Discovering protective T-cell responses by interrogating naturally processed antigenic determinants

Pavlo Gilchuk[1], Charles T. Spencer[1], Stephanie B. Conant[1], Timothy Hill[1], Jennifer J. Gray[1], Xinnan Niu[1], Mu Zheng[1], John J. Erickson[1], Kelli L. Boyd[1], K. Jill McAfee[1], Carla Oseroff[4], Sine R. Hadrup[5], Jack R. Bennink[7], William Hildebrand[8], Kathryn M. Edwards[2], James E. Crowe, Jr.[1,2], John V. Williams[1,2], Søren Buus[6], Alessandro Sette[4], Ton N.M. Schumacher[9], Andrew J. Link[1,3] and Sebastian Joyce[1]

[1]Departments of Pathology, Microbiology and Immunology, [2]Pediatrics, and [3]Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232; [4]Centre for Infectious Diseases, Allergy and Asthma Research, La Jolla Institute of Allergy and Immunology, La Jolla, CA 92037; [5]Centre for Cancer Immunotherapy, Department of Hematology, Herlev Hospital, Herlev, Denmark; [6]Laboratory of Experimental Immunology, University of Copenhagen, Copenhagen, Denmark; [7]Cellular Biology and Viral Immunology Section, Laboratory of Viral Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; [8]Department of Microbiology and Immunology, University of Oklahoma Health Science Centre, Oklahoma City, OK 73190; [9]Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Table of Contents

Methods

Plasmids

Constructs encoding biotin ligase (BirA), human β2m, HLA-A*02;01 and B*07;02 heavy chains for expression in Escherichia coli, and soluble HLA class I production in mammalian cells, were previously described (1-5). Constructs encoding hybrid HLA-A*0201 and -B*0702 heavy chains were designed by replacing their α3 domain residues 206—299 (NCBI RefSeq NM_002116.6 and NM_005514.6) with mouse $H2K^b$ α3 domain residues 203—296 (NCBI RefSeq NM_001001892.2). VACV ORFs were PCR amplified from cloned genome fragments (Addgene) to express truncated subunits $A34R_{21-168}$, $D1R_{565-844}$, $J6R_{188-466}$, $A3L_{62-319}$, $L4R_{33-249}$, $F4L_{1-319}$, $D5R_{330-470}$ and $E2L_{26-301}$ as C-terminal histidine-tagged fusion proteins from the *pET24a* plasmid (Merck; see Fig. S6).

Peptides

Large scale peptide syntheses and RPC purification was performed by the manufacturer (Schafer-N, Copenhagen, Denmark). Sequences of synthetic peptides were confirmed by mass spectrometry. Peptides were reconstituted in 100% DMSO at a concentration of 4.4mM and used at 100pM—20μM for various assays. Measurement of peptide/MHC class I dissociation and determination of $t_{1/2}$ was performed using scintillation proximity assay as previously described (6).

Expansion of epitope-specific T cells in PBMC with antigenic peptides

Frozen aliquots of PBMC were thawed at 37°C and incubated with DNase I (15—30 seconds) before washing with RPMI 1640 medium (Mediatech) supplemented with 5% (vol/vol) heat-inactivated normal human sera (Pel-Freez,), 2mM L-glutamine, 100U/ml of penicillin and 100U/ml of streptomycin (Mediatech). PBMC were rested overnight in 14 ml snap-top round bottom tubes at $3x10^6$/ml at a 30° angle. PBMC were expanded with 1 of 5 pools of peptides (Table S4). Each peptide (22 nM) was added to the rested PBMC along with 5 U/ml rhIL-2 (Teceleukin, NCI-BRB). IL-2 was replenished every other day through the first 7 days of culture at 5 U/ml. After a 5-day rest, PBMCs were washed, counted, and stained with tetramers or restimulated with individual peptides from the stimulating pool in the ELISpot format.

For use as effectors in antigen presentation assay, anti-VACV memory T_{CD8} cells were expanded from mouse spleen with individual antigenic peptides using the same protocol described for the expansion of human T cells from PBMC.

Generation of peptide–HLA class I tetramers

Recombinant heavy and light chain production (1), class I refolding with cognate conditional peptide ligands (2), biotinylation and purification (3), UV-mediated exchange of conditional peptide with VACV-derived peptides and quantification of peptide exchange (7) were performed as described previously. Class I multimerization with PE-, APC-, QDot605-, and QD655-streptavidin conjugated fluorochromes (Invitrogen) were performed as described (7, 8).

Tetramer and antibody staining

Lymphocytes from various mouse tissues were incubated with PE- and APC-labeled hybrid p/class I tetramers (0.1-1 μg/ml) and anti-CD8α-PerCP-Cy5.5 (clone 53-6.7), -B220- FITC (clone RA36B2) and -CD4-FITC (clone H129.19) antibodies for 1hr at room temperature in PBS containing 2% FBS (HyClone) and 50 nM Dasatinib (LC Laboratories). Phenotypic analysis of TCD8 was performed by co-staining with p/class I-QDot605/QDot655 multimers and anti-CD8α-Pacific Blue, -B220-FITC, -CD44-APC (clone IM7), -CD62L-APC-Cy7 (clone MEL-14), -CD127-PE (clone SB/14; all from BD Bioscience) or anti-KLRG-1-APC (clone 2F1; eBioscience). In some experiments p/class I tetramer^{+} TCD8 were analyzed for expression of intracellular GzmB (clone GB11,PE-labeled; Invitrogen). Epitope-specific immune T cell repertoires were analyzed by co-staining magnetically purified TCD8 with p/class I tetramers and a panel of FITC-conjugated anti-TCR-Vβ antibodies (BD Bioscience). Flow cytometric data were acquired using a FACSCalibur or LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Human PBMC were stained with PE- and APC-labeled p/class I tetramers as described previously (8) and with anti-CD8-Alexa Fluor 700 (clone 35B, Invitrogen), -CD4-FITC (clone RPA-T4), -CD14-FITC (clone M5E2), -CD19-FITC (clone HIB19) antibodies and propidium iodide to exclude dead cells (BD Bioscience).

TCD8 re-stimulation and intracellular cytokine staining (ICS)

Splenocytes from immune mice were re-stimulated in vitro for 6 hours with the indicated VACV peptides or irrelevant HLA-B*07;02-restricted HMPV-derived peptide APYAGLIMI at 1—10 μg/ml in the presence of 10μg/ml brefeldin A (Sigma-Aldrich), GolgiStop (BD Bioscience), and anti-CD107a-Alexa Flour 488 (clone eBioH4A3; eBioscience). Cells were then stained with antibodies for surface expression of CD8 (Pacific Blue), CD3 (PerCP Cy5.5; clone 145-2C11) and B220 (APC-Cy7), followed by staining for intracellular IFN-γ (clone XMG1.2, APC), TNF-α (clone MP6-XT22, PE) or IL-2 (clone JES6-5H4, PE; all from BD Bioscience) and analyzed using flow cytometry.

Antigen presentation assay

To prepare stimulators, $2x10^8$ naïve splenocytes were treated overnight with 10 ng/ml LPS. Cell cultures were inoculated with MOI=10 VACV in 7 ml RPMI 1640 + 1% BSA for 1 hour at 37°C followed by dilution to 50 ml RPMI 1640 + 10% FBS. At the indicated time points, brefeldin A was added to 10 μg/ml and cells were placed on ice. To probe for antigen presentation, 10^6 stimulators were incubated for 4 hours with 500-1000 TCD8 expanded *ex vivo* as described above. TCD8 cells were stained for intracellular IFN-γ and TNF-α and analyzed using flow cytometry.

Enumeration of naïve epitope-specific TCD8 *populations*

Enumeration of epitope-specific precursor TCD8 from naïve $B7^{tg}$ mice was adapted from a method described previously (9). In brief, cells from pooled spleens and lymph nodes (LN) were enriched to >85% TCD8 by negative selection (Miltenyi Biotech), stained with anti-CD8α, -CD3ε, -B220, -CD4, -CD44, -CD62L antibodies and various PE-conjugated p/class I tetramers (0.1—1 μg/ml) followed by incubation with anti-PE microbeads (Miltenyi Biotech) to further enrich for the p/class I tetramer-positive fraction by magnetic sorting, and analysed by flow cytometry. p/B7.2 $tetramer^+$ cells were identified as shown in Figure S11. The absolute number of naïve precursors per mouse was determined as described (9) and their frequency was normalized using the following equation: naïve frequency = (number of naïve precursors/$CD8\alpha^+$ T cell number) × 10^6.

Obtaining recombinant VACV proteins

VACV proteins were produced in *E. coli* BL21(DE3), purified from inclusion bodies by Ni-affinity chromatography (GE Healthcare) to >90%, dialyzed in PBS, adjusted to 2 mg/ml with PBS/0.05% SDS and stored at -20°C.

Mouse immunization and lethal VACV challenge

Bone marrow-derived DCs were generated using modification of previously described approach (10). Briefly, bone marrow was obtained from the femurs and tibiae of $B7^{tg}$ mice, erythrocytes lysed, and resuspended at 10^6 cells/ml in DC Media consisting of RPMI-1640 supplemented with 5% (vol/vol) heat-inactivated FBS (HiClone) 2mM L-glutamine, 100U/ml of penicillin, 100U/ml of streptomycin (Mediatech), 20ng/ml rmGM-CSF and 10ng/ml rmIL-4 (Peprotech). After 3 days, 75% of the media was replaced with fresh DC Media. After 6 days, cells were counted and re-plated. On d7, 100ng/ml LPS (Sigma) and 10μM peptide were added to the culture and incubated overnight to mature and to load the DCs with epitope, respectively. On d8, DCs were collected, counted and resuspended in PBS. By this time >85% of cells were $CD11c^{HI}$ as determined by flow cytometry.

For determination of epitope-specific protection, mice were primed s.q. with $1x10^6$ peptide-loaded DCs and then boosted in two weeks later i.v. with 200μg peptide formulated with 50μg of poly-IC (HMW, Invitrogen) and 50μg of anti-CD40 mAb (clone HB14, Bio X cell) in 200μl of PBS. In some experiments mice were primed with peptide, then boosted with peptide-loaded DCs and peptide.

For lethal respiratory challenge, mice were inoculated i.n. with 50μL PBS containing ~10^6 pfu VACV-WR under ketamine/xylazine anesthesia, weighed daily, scored for morbidity (0, no signs of illness or slight fur ruffling; 1, fur ruffling and back arching; 2, extensive fur ruffling and hunched posture; 3, lethargic; 4, death occurred between watch or euthanized after the loss of >30% of initial body weight. Six and 15 days post-challenge, T_{CD8} response was analyzed. Lungs were collected at d6 p.i. for determination of viral titers.

For protein immunization, groups of $B7^{tg}$ mice were injected i.p. twice with 20μg of individual protein subunits formulated with 1μg α-galactosylceramide (Funakoshi Co) in PBS/0.01% SDS buffer. T_{CD8} response was analyzed as described above.

Histology and immunohistochemistry

Harvested lungs were fixed in 10% formalin, embedded in paraffin, 5 μm-thick sections cut, and stained with hematoxylin and eosin (H&E). Immunohistochemistry for T cell infiltration of lungs sections was performed on the Leica Bond Max IHC stainer. Slides were deparaffinized. Heat induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 2 solution for 20 min. Slides were incubated with anti-CD3 (Santa Cruz, Inc. Santa Cruz CA) for one hour at 1:600 dilution. The Bond Intense R detection system was used for visualization. Stained tissue sections were examined using an Olympus BX41 microscope with Plan Achromat objectives 20X/0.5, 60X/0.90; images were captured with a Spot Flex digital camera using Diagnostic Instruments Spot Advanced acquisition software. Adobe Photoshop was utilized for white balancing and resizing of images.

VACV burdens

Lungs from individual mice were homogenized and sonicated prior to plating serial 10-fold dilutions on confluent BSC-40 cells. VACV plaques were visualized as above.

Supplementary Table 1. Characterization of vaccinia viral determinants presented during active infection

A. A*02;01

VACCC ORF[a]	Sequence	Prior reports[b]	Cn[c]	DPI[d]	# Hits[e]	Function[f]	Temporal. Exp[g]	$T_{1/2}$, h[h]	VARV[i]	MONPV	ECTV
$A2L_{114\text{-}122}$	QVKDEKLNL		2.376	1,2,3	7	T	L	14.59			
$A3L_{129\text{-}137}$	LNIMNKLNI		2.820			S	L	14.26			
$A6L_{6\text{-}14}$	VLYDEFVTI	Am	2.018			S	L	14.11			
$A6L_{171\text{-}179}$	ILSDENYLL	AMh	1.925	1,3	6	S	L	24.41			
$A7L_{192\text{-}200}$	KIIQRVQDL		2.021	1,2	3	T	L	1.19			
$A7L_{224\text{-}232}$	ILNDEQLNL		2.951	1,2,3	17	T	L	6.37			
$A7L_{249\text{-}257}$	TVMINNVKL		2.206	1,2,3	4	T	L	0.78			
$A7L_{462\text{-}470}$	QIDVEKKIV		2.106	1,2	4	T	L	13.31			V9I[j]
$A7L_{571\text{-}579}$	LIQEIVHEV	M	2.535	1,3	3	T	E	19.13			
$A8R_{134\text{-}142}$	QVSIIQEKL		2.185	1,2,3	6	T	IE	0.11			
$A10L_{76\text{-}85}$	YLIDTTSREL		2.020	2,3	3	S	L	9.77			E8S
$A10L_{740\text{-}749}$	MLVSQALNSV		1.523			S	L	14.19	N8D	N8D	N8D
$A11R_{260\text{-}269}$	KLGFEEIKGL		2.103	2,3	7	O	L	5.6	E5D		
$A11R_{292\text{-}300}$	KLGIGNSPV		1.626	1	7	O	L	3.13			
$A13L_{61\text{-}70}$	SLYNLVKSSV		1.895	1,3	5	S	L	6.36		V10A	V10A
$A24R_{1117\text{-}1126}$	KIDTTHVSKV		2.190	1,2	4	T	E	4.06			
$A27L_{46\text{-}54}$	TLKQRLTNL	Am	1.607		1	S	L	0.95			
$A29L_{157\text{-}165}$	KLTFLDVEV		1.630	2,3	4	T	E	5.93			
$A31R_{68\text{-}76}$	TINDLKMML		1.822	1,2,3	8	U	E	0.96			
$A36R_{1\text{-}9}$	MMLVPLITV	Amh	1.070			S	E	14.14	M2I		
$A37R_{153\text{-}161}$	YLPLSVFII		1.606			U	IE	2.82	NSH		
$A39R_{13\text{-}21}$	FVDVIIIKV		1.892	1	1	E/V	L	3.57	NSH	NSH	
$A39R_{394\text{-}402}$	MPQMKKILK		2.163			E/V	L	1.05	NSH	NSH	M1I Q3R
$A44L_{306\text{-}314}$	KISNTTFEV		2.495	1,2,3	6	E/V	IE	18.51	V9A		T6A
$A48R_{210\text{-}218}$	IVIEAIHTV	Mh	3.251	1,2,3	10	R	IE	6.66			I6M
$A50R_{458\text{-}466}$	TLRVLQDQL		2.342	2	1	R	E	0.19			
$A51R_{27\text{-}35}$	MIKPCCERV		2.012	1	1	U	E	3.46			
$A52R_{96\text{-}104}$	TIEELKQKL		2.258	1,2,3	6	E/V	IE	14.09		NSH	
$B8R_{101\text{-}109}$	KIGPPTVTL		2.241	1,2	8	E/V	E	1.53			
$B13R_{327\text{-}335}$	HVDGKILFV	Am	2.182	3	2	E/V	IE	4.22			
$B19R_{254\text{-}262}$	LILDPKINV		2.143	1,2,3	5	E/V	E	1.06	P5S		
$B19R_{294\text{-}302}$	WLIGFDFDV	Ah	1.859	3	5	E/V	E	23.56		NSH	
$B20R_{288\text{-}297}$	YLLDRGADIV		2.355	1,2,3	18	U	E	ND			
B23R and $C17L_{264\text{-}272}$	DIEIVKLLL		1.659	1	2	U	N/A	ND	E3D L9M	E3D L9M	NSH

