeded N_{neg} by at least 20 cells / 100,000 $CD4^+$ or $CD8^+$ cells (0.02%), where CD8 and CD4 counts were based on flow cytometric evaluations of the PBMC samples. (2) $R_{vax} \geq 2$, (3) (N_{vax} – 1 SD) ≥ (N_{neg} + 1 SD), and (4) R_{vax} after vaccination ≥ 2 x R_{vax} pre-vaccine.

Fold-increases less than one (e.g., control counts exceed number of responding T-cells, or fold response compared to baseline is less than one) were set equal to one to indicate no response and to prevent overinflating adjusted fold-increases due to pre-vaccine ratios less than one, or division by zero, while not affecting the determination of response. Continuous measures of immune response denoted as fold-increase must satisfy conditions (1)-(4), and were defined as the amount of R_{vax}. Cumulative response over all HLA-appropriate peptides, $CumR_{time}$, was defined, at each time point, as 1 + the sum of fold-increase exceeding 1 over all patient-specific peptides (eg. at week 3, $CumR_3 = 1 +$ (sum over each (R_{vax} -1) for each peptide for which a response was detected). When making comparisons across HLA types, this cumulative response is also calculated for the four peptides restricted by each HLA-Class I allele. When making comparisons across patients overall, this is calculated for all HLA-appropriate peptides in the 12MP, which may be 4 or 8 peptides, depending on HLA type.

Supplemental Results

Completion of study participation. Among the 167 eligible patients, 93 (56%) completed all protocol treatment, and 74 came off study before completing all 10 vaccines (within 1 year): 48 for disease progression, 13 for adverse events, 3 for refusing further therapy, 9 for non-compliance, and one at PI discretion. There were no significant differences among arms in rates of completing treatment or in interrupting treatment for disease progression (Supplemental Table 2).

Autoimmune toxicities. Treatment-related autoimmune toxicities were reported in 10 patients (6%), in 0, 5, 3, and 2 patients in each of the four groups, respectively. Vitiligo was recorded as hypopigmentation of skin and was reported in 8 patients (5%). Serum studies to test for autoimmunity included serum antinuclear antibody (ANA) and rheumatoid factor (RF) tests,

which were run by the participating institutions' clinical laboratories (data not shown). Pretreatment elevations were observed in 17 of 133 patients (13%) for ANA, and in 3 of 124 patients for RF (2%). For those participants with normal levels at time 0, and with repeat testing also at 1 month and/or 1 year, elevations were observed in ANA for 7 of 100 patients (7%), and in RF for none of 101 patients (0%).

Supplemental Discussion

Selection of peptides for this vaccine trial.

There is a range of immunogenicities for the 12 peptides. The justification for using them, as opposed to a more limited set of 4 peptides has been demonstrated in a randomized prospective trial in which 100% of patients had immune responses to the 12MP mix. [1] In that trial, immunogenicity was evaluated after one in vitro sensitization; whereas the present study uses a more stringent assay with direct ex vivo analysis. The definition of clinically relevant rejection antigen is debated; however, all of the 12 peptides restricted by Class I MHC molecules for this trial were selected because of convincing data from our laboratory [2-5] or from colleagues, that they represent epitopes for T cells expanded from tumor infiltrating lymphocytes from melanoma metastases (referenced in [1]). We have confirmed, for 6 of these peptides, that T cells generated in patients vaccinated with these peptides can kill melanoma cells that express the source protein and the appropriate MHC molecule: DAEKSDICTDEY (Tyrosinase $_{240\text{-}251}$), YMDGTMSQV (Tyrosinase $_{369\text{-}377D}$), ALLAVGATK (gp100 $_{17\text{-}25}$), SLFRAVITK (MAGE-A1 $_{96\text{-}104}$), GLYDGMEHL (MAGE-A10 $_{254\text{-}262}$), LIYRRRLMK (gp100$_{614\text{-}622}$) [6,7]. Others have demonstrated the ability of $CD8^+$ T cells induced by vaccination with some of the other peptides, to kill tumor cells expressing those antigens (referenced in [1]).

Criteria for defining immune responses.

Criteria used for definition of T cell responses to vaccination vary among studies in the tumor immunology literature and viral immunology literature. We have used criteria here that are consistent with our prior work with these antigens [8]. A partial survey of these criteria is summarized in Supplemental Table 3. Among 7 studies published in the past 5 years, where IFN-gamma ELIspot assays were performed on PBMC ex vivo, criteria used for defining the lower limit of detection of an immune response range from an increase of approximately 19-50 (median 27) spot forming units per 100,000 CD8+ cells, compared to a mean of negative control wells [9-15]). This compares to our lower limit of 20 per 100,000 CD8+ cells in this manuscript, over the maximum of two sets of negative control wells.

