Human adenovirus-specific T cells modulate HIV-specific T-cell responses to an Ad5-vectored HIV-1 vaccine

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Supplemental methods

IFN-γ ELISpot

For the ELISpot assays, PBMCs were plated in 96-well Immobilon-P plates (Millipore), which had been precoated overnight with 10 μg/ml of αIFN-γ monoclonal antibody (mAb) 1-D1K (Mabtech). 100,000 cells/well were added in 100 μl of R10 [RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin (all but FBS from Invitrogen; FBS from Gemini Bio-Products)]. For negative controls, cells were incubated in medium alone. For positive controls, 0.5 μg/ml phytohemagglutinin (Murex Biotech) was added. Cells were incubated overnight at 37˚C with 5% CO_2. Plates were developed by washing seven times with 0.05% Tween-20 (Sigma-Aldrich) in phosphate buffered saline (DPBS, no Ca & Mg, Invitrogen); then 1 μg/ml of biotinylated αIFN-γ Mab 7-B6-1 (Mabtech) was added for 2·5 hours at room temperature. Plates were washed four times and incubated with a 1:750 dilution of streptavidin-coupled alkaline phosphatase (Mabtech) for 2 hours at room temperature in

the dark. Plates were washed again and IFN-γ production was detected as blue spots after a short incubation with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Pierce). The color reaction was stopped by washing plates with tap water. The number of spots was counted using the CTL ELISpot Reader Unit (Cellular Technology Ltd.) and results were expressed as spot forming cells (SFC) per million input cells.

Intracellular Cytokine Staining

Cryopreserved PBMC samples were thawed and incubated overnight before stimulation as previously described (1). Briefly, PBMCs were stimulated by incubation with 10,000 Ad5 empty vector particles (vector not expressing HIV transgenes) per cell for 24 hours at 37°C in the presence of αCD28 and αCD49d antibodies (BD Biosciences). Brefeldin A (Sigma-Aldrich) was added after 6 hours of stimulation. Vector dilution buffer (GTS buffer: 20mM Tris HCl, 25mM NaCl, 2.5% glycerol, pH8·1) was used as negative control. Intracellular cytokine staining (ICS) was performed using a previously-validated eight-color protocol (2) or a cross-validated ten-color protocol as described (1). Ad5-specific responses were considered positive based on previously described criteria (2). Subjects with high background responses in the negative control (>0.1% of T-cells expressing IFN-γ or IL2) were excluded from the analysis. Representative examples for Ad5-specific $CD4^+$ and $CD8^+$ T-cell immune responses are shown in **Supplemental Figure 1**.