VACCC ORF[a]	Sequence	Prior reports[b]	Cn[c]	DPI[d]	# Hits[e]	Function[f]	Temporal. Exp[g]	$t_{1/2}$, h[h]	VARV[i]	MONPV	ECTV
C1L$_{27-35}$	ILMRHLKNL		2.064	1	1	U	E	16.08	H5Y		NSH
C2L$_{124-132}$	EIINNITAV		2.060	2,3	2	E/V	E	0.68	NSH		
C4L$_{202-209}$	LLVNEFYI		1.833			U	E/L	6.79	I8T		NSH
C5L$_{99-107}$	YINNNIEEI		2.250			U	E/L	4.14			NSH
C7L$_{74-82}$	KVDDTFYYV	AM mh	2.267	1,2,3	7	U	E	12.21		D4Y	
C9L$_{602-610}$	KIDDMIEEV		2.226	1,2,3	8	U	E/L	9.54	D4N E7N	M4N E7D	NSH
C12L$_{16-24}$	VLISPVSIL		1.923	1,2,3	4	E/V	E	0.46			
C12L$_{292-300}$	FLHTTFIDV		1.715	1,2	2	E/V	E	ND	T4K	T5A	
D1R$_{33-41}$	EINNELELV		2.427	1,2	4	T	E	0.83			
D1R$_{251-259}$	RVYEALYYV	AM mh	1.822	1,2	4	R	E	14.44			
D1R$_{764-772}$	YIIKKNDIV		1.582	1	1	T	E	0.78			
D2L$_{18-27}$	ILFPDDVQEL		2.606	1,2,3	6	S	L	5.09	E9K	V7L	
D2L$_{77-85}$	LLFLKDVEP		2.503		0	S	L	29.02	K5E	K5E	K5E
D5R$_{568-577}$	KIRSDNIKKL		2.385	2	1	R	E	6.85			
D8L$_{155-164}$	YLDNLLPSTL		2.189	2	2	S	L	0.98	T9K		
D10R$_{168-176}$	TIINKFFEV		2.419	1	1	O	IE	ND			
D12L$_{174-182}$	SLFKNVRLL	Ah	2.005	1,2,3	3	T	E	1.29			
D12L$_{62-70}$	KLFTHDIML	AM mh	1.602			T	E	22.82			
D13L$_{172-180}$	KLSDSKITV		2.572	1,2,3	13	O	L	27.07			
D13L$_{327-335}$	DVYVKIDNV		1.979	1,2,3	4	O	L	ND			
D13L$_{62-70}$	YITALNHLV		2.567	1	4	O	L	16.66			
D13L$_{70-78}$	VLSLELPEV	M	2.249	1,3	4	O	L	22.73			
E2L$_{249-257}$	KIDYYIPYV	AM mh	2.091	1,3	3	U	E	22.12			V9A
E2L$_{703-711}$	FIFLKKNEL		2.552	2,3	4	U	E	0.15			
E3L$_{42-50}$	EVNKALYDL		1.883	1,2,3	45	E/V	IE	0.89			
E5R$_{117-125}$	KLFSDISAI	Mh	2.338			R	IE	17.75			
E5R$_{252-260}$	LVEKVLKIL		2.120	1,2	11	R	IE	11.08			
E6R$_{462-470}$	YLDGQLARL		2.291	3	1	U	L	14.38			
E9L$_{271-279}$	YITNRLELL		2.256	1,3	4	R	E	2.71			
E9L$_{978-987}$	EIVNLLDNKV		1.853	3	5	R	E	ND			
F1L$_{125-133}$	TVYDINNEV		2.141	2,3	2	U	E	2.84			E8K
F10L$_{254-263}$	PLALYSADKV		1.746	1	2	S	E	ND		A3V	
F10L$_{307-315}$	DLKPDNILL		2.135	1,3	5	S	E	ND			
F11L$_{340-348}$	SLSNLDFRL	M	2.728	1,2,3	65	U	E	23.58	R8Y		
F12L$_{251-260}$	FVNFNSVKNL		2.051	1,3	7	S	E	0.43	N9D	N9D	N9D
F12L$_{286-295}$	NLFDIPLLTV	AM mh	1.639	3	1	S	E	22.59			
F12L$_{404-412}$	FLTSVINRV	AM mh	1.692			S	E	19.28			
F13L$_{140-149}$	SIHTIKTLGV		1.663		2	S	L	0.35			

VACCC ORF[a]	Sequence	Prior reports[b]	Cn[c]	DPI[d]	# Hits[e]	Function[f]	Temporal. Exp[g]	$t_{1/2}$, h[h]	VARV[i]	MONPV	ECTV
F14L$_{39-47}$	ELLNILTEL		2.347	1,2,3	7	U	E	0.88			
G3L$_{99-108}$	DIRTLLPILL		2.248	1,2,3	4	S	L	7.12			
G4L$_{57-66}$	TLIGNFAAHL		2.226	3	4	S	L	9.86			
G5R$_{18-26}$	ILDDNLYKV	Amh	3.437	1,2,3	12	U	IE	26.21			K8N
G5R$_{124-132}$	EMQLKIDKL		2.276	1,2,3	7	U	IE	0.81	M2I		
G5.5R$_{27-35}$	SLKDVLVSV	Mh	2.052	1,2	4	T	E	13.79			
G7L$_{161-169}$	EMKYALINL		2.165	1,2,3	21	S	L	AMB			
G9R$_{113-121}$	VLESCWPDV		1.843	1,2,3	8	S	L	0.93			V1E V9A
G8R$_{251-259}$	KINIFMAFL		1.898	1,3	3	N/A	N/A	1.91			
H3L$_{184-192}$	SLSAYIIRV	Amh	2.669	1,2,3	28	S	L	25.52			
H4L$_{676-684}$	KIEIERKKL		2.309	1,2,3	9	T	L	13.46			
H5R$_{159-168}$	VLEDVQAAGI	Am	1.507			T	E	0.35			
H6R$_{3-11}$	ALFYKDGKL		1.621	2,3	3	T	E/L	0.35			A1P
I1L$_{122-130}$	YIDISDVKV		2.631	3	2	S	L	0.82			
I1L$_{155-163}$	KIEDLINQL		2.444	1,2,3	4	S	L	0.35			
I1L$_{211-219}$	RLYDYFTRV	Amh	2.621	1,2,3	13	S	L	20.03			
I3L$_{212-220}$	KLIIDREVV		1.794	3	3	R	IE	AMB			
I7L$_{364-372}$	KMTLFKSIL		2.416	1,2	3	S	L	0.42			
I8R$_{427-435}$	KVLDIDEIL		2.520	1,2,3	7	T	E	0.55	D4E E7K		
I8R$_{430-438}$	DIDEILEKV		2.510	1	3	T	E	13.38	D1E E4K		
J6R$_{375-384}$	SIIFGRQPSL		2.009	2	1	T	E	0.99			
J6R$_{593-601}$	AINVEKIEL		2.044	1,2	3	T	E	0.20			
J6R$_{653-661}$	LIDNPDNNL		2.230			T	E	0.76			N4D
J6R$_{717-726}$	YILNSLTKGL		2.347	1,2,3	10	T	E	1.66			
J6R$_{1074-1082}$	FVEPEELNL		2.543	1,2	3	T	E	0.94			
K1L$_{191-200}$	SLLFIPDIKL	M	2.487	3	1	U	IE	14.13	S1F		
K3L$_{57-65}$	KLVGKTVKV	M	2.185	1,3	3	E/V	IE	20.50		NSH	T6K
L3L$_{142-150}$	KVVKLTPQV		1.956	1,2,3	4	T	L	3.85	K4R	K4R	K4R
L3L$_{182-191}$	LVSIPRTNIV		1.869	1,3	3	T	L	10.83			
O1L$_{247-255}$	GLNDYLHSV	AM mh	2.493	3	1	U	IE	48.96			
O1L$_{550-559}$	PITDSLSFKL		2.369	2,3	2	U	IE	ND	D4E		
O1L$_{566-574}$	VLNDQYAKV		2.982	1,2	4	U	IE	18.05			
AorfA$_{102-110}$	YLFLICHNL		2.496	1,2,3	12	N/A	N/A	8.23	NSH	NSH	NSH
AorfU$_{59-67}$	RIIYIIRFL		2.543	3	3	N/A	N/A	0.83	NSH	NSH	NSH
IorfA$_{66-74}$	YLSAKITTL		1.652	1	1	N/A	N/A	12.31	NSH	NSH	NSH
VAC WR148$_{433-441}$	KLTELNAEL		2.809	1,3	7	S	L	38.27		A7E	NSH
VAC WR148$_{436-445}$	ELNAELSDKL		2.133	1,2,3	8	S	L	ND		A4E	NSH

B. B*07;02

ORF[a]	Sequence	Prior reports[b]	Cn[c]	DPI[d]	# Hits[e]	Function[f]	Temporal. Exp[g]	$t_{1/2}$, h[h]	VARV[i]	MONPV	ECTV
A3L$_{192-200}$	SPSNHHILL		1.917	1,3	4	S	L	5.45			
A3L$_{453-462}$	SPVIVNGAMM		1.377	1,2	2	S	L	0.62			
A4L$_{83-91}$	VPTATPAPI		1.686	1,2,3	27	S	E/L	0.83	NSH[j]	A4P	NSH
A4L$_{126-135}$	APASSLLPAL		2.110	1,3	6	S	E/L	2.71	NSH	S5T	NSH
A10L$_{111-120}$	NPIINTHSFY		1.732	2,3	3	S	L	ND			S8N
A10L$_{122-130}$	LPPFTQHLL		1.843	2,3	2	S	L	0.24			
A11R$_{22-30}$	YPSNKNYEI		2.005	3	1	O	L	1.19			
A16L$_{330-339}$	EPVVKDKIKL		2.317	1,3	2	S	L	ND		K5N	
A20R$_{162-170}$	IPKYLEIEI		1.960	1,2	14	R	E	0.43	K3N	K3N	
A24R$_{1002-1010}$	KPYASKVFF		1.924	1,2,3	5	T	E	5.43		A4E	
A34R$_{82-90}$	LPRPDTRHL	Am	2.002	1,2,3	14	S	L	7.13		R3G	
A40R$_{3-11}$	KPKTDYAGY		1.941	2	1	E/V	E	ND	D5N	NSH	NSH
A40R$_{88-96}$	NPNSDLIKI		1.137	1	2	E/V	E	ND	NSH	NSH	
A47L$_{227-236}$	KPVSDLYTSM		1.850	1	3	U	IE	3.19		NSH	
B8R$_{70-78}$	FPKNDFVSF		2.237	1,3	3	E/V	E	5.20			K3N
B8R$_{158-167}$	EPVTYDIDDY		2.047	1,3	2	E/V	E	ND	D6N		T4I
B12R$_{251-259}$	EPLELVRYI		2.082	2	1	U	IE	ND	NSH		
B16R$_{226-234}$	LPDGIVTSI		1.932	1,2,3	9	E/V	L	0.59	NSH	D3E I5V	
B17L$_{182-191}$	APLPGNVLVY		1.802	1,3	6	U	E	ND	L3Y	NSH	
B18R$_{305-313}$	RPADSITYL		1.690	1,2,3	6	U	E	3.16	A3L	NSH	R1H A3S
C19L and B25R$_{78-86}$	NPSVLKILL		1.702	2,3	4	U	E	0.22	N1K		N1K
C10L$_{41-49}$	LPMEDNSDI		1.692	1,3	2	U	E	N/A			
C10L$_{41-50}$	LPMEDNSDII		1.829	1	2	U	E	1.38			
C20L and B26R$_{7-16}$	EPIRGYVIIL		2.020	2,3	3	P	E	ND	NSH	NSH	NSH
D11L$_{102-111}$	APEITKDCIF		1.740	2,3	4	T	L	0.94			
D1R$_{384-393}$	GPKSNIDFKI		2.185	1	1	T	E	ND			
D1R$_{808-817}$	RPSTRNFFEL	Am	1.549	1	1	T	E	2.93			
D5R$_{361-369}$	EPLITKLIL		1.578	1,3	3	R	E	ND			
D5R$_{375-383}$	LPKEYSSEL		1.885	1	2	R	E	7.44			
D8L$_{160-169}$	LPSKLDYFTY		1.843	3	1	S	L	ND	T9K	K4T	K4T
D9R$_{26-35}$	IPRSKDTHVF		2.080	2,3	5	O	IE	5.37			
D9R$_{100-109}$	DPHFEELILL		2.066	1	1	O	IE	ND			
D11L$_{506-514}$	MPTVDEDLF		1.652	1,3	2	T	L	0.84			
E1L$_{10-18}$	LPNITLKII		1.539	1,2	3	T	E	0.86			
E2L$_{216-224}$	RPRDAIRFL		2.049	3	1	U	E	4.81			

