Supplementary Data 1: Rationale for age groups

In this study spleens were grouped into subsets according to the age of the donors; the groups included donors under the age of five years, and five years old or greater (**Supplementary Table 1**). Age groups were chosen based on the average age of exposure to wild type AAV and parvovirus B19 [2,3], typically before the age of five years.

Supplementary Data 2: Provided Protein sequences for Proimmune ProPresent® assay

1 Capsid sequence

maadgylpdwledtlsegirqwwklkpgppppkpaerhkddsrglvlpgykylgpfngldkgepvneadaaalehdka
ydrqldsgdnpylkynhadaefqerlkedtsfggnlgravfqakkrvleplglveepvktapgkkrpvehspvepdsssgt
gkagqqparkrlnfgqtgdadsvpdpqplgqppaapsglgtntmatgsgapmadnnegadgvgnssgnwhcdstwm
gdrvittstrtwalptynnhlykqissqsgasndnhyfgystpwgyfdfnrfhchfsprdwqrlinnnwgfrpkrlnfklfni
qvkevtqndgtttiannltstvqvftdseyqlpyvlgsahqgclppfpadvfmvpqygyltlnngsqavgrssfycleyfpsq
mlrtgnnftfsytfedvpfhssyahsqsldrlmnplidqylyylsrtntpsgtttqsrlqfsqagasdirdqsrnwlpgpcyrqq
rvsktsadnnnseyswtgatkyhlngrdslvnpgpamashkddeekffpqsgvlifgkqgsektnvdiekvmitdeeeirt
tnpvateqygsvstnlqrgnrqaatadvntqgvlpgmvwqdrdvylqgpiwakiphtdghfhpsplmggfglkhpppqi
likntpvpanpsttfsaakfasfitqystgqvsveiewelqkenskrwnpeiqytsnynksvnvdftvdtngvyseprpigtr
yltrnl

2 FIX sequence

mqrvnmimaespgliticllgyllsaectvfldhenankilnrpkrynsgkleefvqgnlerecmeekcsfeearevfentert
tefwkqyvdgdqcesnpclnggsckddinsyecwcpfgfegknceldvtcniknngrceqfcknsadnkvvcsctegyrla
enqkscepavpfpcgrvsvsqtskltraetvfpdvdyvnsteaetildnitqstqsfndftrvvggedakpgqfpwqvvlng
kvdafcggsivnekwivtaahcvetgvkitvvagehnieetehteqkrnviriiphhnynaainkynhdialleldeplvlns
yvtpiciadkeytniflkfgsgyvsgwgrvfhkgrsalvlqylrvplvdratclrstkftiynnmfcagfheggrdscqgdsgg
phvtevegtsfltgiiswgeecamkgkygiytkvsryvnwikektklt

3 Altenate open reading frame products to FIX sequence 1

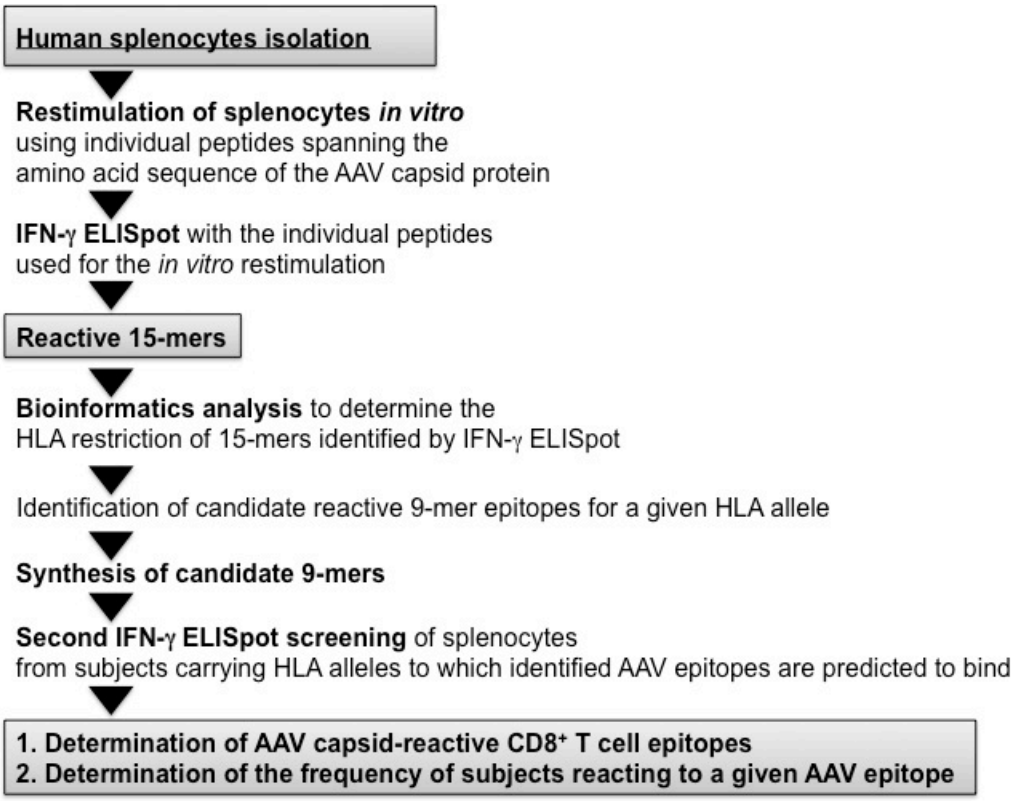
c s a t s w q n h q a s s p s a f d i y s v l n v q f f l i m k t p t k f i g q r g i i q v n w k s l f k g t l r e n v w k k
s v v l k k h e k f l k t l k e q l n f g s s m l m e i s v s p i h v m a a v a r m t l i p m n v g v p l d l k e r t v n m
h v t l r m a d a s s f v k i v l i t r w f a p v l r d i d l q k t r s p v n q q c h f h v e e f l f h k l l s s p v l r l f f l
m w t m i l l k l k p f w i t s l k a p n h l m t s l g l l v e k m p n q v n s l g r l f m v k l m h s v e a l s l m k n
g l l l p t v l k l v l k l q l s q v n i i l r r q n i q s k s e m f e l f l t t t t m q l l i s t t m t l p f w n w t n p c t a t l
h l f a l l t r n t r t s s s n l d l a m v a g e e s s t k g d q l f f s t l e f h l l t e p h v f d l q s s p s i t t c s v l a s
m k e v e i h v k e i v g d p m l l k w k g p v s l e l l a g v k s v q k a n m e y i p r y p g m s t g l r k k q s s l

