Appendix: Supplemental Tables and Figures

Table A1. Vaccine peptides in Env V1/V2 predicted to bind HLA alleles with high affinity

Binding affinity was predicted for peptides in the V1/V2 region of envelope with the class I and class II HLA molecules expressed by trial participants. The table lists the HXB2 start positions and amino acid residues of peptides with predicted binding affinity (IC_{50}) < 100 nM for at least one HLA allele. The predictions were different for the MN subtype-B rgp120 vaccine immunogen and the 92TH023 CRF01_AE ALVAC insert due to differences in their amino acid sequence. These peptides that are predicted to bind with high affinity are not necessarily the same peptides that were considered to be potential epitopes in the T-cell based sieve analysis. This is because a more complex method was used to select appropriate "binder" and "escape" binding affinity thresholds for the analysis (see Materials and Methods for details).

Predicted CD8+ T-cell epitopes		**Predicted CD4+ T-cell epitopes**	
MN immunogen	**CRF01_AE immunogen**	**MN immunogen**	**CRF01_AE immunogen**
147-GTIKGGEMK	151-TDEVRNCSF	154-MKNCSFNITTSIGDK	155-RNCSFNMTTELRDKK
153-EMKNCSFNI	153-EVRNCSFNM	155-KNCSFNITTSIGDKM	156-NCSFNMTTELRDKKQ
157-CSFNITTSI	157-CSFNMTTEL	156-NCSFNITTSIGDKMQ	164-ELRDKKQKVHALFYK
163-TSIGDKMQK	158-SFNMTTELR	157-CSFNITTSIGDKMQK	165-LRDKKQKVHALFYKL
165-IGDKMQKEY	161-MTTELRDKK	158-SFNITTSIGDKMQKE	166-RDKKQKVHALFYKLD
168-KMQKEYALL	168-KKQKVHALF	159-FNITTSIGDKMQKEY	167-DKKQKVHALFYKLDI
169-MQKEYALLY	169-KQKVHALFY	163-TSIGDKMQKEYALLY	168-KKQKVHALFYKLDIV
171-KEYALLYKL	171-KVHALFYKL	164-SIGDKMQKEYALLYK	169-KQKVHALFYKLDIVP
173-YALLYKLDI	174-ALFYKLDIV	165-IGDKMQKEYALLYKL	170-QKVHALFYKLDIVPI
174-ALLYKLDIE	175-LFYKLDIVP	166-GDKMQKEYALLYKLD	171-KVHALFYKLDIVPIE
175-LLYKLDIEP	176-FYKLDIVPI	167-DKMQKEYALLYKLDI	
176-LYKLDIEPI		168-KMQKEYALLYKLDIE	
		169-MQKEYALLYKLDIEP	
		170-QKEYALLYKLDIEPI	

Table A2. HLA class I binding capacity (IC_{50} nM) with V2 peptides

Selected 9-mer peptides were tested for their ability to bind the class I HLA A*02 alleles expressed by RV144 participants. The peptides were selected either because they were included in the RV144 vaccine (either in the vector or the protein boost) or because they were common variants isolated from infected trial participants. The same peptides were also used to assay T-cell responses in IFN-γ ELISpot assays.

Sequence (HXB2 168-176)	A*02:01	A*02:03	A*02:07
KMQKEYALL †	719	12	10585
KKQKVHALF ‡	-	2281	-
KKQQVYALF [V,P]	-	25294	-
KKQKFYALF [V,P]	-	-	-
KRQQVHALF [V]	-	-	-
KQRKVQALF [V]	-	25667	-
RKQQISALF [V]	34021	-	-
KQQKVYALF [V,P]	-	-	-
KQQNFYALF [V]	1768	-	36940
KQQQIHALF [P]	16306	-	37102

"-" indicates IC_{50} > 50,000 nM

† subtype B (MN) sequence

‡ CRF01_AE (92TH023) sequence
(92TH023 is identical to A244 sequence in this region)

[V] sequence isolated from infected vaccine recipient

[P] sequence isolated from infected placebo recipient

34 *Table A3. HLA class II binding capacity (IC_{50} nM) with V2 peptides*

35 Selected 15-mer peptides were tested for their ability to bind class II HLA molecules expressed
36 by RV144 participants. The peptides were selected either because they were part of the RV144
37 vaccine (either in the vector or the protein boost) or because they were common variants isolated
38 from infected trial participants. The same peptides were also used to assay T-cell responses in
39 IFN-γ ELISpot assays.