VACCC ORF[a]	Sequence	Prior reports[b]	Cn[c]	DPI[d]	# Hits[e]	Function[f]	Temporal. Exp[g]	$t_{1/2}$, h[h]	VARV[i]	MONPV	ECTV
E5R$_{131-140}$	NPSKMVYALL		1.567	1,3	2	R	IE	0.39	V6A		
E6R$_{284-293}$	EPEKDIRELL		1.836	3	1	U	L	2.71			
E9L$_{175-183}$	FPSVFINPI		1.594	1,3	2	R	E	2.40			
E9L$_{526-534}$	FPYEGGKVF		1.845	3	3	R	E	6.17			
F2L$_{26-35}$	SPGAAGYDLY		1.537	1,2,3	7	R	E	ND	G3Y		NSH
F2L$_{42-50}$	IPPGERQLI		1.657	1,2	2	R	E	ND			
G2R$_{140-149}$	VPITGSKLIL		2.260	2	1	T	E	1.19	T4A	T4A	NSH
G5R$_{341-349}$	LPCQLMYAL		2.034	2	1	U	IE	3.52			
G7L$_{175-183}$	LPMIIGEPI		2.086	2,3	3	S	L	5.02			
G9R$_{14-23}$	PPPGVPTDEM		1.214	1,3	2	S	L	ND			
G9R$_{69-77}$	GPGGLSALL		1.583	2,3	3	S	L	0.37			G4N
H1L$_{65-73}$	LPNSNINII		2.141	1,2,3	4	T	E/L	1.01			
H3L$_{6-14}$	TPVIVVPVI		2.137	2	1	S	L	ND			V3I
H5R$_{89-97}$	SPSPGVGDI		1.637	1,3	3	T	E	0.65	P2S	P2S	NSH
I1L$_{184-192}$	IPDELIDVL		1.771	3	2	S	L	AMB			
I4L$_{498-507}$	RPIGIGVQGL		2.975	2	3	R	E	ND			
I6L$_{237-245}$	FPTNTLTSI		2.013	2	1	U	E/L	1.52			
I6L$_{272-280}$	IPKKIVSLL		1.752	2	1	U	E/L	5.35			
I6L$_{282-291}$	LPSNVEIKAI		1.769	1	1	U	E/L	0.54			
I7L$_{342-350}$	TPPKSFKSL		1.900	2	2	S	L	0.85			
I8R$_{221-230}$	RPVILSLPRI		1.057	2,3	2	T	E	2.35			
J3R$_{8-16}$	KPFMYFEEI		1.800	1	1	T	E/L	1.24			
J3R$_{253-261}$	YPNQEYDYF		1.656	1,3	2	T	E/L	ND			
J5L$_{53-61}$	LPASLKKNI		2.220	2,3	2	S	L	ND			
J6R$_{1089-1098}$	LPGAANKGKI		1.230	1,2	4	T	E	0.97			
J6R$_{1103-1111}$	IPISDYTGY		2.019	1,3	10	T	E	ND			
K1L$_{151-159}$	IPSTFDLAI		2.020	1,2,3	4	U	IE	0.71	NSH	T4F	
K6L$_{17-25}$	KPITYPKAL		2.603	1,3	14	O	IE	4.99	NSH		
L4R$_{37-45}$	FPRSMLSIF		1.962	1	1	T	L	6.08			
N2L$_{104-113}$	RPNQHHTIDL		2.007	3	1	O	IE	4.73			Q4K

[a]Open reading frames (ORF) and location of epitopes are defined based on Copenhagen reference strain (VACCC, ID 10249)
[b]prior reports according to immune epitope data base (IEDB; www.iedb.org); A, algorithm based; M, discovered by MHC class I ligand elution; m, positive in mouse TCD8 assay; h, positive in human TCD8 assay; blank, this study
[c]correlation coefficient represents the number of peak identities determined between the theoretically and experimentally derived spectra for a given parent ion normalized to the charge state of the peptide
[d]days p.i. of HeLa cultures with VACV at which the peptide was identified

[e]total number of times a given peptide sequence was identified by mass spectrometry
[f]protein function according to (11); S, structural (virion membrane and core); T, transcription; E/V, evasion/virulence; O, other; U, unknown; P, preudogenes
[g]temporality of expression (shortened) according to (12): IE, immediate early; E; early; E/L, early/late; L, late; N/A, unidentified; [h]Half-time of p/MHC stability. ND, no signal obtained; AMB, ambiguous, data from independent experiments were inconsistent. ND, not determined
[i]peptide homologies were identified using Netblast (blastcl3 at www.ncbi.nlm.nih.gov) using the following taxonomy id: VARV, variola virus, 10255; ECTV, ectromelia virus, 12643; MONPV, monkeypox virus, 10244)
[j]Amino acid changes for homologous poxviral epitopes; NSH, no significant homology; blank, conserved sequences with 100 % homology.

Supplementary Table 2: Volunteer demographics, HLA haplotype and vaccination history								
ID	**Gender**	**Ethnicity**	**Age at vaccination**	**HLA-A***		**HLA-B***		**Time since vaccination**
V101	M	Caucasian	22	0101	0301	0702	0801	4.5 months after primary
V111	M	Caucasian	51	0101	0201	0702	0702	4 months after boost
V115	M	Caucasian	34	0201	0201	4001	4101	3 months after boost
V150	F	Caucasian	61	0301	2301	0702	3701	10 months after boost
V154	F	Caucasian	28	0301	3201	0702	1501	3 months after primary
V155	F	Caucasian	30	0201	3201	0702		3 months after primary
V158	F	Caucasian	28	0301	3002	0702	4402	13 days after primary
V163	F	Caucasian	44	0201	0301	0702	1501	6 days after boost
V168	M	Caucasian	32	0205	6801	3902	5301	15 days after primary
V001	F	Caucasian	24	0201		1501	5101	4 years after primary
V003	F	Caucasian	27	0201	2901	0702	0705	4 years after primary
V008	M	Caucasian	60	0201	0301	1402	1404	4 years after primary
V023	F	Caucasian	25	0201	2402	1302	4001	4 years after primary
V034	F	Caucasian	30	0101	0201	0801	1302	3.5 month after boost

Supplementary Table 3. Peptide pools used for screening of TCD8 from VACV immune humans and HLA class I transgenic mice

A. A*02;01			
	ORF[a]	Peptide	Source[b]
Pool 1	$J6R_{593-601}$	AINVEKIEL	MS
	$A23R_{273-281}$	ALDEKLFLI	MS+
	$H6R_{3-11}$	ALFYKDGKL	MS
	$H3L_{142-151}$	AMHDKKIDIL	Predicted
	$A27L_{92-100}$	AMISLAKKI	Predicted
	$A55R_{391-399}$	AMLNGLIYV	Public
	$B15R_{29-37}$	CLTEYILWV	Public
	$G3L_{99-108}$	DIRTLLPILL	MS
	N/A	DVRTLLPILL	dMS
	$F10L_{307-315}$	DLKPDNILL	MS
	N/A	DLKPENILL	dMS
	N/A	DIKPDNILL	dMS
	N/A	DLKPQNILL	dMS
	$E9L_{978-987}$	EIVNLLDNKV	MS
	$VACWR148_{436-445}$	ELNAELSDKL	MS
	$G5R_{124-132}$	EMQLKIDKL	MS
	N/A	EIQLKIDKL	dMS
	$A4L_{247-255}$	ETNDLVTNV	Public
	$E2L_{703-711}$	FIFLKKNEL	MS
	$F12L_{251-260}$	FVNFNSVKNL	MS
	N/A	FVNFNSVKDL	dMS
	$A46R_{142-150}$	GLFDFVNFV	MS+
	$O1L_{247-255}$	GLNDYLHSV	MS+
	$B13R_{327-335}$	HVDGKILFV	MS+
	$G5R_{18-26}$	ILDDNLYKV	MS+
	$D2L_{18-27}$	ILFPDDVQEL	MS
	$A10L_{51-59}$	LLNNSLGSV	Predicted
	$K1L_{159-167}$	ILLSCIHTT	Predicted
	$A7L_{224-232}$	ILNDEQLNL	MS
	$A6L_{171-179}$	ILSDENYLL	MS+
	$A48R_{210-218}$	IVIEAIHTV	MS+
	$A24R_{1117-1126}$	KIDTTHVSKV	MS

Pool 2	N/A	KVDTTHVSKV	dMS
	$E2L_{249-257}$	KIDYYIPYV	MS+
	$H4L_{676-684}$	KIEIERKKL	MS
	$A44L_{306-314}$	KISNTTFEV	MS
	$B8R_{19-27}$	ITSYKFESV	Predicted

	ORF[a]	Peptide	Source[b]
	$E5R_{117-125}$	KLFSDISAI	MS+
	$D12L_{62-70}$	KLFTHDIML	MS+
	$A11R_{260-269}$	KLGFEEIKGL	MS
	N/A	KLGFDEIKGL	dMS
	$A11R_{292-300}$	KLGIGNSPV	MS
	$I3L_{212-220}$	KLIIDREVV	MS
	$B19R_{207-215}$	KLIIHNPEL	Public
	$A29L_{157-165}$	KLTFLDVEV	MS
	$I7L_{364-372}$	KMTLFKSIL	MS
	$C7L_{74-82}$	KVDDTFYYV	MS+
	$A47L_{169-177}$	LLYAHINAL	Public
	$B6R_{108-116}$	LMYDIINSV	Public
	$A3L_{129-137}$	LNIMNKLNI	MS
	$L3L_{182-191}$	LVSIPRTNIV	MS
	$A10L_{740-749}$	MLVSQALNSV	MS
	$A36R_{1-9}$	MMLVPLITV	MS+
	$A10L_{1-10}$	MMPIKSIVTL	Predicted
	$A10L_{773-781}$	NLATSIYTI	Predicted
	$A27L_{53-61}$	NLEKKITNV	Public
	$F12L_{286-295}$	NLFDIPLLTV	MS+
	$A26L_{6-14}$	NLWNGIVPT	Public
	$E3L_{35-43}$	QLNMEKREV	Predicted
	$A4L_{271-280}$	QLVKGFERFQ	Public
	$A2L_{114-122}$	QVKDEKLNL	MS
	$I1L_{211-219}$	RLYDYFTRV	MS+
	$A13L_{61-70}$	SLYNLVKSSV	MS
	$A27L_{4-12}$	TLFPGDDDL	Public

Pool 3	$A27L_{46-54}$	TLKQRLTNL	MS+
	$C7L_{82-90}$	VIYEAVIHL	Predicted
	$H5R_{159-168}$	VLEDVQAAGI	MS+
	$A27L_{78-86}$	VLFRLENHA	Public
	$D6R_{498-506}$	VLPFDIKKL	Public
	$A6L_{6-14}$	VLYDEFVTI	MS+
	$A55R_{78-86}$	YIYGIPLSL	Public
	$D8L_{155-164}$	YLDNLLPSTL	MS
	$A10L_{76-85}$	YLIDTTSREL	MS
	$B20R_{288-297}$	YLLDRGADIV	MS
	$G7L_{250-258}$	YLPEVISTI	Public
	N/A	GLFGFVNFV	dMS
	$J6R_{653-661}$	LIDNPDNNL	MS
	$D2L_{77-85}$	LLFLKDVEP	MS
	$C4L_{202-209}$	LLVNEFYI	MS

	ORF[a]	Peptide	Source[b]
	$A39R_{394\text{-}402}$	MPQMKKILK	MS
	$G5.5R_{27\text{-}35}$	SLKDVLVSV	MS+
	$I4L_{720\text{-}728}$	SMHFYGWSL	Public
	$C16L_{171\text{-}179}$	TLLDHIRTA	Public
	$A27L_{88\text{-}96}$	TLRAAMISL	Public
	$B19R_{294\text{-}302}$	WLIGFDFDV	MS+
	$I8R_{430\text{-}438}$	DIDEILEKV	MS
	$J6R_{1074\text{-}1082}$	FVEPEELNL	MS
	$D13L_{172\text{-}180}$	KLSDSKITV	MS
	$I8R_{427\text{-}435}$	KVLDIDEIL	MS
	$F11L_{340\text{-}348}$	SLSNLDFRL	MS
	$O1L_{566\text{-}574}$	VLNDQYAKV	MS
	$I1L_{122\text{-}130}$	YIDISDVKV	MS
	$D13L_{62\text{-}70}$	YITALNHLV	MS
	$E5R_{252\text{-}260}$	LVEKVLKIL	MS
	$D12L_{251\text{-}259}$	RVYEALYYV	MS+
	$H3L_{184\text{-}192}$	SLSAYIIRV	MS+

Pool 4	$G9R_{113\text{-}121}$	VLESCWPDV	MS
	$C5L_{99\text{-}107}$	YINNNIEEI	MS
	$E9L_{271\text{-}279}$	YITNRLELL	MS
	$F12L_{404\text{-}412}$	FLTSVINRV	MS+
	$G8R_{251\text{-}259}$	KINIFMAFL	MS
	N/A	LLFLEDVEP	dMS
	$VACCA_{177\text{-}186}$	*YLYTEYFLFI	Predicted
	$A7L_{571\text{-}579}$	LIQEIVHEV	MS
	$N2L_{93\text{-}101}$	YVNAILYQI	Public
	$D13L_{327\text{-}335}$	DVYVKIDNV	MS
	$D1R_{33\text{-}41}$	EINNELELV	MS
	$F14L_{39\text{-}47}$	ELLNILTEL	MS
	$G7L_{161\text{-}169}$	EMKYALINL	MS
	$E3L_{42\text{-}50}$	EVNKALYDL	MS
	$A10L_{44\text{-}52}$	FHVPFDILL	Predicted
	$A39R_{13\text{-}21}$	FVDVIIIKV	MS
	$C9L_{602\text{-}610}$	KIDDMIEEV	MS
	$I1L_{155\text{-}163}$	KIEDLINQL	MS
	$B8R_{101\text{-}109}$	KIGPPTVTL	MS
	N/A	KISYYIPYV	dMS
	$VACWR148_{433\text{-}441}$	*KLTELNAEL	MS
	$L3L_{142\text{-}150}$	KVVKLTPQV	MS
	$B19R_{254\text{-}262}$	LILDPKINV	MS
	$A7L_{462\text{-}470}$	QIDVEKKIV	MS
	$A52R_{96\text{-}104}$	TIEELKQKL	MS