4 Altenate open reading frame products to FIX sequence 2

a a r e h d h g r i t r p h h h l p f r i s t q c m y s f s s k r q q n s e s a k e v f r i g r v c s r e p e r m y
g r k v f r s t r s f k h k n n i l e a v c w r s v v q s m f k w r q l q g h f l m l v s l w i r k e l i r c n m
h e w q m r a v l k c q g g l l l y g i s t c r k p e v l t s s a i s m w k s f c f t n f a h p c d c f s c g l c
k f y s n h f g h h s k h p i i l h s g c w w r r c q t r s i p l a g c f e w s c i l w r l y r k m d c n c c p l
c n w c n y s c r r t y g d r t y r a k a k c d s n y s s p q l q c s y v q p h c p s g t g r t l s a k q l r y t
y l h c q g i h e h l p q i w i w l c k w l g k s l p q r e i s f s s s v p s s t c p s h m s s i y k v h h l q h
v l c w l p r r r f m s r r w g t p c y s g r d q f l n w n y l g r v c n e r q i w n i y q g i p v c q l d g k
n k a h l

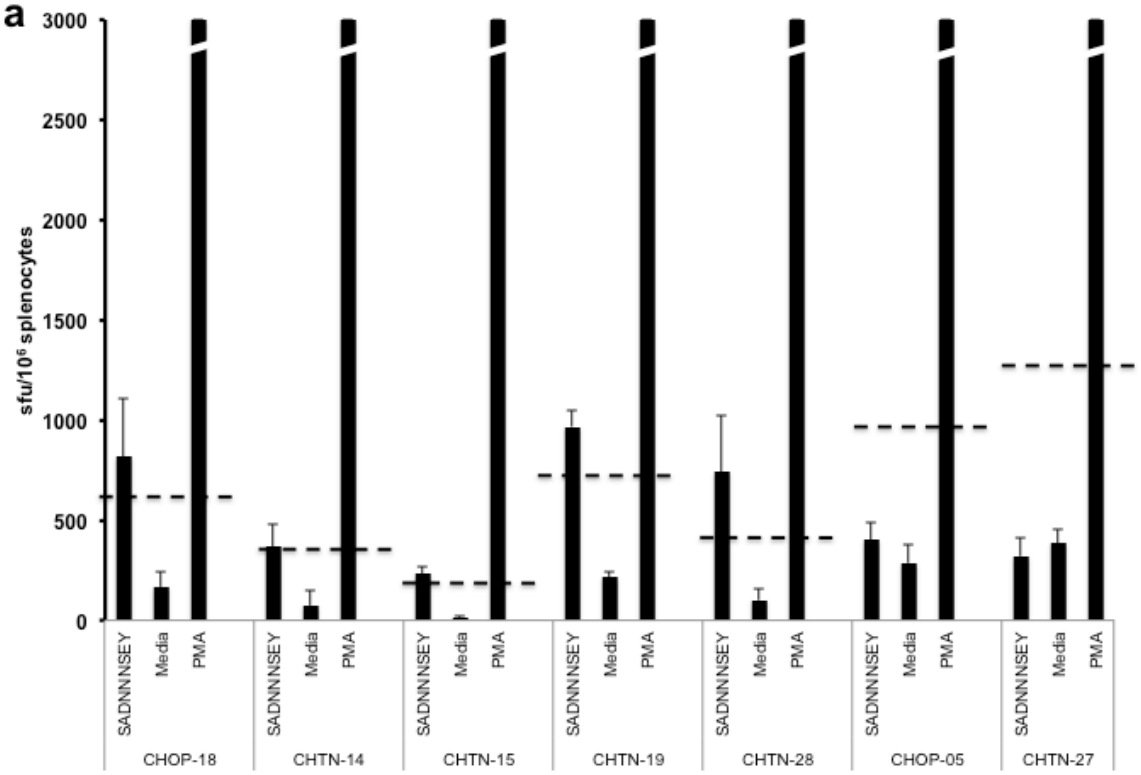
Supplementary Figure 1. Experimental steps in AAV capsid T cell epitope discovery. Flow diagram of experimental steps for the high-throughput mapping of MHC class I epitopes within the AAV capsid. A peptide library of 15-mers overlapping in sequence by 10 amino acids and spanning the AAV capsid protein VP1 is synthesized. Splenocytes are isolated and restimulated in vitro in 96 well plates; in each well cells are incubated with one single peptide form the AAV peptide library. After restimulation, cells are tested for reactivity against AAV peptides in an ELISpot assay. At this step, reactive AAV capsid 15-mers are identified. Using the HLA type of the splenocytes' donor and bioinformatics analysis MHC restriction of the 15-mer and binding 9-mer subsequence are determined. Candidate 9-mer epitopes are synthesized and used to screen splenocytes from donors selected based on the cognate HLA allele of the predicted epitopes. The final outputs of this analysis are the $CD8^+$ T cell epitopes for a given HLA allele, and the frequency of subjects reacting to the epitope.

