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ARTICLE

AAV capsid CD8+ T-cell epitopes are highly conserved across AAV serotypes

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Adeno-associated virus (AAV) has become one of the most promising vectors in gene transfer in the last 10 years with successful translation to clinical trials in humans and even market approval for a first gene therapy product in Europe. Administration to humans, however, revealed that adaptive immune responses against the vector capsid can present an obstacle to sustained transgene expression due to the activation and expansion of capsid-specific T cells. The limited number of peripheral blood mononuclear cells (PBMCs) obtained from samples within clinical trials allows for little more than monitoring of T-cell responses. We were able to identify immunodominant major histocompatibility complex (MHC) class I epitopes for common human leukocyte antigen (HLA) types by using spleens isolated from subjects undergoing splenectomy for non-malignant indications as a source of large numbers of lymphocytes and restimulating them with single AAV capsid peptides *in vitro*. Further experiments confirmed that these epitopes are naturally processed and functionally relevant. The design of more effective and less immunogenic AAV vectors, and precise immune monitoring of vector-infused subjects, are facilitated by these findings.

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INTRODUCTION

Recombinant adeno-associated viral (AAV) vectors are among the most promising gene transfer vectors due to their ability to transduce a wide range of dividing and non-dividing cells,[1] their lack of pathogenicity, and their relatively benign immunogenicity profile.[2–5] A wealth of preclinical data shows sustained expression of a transgene at therapeutic levels after a single administration of an AAV vector.[6] More recently, results have been successfully translated to the clinical setting, and have resulted in the approval of the first gene therapy drug in Europe.[7] However, concerns over the immunogenicity of the AAV capsid in humans were raised in the first clinical trial for hepatic delivery of an AAV serotype 2 vector encoding the coagulation factor IX transgene (AAV2-F.IX) in severe hemophilia B subjects.[8] In this study, therapeutic levels of transgene expression were reached, but expression was unexpectedly short-lived, with F.IX levels remaining therapeutic for ~4 weeks, then gradually decreasing to baseline over the ensuing 8 weeks, concomitant with a transient, self-limited elevation of liver enzymes.[8] In this study, a population of AAV capsid-specific $CD8^+$ T cells was identified, which expanded upon AAV2-F.IX vector delivery and then contracted with kinetics similar to that of the rise and fall in serum transaminases.[9] Following this initial observation, we and other groups documented the activation and expansion of capsid-specific T cells following the administration of AAV vectors in humans.[10–14] In a more recent study with systemic delivery of a self-complementary AAV serotype 8 vector encoding the coagulation factor IX transgene (AAV8-F.IX) in severe hemophilia B subjects, the previous observations were confirmed albeit with slightly different kinetics.[15] In this trial the T cell response could be managed with a short course of steroids, which preserved long-term transgene expression, but at the same time confirmed that expression levels are very sensitive to the cellular immune response.

In previous reports we showed that initial AAV exposure can lead to the development of humoral and cellular immunity against AAV capsid.[8] We also showed that AAV transduced cells present capsid antigen via major histocompatibility complex (MHC) class I molecules on the surface of transduced cells.[16,17]

While several reports in the literature describe the prevalence of antibodies to AAV in the human population,[18,19] and the absence of a correlation between antibody titers and ELISpot responses to AAV1,[20] T-cell responses to the AAV capsid in humans are somewhat less studied.[21]

The current work consists of two major parts: (i) the identification and characterization of CD8+T-cell epitopes on the AAV capsid, including AAV1, 2, and 8 and (ii) the demonstration that these epitopes are processed, presented, and trigger cytotoxic T-cell responses when presented. To carry out the first part, we used 44 spleens isolated from pediatric and adult human subjects undergoing splenectomy for nonmalignant indications.[9] Because of the large number of recovered cells, high-throughput mapping of AAV capsid

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MHC class I epitopes binding to several common human leukocyte antigen (HLA) alleles was possible. Epitope mapping was performed with a library of 15-mers spanning the entire amino acid sequence of the AAV capsid protein VP1. Capsid T-cell responses were initially detected by IFN-γ ELISpot assay, evaluated, and further characterized by polyfunctional analysis and phenotyping of capsid-reactive $CD8^+$ T cells including functional cytotoxic T lymphocyte assays. A ProPresent assay, commercially available from Proimmune[22] and nucleofection experiments confirmed that the capsid protein is indeed processed to yield the detected peptide epitopes and further characterized the epitope binding affinity.

These results are of interest for future AAV gene transfer studies in humans as the identification of AAV MHC class I epitopes for several common HLA alleles will facilitate development of AAV-specific reagents for efficient immunomonitoring of capsid T-cell responses. Ultimately, this information may lead to the development of less immunogenic vectors by epitope reduction in the capsid.