	ORF[a]	Peptide	Source[b]
	$C12L_{16-24}$	VLISPVSIL	MS
	$J6R_{717-726}$	YILNSLTKGL	MS
	$A37R_{153-161}$	YLPLSVFII	MS
	I orf A_{66-74}	YLSAKITTL	MS
	B23R and $C17L_{264-272}$	DIEIVKLLL	MS
	$C12L_{292-300}$	FLHTTFIDV	MS

Pool 5	$C2L_{124-132}$	EIINNITAV	MS
	$C1L_{27-35}$	ILMRHLKNL	MS
	$A7L_{192-200}$	KIIQRVQDL	MS
	$VACWR148_{433-441}$	KLTELNAEL	MS
	$A7L_{571-579}$	LIQEIVHEV	MS
	$A51R_{27-35}$	MIKPCCERV	MS
	$F10L_{254-263}$	PLALYSADKV	MS
	A orf U_{59-67}	RIIYIIRFL	MS
	$F13L_{140-149}$	SIHTIKTLGV	MS
	$J6R_{375-384}$	SIIFGRQPSL	MS
	$K1L_{191-200}$	SLLFIPDIKL	MS
	$D10R_{168-176}$	TIINKFFEV	MS
	$G4L_{57-66}$	TLIGNFAAHL	MS
	$A50R_{458-466}$	TLRVLQDQL	MS
	$F1L_{125-133}$	TVYDINNEV	MS
	$D13L_{70-78}$	VLSLELPEV	MS
	$I1L_{122-130}$	YIDISDVKV	MS
	$D1R_{764-772}$	YIIKKNDIV	MS
	$D13L_{62-70}$	YITALNHLV	MS
	$E6R_{462-470}$	YLDGQLARL	MS
	$A31R_{68-76}$	TINDLKMML	MS
	$A8R_{134-142}$	QVSIIQEKL	MS
	N/A	TVMINNVKI	dMS
	$A7L_{249-257}$	TVMINNVKL	MS
	$K3L_{57-65}$	KLVGKTVKV	MS
	$O1L_{550-559}$	PITDSLSFKL	MS
	$D12L_{174-182}$	SLFKNVRLL	MS+
	A orf $A_{102-110}$	YLFLICHNL	MS
	$D5R_{568-577}$	KIRSDNIKKL	MS

B. B*07;02			
	ORF	**Peptide**	**Source**
Pool 1	$D11L_{102-111}$	APEITKDCIF	MS
	$B17L_{182-191}$	APLPGNVLVY	MS
	N/A	APYPGNVLVY	dMS
	$F4L_{6-14}$	APNPNRFVI	Public
	$B8R_{158-167}$	EPVTYDIDDY	MS
	N/A	EPVTYNIDDY	dMS
	N/A	B35	MS
	$B8R_{70-78}$	FPKNDFVSF	MS
	N/A	B35	MS
	$E9L_{175-183}$	FPSVFINPI	MS
	N/A	FPSVFINPV	dMS
	$E9L_{526-534}$	FPYEGGKVF	MS
	N/A	B35	MS
	$I1L_{184-192}$	IPDELIDVL	MS
	$J6R_{1103-1111}$	IPISDYTGY	MS
	N/A	B35	MS
	$F2L_{42-50}$	IPPGERQLI	MS
	$D9R_{26-35}$	IPRSKDTHVF	MS
	$C19L/B25R_{78-86}$	NPSVLKILL	MS
	$G9R_{14-23}$	PPPGVPTDEM	MS
	$F2L_{26-35}$	SPGAAGYDLY	MS
	$G9R_{69-77}$	GPGGLSALL	MS
	$B15R_{91-101}$	IPDEQKTIIGL	Predicted
	$A20R_{162-170}$	IPKYLEIEI	MS
	N/A	B35	MS
	N/A	B35	MS

Pool 2	$J5L_{53-61}$	LPASLKKNI	MS
	$H1L_{65-73}$	LPNSNINII	MS
	$A10L_{122-130}$	LPPFTQHLL	MS
	$J6R_{303-311}$	MPAYIRNTL	Public
	$A40R_{88-96}$	NPNSDLIKI	MS
	$E5R_{131-140}$	NPSKMVYALL	MS
	N/A	NPSKMAYALL	dMS
	N/A	B35	MS
	N/A	NPVTIINEY	dMS
	$B18R_{305-313}$	RPADSITYL	MS
	N/A	RPLDSITYL	dMS
	$D1R_{808-817}$	RPSTRNFFEL	MS+
	$I8R_{221-230}$	RPVILSLPRI	MS
	N/A	B35	MS

	ORF[a]	**Peptide**	**Source**[b]
	A3L$_{192-200}$	SPSNHHILL	MS
	H5R$_{89-97}$	SPSPGVGDI	MS
	N/A	B35	MS
	G7L$_{175-183}$	LPMIIGEPI	MS
	N/A	B35	MS
	I7L$_{342-350}$	TPPKSFKSL	MS
	B22R$_{72-80}$	TVADVRHCL	Public
	J3R$_{253-261}$	YPNQEYDYF	MS
	A4L$_{126-135}$	APASSLLPAL	MS
	B12R$_{251-259}$	EPLELVRYI	MS
	A16L$_{330-339}$	EPVVKDKIKL	MS
	O1L$_{549-557}$	IPITESLSF	Public

Pool 3	N/A	IPNYLEIEI	dMS
	N/A	B35	MS
	C1L$_{102-111}$	KPKPAVRFAI	Public
	A24R$_{1002-1010}$	KPYASKVFF	MS
	C10L$_{41-49}$	LPMEDNSDI	MS
	E1L$_{10-18}$	LPNITLKII	MS
	D11L$_{506-514}$	MPTVDEDLF	MS
	C20L/B26R$_{7-16}$	EPIRGYVIIL	MS
	G5R$_{341-349}$	LPCQLMYAL	MS
	D5R$_{361-369}$	EPLITKLIL	MS
	N/A	B35	MS
	D1R$_{686-694}$	HPRHYATVM	Public
	J6R$_{1089-1098}$	LPGAANKGKI	MS
	A10L$_{111-120}$	NPIINTHSFY	MS
	O1L$_{335-344}$	RPMSLRSTII	Public
	A3L$_{453-462}$	SPVIVNGAMM	MS
	I6L$_{237-245}$	FPTNTLTSI	MS
	D1R$_{384-393}$	GPKSNIDFKI	MS
	K6L$_{17-25}$	KPITYPKAL	MS
	I4L$_{498-507}$	RPIGIGVQGL	MS
	N2L$_{104-113}$	RPNQHHTIDL	MS
	A34R$_{82-90}$	LPRPDTRHL	MS+
	E2L$_{216-224}$	RPRDAIRFL	MS
	H3L$_{6-14}$	TPVIVVPVI	MS
	G2R$_{140-149}$	VPITGSKLIL	MS
	A11R$_{22-30}$	YPSNKNYEI	MS

Pool 4	N/A	APAAPGLSL	self
	N/A	APIDRVGQTL	self

	ORF[a]	Peptide	Source[b]
	N/A	APKCDVSFL	self
	N/A	EPTPGPVRIL	self
	N/A	GPSGLGKTAI	self
	N/A	GPVRPYFSL	self
	N/A	IPRDPSQQEL	self
	$K1L_{151-159}$	IPSTFDLAI	MS
	N/A	KPRPDVTNEL	self
	N/A	LPAESLGPRI	self
	$B16R_{226-234}$	LPDGIVTSI	MS
	N/A	LPNELPAHLL	self
	N/A	LPQANRDTL	self
	N/A	LPRKPVAGAL	self
	N/A	RPFPKLRIL	self
	N/A	LPVSCTPGPL	self
	N/A	RPGSSDRVL	self
	N/A	RPQASHQLL	self
	N/A	RPQVAKTLL	self
	N/A	RPRPVSPSSL	self
	N/A	RPSPPNPEL	self
	N/A	SPAGSTRVL	self
	N/A	SPNDKSINAL	self
	N/A	SPRAAEPVQL	self
	N/A	SPSSKYVKL	self
	N/A	APRKGSFSAL	self

Pool 5	$A4L_{83-91}$	VPTATPAPI	MS
	$E6R_{284-293}$	EPEKDIRELL	MS
	N/A	B35	MS
	$D1R_{384-393}$	GPKSNIDFKI	MS
	$I6L_{272-280}$	IPKKIVSLL	MS
	$A40R_{3-11}$	KPKTDYAGY	MS
	$A47L_{227-236}$	KPVSDLYTSM	MS
	N/A	B35	MS
	$D5R_{375-383}$	LPKEYSSEL	MS
	N/A	B35	MS
	$D8L_{160-169}$	LPSKLDYFTY	MS
	N/A	APIQGNREEL	self
	N/A	APSAFGMLL	self
	N/A	LPKTGTVSL	self
	N/A	SPSSPGSSL	self
	N/A	SPVYLDLAYL	self
	N/A	TPSNTPTGPL	self
	$L4R_{37-45}$	FPRSMLSIF	MS

	ORF[a]	Peptide	Source[b]
	$J3R_{8-16}$	KPFMYFEEI	MS
	N/A	KPYFPPRIL	self
	N/A	LPSKPSSTL	self
	$D9R_{100-109}$	DPHFEELILL	MS
	$G5R_{341-349}$	LPCQLMYAL	MS
	$C10L_{41-50}$	LPMEDNSDII	MS
	$I6L_{282-291}$	LPSNVEIKAI	MS
	N/A	B35	MS
	N/A	B35	MS
	N/A	B35	MS
	$A11R_{22-30}$	YPSNKNYEI	MS

[a]ORFs are defined as in Table 1, asterisks indicated peptides homologous to Western Reserve (VACWR, ID 696871) or Ankara (VACCA, ID 126794) vaccinia strains;

[b]Prior reports according to the immune epitope data base (IEDB; www.iedb.org). MS, this study; dMS, derivatives of MS-discovered VACV peptides generated as homologous to variola virus (Reference ID 10255). MS+, reported from MHC class I ligand elution; public, reported as positive in human or HLA class I transgenic mouse TCD8 assay; predicted, reported as predicted VACV epitopes; B35, naturally processed HLA-B35 restricted VACV peptides found in this study (unpublished data); self, self peptides found to be naturally presented by VACV-infected HeLa cells in this study

Supplementary Table 4. Summary of immune epitopes identified in smallpox-vaccinated volunteers

A*02;01

Sequence	V001	V008	V023	V034	V115	V163	V168
Novel immune epitopes identified in this study							
AINVEKIEL							224
DIDEILEKV		113					
MIKPCCERV	155						
KIEDLINQL		153					
LILDPKINV		100					
RIIYIIRFL	124				112		
TLIGNFAAHL	653	207					
VLISPVSIL		231	189				
YIDISDVKV	282						
KLTFLDVEV	111						
ILNDEQLNL	408	1222					
SLYNLVKSSV						263	
YITALNHLV					225		
KISNTTFEV	115						
YLDGQLARL					225		
KIDDMIEEV		116					
KLVGKTVKV					112		
SLSNLDFRL	*226	*100	*119				115
SLLFIPDIKL	520						
LIQEIVHEV	226						
Known immune epitopes also found as naturally processed in this study							
VLEDVQAAGI		133					
FLTSVINRV	*877	*136	*249	*245			
ILDDNLYKV	*1162	*2602	*5362	*2839			
SLFKNVRLL					112		
RVYEALYYV	*297						
WLIGFDFDV	188						
IVIEAIHTV			*288	*625			
KVDDTFYYV	*453		*928	*156			
NLFDIPLLTV	*388	*256	*171	*903			
KIDYYIPYV	*1836	*344	*925	*379			
GLNDYLHSV	*308	*518	*1069	*778			

Supplementary Table 4 contd…

Known immune epitopes not found as naturally processed in this study

VLPFDIKKL		107				
TLFPGDDDL						182
TLLDHIRTA	*320		*1036			
YIYGIPLSL			2909			
CLTEYILWV	*1360	*2229	*1892	*2009		
YLPEVISTI		176				
ALDEKLFLI			*445	*195		
GLFDFVNFV			*195			

The HLA class I allotype for each donor is provided in Table S2

The numbers of spot forming cells (SFC) over background per million PBMCs that secrete IFN-γ in response to individual peptides are presented

Responses are considered positive if SFC cells per million PBMC were at least 100 over background

*Epitopes also found as positive in tetramer binding assay

Supplementary Table 5. Summary of immune epitopes identified in HLA class I transgenic mice

A*02;01		
Sequence[a]	**ORF**	**$t_{1/2}$, h**
Novel immune epitopes identified in this study		
YIDISDVKV	$I1L_{122-130}$	0.82
SLSNLDFRL*	$F11L_{340-348}$	23.58
TLIGNFAAHL	$G4L_{57-66}$	9.86
TLRVLQDQL	$A50R_{458-466}$	0.19
KIDDMIEEV*	$C9L_{602-610}$	9.34
VLSLELPEV*	$D13L_{70-78}$	22.73
DLKPDNILL*	$F10L_{307-315}$	ND
Known immune epitopes also found as naturally processed in this study		
ILSDENYLL*	$A6L_{171-179}$	24.41
KLFSDISAI*	$E5R_{117-125}$	17.75
RLYDYFTRV*	$I1L_{211-219}$	20.04
VLYDEFVTI*	$A6L_{6-14}$	14.04
ILDDNLYKV*	$G5R_{18-26}$	26.21
HVDGKILFV*	$B13R_{327-335}$	4.22
SLSAYIIRV*	$H3L_{184-192}$	25.52
RVYEALYYV*	$D12L_{251-259}$	14.44
NLFDIPLLTV*	$F12L_{286-295}$	22.59
IVIEAIHTV*	$A48R_{210-218}$	6.66
KVDDTFYYV*	$C7L_{74-82}$	12.21
Known immune epitopes not found as naturally processed in this study		
ALDEKLFLI*	$A23R_{273-281}$	ND
GLFDFVNFV*	$A46R_{142-150}$	24.76