Supplementary Figure 1

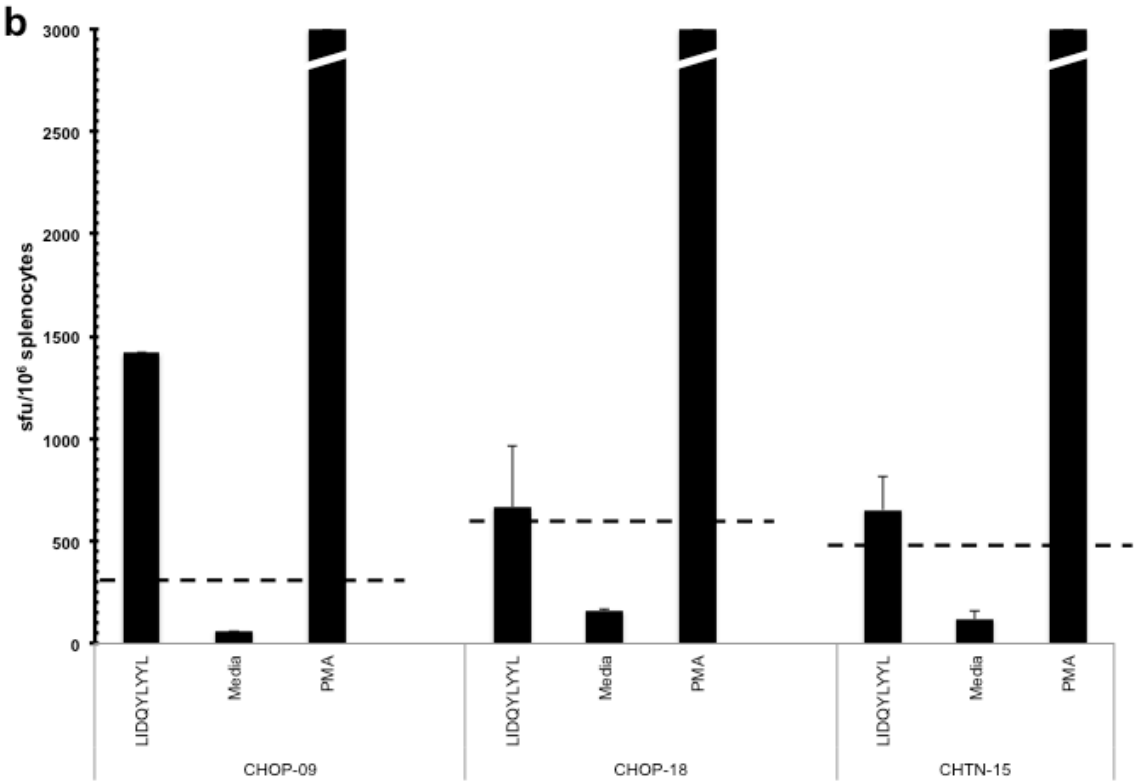


Supplementary Figure 2. Validation of identified AAV epitopes.

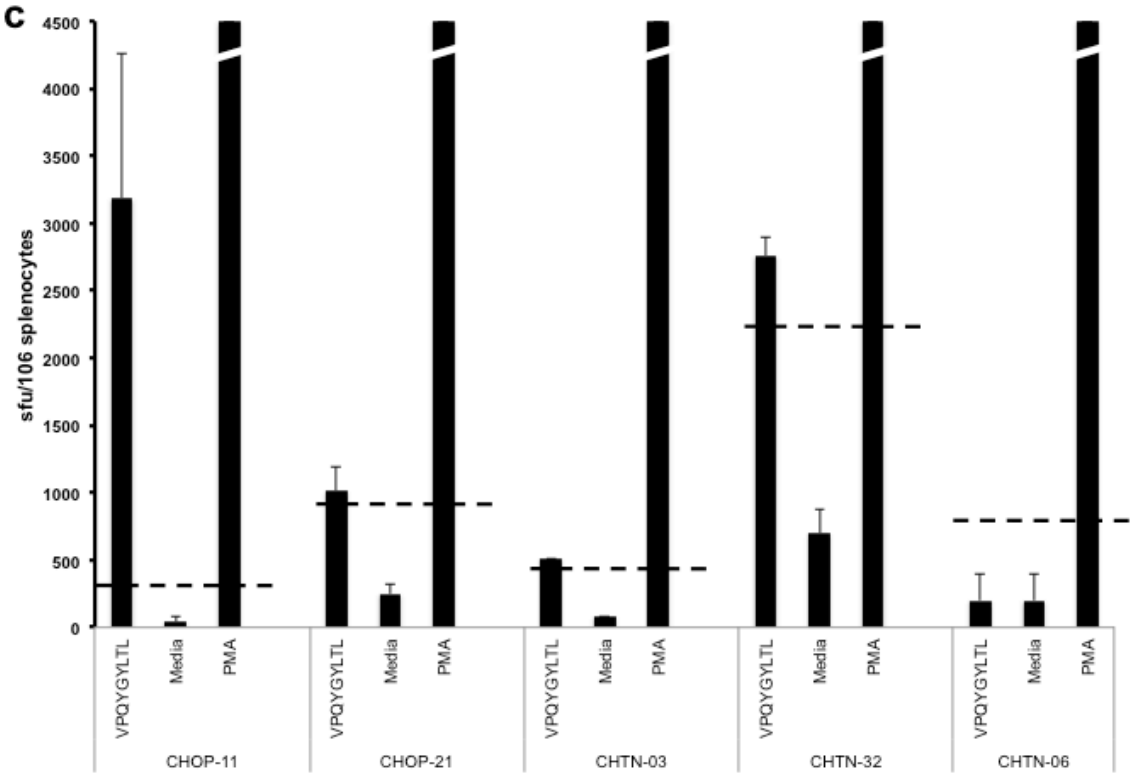
HLA restriction of 9-mer epitope sequence of peptides identified with the high-throughput screening of the AAV-2 peptide library was determined. MHC class I binder 9-mers identified were then synthesized and used in an IFN-γ ELISpot screening. (**a**) HLA-A*0101 subjects tested against the SADNNNSEY epitope; (**b**) HLA-A*0201/0202 subjects tested against the LIDQYLYYL epitope; (**c**) HLA-B*0702 subjects tested against the VPQYGYLTL epitope; (**d**) HLA-B*0801 subjects tested against the TTSTRTWAL epitope; (**e**) HLA-B*1501 subjects tested against the YHLNGRSSF epitope; (**f**) HLA-B*44 subjects tested against the SQAVGRSSF epitope; (**g**) HLA-B*51 and HLA-B*53 subjects tested against the VPANPSTTF, FPQSGVLIF, and QPAKKRLNF epitopes. Sfu, spot forming units; Medium, negative control; PMA, phorbol 12-myristate 13-acetate (positive control). Dashed lines indicate threshold for positivity; both positive and negative results are displayed.


