

Supplemental Materials and Methods

ICS Criteria

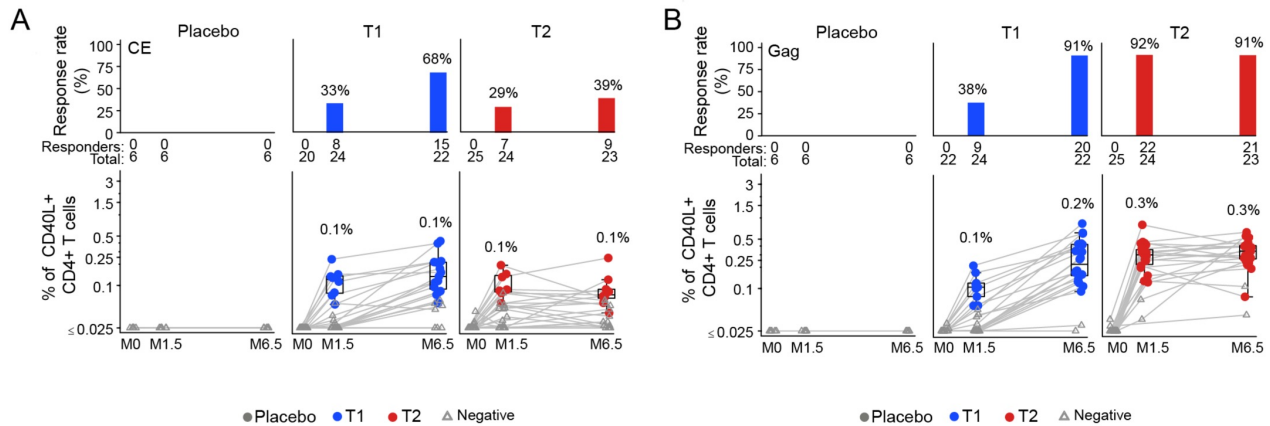
Several criteria were used to determine if data from an assay were acceptable and could be statistically analyzed. The blood draw date must have been within the allowable visit window as determined by the protocol. After sample thawing and overnight incubation, the viability of the PBMCs must have been 66% or greater. If it was not, a new specimen for that participant at that time point was thawed for testing. If the PBMC viability of the second thawed aliquot was below this threshold, the ICS assay was not performed, and no data were reported to the statistical center for the participant and time point. For the negative control acceptance criteria, if the average cytokine response for the negative control wells was above 0.1% for either the CD4+ or CD8+ T cells, the sample was retested. If the retested results were above 0.1%, the data were excluded from analysis; otherwise, the retest data were used. The total numbers of CD4+ and CD8+ T cells must also have exceeded certain thresholds. If the number of CD8+ T cells was less than 5,000 or CD4+ T cells was less than 10,000 for any of the HIV-1 peptide pools or for one of the negative control replicates for a particular sample, data for that stimulation were filtered. If both negative control replicates failed for the number of T cells, the sample was retested. If 1 negative control replicate failed for number of T cells, the negative control replicate with sufficient T cells was used.

ICS assay

To assess positivity for a peptide pool within a T cell subset, a 2-by-2 contingency table was constructed comparing the HIV-1 peptide-stimulated and negative control data. The 4 entries in each table were the number of cells positive for IFN- γ and the number of cells negative for IFN- γ , for both the stimulated and the negative control data. If both negative control replicates were

included, then the average number of total cells and the average number of positive cells were used. A 1-sided Fisher's exact test was applied to the table, testing whether the number of cytokine-producing cells for the stimulated data was equal to that for the negative control data. Typically, multiplicity adjustment was made to the individual peptide pool P values since multiple individual tests (for each peptide pool) were conducted simultaneously; this was not done for mapping studies to provide higher sensitivity to detect responses. If the P value for a peptide pool was no more than 0.00001, the response to the peptide pool for the T cell subset was considered positive. Because the sample sizes (i.e., total cell counts for the T cell subset) were large (up to 100,000 cells), the Fisher's exact test has high power to reject the null hypothesis for very small differences. The P value significance threshold was chosen stringently (≤ 0.00001).

Supplemental Figure 1



Supplemental Figure 1. Flow cytometric analysis of CD40L⁺ CD4⁺ T-cell responses to CE and full-length p55^{Gag}. CD40L⁺ CD4⁺ T-cell responses to (A) total CE and (B) p55^{Gag} were measured in PBMC. Upper panels indicate the response rate (number of responders/number of participants tested). The lower panels show the distribution of background-adjusted magnitude of CD40L⁺ T-cell responses are displayed graphically on the log scale y-axis truncated at 0.025%; any values below this level are censored. Plots include data from responders in color and non-responders in grey, with data points for each participant are connected by a grey line. Box plot whiskers extend to the most extreme data points that are no more than 1.5 times the interquartile range (i.e., height of the box) or if no value meets this criterion, to the data extremes. Median response magnitudes are shown as % of CD40L⁺ response.

Supplemental Table 1. p24CE1 and p24CE2 proteins.