RESULTS

Identification of AAV capsid T-cell epitopes in human splenocytes

In the current study, we used human splenocytes from 44 donors to develop a comprehensive characterization of human immune responses to AAV. First, we measured T-cell responses directed to the AAV-2 or AAV-1 capsid using an IFN-γ ELISpot assay on either unexpanded cells or on splenocytes restimulated *in vitro* with a peptide library spanning the VP1 protein sequence of the AAV capsid.[8] A low frequency of IFN-γ secretion was observed in unexpanded splenocytes using the ELISpot assay, with only 2 out of 44 subjects showing a positive T-cell response to the AAV capsid (Table 1), consistent with a previous result published by our group.[9]

After expansion, 20 out of 32 tested spleen samples scored positive in the IFN-γ assay; a statistically significant difference in frequency of T-cell responses to the AAV capsid was measured for the young age group (<5 years old, 16.67% positive response) compared to the ≥5-year-old group (73.08%); only one positive sample came from a donor aged <5 years (Supplementary Data 1; Table 1).

The large number of cells isolated and stored from each spleen, in combination with bioinformatics algorithms, allowed us to specify and confirm single 9-mer peptides as capsid T-cell epitopes experimentally (Figure 1; Supplementary Figure S1) and to define their predicted binding affinity to the cognate MHC class I molecules (Table 2). We also gained insight into the frequency of subjects responding to a specific epitope (Table 2; Supplementary Figure S2). All epitopes identified with the ELISpot assay exhibited a binding affinity to MHC class I of at least 50% of the maximum binding affinity of the consensus sequence defined by the position-specific scoring matrix (PSSM) for the cognate HLA allele.[23] Most, but not all epitopes, could be confirmed, and there was no obvious relationship between the binding affinity score for a specific HLA allele and the frequency of subjects responding to the specific epitope (Table 2). Not all epitopes could be confirmed because bioinformatics analysis was restricted to HLA alleles present in the databases, and thus, further analysis and specification of some reactive peptides was not possible based on the rare haplotype (Table 2, footnote d). In several instances, subjects sharing the same HLA alleles showed reactivity to the same AAV-derived epitopes. In one instance, two different epitopes binding to the same HLA (HLA-B*51, Table 2) were identified.

Table 1 Capsid-specific T-cell responses in splenocytes before and after *in vitro* restimulation

Age	*N*	*Reactivity unstimulated*	*Reactivity after* in vitro *restimulation*
<5	7	0/7 (0.00%)	1/6 (16.67%)
≥5	37	2/37 (5.41%)	19/26 (73.08%)
Total	44	2/44 (4.55%)	20/32 (62.5%)

Two of the epitopes identified in spleens (SADNNNSEY binding to the HLA-A*0101 and VPQYGYLTL binding to the HLA-B*0702) were previously identified in peripheral blood mononuclear cells (PBMCs) isolated from hemophilia B subjects undergoing liver-directed AAV-2 gene transfer.[9] Comparison of identified epitopes across alternate AAV serotypes showed a high degree of conservation for several of the identified peptides (Supplementary Table S2).

Capsid-specific $CD8^+$ T cells derived from spleen are functional and recognize conserved epitopes within alternate AAV capsid serotypes

MHC class I pentamer staining was performed in those samples for which these reagents were available to confirm the expansion of capsid-specific $CD8^+$ T cells (Figure 2a). Polyfunctional analysis of markers of T-cell activation and function was performed after *in vitro* restimulation with AAV capsid; intracellular levels of IL-2, IFN-γ, TNF-α, and the degranulation marker CD107a in response to the AAV capsid antigen were normalized to background levels (medium only control). All positive samples presented a population of capsid-reactive $CD8^+$ T cells secreting high levels of IL-2, IFN-γ, and TNF-α and displaying the degranulation marker CD107a (Figure 2b; Supplementary Figure S3).