a
3000
2500
2000
1500
1000
500
0
sfu/10^6 splenocytes
SADNNNSEY
Media
PMA
CHOP-18
CHTN-14
CHTN-15
CHTN-19
CHTN-28
CHOP-05
CHTN-27

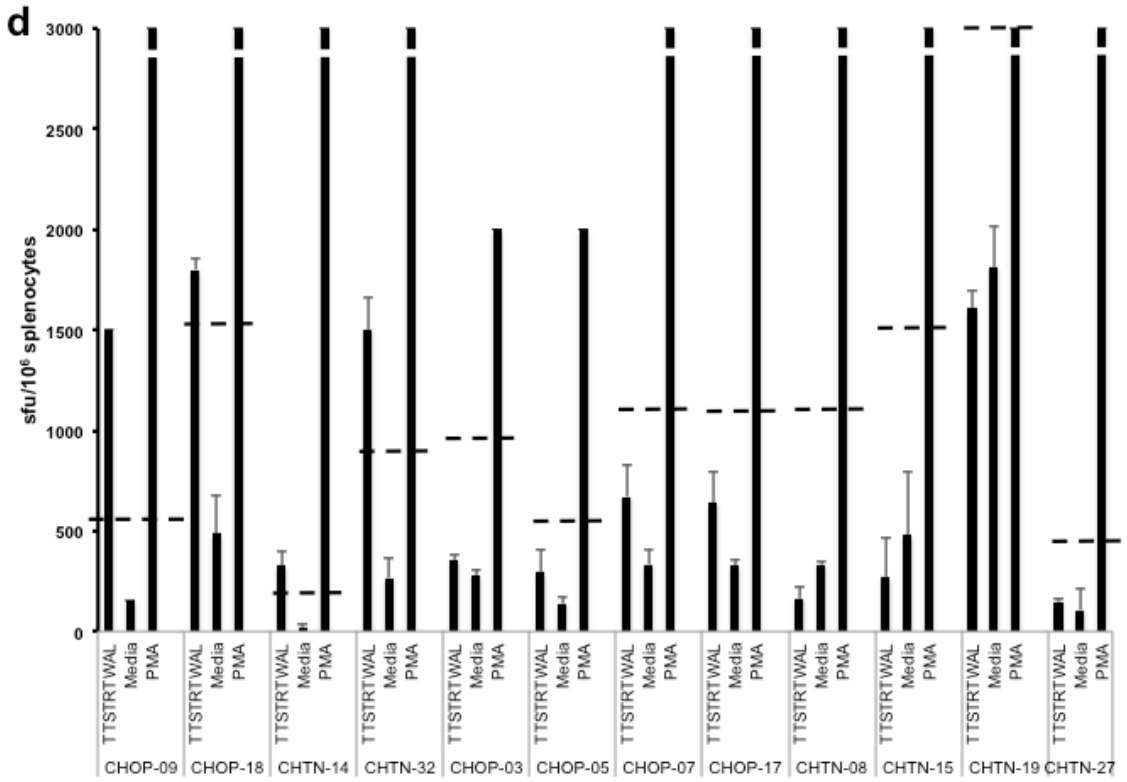
Supplementary Figure 2



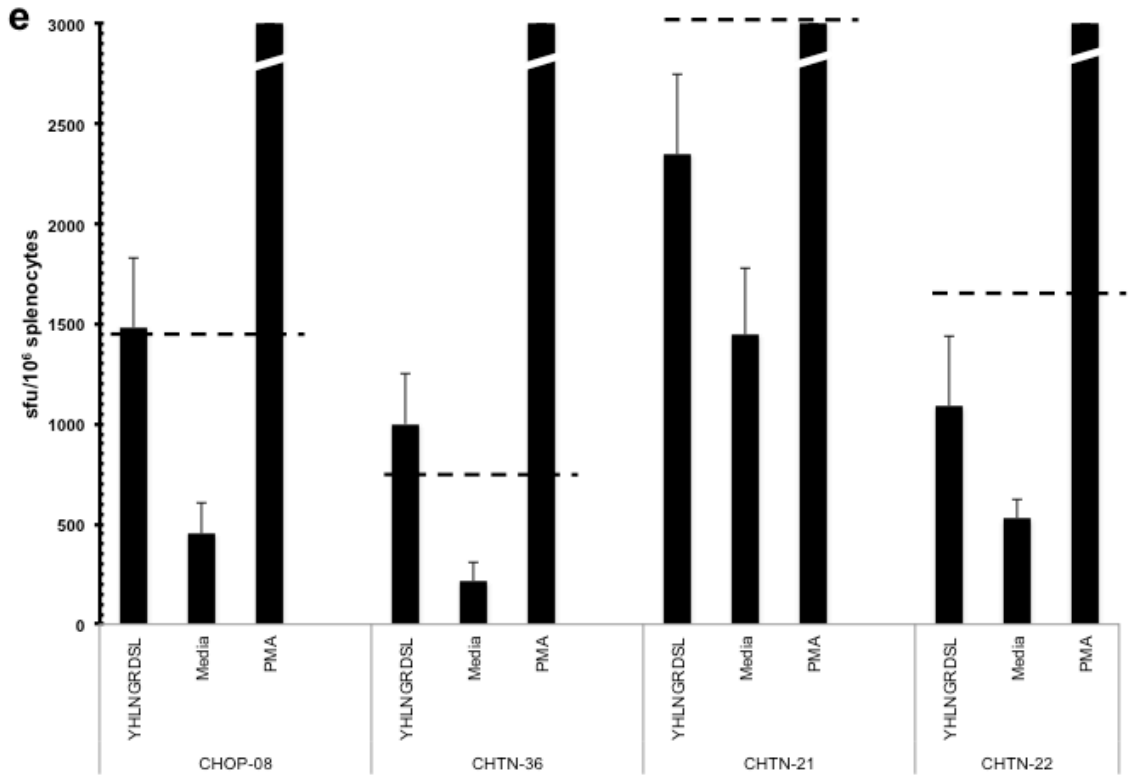
Supplementary Figure 2



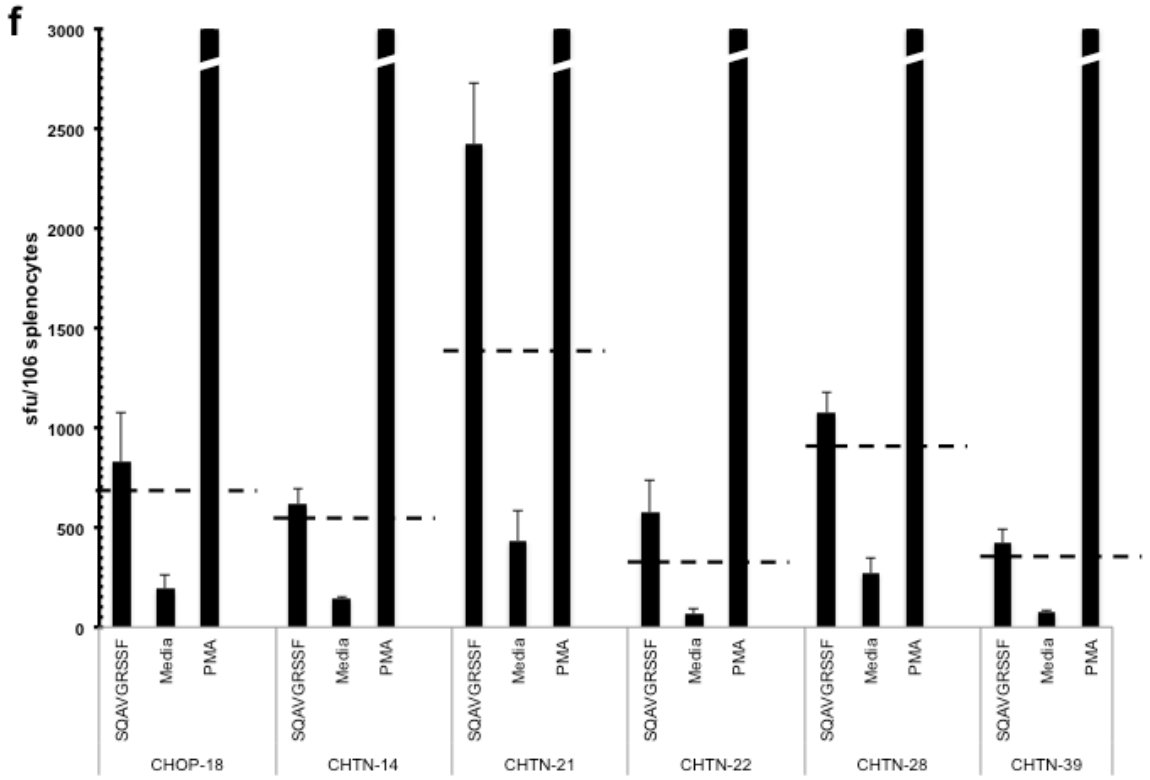
Supplementary Figure 2



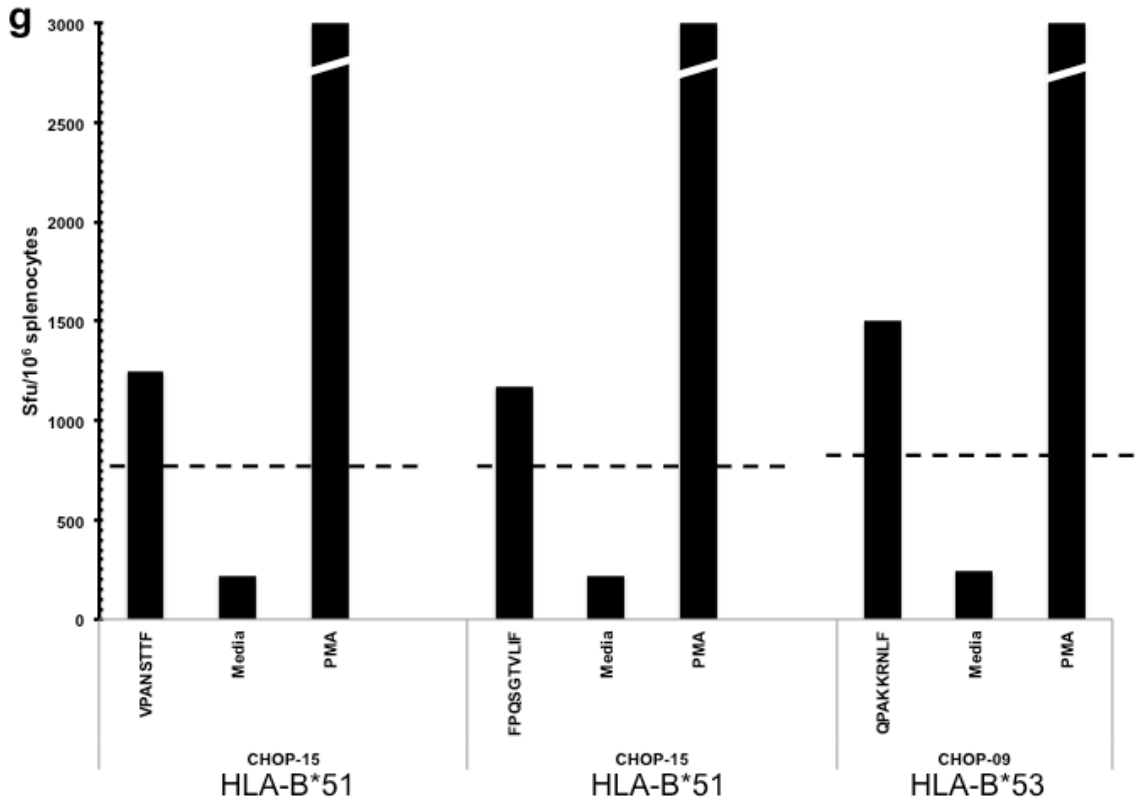
Supplementary Figure 2



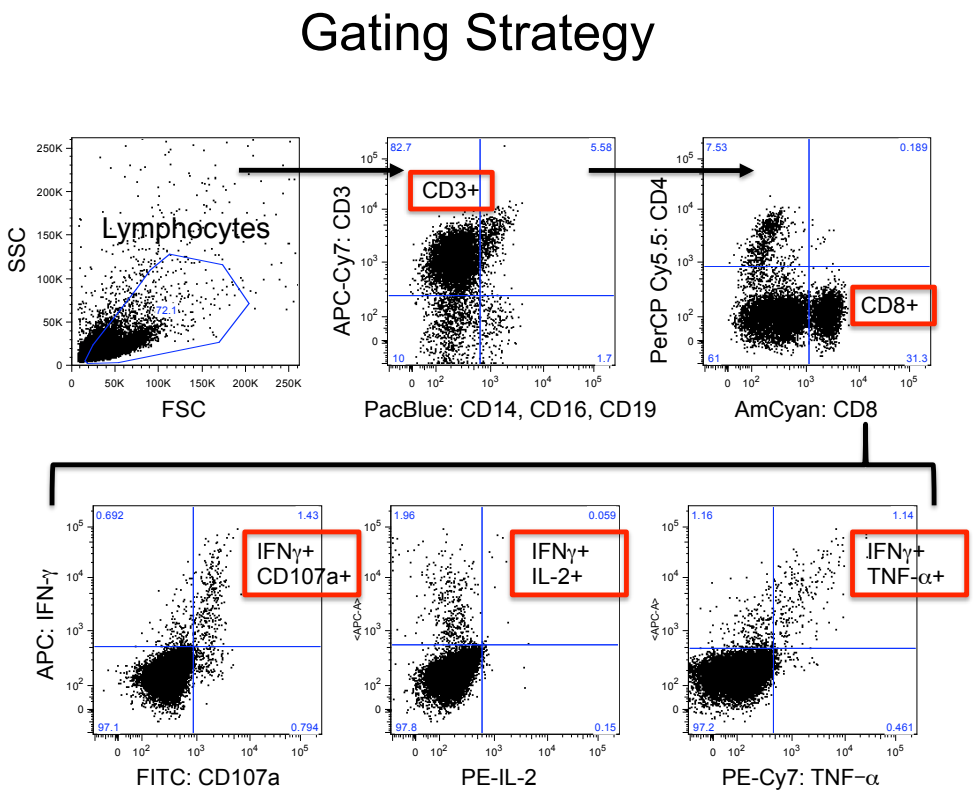
Supplementary Figure 2



Supplementary Figure 2



Supplementary Figure 3. Gating strategy for polyfunctional analysis of capsid-specific CD8$^+$ T cells expanded from splenocytes. Lymphocyte fraction of PBMC was first gated on CD3$^+$ T cells that were CD14$^-$CD16$^-$CD19$^-$ (dump channel), then on CD4$^-$ CD8$^+$ T cells. CD8$^+$ T cells gated with this strategy were analyzed for double expression of IFN-γ with CD107a, IL-2 and TNF-α.