Those papers also required a responder to have 2-4 fold increase over negative control wells; in our manuscript, we require a 2-fold increase. In one of the 7 papers surveyed, the only criterion for a positive response was an increase over the negative control by 3 standard deviations of that negative control value [16]. The present manuscript uses 4 criteria, all of which must be met to define a positive immune response: increase over negative controls by at least 20 cells per 100,000 CD8+ cells, and by at least 2 fold, and by at least the sum of the standard deviations of both the negative control and the experimental wells, as well as by an increase over any pre-existing response, by at least 2-fold. The combination of these 4 criteria increase the stringency of these requirements, compared to other reported criteria. It is also noteworthy that in one of the surveyed reports, a high responder is considered to have approximately 175 responding cells per 100,000 CD8+ cells [9]. As shown in Figure 3 and Supplemental Figure 1, for patients in groups A and B, the median magnitude of the response to 12MP exceeds 0.2% (200 cells per 100,000 CD8+ cells), and 5-fold the negative control, after correcting for any prevaccine response. The mean values are approximately 400-500 spots per 100,000 CD8+ cells and

approach 10-fold the negative controls. Also, by comparison to the present study, multiple reports of immune response to cancer vaccines and HIV vaccines have used minimal criteria of a 2-4 fold increase and 5-60 cells per 100,000 CD8+ cells, after in vitro sensitization [17-20]. In prior work, we have used criteria identical to those of the present report for direct ex vivo analyses [8], and have used criteria for IVS (stimulated) ELISpot assays, matching the current study, but with a more stringent criterion of 150 IFN-gamma secreting cells per 100,000 CD8+ cells [1].

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Supplemental Table 1. Treatment-related adverse events, by study group and overall: 170 patients overall.

		% with toxicity, any grade					Number (of 170) with toxicity by maximum grade			
Tox Group	**Toxicity**	**Overall**	**Group A**	**Group B**	**Group C**	**Group D**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
ANY TOXICITY	Maximum grade toxicity by patient	99	100	100	95	100	25	126	16	1
ALLERGY/IMMUNOLOGY		30	20	47	36	18	51	.	.	.
	Allergic reaction	2	2	5	.	.	3	.	.	.
	Autoimmune reaction	6	.	12	7	5	10	.	.	.
	Rhinitis	25	20	33	33	14	42	.	.	.
AUDITORY/EAR		4	2	2	2	7	.	5	1	.
	Tinnitus	3	2	2	2	5	.	5	.	.
BLOOD/BONE MARROW		51	63	44	40	57	73	11	3	.
	Hemoglobin	34	44	35	19	36	55	2	.	.
	Leukocytes	18	20	14	12	25	24	5	1	.
	Lymphopenia	16	22	19	10	16	18	7	3	.
	Neutrophils	7	5	9	10	5	8	4	.	.
	Platelets	3	2	.	7	2	5	.	.	.
CARDIAC ARRHYTHMIA		2	.	.	2	5	1	2	.	.
	Palpitations	1	.	.	.	2	1	.	.	.
	Vasovagal episode	1	.	.	2	2	.	2	.	.
CONSTITUTIONAL SYMPTOMS		86	83	91	86	84	113	31	2	.
	Fatigue	69	54	88	67	68	97	19	2	.
	Fever	42	37	65	33	32	52	19	.	.
	Rigors/chills	55	63	77	40	41	90	4	.	.
	Sweating	43	39	58	38	36	70	3	.	.
	Weight loss	1	5	.	.	.	2	.	.	.
DERMATOLOGY/SKIN		96	98	100	90	98	28	126	10	.
	Alopecia	5	.	5	10	5	8	.	.	.
	Dry skin	1	.	2	2	.	2	.	.	.
	Flushing	19	20	21	24	14	33	.	.	.
	Hyperpigmentation	1	2	.	.	2	2	.	.	.
	Hypopigmentation	5	7	2	5	5	8	.	.	.
	Injection Site Reaction/Induration	96	98	100	90	98	29	128	7	.
	Pruritus	16	12	19	14	18	24	3	.	.
	Rash	21	22	30	14	16	30	5	.	.
	Ulceration	26	29	44	7	23	1	33	10	.
	Urticaria	1	2	.	2	.	2	.	.	.
GASTROINTESTINAL		67	59	79	60	70	101	12	1	.
	Anorexia	36	34	51	33	27	59	3	.	.
	Constipation	7	2	9	5	11	12	.	.	.
	Diarrhea	28	32	30	26	25	46	2	.	.
	Mucositis (clinical exam) - Oral cavity	12	17	14	12	7	21	.	.	.
	Mucositis (clinical exam) – Pharynx	1	.	2	.	.	1	.	.	.
	Mucositis (funct/sympt) - Oral cavity	1	2	.	.	2	2	.	.	.
	Nausea	48	41	56	36	57	72	9	.	.
	Taste alteration	1	.	.	2	2	2	.	.	.