	CE 2	CE 3	CE 4	
p24CE1	VIPMF S ALSEGATPQDLN AA VGGHQAA M QMLK D TINEEAAEWDR AAAE <u>PRGSDIAGTTSTLQEQI</u> G W AAA			
p24CE2	VIPMF T ALSEGATPQDLN AA VGGHQAA M QMLK E TINEEAAEWDR AAAE <u>PRGSDIAGTTSTLQEQI</u> A W AAA			
	CE 5	CE 6	CE 7	CE 1
p24CE1	KRWIILGLNKIVRMYS P T SIAAKY VDRF Y KTLRAEQ AAGLEEMMTACQGVGGP GHKAA <u>I</u> SPRTLNAWVKV			
p24CE2	KRWIILGLNKIVRMYS V SIAAKY VDRF F KTLRAEQ AAGLEEMMTACQGVGGP SHKAA <u>L</u> SPRTLNAWVKV			

Notes:

Differences between p24CE1 and p24CE2 are shown in red vs blue type, respectively.

Linker sequences are shown in green type.

Linker sequences that were conserved in HIV, when adjacent to CE are underlined.

CE 1 was placed at the C-terminus of each construct to enhance gene expression (Kulkarni V, et al. *PLoS One*. 2013;8:e60245).

Supplemental Table 2. Reason for missing ICS data.

Visit	Treatment	Expected n	Assayed n	Reason for unavailability	Data reported		
					CD4	CD8	Reason missing
M0	Placebo	6	6	-	6	6	-
	T1	25	22	1 no PBMC collected, 1 PBMC not shipped before assays performed, 1 no post- baseline PBMC (terminated)	20	20	1 Low CD4 and CD8 T cell number, 1 CD4 and CD8 unreliable*
	T2	25	25	-	25	24	1 high CD8 background
M1.5	Placebo	6	6	-	6	6	-
	T1	25	24	1 missed visit	24	23	1 high CD8 background
	T2	25	24	1 no PBMC collected	23	23	1 high CD4 and CD8 background
M6.5	Placebo	6	6	-	6	6	-
	T1	25	22	2 missed visits, 1 out of window	22	22	-
	T2	25	23	1 missed visit 1 terminated	21	23	2 high CD4 background

*Missing both CD4 and CD8 only for Total CE; data for 22 available for p55^{Gag}

Supplemental Table 3. Reagents in the 28-color ICS panel.

Antibody	Manufacturer	Catalog Number
UViD	Invitrogen	L34962
CD3 BUV395	BD Biosciences	563546
CD4 BV480	BD Biosciences	566165
CD8a BUV805	BD Biosciences	564912
CD45RA BUV496	BD Biosciences	CUSTOM
CD154 (CD40L) BUV737	BD Biosciences	748963
CD25 BUV563	BD Biosciences	565699
TNF- α FITC	Invitrogen	11-7349-82
CD16 BV570	BioLegend	302036
Perforin PE	BioLegend	353304
KLRG1 PE-Vio615	Miltenyi	130-108-366
CXCR5 BV785	BioLegend	356936
PD-1 BV650	BioLegend	329950
IL-4 BB630 ^A	BD Biosciences	CUSTOM
IL-13 BB630 ^A	BD Biosciences	CUSTOM
HLA-DR BUV661	BD Biosciences	565073
CD14 BB660	BD Biosciences	624295
CTLA-4 PE-Cy5	BD Biosciences	555854
FOXP3 PE-Cy5.5	Invitrogen	35-4776-42
IL-2 BB700	BD Biosciences	566405
CCR5 BV711	BD Biosciences	563395
Granzyme B Alx700	BD Biosciences	560213
IFN- γ V450	BD Biosciences	560371
IL-17a PE-Cy7	BioLegend	512315
IL-17F PE-Cy7	Invitrogen	25-7169-42
CD56 BV750	BioLegend	362556
LAG-3 APC ^B	BioLegend	369212
TIM-3 APC ^B	BioLegend	345012
CCR7 (CD197) BV605	BioLegend	353224
CD127 BB790	BD Biosciences	CUSTOM
CD69 APC-Fire750	BioLegend	310946

^AIL-4 and IL-13 are detected in the same channel.

^BLAG-3 and TIM-3 are detected in the same channel.

Supplemental Table 4. Reagents in the 17-color ICS panel.

Antibody	Manufacturer	Catalog Number
AViD ^A	Invitrogen	L34957
CD3 BUV737	BD Biosciences	564307
CD4 BUV395	BD Biosciences	563550
CD8 BV650	BD Biosciences	563821
CD14 BV510 ^A	BioLegend	301842
CD56 BV570	BioLegend	318330
CXCR5 PE-Dazzle594	BioLegend	356928
PD-1 (CD279) BV605	BioLegend	329924
ICOS (CD278) BV711	BD Biosciences	563833
CD45RA APC H7	BD Biosciences	560674
CCR7 BV785	BioLegend	353229
IFN- γ V450	Becton Dickinson	560371
TNF α FITC	eBiosciences	11-7349-82
IL-2 PE	BD Biosciences	559334
IL-4 PerCP-Cy5.5	BD Biosciences	500822
IL-17a PE-Cy7	BioLegend	512315
CD154 APC	BD Biosciences	555702
Granzyme B Alx700	BD Biosciences	560213

^AAViD and CD14 are detected in the same channel.

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