Supplementary Table 1. Age and HLA haplotype of spleen donors.

Age range	Sample ID	Age	HLA-A	HLA-B
<5	CHOP-05	0 yrs 9 mo	0101, 6801	0801, 4402
	CHOP-04	2 yrs	0205, 2301	1516, 4201
	CHOP-06	2 yrs 8 mo	03xx, 23xx	60xx, 65xx
	CHOP-10	2 yrs 8 mo	0301, 2301	45xx
	CHOP-17	2 yrs 11 mo	68xx, 74xx	0801, 53xx
	CHOP-12	3 yrs 7 mo	03xx, 31xx	57xx, 64xx
	CHOP-03	4 yrs	3002, 6802	0801, 5801
≥5	CHOP-01	5 yrs	23xx, 34xx	53xx, 72xx
	CHOP-09	5 yrs	0101, 0202	0801, 53xx
	CHOP-14	5 yrs	03xx, 26xx	27xx, 49xx
	CHOP-15	5 yrs	0201, 0202	51xx
	CHOP-16	8 yrs	25xx, 29xx	55xx, 65xx
	CHOP-07	12 yrs	0201, 24xx	0801, 18xx
	CHOP-18	12 yrs	0101, 30xx	0801, 44xx
	CHOP-08	13 yrs	0201	1501, 44xx
	CHOP-11	13 yrs	0201, 30xx	0702, 53xx
	CHOP-20	13 yrs	0201, 23xx	41xx, 44xx
	CHOP-21	16 yrs	03xx, 23xx	0702
	CHOP-02	17 yrs	Unknown	Unknown
	CHOP-13	18 yrs	30xx, 68xx	71xx, 72xx