		% with toxicity, any grade					Number (of 170) with toxicity by maximum grade			
Tox Group	**Toxicity**	**Overall**	**Group A**	**Group B**	**Group C**	**Group D**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
	Vomiting	14	12	23	5	16	20	3	1	.
INFECTION		2	2	7	.	.	.	4	.	.
	Infection (documented clinically) - Skin (cellulitis)	1	2	2	.	.	.	2	.	.
	Infection with normal ANC - Skin (cellulitis)	1	.	2	.	.	.	1	.	.
	Infection with unknown ANC - Skin (cellulitis)	1	.	2	.	.	.	1	.	.
LYMPHATICS		7	7	9	10	2	12	.	.	.
	Edema: limb	5	5	7	10	.	9	.	.	.
	Edema: trunk/genital	2	2	2	.	2	3	.	.	.
METABOLIC/ LABORATORY		62	49	77	60	64	99	6	.	1
	ALT	3	2	.	2	7	5	.	.	.
	AST	8	10	5	10	9	14	.	.	.
	Alkaline phosphatases	4	2	5	2	5	6	.	.	.
	Bilirubin	6	5	7	10	5	11	.	.	.
	Creatinine	2	5	.	2	2	4	.	.	.
	Hypercalcemia	2	.	.	7	2	4	.	.	.
	Hyperglycemia	35	24	49	29	36	54	5	.	.
	Hyperkalemia	18	22	21	17	14	30	1	.	.
	Hypernatremia	2	5	2	.	.	3	.	.	.
	Hypoalbuminemia	1	.	5	.	.	1	1	.	.
	Hypocalcemia	2	.	7	.	.	2	1	.	.
	Hypoglycemia	8	5	7	10	11	13	.	.	1
	Hypokalemia	4	2	7	.	7	7	.	.	.
	Hypomagnesemia	2	.	5	2	.	3	.	.	.
	Hyponatremia	6	2	9	2	9	10	.	.	.
MUSCULOSKELETAL/ SOFT TISSUE		4	5	2	5	2	6	.	.	.
	Arthritis	1	.	2	.	.	1	.	.	.
	Muscle weakness - Extremity-upper	1	.	.	5	.	2	.	.	.
	Muscle weakness - Whole body/generalized	1	2	.	.	.	1	.	.	.
	Musculoskeletal - Other (Specify)	1	2	.	.	2	2	.	.	.
NEUROLOGY		31	37	33	24	30	50	2	.	.
	Dizziness	24	29	26	19	20	39	1	.	.
	Mood alteration – Agitation	5	5	.	7	9	8	1	.	.
	Mood alteration – Anxiety	4	5	5	7	.	7	.	.	.
	Mood alteration – Depression	4	7	5	2	2	7	.	.	.
	Neuropathy-motor	1	2	.	.	.	1	.	.	.
	Neuropathy-sensory	1	.	2	.	2	2	.	.	.
OCULAR/VISUAL		5	5	7	5	2	7	1	.	.
	Blurred vision	1	.	2	2	.	2	.	.	.
	Dry eye	3	5	5	.	2	4	1	.	.
	Ocular - Other (Specify)	2	.	2	5	.	2	1	.	.