Ad5 and HIV peptide pool-specific immune responses

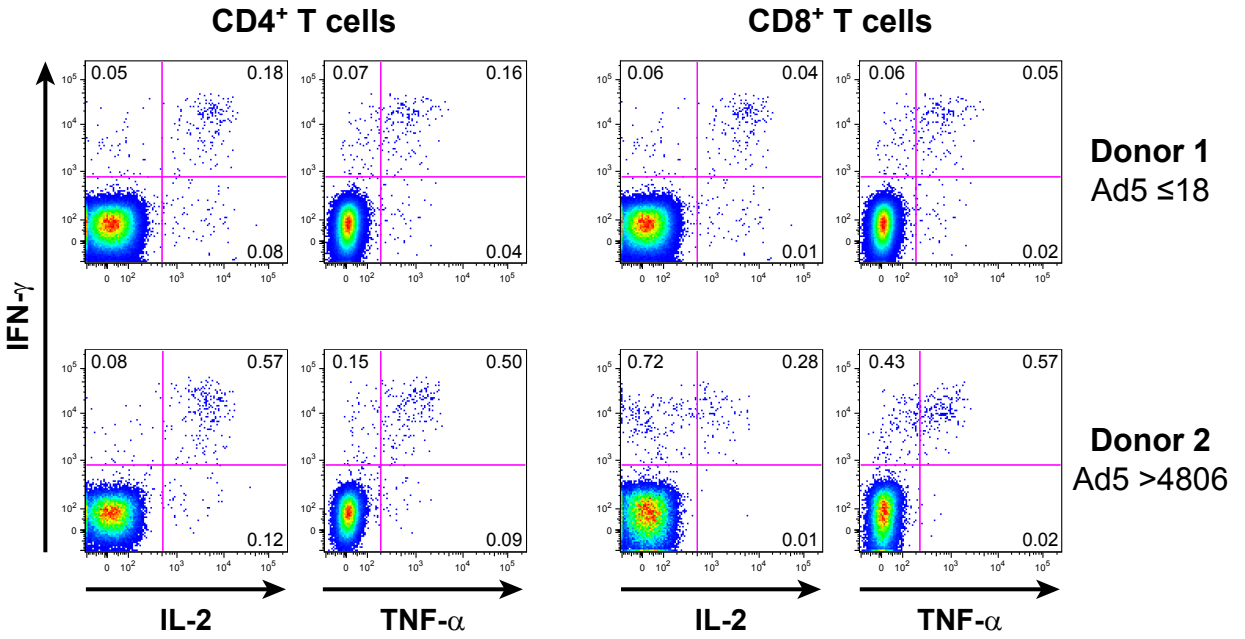
Cryopreserved PBMC samples were thawed and incubated overnight before stimulation as previously described (1). PBMCs were stimulated by incubation with 1 μg/ml of peptide pools in the presence of Brefeldin A and αCD28 and αCD49d antibodies for 6 hours at 37°C. Duplicate wells of PBMCs incubated in media with 0.5% DMSO were used as a negative control, while PBMCs stimulated with SEB or a CMV peptide pool (15-mer peptides overlapping by 11 amino acids spanning pp65) were used as positive controls. Cells were stained with the ten-color ICS protocol (described above).

Adenovirus Neutralizing Antibody Assays

Ad5 nAb titers were measured as previously described (3). To determine Ad1 and Ad2 nAb titers, serial 1:4 dilutions of serum from 1:18 to 1:4608 were incubated with $1{\cdot}25{\times}10^{7}$ viral particles of wild-type adenovirus serotypes 1 (ATCC# VR-1) or 2 (ATCC# VR-846) for one hour. The neutralization mixtures were then transferred to Human Embryonic Kidney (HEK) 293 cell monolayers in 96-well plates and incubated for one hour. Following infection, complete DMEM medium was added to the cells and the plates were incubated for 48 hours in a tissue culture incubator. The cell monolayers were then washed, fixed with 100% methanol and stained with a 1:1000 dilution of hexon-specific antibody that binds to both Ad1 and Ad2 hexon (Genway). Infected cells were visualized by a secondary peroxidase-conjugated antibody (BD Biosciences) followed by incubation with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). The infected cells were counted on a CTL Immunospot reader. Titers were measured as the inverse of the 50% neutralization endpoint.

Supplemental Figure 1. Representative examples for Ad5 vector-specific $CD4^+$ and $CD8^+$ T-cell responses.