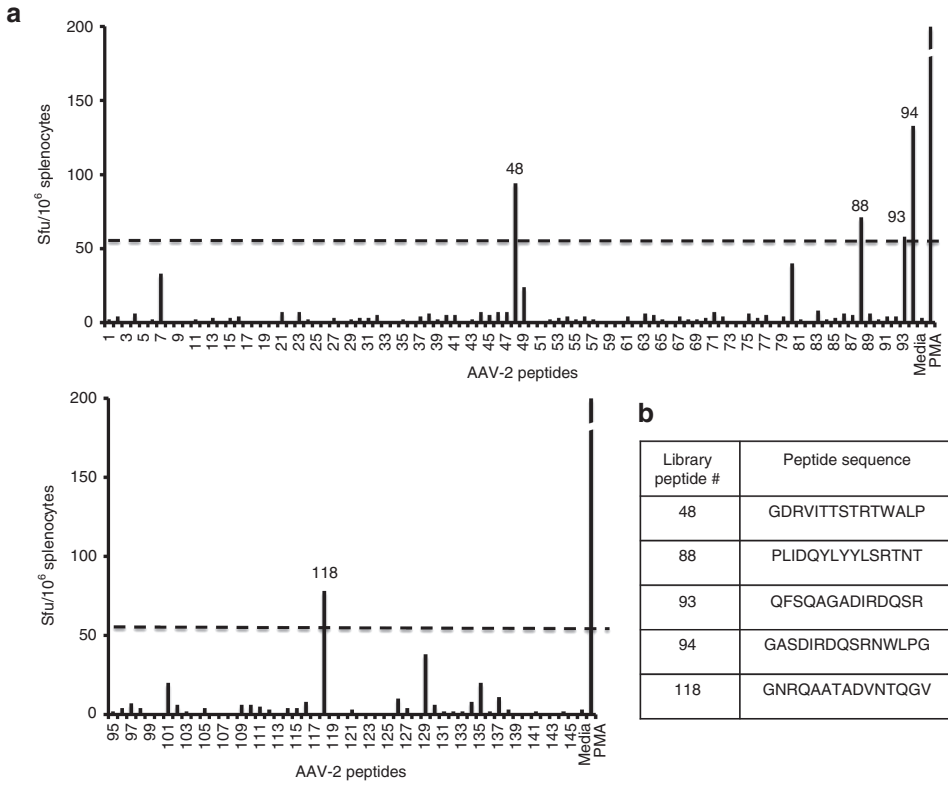
This polyfunctional T-cell analysis suggested that capsid-specific $CD8^+$ T cells expanded from splenocytes were functional. An *in vitro* cytotoxic T lymphocyte (CTL) assay with PBMC-derived effector cells was used to confirm the ability of capsid-specific effector T cells to kill AAV transduced target cells; efficient target cell killing was measured in 8 out of 10 samples tested (Supplementary Table S3). The same CTL assay with splenocyte-derived effector cells was used to test cytotoxicity as well as cross-reactivity of capsid $CD8^+$ T cells. Splenocyte samples from two donors bearing the HLA-A*0101 or HLA-B*0702 alleles were used; cells were restimulated *in vitro* with either the AAV-2 peptide SADNNNSEY (binding to HLA-A*0101) or the AAV-2 peptide VPQYGYLTL (binding to HLA-B*0702) and then used in CTL assays against target cells transduced with either AAV-2 or AAV-6 vectors, or targets loaded with either the AAV-2 or AAV-6 peptide epitopes.

Efficient CTL killing was observed with AAV-2 transduced or AAV-2 peptide-loaded target cells (Figure 3a–d). CTL killing was also observed with AAV-6 capsid or peptide when effectors from the HLA-B*0702 donor were used (Figure 3a,c), reflecting the high degree of conservation of the epitope VPQYGYLTL across alternate serotypes (Figure 3e). In contrast, no killing was measured with AAV-6–transduced or AAV-6 peptide–loaded targets with effectors expanded against the nonconserved SADNNNSEY epitope from AAV-2 (Figure 3b,d).

Cytotoxic capsid-specific $CD8^+$ T-cell responses are derived from memory T cells

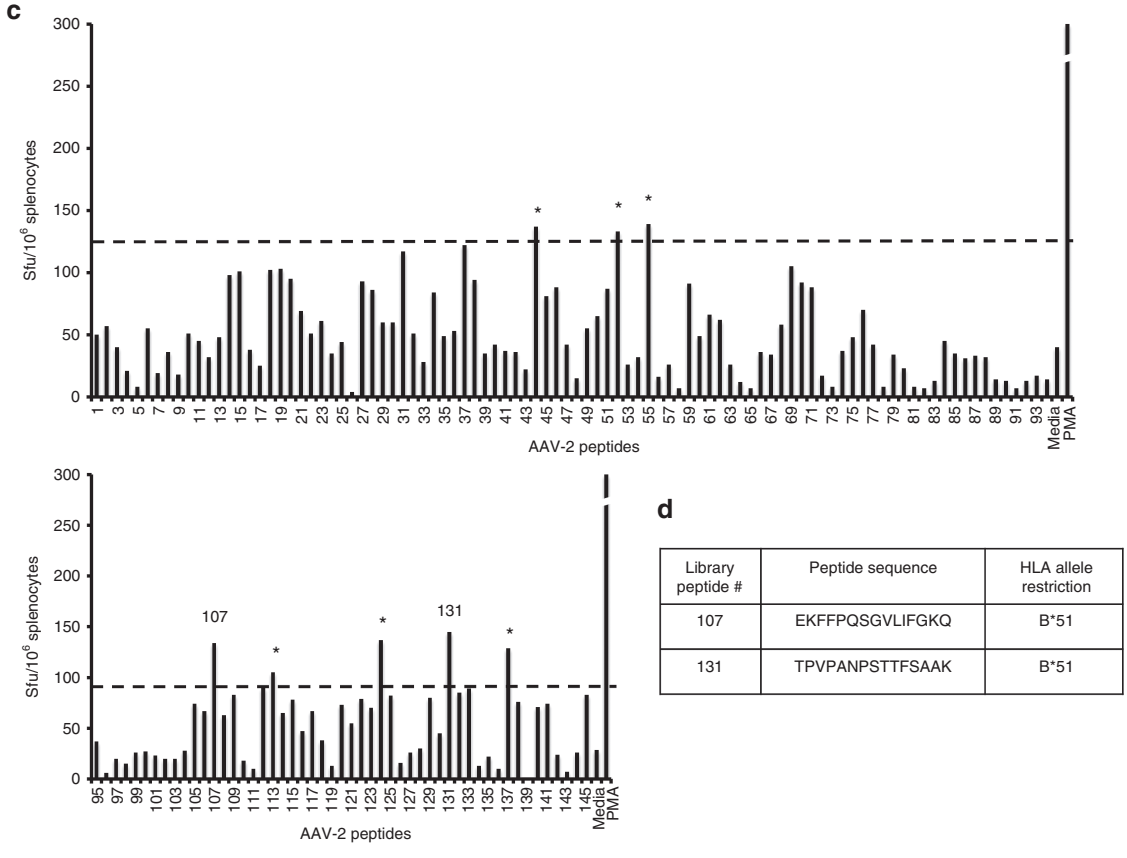
The fact that T-cell responses are more frequent in the >5-years-old age cohort (presumably after initial infection) suggests that IFN-γ–producing cells might be derived from capsid-specific memory