		% with toxicity, any grade					Number (of 170) with toxicity by maximum grade			
Tox Group	**Toxicity**	**Overall**	**Group A**	**Group B**	**Group C**	**Group D**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
PAIN		73	80	81	71	59	115	7	2	.
	Pain - Abdomen NOS	1	.	2	.	2	2	.	.	.
	Pain – Back	2	2	.	5	2	4	.	.	.
	Pain – Buttock	1	.	.	.	2	1	.	.	.
	Pain – Chest wall	2	.	.	2	5	3	.	.	.
	Pain – Chest/thorax NOS	1	.	.	2	.	1	.	.	.
	Pain - Extremity-limb	3	.	7	5	.	5	.	.	.
	Pain - Eye	1	2	.	.	.	1	.	.	.
	Pain - Head/headache	51	59	58	50	36	82	4	.	.
	Pain – Joint	33	39	33	29	32	51	5	.	.
	Pain – Larynx	2	2	2	2	.	3	.	.	.
	Pain – Muscle	39	51	51	29	27	63	3	1	.
	Pain - Neck	1	.	2	.	.	1	.	.	.
	Pain – Oral cavity	1	.	.	2	.	1	.	.	.
	Pain - Other (Specify)	4	.	7	.	9	7	.	.	.
	Pain – Pain NOS	1	.	.	2	.	.	.	1	.
	Pain – Sinus	1	.	2	.	.	1	.	.	.
	Pain - Throat/pharynx/larynx	19	20	12	21	23	32	.	.	.
PULMONARY/UPPER RESPIRATORY		47	54	51	45	39	75	3	2	.
	Bronchospasm	1	.	.	.	2	.	1	.	.
	Cough	32	37	33	36	23	52	2	.	.
	Dyspnea	19	29	26	12	9	29	2	1	.
	Hypoxia	1	.	2	.	.	.	1	.	.
	Nasal/paranasal reactions	27	24	33	26	25	46	.	.	.
	Pneumonitis	2	5	5	.	.	2	1	1	.
	Pulmonary - Other (Specify)	2	5	.	2	2	4	.	.	.
	Voice changes	1	2	.	2	.	2	.	.	.
SYNDROMES		21	24	33	14	14	26	9	1	.
	Cytokine release syndrome	1	.	2	.	2	.	1	1	.
	Flu-like syndrome	20	24	30	14	11	26	8	.	.

Supplemental Table 2. Reasons for discontinuing study treatment, by arm.

	Arm				Total
	A	B	C	D	
	N (%)	N (%)	N (%)	N (%)	N (%)
Off-TX					
Completed Treatment	21 (51.2)	19 (46.3)	25 (59.5)	28 (65.1)	93 (55.7)
Disease Progression	14 (34.1)	10 (24.4)	12 (28.6)	12 (27.9)	48 (28.7)
Unacceptable AEs	3 (7.3)	7 (17.1)	2 (4.8)	1 (2.3)	13 (7.8)
Non-protocol treatment	2 (4.9)	.	3 (7.1)	1 (2.3)	6 (3.6)
Protocol Violation(s)	.	3 (7.3)	.	.	3 (1.8)
Refused Further Treatment	1 (2.4)	1 (2.4)	.	1 (2.3)	3 (1.8)
PI Discretion	.	1 (2.4)	.	.	1 (0.6)
Total	41 (100.0)	41 (100.0)	42 (100.0)	43 (100.0)	167 (100.0)

Supplemental Table 3. Published criteria for immune response by ELIspot assay.

Type of ELIspot assay	Days stim	Fold increase	Counts	Per	Cells	Calc # per 100,000 CD8*	Notes and other criteria	Ref
Ex vivo	0	ns	1	2850	PBMC	175	High responder	[1]
Ex vivo	0	ns	1	19000	PBMC	26	Low** responder	[1]
Ex vivo	0	>2	> 5	100K	PBMC	25	3% false positive	[2]
Ex vivo	0	>4	>38	1,000,000	PBMC	19	IAVI criteria***	[3]
Ex vivo	0		1	10,000	PBMC	50		[4]
Ex vivo	0	>4	>55	1,000,000	PBMC	28		[5]
Ex vivo	0	--	--	--	--	--	3 SD over mean neg	[6]
Ex vivo	0	>=4	>=11	200,000	PBMC	28	Per Mogg	[7]
Ex vivo	**0**	**>=2**	**>=20**	**100,000**	**CD8+**	**20**	**Sum of SD over max neg; >2x vs prevax**	**This study**
Stim	4						2x prevax = strong responder	[8]
Stim	8-9	>4	>10	100,000	Cells	50		[9]
Stim	10-14	>3	>30	50,000	CD8	60		[10]
Stim	ns	>2	>50	1,000,000	CD8	5		[11]

Days stim = days stimulated with antigen in vitro prior to assay; ns = not specified
* based on an average that CD8 cells represent 20% of PBMC
** Britten refers to 1/2650 PBMC as a high responder, and <1/19,000 PBMC as a low responder. [1]
*** IAVI = International AIDS Vaccine Initiative