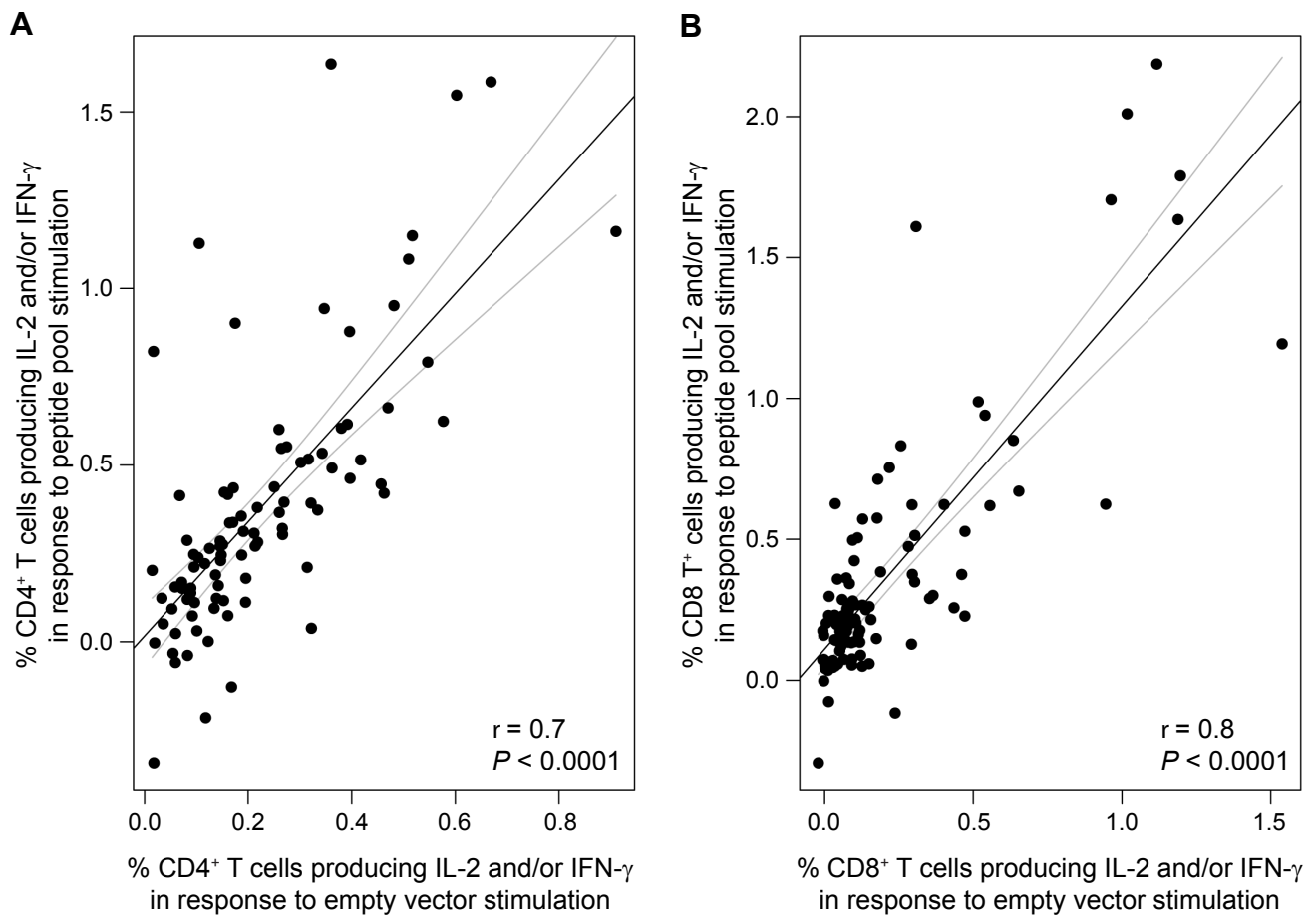
Cytokine secretion after 24h stimulation with the empty Ad5 vector is shown for an ~~Ad5~~ Ad5-seronegative placebo recipient (top panel) and Ad5-seropositive vaccine recipient (bottom panel) from the Step Study.


CD4+ T cells
CD8+ T cells
IFN-γ
IL-2
TNF-α
IL-2
TNF-α
0.05
0.18
0.08
0.07
0.16
0.04
0.06
0.04
0.01
0.06
0.05
0.02
0.08
0.57
0.12
0.15
0.50
0.09
0.72
0.28
0.01
0.43
0.57
0.02
Donor 1
Ad5 ≤18
Donor 2
Ad5 >4806

Supplemental Figure 2. Correlation of Ad5-specific T-cell responses measured by stimulation with empty vector and peptide pools.