T cells (Table 1). To test this hypothesis, total splenocytes from an HLA-B*0702 reactive donor were sorted into two different fractions, $CD4^+$-$CD8^+$-$CD45RO^+$ and $CD4^+$-$CD8^+$-$CD45RO^-$. Cells were then restimulated *in vitro* with the HLA-B*0702–restricted epitope VPQYGYLTL and assayed in an IFN-γ ELISpot assay. The sorting resulted in the detection of a robust response to the peptide epitope and to the whole AAV capsid antigen only in total splenocytes and in the $CD45RO^+$ fraction of splenocytes (Figure 4). Furthermore, CTL-mediated killing of AAV-transduced target cells was observed only when $CD45RO^+$ cells of a reactive donor were used as effectors.



b

Library peptide #	Peptide sequence	HLA allele restriction
48	GDRVITTSTRTWALP	B*0801
88	PLIDQYLYYLSRTNT	A*0201/0202
93	QFSQAGADIRDQSR	N/D
94	GASDIRDQSRNWLPG	N/D
118	GNRQAATADVNTQGV	N/D



d

Library peptide #	Peptide sequence	HLA allele restriction
107	EKFFPQSGVLIFGKQ	B*51
131	TPVPANPSTTFSAAK	B*51

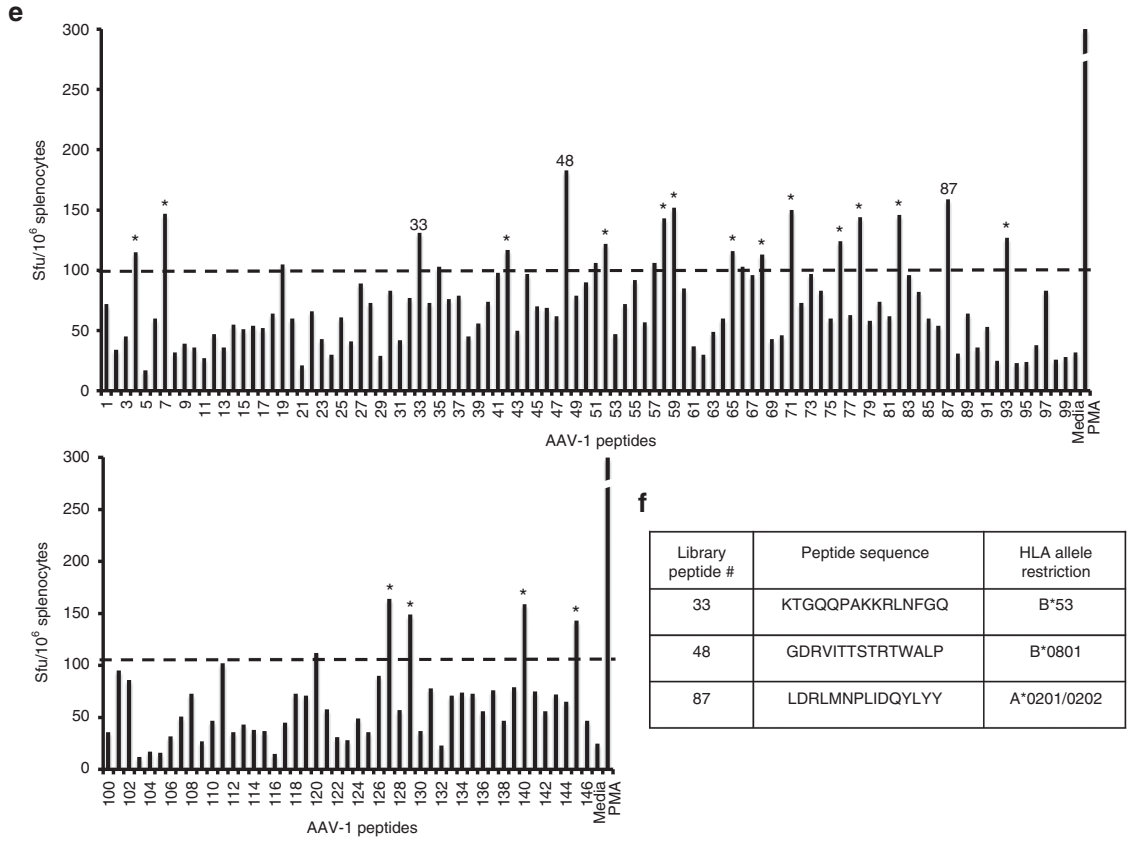
Identified epitopes are naturally processed and presented

With the combined approach of bioinformatics, IFN-γ ELISpot, functional analysis via flow cytometry and CTL assay, we identified and confirmed several AAV epitopes. Next we wanted to demonstrate that these epitopes were indeed naturally processed and presented.