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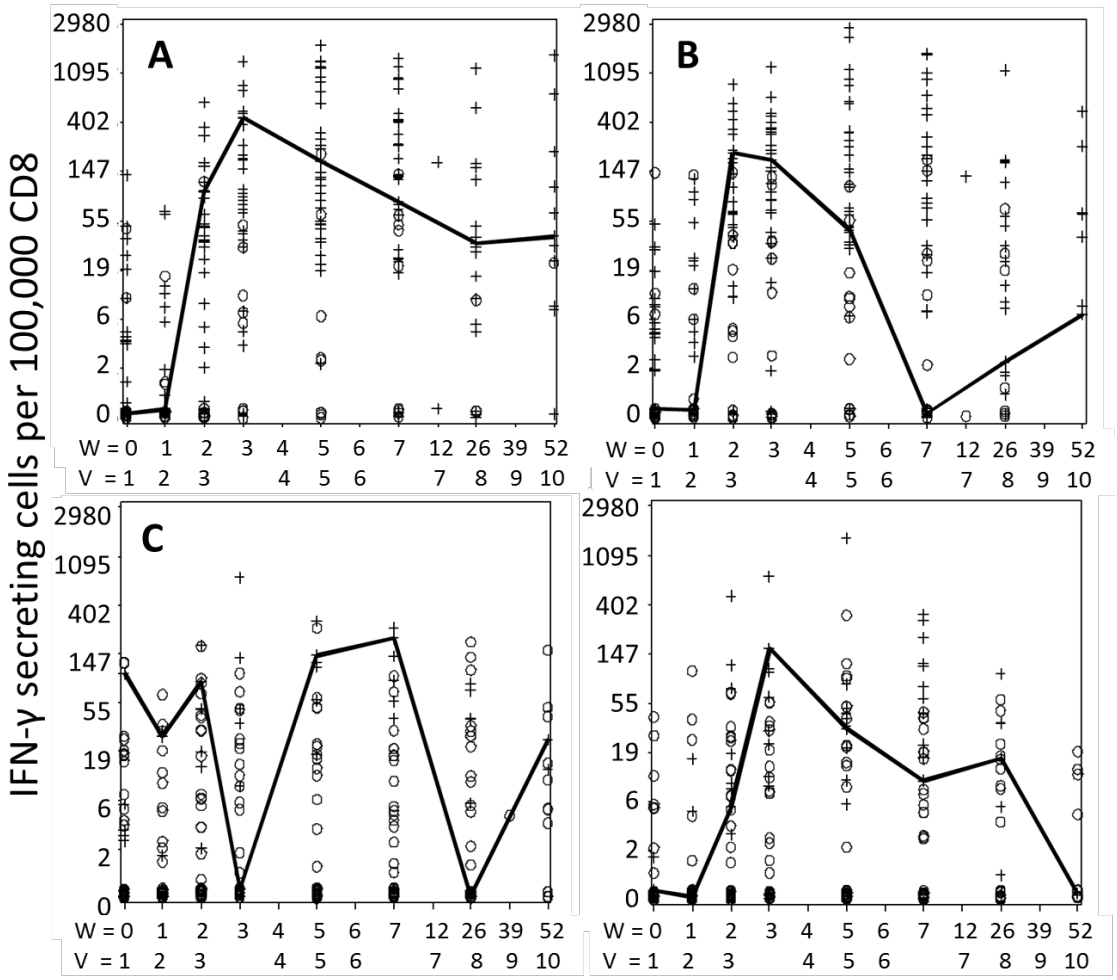

Supplemental Figure legend

Raw data for ELIspot measures of CD8+ T cell response to 12MP. This figure plots (on a natural log, minus 1, scale) the number of interferon-gamma secreting cells per 100,000 $CD8^+$ cells responding to the 12MP peptide pool, after subtracting the number of interferon-gamma-secreting cells in negative control wells. The mean value for negative controls across the whole study was 19.3 spots (95% CI 15.9, 22.7) per 100,000 PBMC, or 99.3 (95% CI 83.1, 115.5) per 100,000 $CD8^+$ cells. These raw data are shown for all patients in each arm of the study (Arms A-D, in panels A-D, respectively) over time from pretreatment week (W) 0, on the day of the first vaccine (V), through month 12, at the day of the last vaccine. For each data point, the symbol signifies whether this patient was considered to be an immune responder (+) or not (empty circle) based on the criteria provided in the Methods. Each value represents the mean of quadruplicate wells. The solid line in each graph represents data for the patient whose peak response was at the 75th percentile for that group. Peak values ranged to more than 1000 spots per 100,000 $CD8^+$ cells for patients in Arm A and B, with most values (for weeks 3-7) substantially exceeding the level of negative controls, whereas for patients in Arms C and D, few values exceeded the level of negative controls by more than 1 natural log.