40

Sequence (HXB2 157-181)	DRB1*03:01	DRB1*12:01	DRB1*15:01	DRB3*02:02	DRB4*01:01	DRB5*01:01
DKMQKEYALLYKLDI †	80	71	93	-	124	2090
DKKQKVHALFYKLDI ‡	39312	10	71	-	371	-
DKKQKVHALFYRLDI [P]	26963	6.7	60	-	486	18896
DKQQKVHALFYRLDI [V]	-	16	324	-	697	23743
DKQHKVHALFYKLDI [V]	-	11	366	-	222	-
DKRRMVHALFYRLDI [V]	16654	5.3	15	42166	72	235
DKKQKVRALFYKLDI [P]	-	3.6	20	-	303	958
DKQQKVYALFYNLDI [P]	64	47	3964	21290	18920	13113

41
42 "-" indicates IC_{50} > 50,000 nM

43 † subtype-B (MN) sequence

44 ‡ CRF01_AE (92TH023) sequence
45 (92TH023 is identical to A244 sequence in this region)

46 [V] sequence isolated from infected vaccine recipient

47 [P] sequence isolated from infected placebo recipient

48

49 *Table A4. HLA associations with A*02 in RV144 uninfected vaccine and placebo recipients*

50 To determine if the associations with A*02 in the trial could be attributed to other HLA alleles
51 we performed a genetic analysis to identify HLA alleles in linkage-disequilibrium with the A*02
52 allele. All HLA alleles found in RV144 participants that were outside the HLA-A locus were
53 tested for an association with the A*02 allele. This included alleles at the HLA- B, Cw, DRB1-5,
54 DPA1, DPB1, DQA1, DQB1 loci (92 alleles total). The analysis was performed using the HLA
55 types of uninfected participants only (vaccine and placebo recipients). Each allele was tested
56 using a Fisher's exact test of the 2 x 2 contingency table containing the number of participants
57 carrying the alleles (both, neither or one of each). The p-values were adjusted for multiple tests
58 using the Bonferroni method. All q-values < 0.1 are shown in the table. The analysis revealed an
59 HLA haplotype A*0207:B*4601:Cw*0102 that is common in the RV144 cohort (est. ~14%) and
60 has been previously documented as one of the most common haplotypes in ethnic northeast
61 Thais [75] and other southeast Asian ethnicities [83,84]. This led us to test if any four-digit HLA
62 alleles were associated with A*0207 in trial participants (196 four-digit alleles total). This further
63 indicated that two class II alleles DRB1*0901 and DQB1*0303 may also be associated with
64 A*0207 and this haplotype, but the estimated frequency is lower in RV144 participants (6%) and
65 in ethnic northeastern Thais (~4%) [85].

66

A*02 associations with two-digit alleles	**p-value**	**q-value**
B*46	5.6e-9	5.2e-7
Cw*01	1.1e-8	1.0e-6

A*0207 associations with four-digit alleles	**p-value**	**q-value**
Cw*0102	1.5e-19	2.9e-17
B*4601	2.6e-18	5.0e-16
DQB1*0303	5.6e-05	0.011
DRB1*0901	3.1e-04	0.060

67

68 *Table A5. Vaccine efficacy in HLA-defined subgroups associated with A*02*

69 Vaccine efficacy was estimated in several HLA-determined subgroups to address the hypothesis
70 that higher VE associated with HLA-A*02 may be attributed to an association with other HLA
71 alleles in a haplotype with HLA-A*02. The frequency of each subgroup in the RV144 study was
72 estimated from the fraction of 280 HLA-typed uninfected vaccine and placebo recipients
73 carrying all alleles indicated (number of individuals indicated in parentheses). VE is estimated
74 using the "case-only" method, with a p-value testing that null hypothesis that VE(HLA+) = 0.
75 The interaction-p is for the test that VE(HLA+) = VE(HLA-) where "HLA+" is the subgroup
76 carrying all alleles and "HLA-" is the remainder of the cohort. None of the p-values were
77 adjusted for multiple tests.

78

Subgroup	**Est. frequency in RV144**	**VE(HLA+)**	**95% CI**		**p-value**	**interaction-p**
A*02	51% (142)	54%	20%	73%	0.006	0.0495
A*0207 & B*4601 & Cw*0102	14% (38)	71%	13%	91%	0.029	0.095
A*0207 & B*4601 & Cw*0102 & DQB1*0303	6% (18)	80%	-71%	98%	0.14	0.249
A*0207 & B*4601 & Cw*0102 & DRB1*0901	6% (16)	80%	-71%	98%	0.14	0.249
A*0207	16% (45)	71%	20%	89%	0.018	0.067
A*0203	22% (63)	31%	-62%	70%	0.398	0.991
A*0201	12% (33)	50%	-100%	87%	0.329	0.638
Cw*0102	32% (90)	31%	-22%	61%	0.204	0.998
B*4601 (subset of Cw*0102)	28% (78)	28%	-32%	61%	0.29	0.862
DQB1*0303	22% (62)	41%	-28%	73%	0.185	0.654
DRB1*0901	17% (48)	36%	-49%	72%	0.303	0.857
non-A*0207 & Cw*0102 & B*4601	18% (49)	-7%	-116%	47%	0.858	0.163
non-A*0207 & DQB1*0303	15% (41)	17%	-93%	64%	0.671	0.626