As classic MHC class I presentation occurs with peptides synthesized by the presenting cell, first we addressed the question by nucleofection experiments followed by the previously described CTL assay as a read-out. Nucleofection with capsid encoding plasmids was used instead of infection with the different AAV serotypes in order to avoid influences of variability of infectivity and intracellular processing on the results. Splenocytes were depleted of $CD8^+$ T cells (human $CD8^+$ T-cell depletion kit by Miltenyi, San Diego, CA). The remaining cells were nucleofected (Amaxa Nucleofection kit; Lonza, Basel, Switzerland) with plasmids encoding AAV1, AAV2, or AAV8 capsid as well as a control GFP plasmid. Forty-eight hours after nucleofection, the cells were used as antigen-presenting cells (APCs) to generate effector T cells in *in vitro* expansions (see Materials and Methods). After two rounds of stimulation, the generated effector T cells were used in CTL assays. Target cells were hepatocyte cell lines loaded with the relevant peptide epitopes or an irrelevant control peptide. The experiments were conducted with HLA matched as well as mismatched (control) target cells. The results are depicted in a heat map, which shows induction of cytotoxicity over background. Significant cytotoxicity could only be detected when using HLA-matched target cells (Figure 5). The nucleofected APCs led to the expansion of functional effector cells that were able to recognize and kill target cells loaded with the identified peptides but not an irrelevant peptide. Consistent with the findings from the cytotoxicity assays, a striking amount of cross-reactivity between AAV serotypes could be detected. Expansion with the control protein GFP-nucleofected splenocytes did not create functional effector cells.

One argument against the approach with nucleofection is that there might be a difference in presented peptides between the classical pathway of *de novo* synthesis by the cell (nucleofection with plasmid) compared to the presentation after uptake and cleavage of capsid proteins (cross-presentation) after transduction with AAV. Another argument is that unphysiological high levels of peptide presentation are achieved this way.

More recently, the availability of a commercial assay (ProPresent) allows the identification of peptides which are presented in the context of MHC class I. Briefly, this assay employs the use of a pan-MHC antibody to specifically pull down MHC class I molecules in order to recover and identify bound peptides via high-resolution mass spectrometry (ProPresent; Proimmune, Oxford, UK). HHL5-B7 hepatocytes were transduced with an AAV-2 vector encoding human F.IX or an AAV-2 vector encoding the entire AAV-2 capsid sequence. The search against a



Library peptide #	Peptide sequence	HLA allele restriction
33	KTGQQPAKKRLNFGQ	B*53
48	GDRVITTSTRTWALP	B*0801
87	LDRLMNPLIDQYLYY	A*0201/0202

Figure 1 High-throughput mapping of AAV capsid T cell epitopes. (**a, c, e**) Single-peptide IFN-γ ELISpot on *in vitro* expanded splenocytes. Splenocytes from normal donors were restimulated *in vitro* with 15-mers derived from the AAV-2 (**a**, **c**) or AAV-1 (**e**) capsid VP-1 amino acid sequence. Each of the 145 peptides from the AAV libraries was used individually in the restimulation. After restimulation, cells were rechallenged with the same peptides in an ELISpot assay. Positive peptides were selected for further validation. Dashed line, threshold for positivity; numbers 1 through 145, 15-mers from the AAV peptide library; PMA, phorbol 12-myristate 13-acetate (positive control); Sfu, spot-forming units. *Peptides positive but not further confirmed. (**b, d, f**) Peptides identified with relative HLA restriction. N/D, not determined.

Table 2 Identified AAV capsid MHC class I epitopes

	HLA allele	*Epitope*	*Serotype*	*Rankpep score*[a]	*Number of subjects reactive to capsid*[c]
A locus	0101	SADNNNSEY	AAV-2	63.03%	4/7
	0201/0202	LIDQYLYYL	AAV-2	52.46%	3/3
		LDRLMNPLI	AAV-1	40.14%	1/1
B locus	0702	VPQYGYLTL	AAV-2	78.92%	5/7
	0801	TTSTRTWAL	AAV-2	55.94%	4/12
		TTSTRTWAL	AAV-1	55.94%	1/1
	1501	YHLNGRDSL	AAV-2	N/D[b]	2/4
	44	SQAVGRSSF	AAV-2	60.24%	6/6
	51	VPANPSTTF	AAV-2	70.75%	1/1
		FPQSGVLIF	AAV-2	67.35%	1/1
	53	QPAKKRLNF	AAV-1	58.02%	1/1
N/D[d]	N/D[d]	YFDFNRFHCHFSPRD	AAV-2	N/D	2
		VGNSSGNWHCDSTWM	AAV-2	N/D	1
		QFSQAGASDIRDQSR	AAV-2	N/D	1
		GASDIRQSRNWLP	AAV-2	N/D	1
		GNRQAATADVNTQGV	AAV-2	N/D	1

N/D, not determined.
[a]Percent of maximum binding affinity to the HLA allele of a consensus sequence. [b]HLA restriction determined via alternate bioinformatics algorithm (SYFPEITHI). [c]Ratio of number of subjects with positive T-cell responses to the AAV capsid over the total number of subjects tested sharing the same HLA allele. [d]No match via bioinformatics.