[a]Identified by IFN-γ ELISpot assay

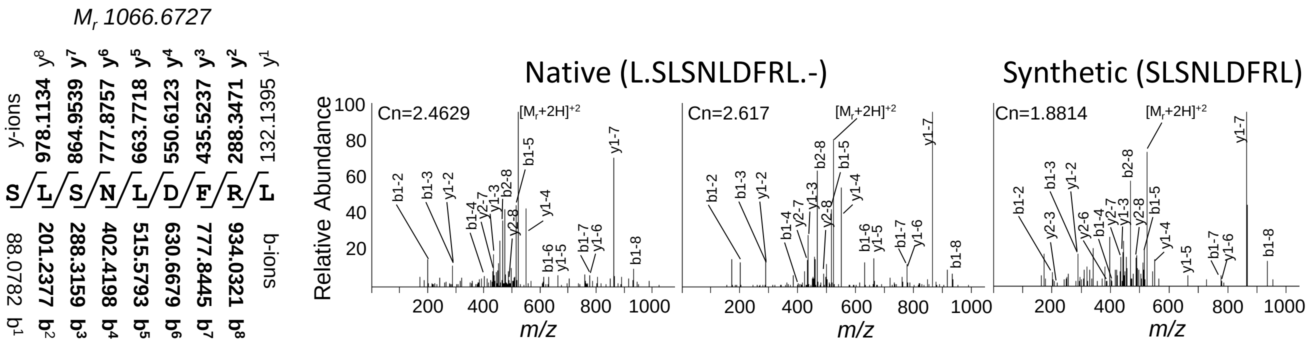
*Epitopes also found as positive in tetramer binding assay

Supplementary Figure 1. Identification of vaccinia viral determinants presented during active infection.

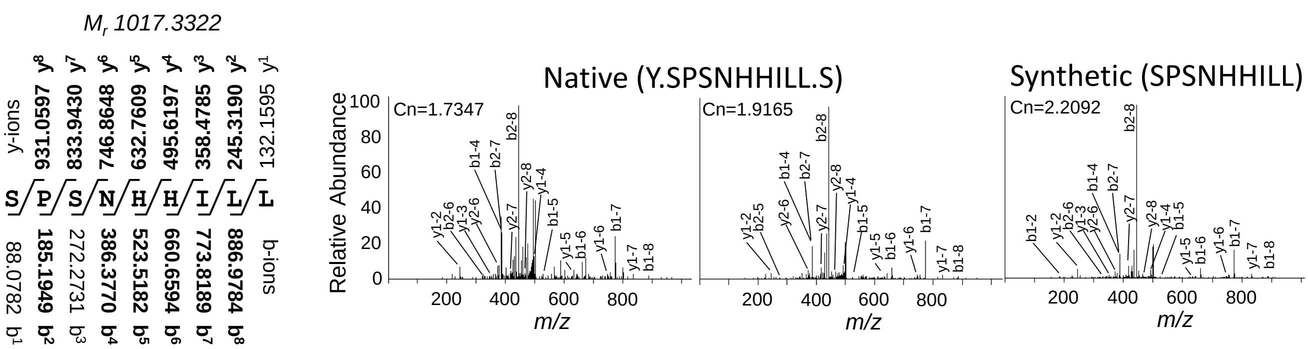
Representative mass spectra comparing synthetic peptides with the peptides eluted from purified sA2.1 (**A**) and sB7.2 molecules (**B**; Native). For each spectrum, the *b*- and *y*-ion fragments are indicated along with the Sequest cross-correlation score (Cn) showing the degree of concordance between the observed and expected fragment patterns. To the left of the spectra are the ion values for each peptide (**bold**, observed ion masses).

Supplementary Figure 1

A



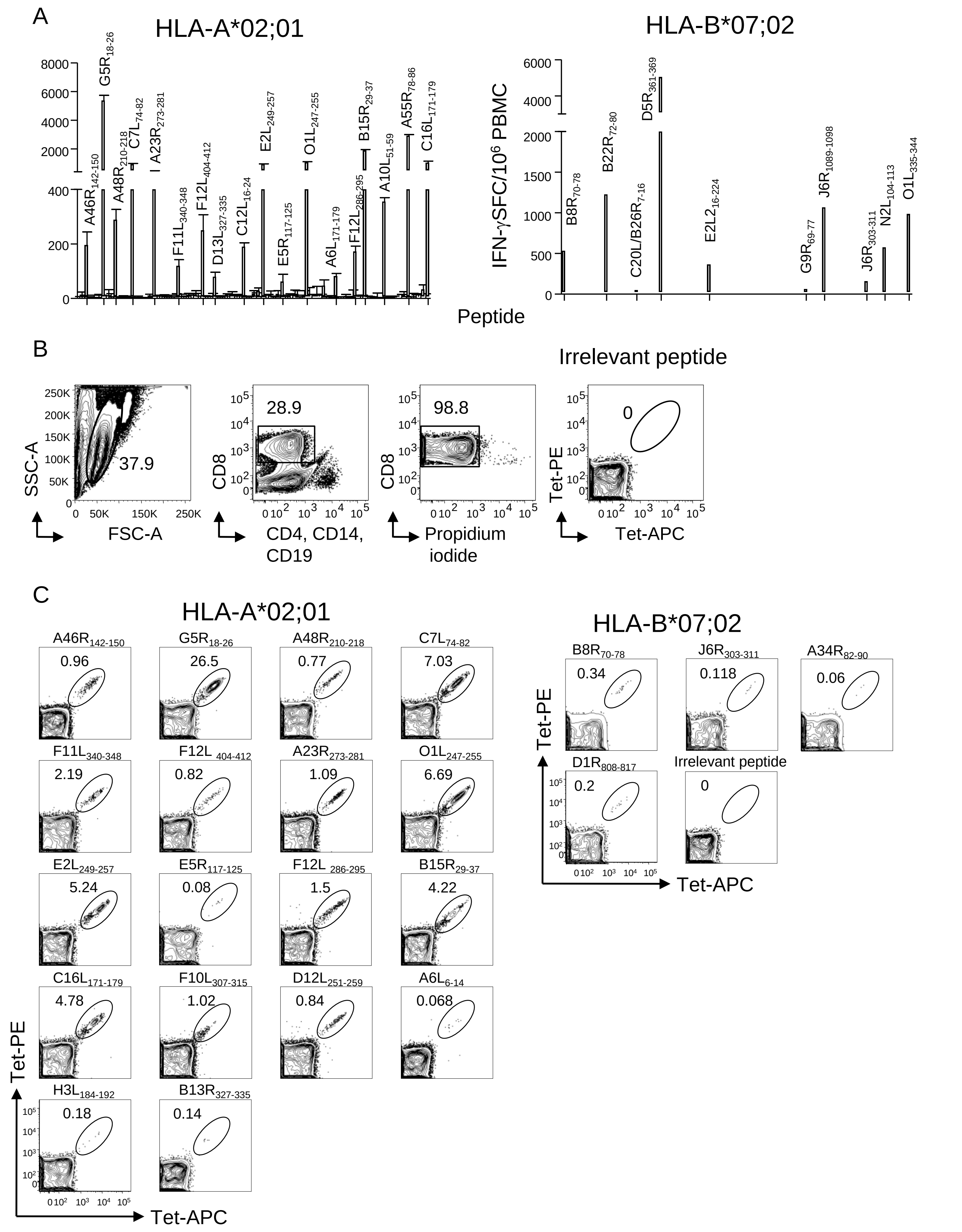
B



Supplementary Figure 2. Identification of vaccinia-specific T_{CD8} responses in smallpox-vaccinated volunteers.

PBMCs were expanded ex vivo with peptide pools (Table S4) and the immune epitopes were identified using IFN-γ ELISpot assay (**A**) and by dual-flourochrome encoding using the indicated PE- and APC-conjugated p/B7.2 tetramers (**B—C**). Bar graph indicates mean±SD of the assay duplicates. Contour plots were gated on live $CD8^+$ T lymphocytes as shown in (**B**). Irrelevant peptide is a B*07;02-restricted HMPV immune epitope (13).

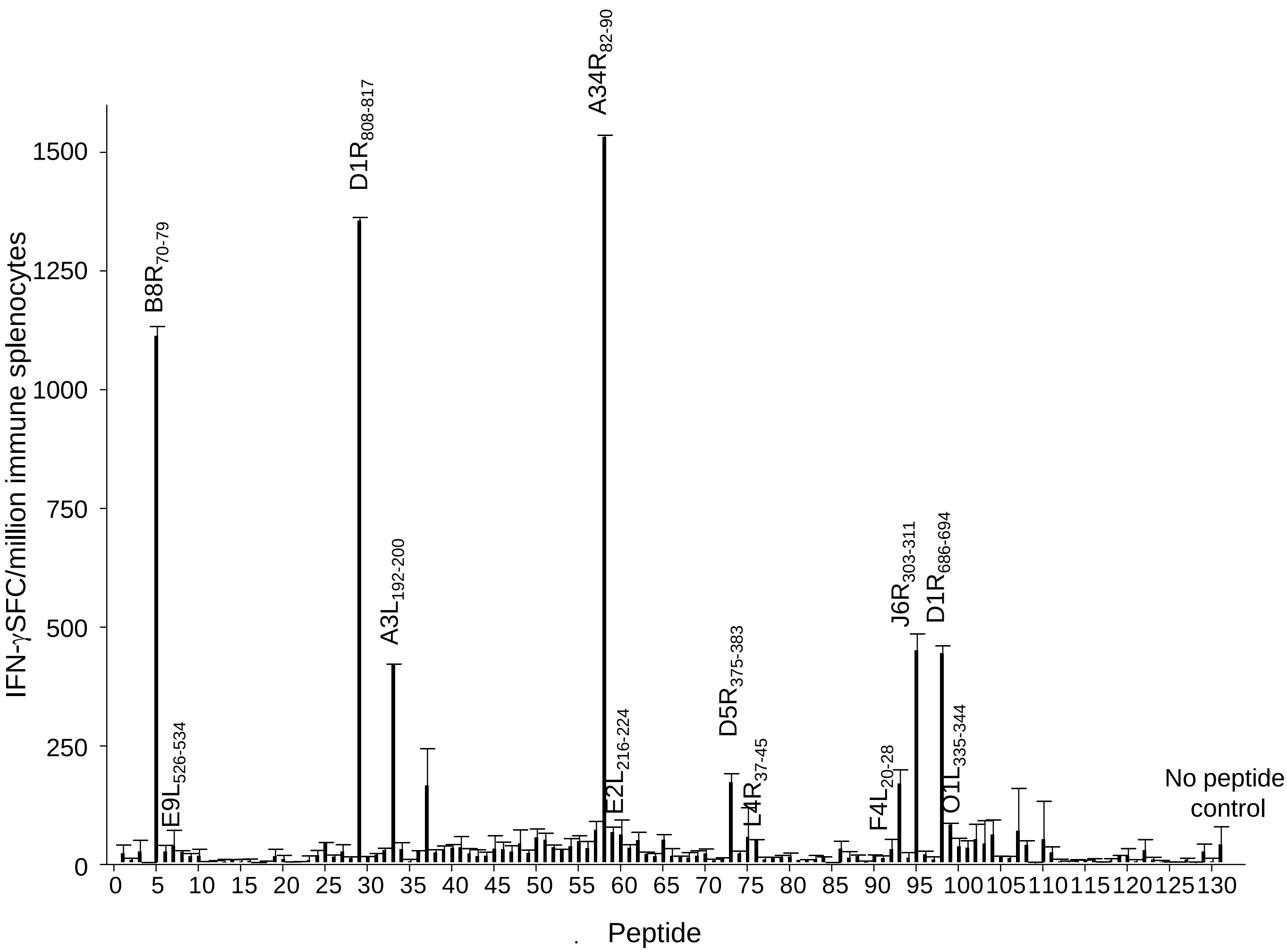
Supplementary Figure 2



Supplementary Figure 3. Identification immune T_{CD8} elicited by VACV in HLA class I transgenic mice.

A2^{tg} mice were inoculated i.p. with VACV and splenic T_{CD8} responses were analyzed on d7 p.i. using IFN-γ ELISpot assay. Data are representative of at least three independent experiments; *n*=3—5 mice/experiment; mean±SD.

Supplementary Figure 3



Supplementary Figure 4. Validating VACV-specific T_{CD8} responses in HLA A02;01 transgenic mice.

A2^{tg} mice were inoculated i.p. with VACV and splenic T_{CD8} responses were analyzed on d7 p.i.