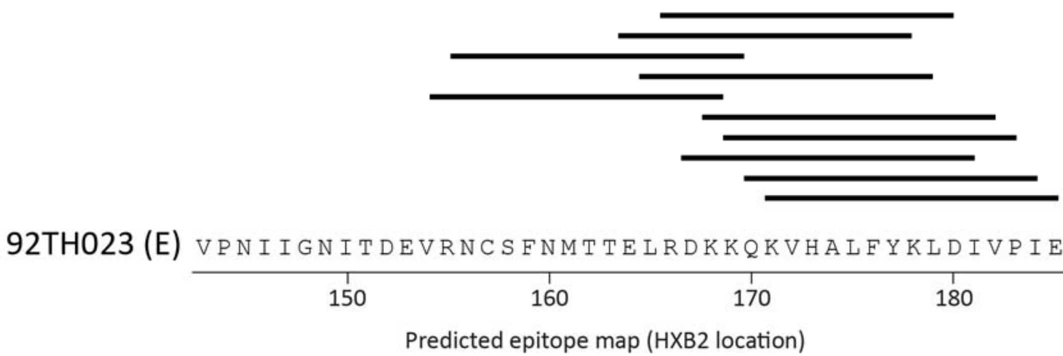
79

80

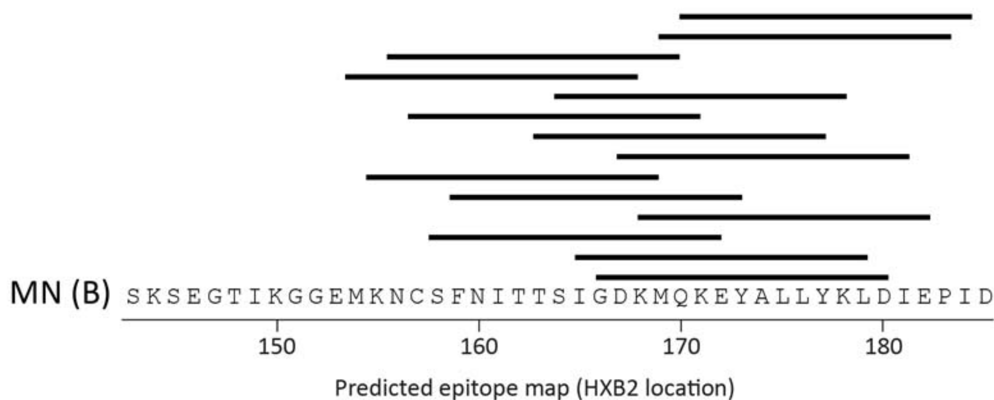
Figure A1. Predicted vaccine CD4+ T-cell epitopes in the V1/V2 region of envelope. Potential vaccine-induced CD4+ T-cell epitopes were identified using computational prediction of peptide binding with HLA class II molecules (NetMHCIIpan, [35]). In the analysis we considered the HLA alleles found in each RV144 participant and all 15-mer peptides in the V1/V2 region of envelope of both the CRF01_AE (92TH023) and the subtype-B (MN) vaccine immunogens. Peptides with a predicted IC_{50} < 100 nM were considered potential epitopes (solid bars). A T-cell based sieve analysis was performed using these predictions, but it did not detect a difference in the number of predicted binding “escapes” in vaccine versus placebo recipients.

Figure A1. Predicted vaccine CD4+ T-cell epitopes in the V1/V2 region of envelope

A



B



Appendix: Supplemental Methods

*Experimental validation of the A*02-KMQKEYALL T-cell epitope*

Subjects. The subjects were long term non-progressors (LTNPs) recruited and enrolled at the HIV Vaccine Trials Units (M.J.M., principle investigator). The appropriate Institutional Review Boards approved the studies, and volunteers provided written consent. They were defined as HIV infected for more than 11 years, with repeated CD4+ T cell counts over 500 cells per μl or CD4% over 28% and viral load <10,000 copies per ml in the absence of antiretroviral therapy.

Peptides. Peptides for assaying T-cell responses to the V2 region were chosen based on the locations of the predicted CD8+ (HXB2 168-176) and CD4+ (HXB2 167-181) epitopes. Two variants of these peptides corresponded to the MN strain (KMQKEYALL and DKMQKEYALLYKLDI) and the 92TH023 strain (same as CM244 strain) (KKQKVHALF, DKKQKVHALFYRLDI) that were present in the RV144 vaccine. Eight other variants (see **Table S2**) corresponded to breakthrough viruses isolated from infected RV144 participants. Biosynthesis Inc (Lewisville, TX) synthesized the peptides. Note that due to the high variability of V2 it is unlikely that the peptides matched the sequences of viruses that were present within each subject.