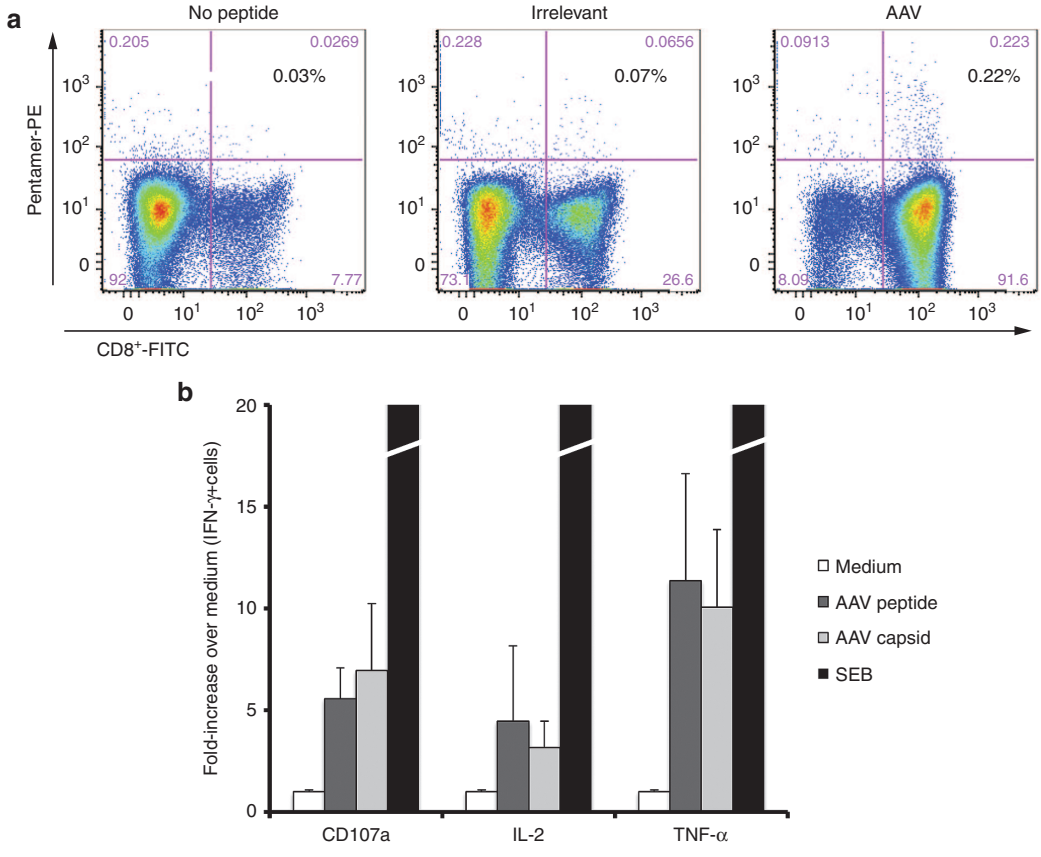
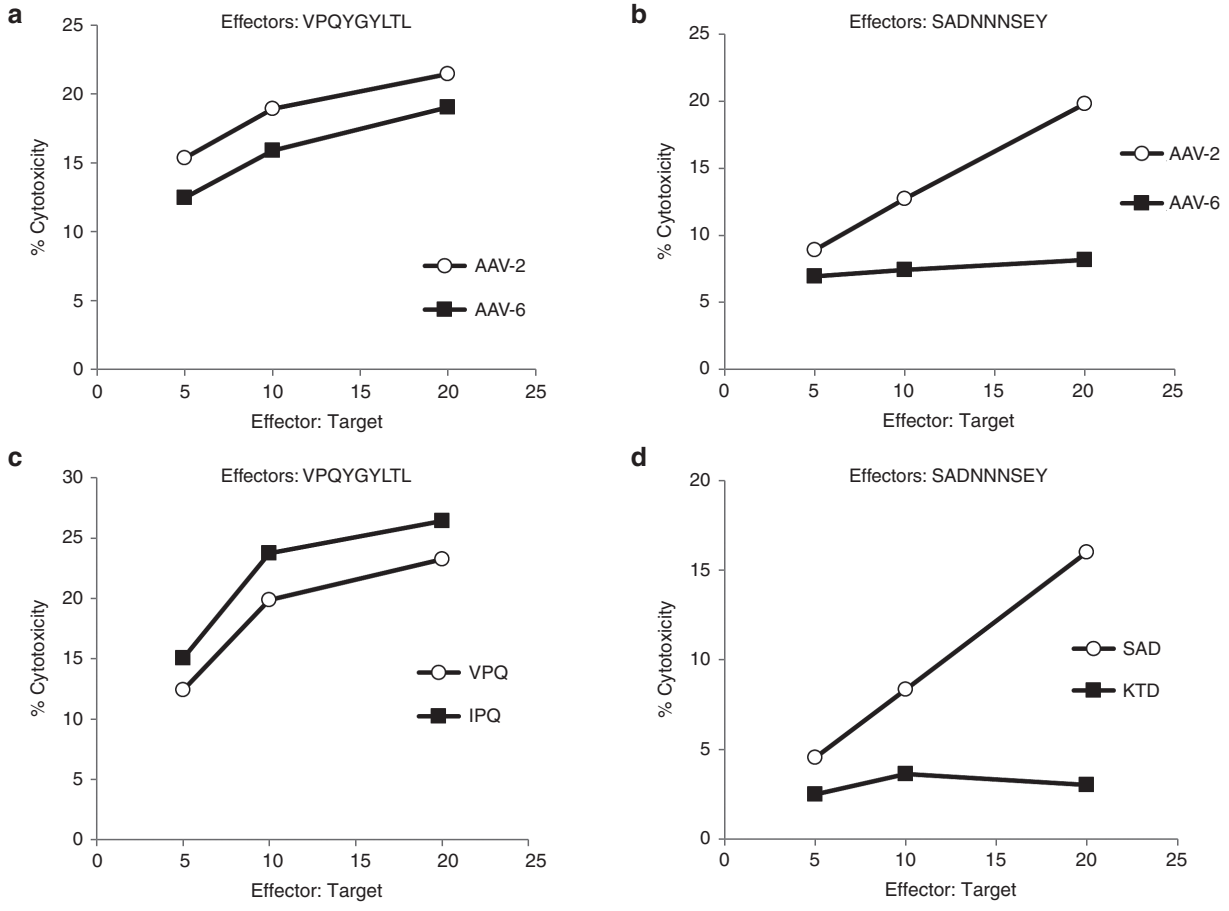