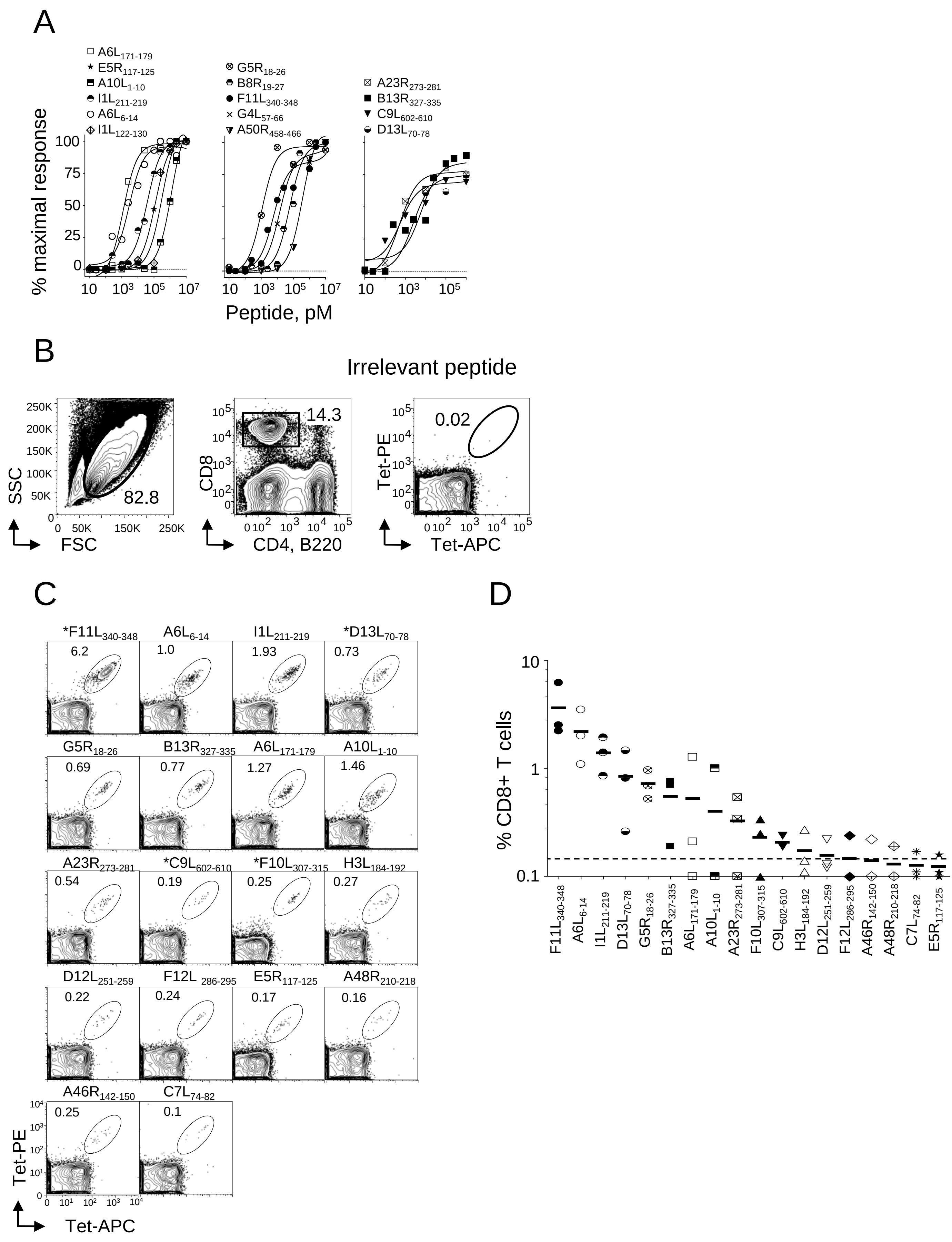
(**A**) Dose-dependent IFN-γ response by VACV-immune splenocytes measured by epitope titration in the ELISpot assay. Data represent the mean of triplicate wells; representative of two independent experiments (*n*=5).

(**B**) Gating strategy utilized for identification of individual T_{CD8} specificities with tetramers from mouse immune splenocytes.

(**C**) T_{CD8} responses detected using p/class I tetramers on VACV immune splenocytes. Representative of 3 independent experiments using pooled spleens (*n*=5—10). Numbers indicate % CD8$^+$ p/class I tetramer+ cells; *, novel immune epitopes.

D) VACV-specific T_{CD8} hierarchy defined by p/class I tetramer staining of VACV immune splenocytes. Cumulative data from 3 experiments (*n*=5—8). Each dot represents T_{CD8} frequency from one experiment. Dotted line indicates assay background defined by irrelevant HMPV-derived T_{CD8} epitope/B7.2 tetramer binding (13).

Supplementary Figure 4

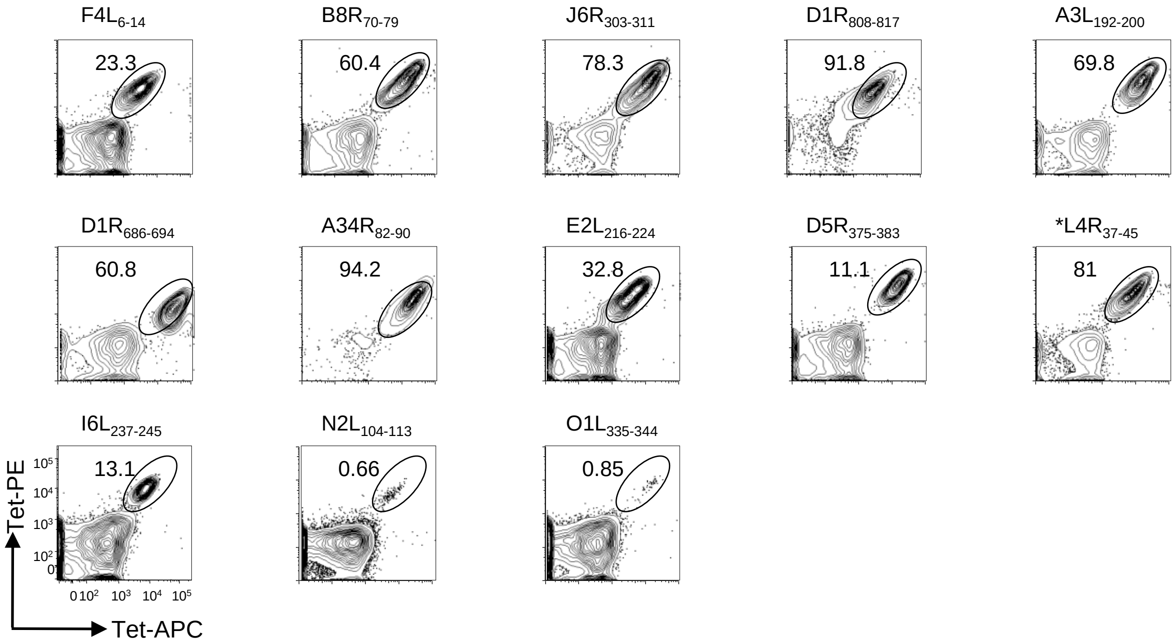


Supplementary Figure 5. Validating immune TCD8 identified in acutely infected B7^{tg} mice by their ability to expand in splenic cultures after re-stimulation with individual antigenic peptides.

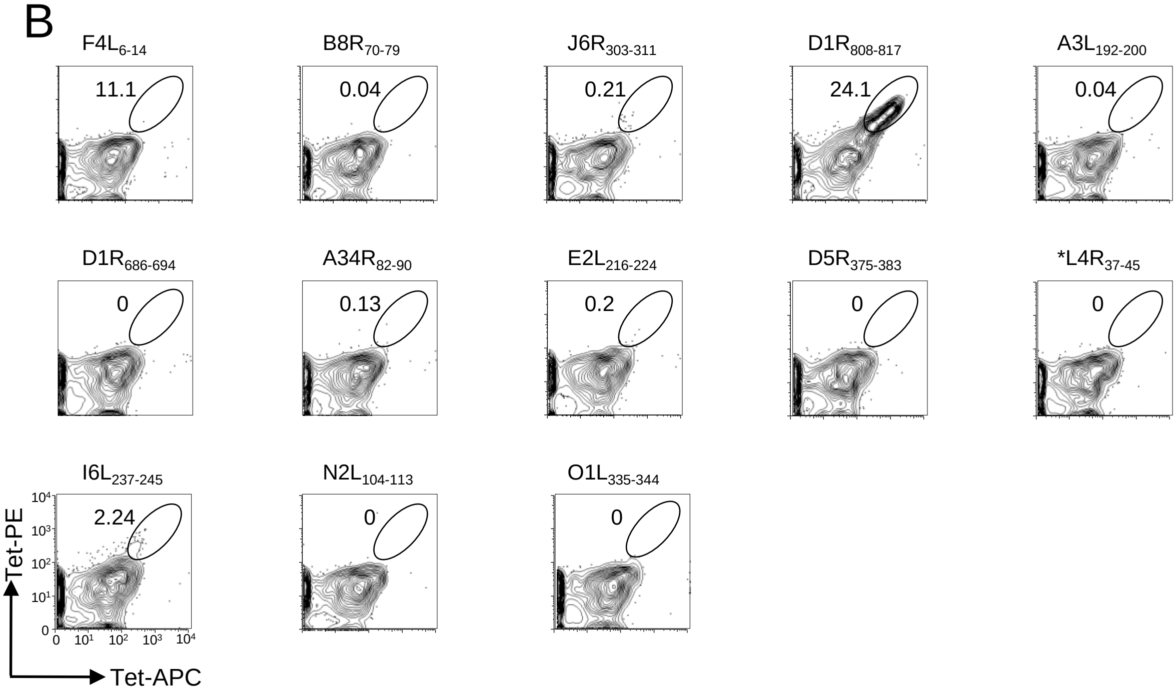
Splenic cells of VACV immune or uninfected mice were expanded in vitro with individual antigenic peptides (100nM) in the presence of rhIL-2 (5U/ml). On d7, expansion of epitope specific TCD8 from VACV immune (**A**) and naïve (**B**) splenic cultures was monitored by flow cytometry using p/B7.2 tetramers. Contour plots are gated on live CD8^{+} T lymphocytes. Numbers indicate percent epitope-specific TCD8. Data are representative of two independent experiments; *n*=3 pooled spleens/experiment.

Supplementary Figure 5

A



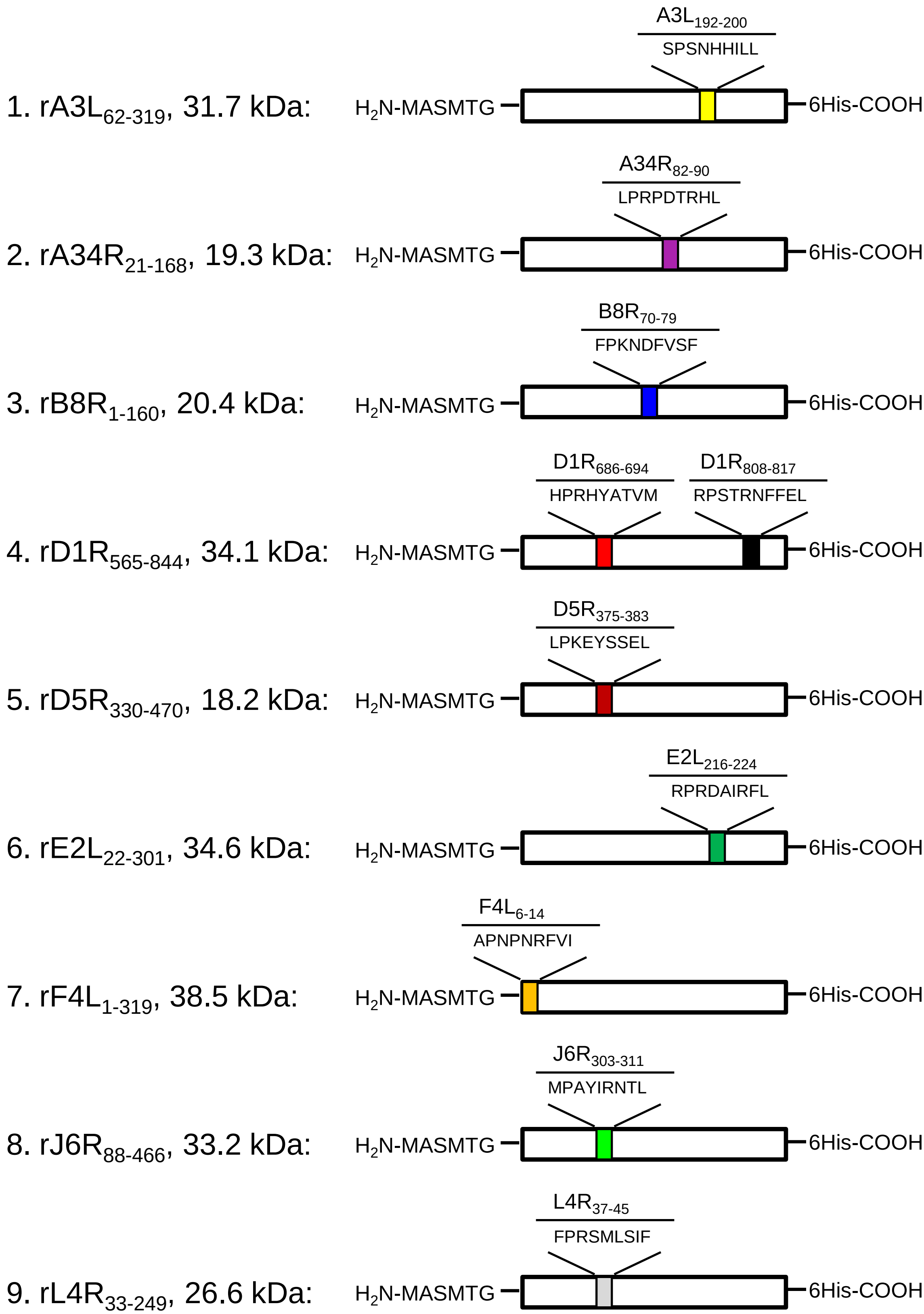
B



Supplementary Figure 6. Design of recombinant VACV-derived proteins encompassing targeted immune determinants.

Schematic diagram of engineered proteins showing nine VACV antigens with amino acid positions selected for the design of truncated recombinant subunit. Location and sequences of immune epitopes, and also their N- and C-terminal tag sequences introduced to facilitate expression and purification of recombinant proteins from *E. coli* are shown. Note that recombinant $B8R_{1-160}$ and $A34R_{21-168}$ were produced in undetectable amounts by *E. coli*.