	CHOP-19	20 yrs	0201, 26xx	38xx, 44xx
	CHTN-11	26 yrs	0201	51xx, 57xx
	CHTN-15	27 yrs	0101, 0201	0801, 35xx
	CHTN-36	29 yrs	0201, 03xx	1501, 44xx
	CHTN-06	37 yrs	0201, 11xx	0702, 1503
	CHTN-16	40 yrs	33xx, 68xx	1503, 53xx
	CHTN-27	41 yrs	0101, 24xx	0801, 55xx
	CHTN-05	54 yrs	0201, 33xx	44xx, 57xx
	CHTN-04	55 yrs	0201, 30xx	18xx, 52xx
	CHTN-07	55 yrs	0201, 03xx	0702, 14xx
	CHTN-14	58 yrs	0101, 68xx	0801, 44xx
	CHTN-03	59 yrs	03xx	0702, 35xx
	CHTN-02	63 yrs	32xx, 66xx	44xx, 56xx
	CHTN-28	65 yrs	0101, 03xx	18xx, 44xx
	CHTN-32	67 yrs	0201, 03xx	0702, 0801
	CHTN-19	69 yrs	0101, 03xx	0801, 14xx
	CHTN-10	73 yrs	0201, 03xx	0702, 40xx
	CHTN-20	77 yrs	24xx, 25xx	1501, 39xx
	CHTN-21	79 yrs	0201, 0301	1501, 44xx
	CHTN-39	81 yrs	0201, 24xx	44xx
Unknown	CHTN-01	Unknown	0201, 03xx	47xx, 64xx
	CHTN-08	Unknown	03xx, 29xx	0801, 44xx
	CHTN-09	Unknown	03xx, 24xx	0702, 39xx