Background-adjusted $CD4^+$ (A) and $CD8^+$ (B) Ad5-specific T-cell responses measured by stimulation with empty Ad5 vector (x-axis) or peptide pools (y-axis) are shown for 102 Step study participants. The magnitude of Ad5-specific T-cell responses detected using empty vector stimulation versus the sum of magnitudes measured by stimulation with Ad5 peptide pools were highly correlated for $CD4^+$ (A; ρ=0.7, p<0.0001, Spearman rank correlation) and $CD8^+$ T cells (B; ρ=0.8, p<0.0001), although the regression line slopes indicate moderately higher magnitudes of responding cells detected by the peptide pools (CD4 slope=1·6, CD8 slope=1·2). This difference could be due to a higher density of

MHC-peptide complexes following incubation with peptides compared to that achieved after intracellular processing of vector particles.


A
% CD4+ T cells producing IL-2 and/or IFN-γ in response to peptide pool stimulation
% CD4+ T cells producing IL-2 and/or IFN-γ in response to empty vector stimulation
r = 0.7
P < 0.0001
B
% CD8 T+ cells producing IL-2 and/or IFN-γ in response to peptide pool stimulation
% CD8+ T cells producing IL-2 and/or IFN-γ in response to empty vector stimulation
r = 0.8
P < 0.0001

Supplemental Table 1. Ad5 peptide distribution in protein pools.

Protein	# peptides	# pools	# peptides per pool
hexon	266	2	133
penton	159	1	159
fiber	161	1	161
pV	100	1	152
pVII	52		
100K	282	2	141
E2 DNA polymerase (pol)	300	2	150
E2 preterminal protein (pTP)	186	2	166
E2 ssDNA binding protein (DBP)	146		
E3 gp19	43	1	121
E4 orf 6	78		
Total	**1773**	**12**	

Supplemental Table 2. Ad5 hexon-specific responses.

Protocol	PTID	Ad5 nAb titer	Sequence	SFC/million
HVTN 071	121360037	431	PQKFFAIKNLLLL	178
		431	GSYTYEWNFRKDVNM	193
HVTN 071	123360032	4103	PQKFFAIKNLLLL	223
HVTN 071	123360058	<18	KKFLCDRTLWRIPF	78
		<18	LTDLGQNLLYANSAH	438

Protocol	PTID	Ad5 nAb titer	Sequence	SFC/million
HVTN 071	125360010	<18	PQKFFAIKNLLLL	143
		<18	KFFAIKNLLLLPGSY	618
		<18	PSRNWAAFRGWAFTR	103
		<18	PSLGSGYDPYYTY	78
		<18	YDPYYTYSGSIPYL	103
		<18	YYTYSGSIPYLDGTF	93
HVTN 071	126360012	<18	GSYTYEWNFRKDVNM	133
HVTN 071	126360047	<18	SMMPQWSYMHISGQDA	80
		<18	WSYMHISGQDASEYL	65
HVTN 071	126360051	<18	ESYKDRMYSFFRNF	80
		<18	WSYMHISGQDASEYL	60
		<18	MPNRPNYIAFRDNFI	135
		<18	PNYIAFRDNFIGLMY	90
		<18	IGDRTRYFSMWNQAV	155
		<18	TRYFSMWNQAVDSY	135
		<18	NHHRNAGLRYRSMLL	105
		<18	NAGLRYRSMLLGNGR	70
		<18	PQKFFAIKNLLLL	50
HVTN 071	126360070	<18	LLLPGSYTYEWNFRK	173
		<18	GSYTYEWNFRKDVNM	83
		<18	GTFYLNHTFKKVAI	63
HVTN 071	126360104	<18	GTFYLNHTFKKVAI	68
HVTN 071	126360133	707	FARATETYFSLNNKF	65
		707	ETYFSLNNKFRNPTV	90
		707	TRYFSMWNQAVDSY	60
HVTN 071	126360133	707	GLVDCYINLGARWSL	75
		707	IQVPQKFFAIKNLLL	100
		707	PQKFFAIKNLLLL	50
		707	LTDLGQNLLYANSAH	260
		707	PTLLYVLFEVFDVVR	95
HVTN 071	126360157	<18	YSGINITKEGIQIGV	78
		<18	MPNRPNYIAFRDNFI	158
		<18	PNYIAFRDNFIGLMY	198
		<18	GLMYYNSTGNMGVLA	103
		<18	NNFAMEINLNANLWR	63