Figure 2 MHC class I pentamer staining and polyfunctional analysis of capsid-specific CD8$^+$ T cells expanded from splenocytes. (**a**) Capsid-specific MHC class I pentamer staining of HLA-A*0101 cells expanded without peptide, an irrelevant peptide (HIV-1 env gp120 848–856), or AAV capsid. Cells were gated on lymphocytes and CD14$^+$, CD16$^+$ and CD19$^+$ cells were gated out. (**b**) Polyfunctional analysis of T-cell activation. Histograms show the fold increase in the percent of double-positive CD8$^+$ cells (CD8$^+$IFN-γ$^+$CD107a$^+$, CD8$^+$IFN-γ$^+$IL-2$^+$, or CD8$^+$IFN-γ$^+$TNF-α$^+$) over medium only control. SEB, *Staphylococcal enterotoxin B* (positive control). Cells were gated on lymphocytes and CD8$^+$ T cells.



e

Serotype	HLA-B*0702 epitope	HLA-A*0101 epitope
AAV-2	VPQYGYLTL	SADNNNSEY
AAV-6	**I**PQYGYLTL	**KT**DNNNS**NF**

Figure 3 Cross-reactivity of capsid-specific T-cell–mediated killing. (**a, c**) HLA-B*0702 splenocytes expanded against the conserved AAV-2 epitope VPQYGYLTL and tested against (**a**) AAV-2 or AAV-6 transducted target cells, or tested against (**c**) VPQYGYLTL (AAV-2) or IPQYGYLTL (AAV-6) peptide-loaded targets (10 µg/ml). (**b, d**) HLA-A*0101 splenocytes expanded against the nonconserved AAV-2 epitope SADNNNSEY and tested against (**b**) AAV-2 or AAV-6–transduced target cells, or tested against (**d**) SADNNNSEY (AAV-2) or KTDNNNSNF (AAV-6) peptide-loaded targets (10 µ g/ml). Results are shown as percent cytotoxicity after background exclusion. (**e**) Amino acid sequence conservation of the epitopes tested across AAV serotype 2 and 6.

database containing the complete human proteome and protein sequences 1–4 (from the capsid, from the Factor IX sequence, or from alternate open reading frames, *vide supra*) did not identify a significant MHC-associated peptide. However, a restricted database search of peptide sequences that excluded the human proteome (to exclude endogenous peptides) and included only the AAV-2 capsid and control proteins, returned some corresponding peptides. One particular peptide did achieve a significantly high score (Table 3). This peptide, SLDRLMNPL, was identified as a high binder in our previous bioinformatics analysis and has been subsequently confirmed via ELISpot assay using an AAV-1 capsid peptide library (Table 2).