Supplementary Table 2. Homology between identified AAV capsid MHC class I epitopes.

HLA Allele		A*0101	A*0201/0202	B*0702	B*0801	B*1501	B*44xx	B*51xx
AAV Serotype	AAV-1	KTDNNNSNF	LIDQYLYYL	IPQYGYLTL	TTSTRTWAL	YNLNGRESI	SQA---VGRSSF	FPMSGVMIF
	AAV-2	SADNNNSEY	LIDQYLYYL	VPQYGYLTL	TTSTRTWAL	YHLNGRDSL	SQA---VGRSSF	FPQSGVLIF
	AAV-3a	ANDNNNSNF	LIDQYLYYL	VPQYGYLTL	TTSTRTWAL	YHLNGRDSL	SQA---VGRSSF	FPMHGNLIF
	AAV-3b	ANDNNNSNF	LIDQYLYYL	VPQYGYLTL	TTSTRTWAL	YHLNGRDSL	SQA---VGRSSF	FPMHGNLIF
	AAV-4	IPATGSDSL	LIDQYLYYL	VPQYGYCGL	TTSTRTWVL	STLDGRWSA	NTSQQQTDRNAF	S-NSQLIFA
	AAV-5	SGVNRASVS	LVDQYLYRF	LPQYGYATL	TKSTRTWVL	MELEGASYQ	ENP---TERSSF	YALENTMIF
	AAV-6	KTDNNNSNF	LIDQYLYYL	IPQYGYLTL	TTSTRTWAL	YNLNGRESI	SQA---VGRSSF	FPMSGVMIF
	AAV-7	LDQNNNSNF	LIDQYLYYL	IPQYGYLTL	TTSTRTWAL	YHLNGRNSL	SQS---VGRSSF	FPSSGVLIF
	AAV-8	TGQNNNSNF	LIDQYLYYL	IPQYGYLTL	TTSTRTWAL	YHLNGRNSL	SQA---VGRSSF	FPSNGILIF

HLA Allele		B*51xx	B*53xx	unknown	unknown	unknown	unknown	unknown
AAV Serotype	AAV-1	VPANPPAEF	QPAKKRLNF	VGNASGNWHCDSTWL	YFDFNRFHCHFSPRD	LFSRGSPAGMSVQPK	SPAGMSVQPKNWLPG	SSTDPATGDVHAMGA
	AAV-2	VPANPSTTF	QPARKRLNF	VGNSSGNWHCDSTWM	YFDFNRFHCHFSPRD	QFSQAGASDIRDQSR	GASDIRDQSRNWLPG	GNRQAATADVNTQGV
	AAV-3a	VPANPPTTF	QPARKRLNF	VGNSSGNWHCDSQWL	YFDFNRFHCHFSPRD	LFSQAGPQSMSVLQAR	GPQSMSVLQARNWLPG	SNTAPTTGTVNHQGA
	AAV-3b	VPANPPTTF	QPARKRLNF	VGNSSGNWHCDSQWL	YFDFNRFHCHFSPRD	LFSQAGPQSMSVLQAR	GPQSMSVLQARNWLPG	SNTAPTTRTVNDQGA
	AAV-4	VPANPATTF	QPAKKKLVF	VGNASGDWHCDSTWS	YFDFNRFHCHFSPRD	NFTKLRPTNFSNFKK	RPTNFSNFKKNWLPG	NSNLPTVDRLTALGA
	AAV-5	VPGNI-TSF	KPST-----	VGNASGDWHCDSTWM	YFDFNRFHSHWSPRD	QFNKNLAGRYANTYK	LAGRYANTYKNWFPG	STTAPATGTYNLQEI
	AAV-6	VPANPPAEF	QPAKKRLNF	VGNASGNWHCDSTWL	YFDFNRFHCHFSPRD	LFSRGSPAGMSVQPK	SPAGMSVQPKNWLPG	SSTDPATGDVHVMGA
	AAV-7	VPANPPEVF	QPARKRLNF	VGNASGNWHCDSTWL	YFDFNRFHCHFSPRD	QFYQGGPSTMAEQAK	GPSTMAEQAKNWLPG	ANTAAQTQVVNNQGA
	AAV-8	VPADPPTTF	QPARKRLNF	VGSSSGNWHCDSTWL	YFDFNRFHCHFSPRD	GFSQGGPNTMANQAK	GPNTMANQAKNWLPG	QNTAPQIGTVNSQGA

Supplementary Table 3. Expanded capsid-specific PBMCs are cytotoxic in an *in vitro* CTL assay.