Supplementary Figure 6



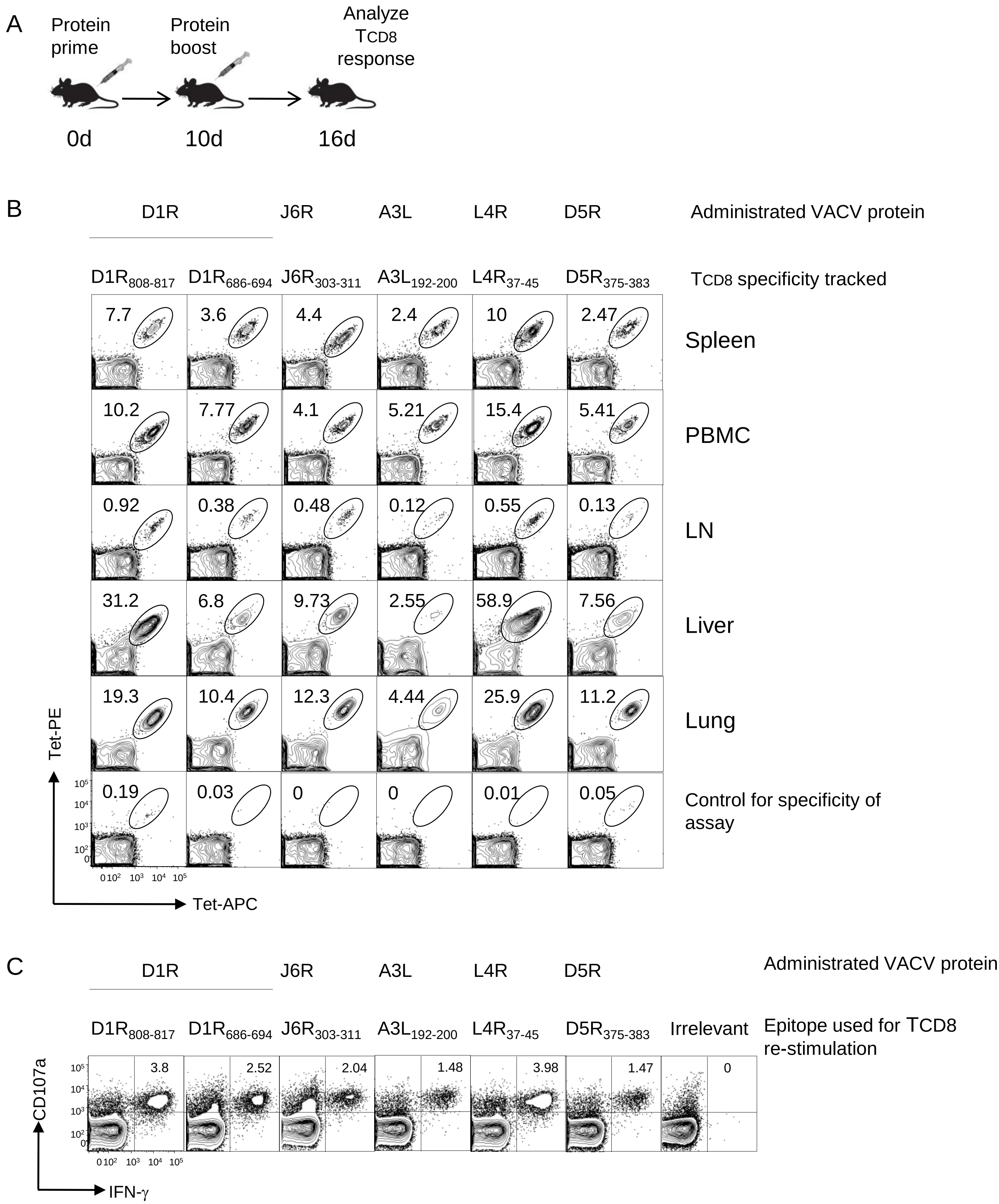
Supplementary Figure 7. Efficient processing of immune determinants from cognate VACV proteins

(**A**) Immunization strategy: Recombinant VACV-derived proteins containing targeted antigenic determinants were formulated with αGalCer and administrated to mice i.p. as indicated.

B) Robust systemic epitope-specific T_{CD8} response detected was with p/class I tetramers after prime-boost with individual VACV proteins. Data are representative of at least two independent experiments using pooled samples (*n*=3—7).

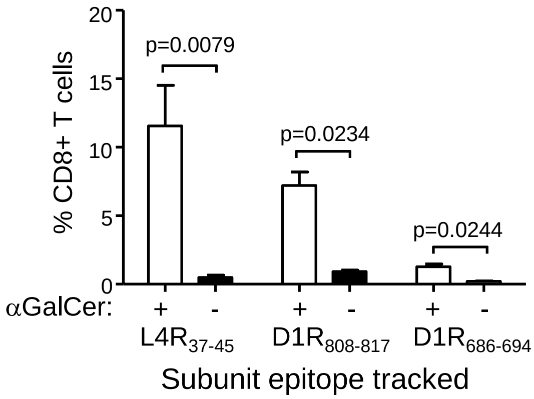
(**C**) T_{CD8} elicited after protein immunization functionally responded to re-stimulation with appropriate synthetic peptides by degranulation and production of intracellular IFN-γ.

Supplementary Figure 7



Supplementary Figure 8. Priming of T_{CD8} response with antigenic protein requires αGalCer in the protein-adjuvant formulation. Mice (*n*=5/group) were primed once i.p. with 20μg of individual recombinant subunits ($L4R_{33-249}$ or $D1R_{565-844}$) with or without αGalCer (1μg/mouse) and epitope-specific T_{CD8} against targeted epitopes were enumerated on d10 p.i. from blood with p/B7.2 tetramers. Each dot represents individual mouse. **, $p<0.01$ (Mann-Whitney); nd, not detected. Representative of two independent experiments.

Supplementary Figure 8

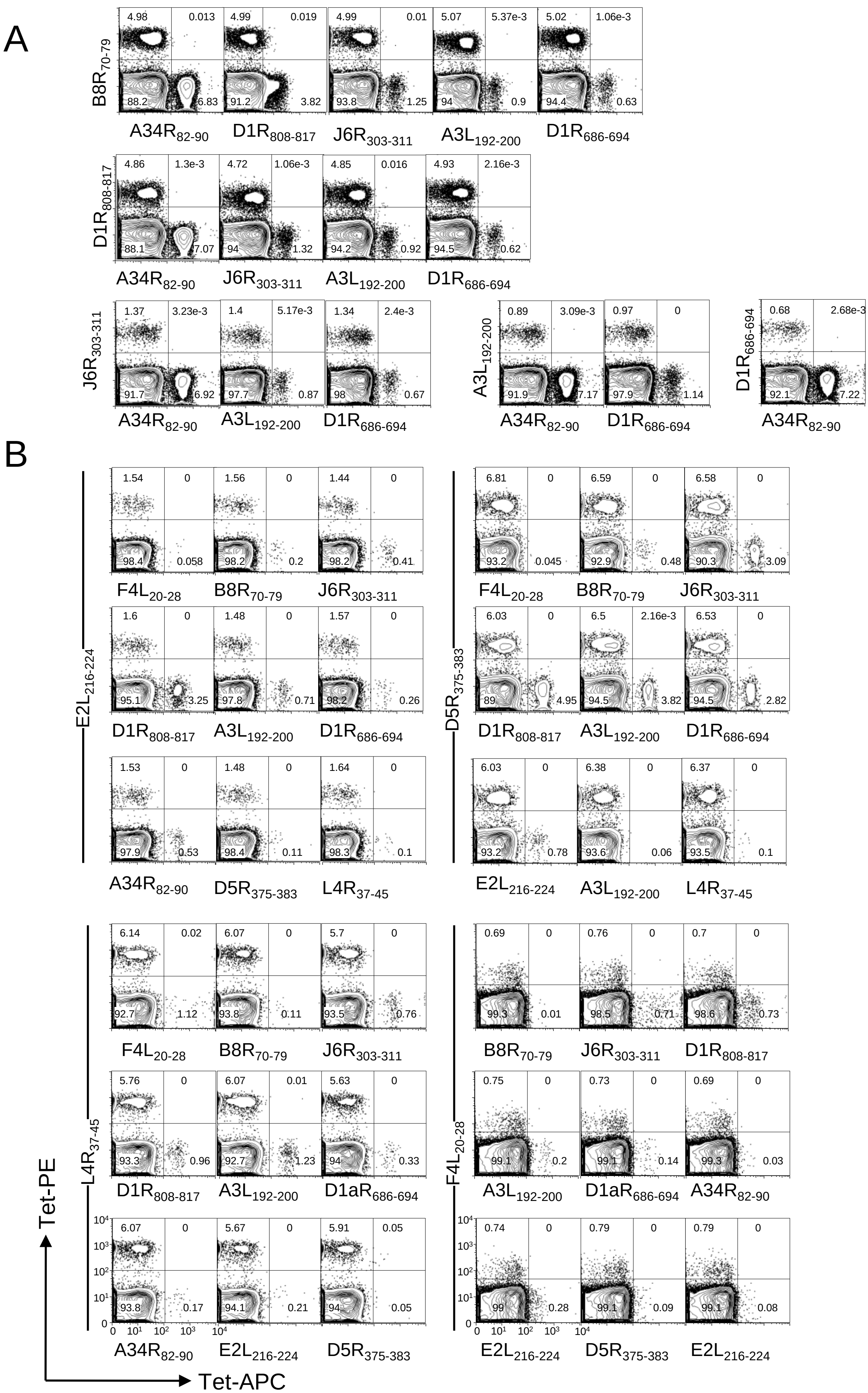


Supplementary Figure 9. Validating individual T_{CD8} specificities elicited by VACV using combinatorial exclusion flow cytometry with dual-fluorochome-encoded p/B7.2 tetramers.

(**A**) Primary T_{CD8} response analyzed on d7 p.i.

(**B**) Four subdominant T_{CD8} were enhanced by priming with peptides followed by VACV challenge before analysis. Contour plots are gated on live $CD8^+$ T lymphocytes. Gate numbers indicate percent epitope-specific T_{CD8}. Data are representative of two independent experiments; *n*=5 mice/experiment.

Supplementary Figure 9



Supplementary Figure 10. Characterization of T_{CD8} response elicited in acutely infected B7^{tg} mice.

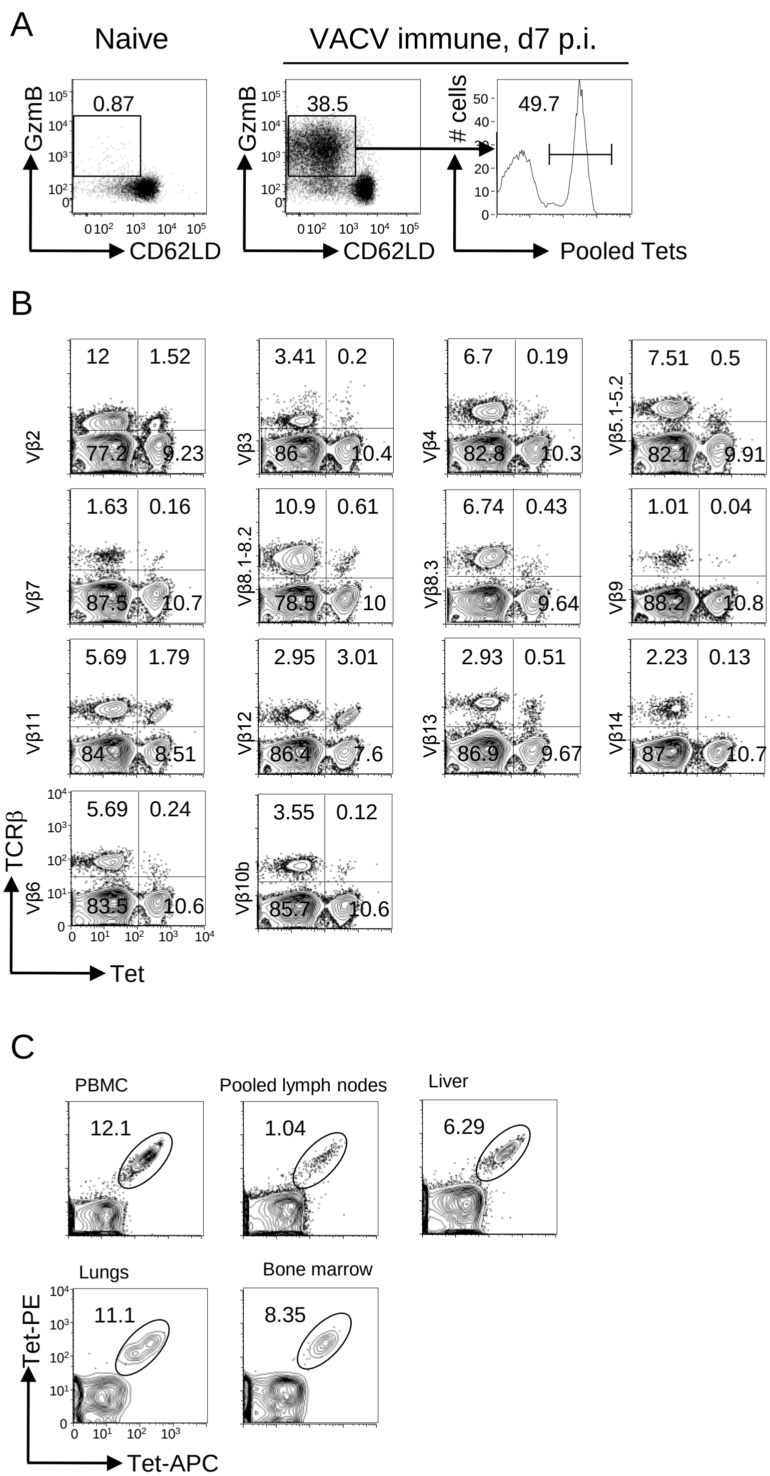
Mice were inoculated i.p. with $2x10^5$ pfu VACV and T_{CD8} response was analyzed as indicated below.

(**A**) Coverage of the overall anti-VACV response by identified T_{CD8} specificities determined as the fraction of the CD44^{HI}CD62L^{LO}GzmB$^+$ population within splenic T_{CD8} population stained by pooled p/B7.2 tetramers against 10 immune determinants as in Figure 3. Measurement was performed on individual mouse (*n*=5). Data are representative of two independent experiments.

(**B**) Analysis of TCR Vβ usage within a given T_{CD8} specificity revealed multiple clonotypes. Cells were gated on live CD8$^+$ T lymphocytes.

(**C**) Anti-VACV epitope-specific T_{CD8} detected in lymphoid and peripheral tissues. Data are representative of at least three independent experiments; *n*=3—5 mice/experiment for A34R$_{82\text{-}90}$-specific T_{CD8} in **B** and **C**.