Protocol	PTID	Ad5 nAb titer	Sequence	SFC/million
		<18	CYINLGARWSLDYM	58
		<18	IQVPQKFFAIKNLLL	98
		<18	PQKFFAIKNLLLL	88
		<18	VDGASIKFDSICLYA	73
		<18	SIKFDSICLYATFF	63
		<18	PMSRQVVDDTKYKDY	88
		<18	PLIGKTAVDSITQKK	93
		<18	LTDLGQNLLYANSAH	468
		<18	LYANSAHALDMTFEV	98
		<18	PTLLYVLFEVFDVVR	173
SAC	205585	1579	PQKFFAIKNLLLL	58
		<18	GPTFKPYSGTAYNAL	75
		<18	MPNRPNYIAFRDNFI	315
		<18	PNYIAFRDNFIGLMY	445
		<18	LDSIGDRTRYFSMW	65
		<18	IGDRTRYFSMWNQAV	280
		<18	TRYFSMWNQAVDSY	280
		<18	GLVDCYINLGARWSL	65
SAC	214817	<18	CYINLGARWSLDYM	90
		<18	IQVPQKFFAIKNLLL	175
		<18	PQKFFAIKNLLLL	215
		<18	YDPYYTYSGSIPYL	115
		<18	YYTYSGSIPYLDGTF	85
		<18	AQCNMTKDWFLVQML	55
		<18	PMDEPTLLYVLFEVF	75
		<18	PTLLYVLFEVFDVVR	115
SAC	215051	805	PQKFFAIKNLLLL	195
		805		55
SAC	224600	>4608	GSYTYEWNFRKDVNM	80
SAC	228071	<18	MPNRPNYIAFRDNFI	83
		<18	YDPYYTYSGSIPYL	353
		<18	YYTYSGSIPYLDGTF	288
SAC	246728	2627	PNYIAFRDNFIGLMY	55
		2627	YDPYYTYSGSIPYL	95
		2627	YYTYSGSIPYLDGTF	95
		2627	PMDEPTLLYVLFEVF	55

Protocol	PTID	Ad5 nAb titer	Sequence	SFC/million
SAC	274012	<18	GQQSMPNRPNYIAFR	98
		<18	MPNRPNYIAFRDNFI	68
		<18	QASQLNAVVDLQDRNTEL	53
		<18	VVDLQDRNTELSYQL	58
		<18	LQDRNTELSYQLLL	68
		<18	FSMWNQAVDSYDPDV	123
		<18	NFLYSNIALYLPDKL	53
		<18	PQKFFAIKNLLLL	63
		<18	GSYTYEWNFRKDVNM	238
		<18	PMDEPTLLYVLFEVF	73
		<18	PTLLYVLFEVFDVVR	108
SAC	291114	117	ESYKDRMYSFFRNF	58
		117	MYSFFRNFQPMSRQV	63
		117	PTLLYVLFEVFDVVR	68
SAC	298998	45	IGDRTRYFSMWNQAV	58
			CYINLGARWSLDYM	68

Supplemental Table 3. Ad1/Ad2 hexon-specific responses.