Donor ID	Donor #	Age	Gender	HLA-A	HLA-B	Epitope	CTL Killing
20060419M	16	43	Male	A0201, A31xx	B0702, 57xx	VPQYGYLTL	yes
20060518M	none	52	Male	A0201, A11xx	B0702, B35xx	VPQYGYLTL	yes
20060817	none	52	Male	A0201, A03xx	B0702, B0801	VPQYGYLTL	yes
20060629P	26	52	Male	A0201, A0301	B0702, B0801	VPQYGYLTL	yes
20060810	33	27	Male	A0201, A0301	B0702, B2705	VPQYGYLTL	yes
20061130	36	45	Male	A0201, A6801	B0702, B4402	VPQYGYLTL	yes
20080213	44	57	Male	A0101, A3301	B0801, B1402	SADNNNSEY	yes
20060524J	22	39	Male	A0101, A0201	B0801, B61*4002	SADNNNSEY	yes
20060503G	20	49	Male	A0101, A6801	B5701, B5703	SADNNNSEY	no
20060601F	24	46	Female	A0101, A0201	B1402, B4102	SADNNNSEY	no
							8/10 (80%)

Supplementary Table 4. Binding affinity of identified AAV capsid epitopes is comparable to other viral epitopes

After we confirmed the natural processing and presentation of some of the peptide epitopes that we identified, we went on to determine the binding affinity of these by a fluorescent MHC I binding affinity assay.

We determined the apparent Kd values for some peptides for a number of HLA types (**Table 4a**) and the HIV A0201 epitope SLYNTVATL from HIV-1 GAG and compared them to the published values for the HIV epitope (**Table 4b**). Apparent Kd values for AAV peptides ranged between 8.2 µM and 1.29 µM and 2.64µM for a reference HIV peptide (SLYNTVATL), which was right in the range between reported values for this peptide (6.06 µM and 1.50 µM respectively)[5,8].

a

HLA Haplotype	AAV epitope:	AAV serotype:	Kd (apparent):
A*0101	KTDNNNSNF	AAV-1	8.20 µM
	SADNNNSEY	AAV-2	6.75 µM
	TGQNNNSNF	AAV-8	3.59 µM
A*0201	LIDQYLYYL	AAV-1,2,8	4.54 µM
B*0702	IPQYGYLTL	AAV-1,8	1.29 µM
	VPQYGYLTL	AAV-2	1.32 µM
B*0801	TTSTRTWAL	AAV-1,2,8	7.45 µM
B*1501	YNLNGRESI	AAV-1	3.41 µM
	YHLNGRDSL	AAV-2	2.70 µM
	YHLNGRNSL	AAV-8	3.03 µM

	EPRPIGTRY	AAV-1,2,8	3.69 μM
B*51	FPMSGVMIF	AAV-1	4.61 μM
	FPQSGVLIF	AAV-2	3.21 μM
	FPSNGILIF	AAV-8	3.93 μM
	VPANPPAEF	AAV-1	4.59 μM
	VPANPSTTF	AAV-2	3.06 μM
	VPADPPTTF	AAV-8	2.92 μM

b

HLA Haplotype	**Reference Peptide:**	**Peptide Source:**	**Kd (apparent):**
A*0201	**SLYNTVATL**	**HIV-1 GAG**	**2.64 μM**
A*0201	**SLYNTVATL**	**HIV-1 GAG**	**6.09 μM[1]**
A*0201	**SLYNTVATL**	**HIV-1 GAG**	**1.50 μM[2]**

Supplementary Materials and Methods.

Spleen as a source of lymphocytes

Research on splenic samples was conducted in accordance with procedures approved by The Children's Hospital of Philadelphia Institutional Review Board. Spleens were processed fresh within 12 hours after surgery as previously described[1]; specimens derived from subjects affected by malignant or immunological disorders were not collected for this study. No matched PBMC were available from any of the subjects included in this study. All specimens were de-identified with only the age of the donor and the diagnosis recorded. Donors' ages ranged from 9 months to 81 years, with 7 subjects less than 5 years old and 37 subjects greater than or equal to 5 years of age. HLA haplotype was obtained for all samples at the University of Pennsylvania Medical Center Histocompatibility Laboratory. For each sample $>10^8$ cells were isolated and cryopreserved until assayed.

MHC-binding Affinity Measurements:

Binding affinity was measured using a modified antibody capture assay against MHC Class I molecules as previously described [4]. Briefly, 250 nM purified MHC Class I molecules (provided by the NIH Tetramer Core Facility, Emory U. and Immudex LLC, Copenhagen, Denmark) were captured overnight on a 96-well plate (Corning, Corning, NY) coated with an anti-human HLA-ABC MHC Class I antibody W6/32 (Ebioscience, San Diego, CA). MHC Class I molecules were activated for 15 minutes at 56°C[5]. Varying concentrations (0-5mM) of 6-FAM fluorescently-labeled peptide (Genemed Synthesis, San Antonio, TX) were incubated in the presence of 2μM beta-2-

microglobulin (Lee Biosolutions, St. Louis, MO) and protease inhibitor cocktail (Sigma, St. Louis, MO) for 2 days at room temperature. Following incubation, the plate was washed and bound peptide measured using a Spectramax M2 (Molecular Devices, Sunnyvale, CA) at 495 nM Abs, 520 nM emission. Binding affinity was calculated using referenced equations by nonlinear least squares regression analysis using the Marquadt algorithm. The qualities of the fits were assessed by the criteria described [6]. Dissociation constants ($Kd_{apparent}$) for the interaction between peptide and membrane bound MHC molecule were obtained from the dependence of the fluorescence intensity on the concentration of cofactor [7].

Supplemental references

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