Supplementary Figure 10



Supplementary Figure 11. Enumeration of naïve pTCD8 specific to VACV determinants in B7^{tg} mice.

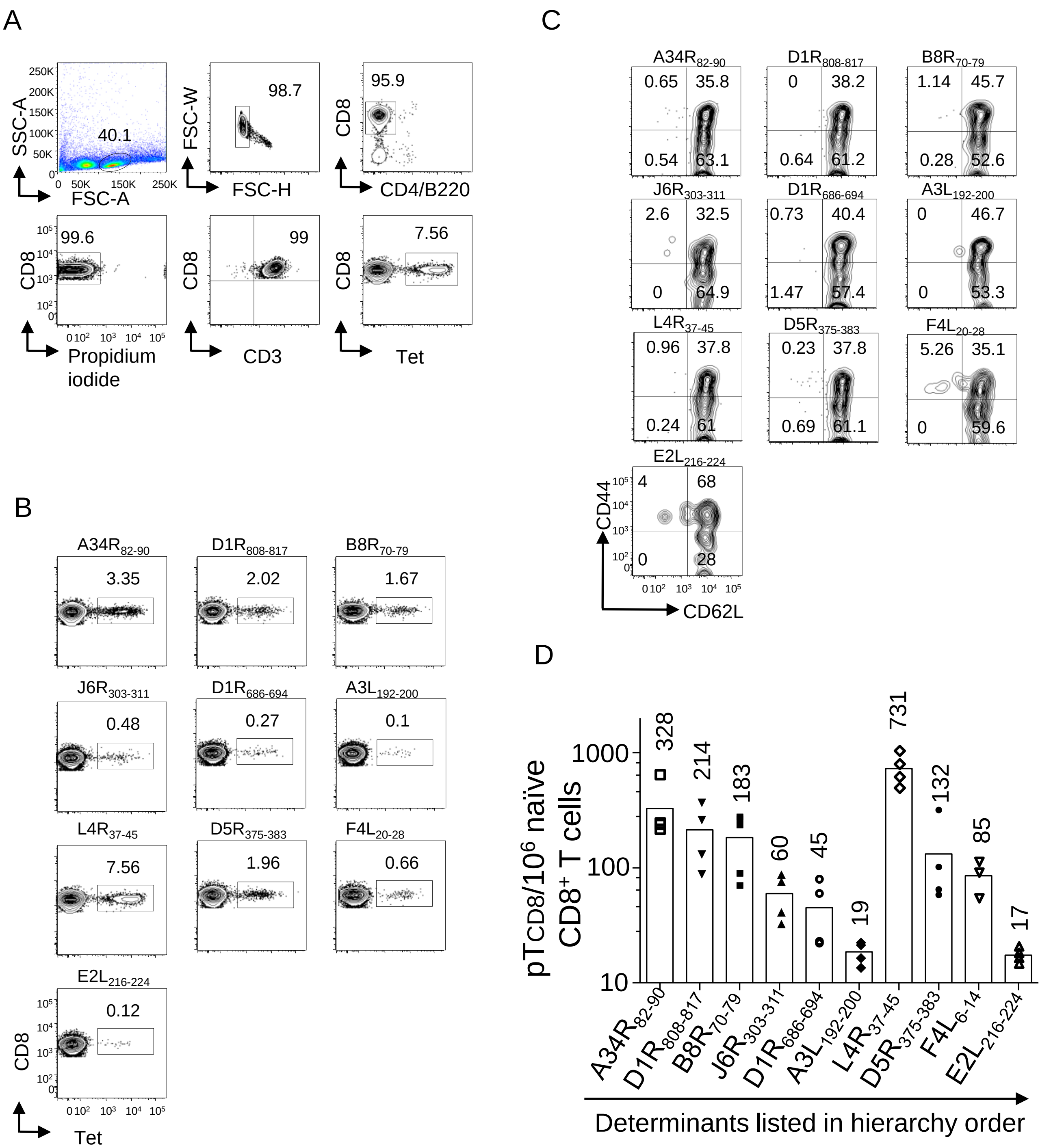
(A) Flow cytometry gating strategy used to identify naïve TCD8 from total pooled spleen and lymph node cells: FSC/SSC-A, FSC-(W/H)^{LO} SSC-(W/H)^{LO}, B220^{NEG}/CD4^{NEG}/CD8α^{+}/CD3ε^{+}, PI^{LO} and p/B7.2 tetramer^{+}. Representative flow plots to identify L4R$_{37\text{-}45}$-specific pTCD8 are shown.

(B) Representative flow plots for ten pTCD8 specificities enriched with p/B7.2 tetramers are shown.

(C) Phenotype of epitope-specific naïve pTCD8 enriched with p/B7.2 tetramers. Contour plots are gated on p/B7.2 class I tetramer^{+} cells.

(D) Normalized frequency of naïve pTCD8 for the naturally processed VACV determinants identified in B7^{tg} mice (HLA-B7^{tg} mouse has ~8.3x10^{6} total naïve CD8^{+} T cells compared to C57BL/6 mice, which have ~17x10^{6} total naïve CD8^{+} T cells). Data represent 3—5 independent experiments from pooled spleen and macroscopical LNs. Each dot represents one experiment; number indicates mean pTCD8 frequency per million CD8^{+} T cells.

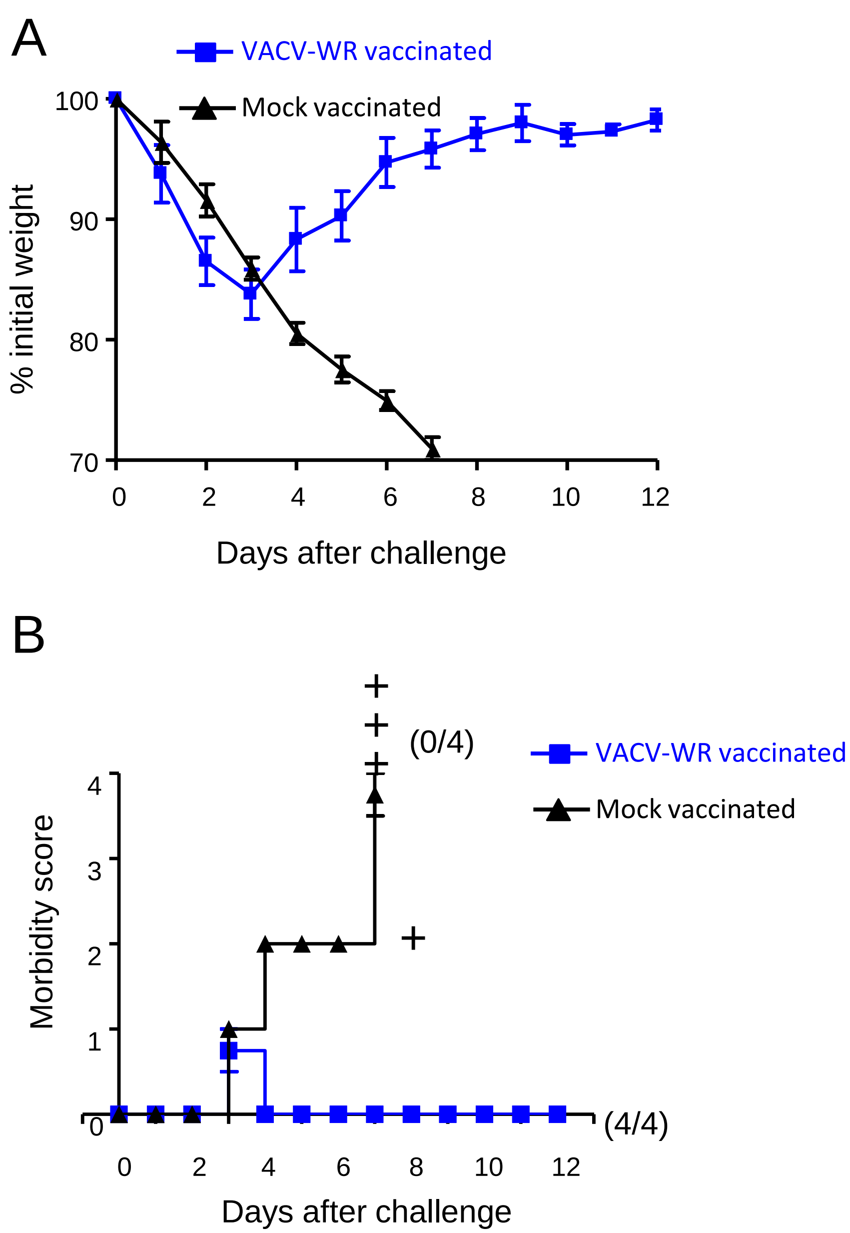
Supplementary Figure 11



Supplementary Figure 12. Validation of VACV-WR inoculation conditions used for lethal respiratory challenge of epitope-vaccinated B7^{tg} mice shown in Figure 5A.

Two groups of B7^{tg} mice (*n*=4—7/group) were vaccinated with 10^6 pfu VACV-WR in 100μl PBS (i.p.) or 100μl PBS (i.p.). On d14 p.i., mice were anesthetized with ketamine/xylazine, challenged i.n. with 10^6 pfu VACV-WR in 50μl PBS and observed during next 12 days for weight loss (**A**) and morbidity (**B**); mean$\pm$SEM. +, the time points at which death occurred between watch; the number of surviving/total animals are in parenthesis.

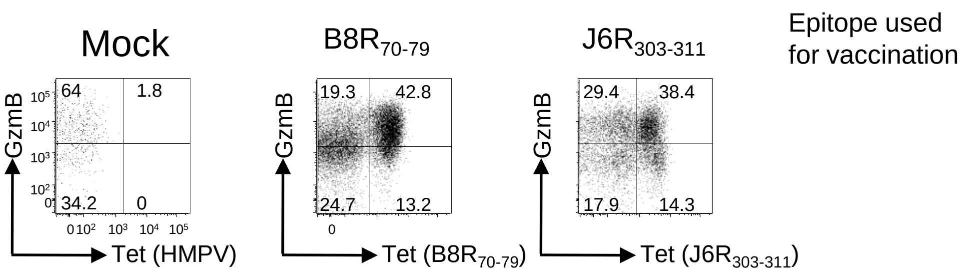
Supplementary Figure 12



Supplementary Figure 13. Epitope-specific T_{CD8} elicited in lungs by peptide vaccination contain GzmB.

p/B7.2 tetramer staining of T_{CD8} harvested from infected lungs of non-vaccinated (mock) and epitope-vaccinated ($B8R_{70-79}$ and $J6R_{303-311}$) mice as in Figures 5, 6. The majority of T_{CD8} infiltrating lungs are specific for the single epitope targeted by vaccination and produce GzmB. Data represent one of five mice from each group.

Supplementary Figure 13



Literature Cited

1. Rodenko, B., Toebes, M., Hadrup, S.R., van Esch, W.J., Molenaar, A.M., Schumacher, T.N., and Ovaa, H. 2006. Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* 1:1120-1132.

2. Bakker, A.H., Hoppes, R., Linnemann, C., Toebes, M., Rodenko, B., Berkers, C.R., Hadrup, S.R., van Esch, W.J., Heemskerk, M.H., Ovaa, H., et al. 2008. Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Proc Natl Acad Sci U S A* 105:3825-3830.

3. Toebes, M., Rodenko, B., Ovaa, H., and Schumacher, T.N. 2009. Generation of peptide MHC class I monomers and multimers through ligand exchange. *Curr Protoc Immunol* Chapter 18:Unit 18 16.

4. Hickman, H.D., Batson, C.L., Prilliman, K.R., Crawford, D.L., Jackson, K.L., and Hildebrand, W.H. 2000. C-terminal epitope tagging facilitates comparative ligand mapping from MHC class I positive cells. *Hum Immunol* 61:1339-1346.

5. Prilliman, K., Lindsey, M., Zuo, Y., Jackson, K.W., Zhang, Y., and Hildebrand, W. 1997. Large-scale production of class I bound peptides: assigning a signature to HLA-B*1501. *Immunogenetics* 45:379-385.

6. Harndahl, M., Rasmussen, M., Roder, G., and Buus, S. 2010. Real-time, high-throughput measurements of peptide-MHC-I dissociation using a scintillation proximity assay. *J Immunol Methods*.

7. Hadrup, S.R., Toebes, M., Rodenko, B., Bakker, A.H., Egan, D.A., Ovaa, H., and Schumacher, T.N. 2009. High-throughput T-cell epitope discovery through MHC peptide exchange. *Methods Mol Biol* 524:383-405.

8. Hadrup, S.R., Bakker, A.H., Shu, C.J., Andersen, R.S., van Veluw, J., Hombrink, P., Castermans, E., Thor Straten, P., Blank, C., Haanen, J.B., et al. 2009. Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Methods* 6:520-526.

9. Moon, J.J., Chu, H.H., Hataye, J., Pagan, A.J., Pepper, M., McLachlan, J.B., Zell, T., and Jenkins, M.K. 2009. Tracking epitope-specific T cells. *Nat Protoc* 4:565-581.

10. Matheu, M.P., Sen, D., Cahalan, M.D., and Parker, I. 2008. Generation of bone marrow derived murine dendritic cells for use in 2-photon imaging. *J Vis Exp*.

11. Assarsson, E., Sidney, J., Oseroff, C., Pasquetto, V., Bui, H.H., Frahm, N., Brander, C., Peters, B., Grey, H., and Sette, A. 2007. A quantitative analysis of the variables affecting the repertoire of T cell specificities recognized after vaccinia virus infection. *J Immunol* 178:7890-7901.

12. Assarsson, E., Greenbaum, J.A., Sundstrom, M., Schaffer, L., Hammond, J.A., Pasquetto, V., Oseroff, C., Hendrickson, R.C., Lefkowitz, E.J., Tscharke, D.C., et al. 2008. Kinetic analysis of a complete poxvirus transcriptome reveals an immediate-early class of genes. *Proc Natl Acad Sci U S A* 105:2140-2145.

13. Erickson, J.J., Gilchuk, P., Hastings, A.K., Tollefson, S.J., Johnson, M., Downing, M.B., Boyd, K.L., Johnson, J.E., Kim, A.S., Joyce, S., et al. 2012. Viral acute lower respiratory infections impair CD8+ T cells through PD-1. *J Clin Invest*.