Protocol	PTID	Ad1/Ad2	Sequence	SFC/million
HVTN 071	123360058	Ad1	GALESKVEMQFF	85
SAC	205585	Ad1	SCEWEQEEPTQEMAE	63
		Ad1	TPMKPCYGSYAR	93
		Ad1	ANNQGALESKVEMQF	53
		Ad1	ALESKVEMQFFAPSGT	53
		Ad2	LSGETITKSGLQIGS	53
SAC	214817	Ad1	ANNQGALESKVEMQF	93
SAC	215051	Ad1	EQEEPTQEMAEELEDEE	85
		Ad1	PTQEMAEELEDEEEAEE	95
		Ad1	LAGEKITANGLQIVS	55
		Ad1	KITANGLQIVSDTQTEGNPV	75
		Ad1	PMKPCYGSYARPTNK	125
		Ad1	QPSIVLYSEDVNM	145
		Ad1	AMLGQQAMPNRPNYIA	85
		Ad2	EDEEEEDEDEEEEEE	555
		Ad2	EEDEDEEEEEEEQNARDQATKK	85
		Ad2	DEEEEEEEQNARDQATKKTHVY	65
		Ad2	KKTHVYAQAPLS	75
		Ad2	AQAPLSGETITKSGL	105
		Ad2	EAEATASGGRVLKK	75
		Ad2	CYGSYARPTNKNGGQGI	58
		Ad2	RPTNKNGGQGILVA	88
		Ad2	GVPLPKVDLQFF	78
		Ad2	VPLPKVDLQFFSNTT	68
		Ad2	LQFFSNTTSLNDRQGNAT	68
		Ad2	LNDRQGNATKPKVVLY	248
		Ad2	KPKVVLYSEDVNM	88
		Ad2	EDVNMETPDTHLSYK	138
		Ad2	METPDTHLSYKPGKG	128
		Ad2	AMLGQQSMPNRPNYIA	1518
SAC	224600	Ad2	NPFGGQSVLVPDEKGV	73
SAC	228071	Ad1	EQEEPTQEMAEELEDEE	103
		Ad1	QPSIVLYSEDVNM	73
		Ad1	SKTDENSKAMLGQQAM	123
		Ad1	NSKAMLGQQAMPNR	273
		Ad1	AMLGQQAMPNRPNYIA	133

Protocol	PTID	Ad1/Ad2	Sequence	SFC/million
		Ad2	AVAEDEEEEDEDEE	63
SAC	228071	Ad2	YQPEPQIGESQWN	603
		Ad1	APNSCEWEQEEPTQ	185
		Ad1	SCEWEQEEPTQEMAE	195
		Ad1	MAEELEDEEEAEEEEA	155
		Ad1	EEAEEEAEAPQADQKVKKTHVY	55
		Ad1	LQIVSDTQTEGNPVFADP	65
		Ad1	YQPEPQVGESQWN	55
		Ad1	CYGSYARPTNKNGGQGI	55
		Ad1	GILVANNQGALESKV	175
		Ad1	ANNQGALESKVEMQF	95
SAC	246281	Ad1	YKPSKTDENSKAML	65
		Ad2	EQTEDSGRAVAEDEE	65
		Ad2	EDEEEEDEDEEEEEE	65
		Ad2	EEDEDEEEEEEEQNARDQATKK	75
		Ad2	KTHVYAQAPLSGETI	85
		Ad2	AQAPLSGETITKSGL	55
		Ad2	LSGETITKSGLQIGS	55
		Ad2	SDNAETQAKPVYADPSY	155
		Ad2	AETQAKPVYADPSYQPEPQI	65
		Ad2	TPMKPCYGSYAR	95
		Ad2	CYGSYARPTNKNGGQGI	65
SAC	247354	Ad2	KVVLYSEDVNMETPDTH	55
		Ad2	METPDTHLSYKPGKG	55
		Ad1	MAEELEDEEEAEEEEA	135
		Ad2	LAPKGAPNSCEW	445
SAC	248433	Ad2	APKGAPNSCEWEQTE	75
		Ad2	KVVLYSEDVNMETPDTH	115
		Ad2	YKPGKGDENSKAML	55

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