IFN-γ Elispot assay. Cryopreserved PBMCs were thawed at 37°C and re-suspended (2×10^6 cells/ml) in R10 medium (RPMI-HEPES with 10% fetal bovine serum supplemented with L-glutamine, and penicillin-streptomycin). Cell viability and recovery were both determined after initial thawing and overnight incubation at 37°C and 5% CO2. Multiscreen (Millipore, Bedford, MA.) filtration plates were coated with 100μl of the anti-IFN-γ monoclonal antibody 1-D1K/well (10 μg/ml; Mabtech, Nacka, Sweden), incubated overnight at 4°C, washed with phosphate-buffered saline (PBS), and blocked (37°C for 2 h) with R10 medium. PBMCs were washed, and 200,000 cells suspended in 75 μl of medium were plated in each well. Peptides were diluted in 25 μl of RPMI-HEPES and added to appropriate wells at a 2 μg/ml final concentration. The peptide solvent, dimethyl sulfoxide, was kept below 0.01% final concentration in the peptide mixture. Cells were stimulated with individual peptides overnight at 37°C (5% CO2). IFN-γ secretion was detected colorimetrically by sequential incubations with secondary biotinylated anti-IFN-γ 7-B6-1 monoclonal antibody (1

126 μg/ml; Mabtech), strepatavidin-alkaline phosphatase (BD Pharmingen, San Diego, CA.), and
127 nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
128 substrate (Thermo Scientific Rockford, IL). Phytohemagglutinin (2 μg/ml; Murex Biotech,
129 Dartford, UK) or CEF peptide pool (Mabtech) stimulations were used as a positive control. For
130 the negative control, peptide dilutent was used. Spots formed by IFN-γ-secreting cells were
131 counted with an automated ImmunoSpot plate reader (Cellular Technology LTD, Shaker
132 Heights, Ohio), and results are presented as spot-forming cells (SFC) per 10^6 PBMCs. A
133 response was considered positive when the mean SFC for the experimental wells was at least
134 two-fold greater than the mean SFC for the negative control wells and the mean SFC/10^6 cells in
135 the experimental wells was >50 after subtraction of the mean SFC/10^6 cells of the negative
136 control wells.
137
138 *Ex vivo* polychromatic flow cytometry (ICS). PBMC were washed in R10 and rested overnight.
139 The next day, cells were washed and stimulated for 5 h in the presence of brefeldin A (10 μg/ml)
140 with either staphylococcal enterotoxin B (1 μg/ml; Sigma-Aldrich) as a positive control, HIV-1
141 peptides (2 μg/ml each peptide/sample), or no peptide as a negative control. Cells were washed
142 twice with PBS and stained with ViViD cell viability reagent (Life Sciences, Grand Island, NY)
143 Intracellular staining was performed using standard techniques (BD Biosciences; cytokine flow
144 cytometry protocol) using previously titrated monoclonal (mAb) reagents. Anti-CD3-APCCy7, -
145 CD8-PerCPCy5.5, -IFN-γ -PE-Cy7, -IL-2-PE, and -TNF-α-allophycocyanin (BD/Pharmingen.).
146 Data acquisition was performed on an LSRII flow cytometer (BD Biosciences, San Jose, CA),
147 collecting 100,000 –200,000 viable lymphocyte gated, or 10,000–40,000 CD8+ events per
148 sample, and analyzed with FlowJo software (Tree Star). Positive responses were designated
149 when the percentage of bright cytokine+/CD8+ T cells was twice that of the negative control.
150
151 *Criteria for selecting immune correlates to compare in A*02(+/-) subgroups*
152 After finding the association between HLA A*02 and VE we decided to repeat the immune
153 correlates analysis in subgroups of participants with and without the A*02 allele, to test if the
154 allele was also associated with the established immune correlates of risk (CoRs). For this reason
155 and to preserve statistical power we limited the analysis to immunological assays that were found
156 to be significant correlates of risk prior to multiplicity adjustment in either the primary or

157 secondary analysis of the Haynes et al. study [15]. Specifically, we tested the following assays,
158 which had the following CoR results: (1) Env V1/V2 IgG antibody binding (OR=0.57, p = 0.01,
159 q=0.08) (2) IgA binding to a panel of Env isolates (M-B gD) (OR=1.54, p = 0.03, q=0.08) (3)
160 IgA binding to consensus A Env gp140 (OR=3.71, p = 0.001, q = 0.1) (4) IgA antibody binding
161 to gp120-C1 MQEDVISLWDQSLKPCVKLTPLCV (OR=3.15, p = 0.003, q=0.13) (5) V2
162 hotspot peptide microarray (OR = 0.64, p = 0.03), (6) PBMC Luminex cytokine score (OR =
163 0.31, p = 0.02, q = 0.27).