Finally, we conducted studies using a fluorescent MHC class I binding affinity assay to determine apparent Kd values for identified AAV capsid epitopes. Apparent Kd values for AAV peptides ranged between 8.2 and 1.29 µM and 2.64 µM for a reference HIV peptide (SLYNTVATL), which was right in the range between reported values for this peptide (6.06 and 1.50 µM, respectively; Supplementary Table S4).[24,25]

DISCUSSION

In the first gene transfer study with an AAV vector infused into liver in men with severe hemophilia, a cellular immune response toward the capsid eventually led to the loss of transgene expression when left untreated[8] while prompt treatment of a cellular response with steroids rescued the expression in a more recent trial.[15] With the treatment of more subjects in current AAV trials, the cellular immune response has been identified as pivotal for success or failure of long-term gene transfer with AAV, particularly in the setting of liver-directed trials.

Lack of a reliable animal model of human T-cell responses against the AAV capsid has been and remains an obstacle to the study of capsid immune responses,[4,26,27] underscoring the importance of immunomonitoring of T-cell responses against the capsid in healthy humans and in AAV-injected subjects.

Lymphocytes continuously recirculate through peripheral lymphoid organs, and thus, monitoring of an ongoing immune response can be readily performed by assaying PBMC for reactivity to a specific antigen, e.g., using an ELISpot assay.[28] While PBMCs

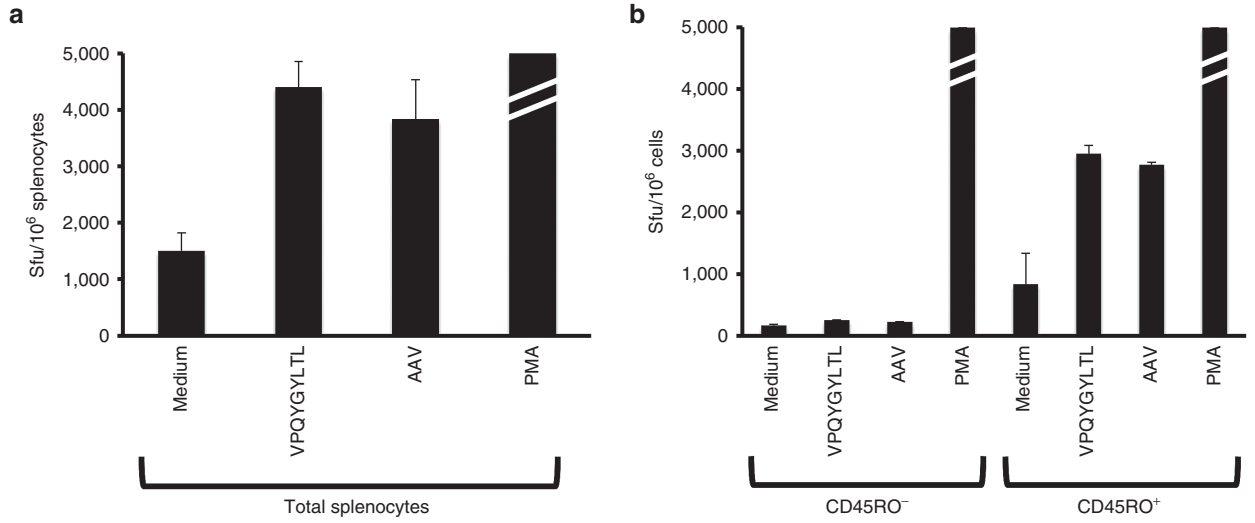


Figure 4 AAV capsid reactivity of memory (CD45RO$^+$) T cells. (**a**) Total splenocytes, (**b**) CD45RO$^-$, or CD45RO$^+$ T cells from an HLA-B*0702 donor were restimulated *in vitro* against the corresponding HLA-restricted AAV epitope (VPQYGYLTL) and tested on IFN-γ ELISpot assay. AAV, AAV whole capsids; Medium, negative control; PMA, phorbol 12-myristate 13-acetate (positive control); Sfu, spot-forming units.

are routinely used for immunomonitoring purposes, this approach may fail to detect reactive T cells that circulate at very low frequencies and that may be found in higher numbers in primary lymphoid organs. Furthermore, the large numbers of lymphocytes that can be isolated from spleens allows experimental approaches such as fine mapping of MHC class I epitopes that would be difficult or impossible with peripheral blood samples.

The aim of this study was to perform an *ex vivo* characterization and validation of HLA-restricted AAV capsid epitopes. This knowledge can be used for improved immunomonitoring through the design of pentamers for specific HLA alleles, as well as for future bioengineering efforts to alter the vector to reduce immunogenicity.

For this study, we used splenocytes from a cohort of healthy pediatric and adult subjects undergoing splenectomy for nonmalignant indications.

Using this approach, we delineated several novel aspects of the immune response to AAV. First, the current study establishes that similar to AAV capsid-reactive T cells in the periphery, unexpanded splenocytes fail to manifest a significant response to AAV capsid in an ELISpot assay. However, after *in vitro* expansion with capsid peptides, we did see a significant age-dependent increase in T-cell reactivity to the AAV capsid. This is in agreement with seroprevalence data of AAV, showing a steep increase in anti-AAV antibodies from 1 to 10 years of age.[18] Our study supports the hypothesis that the capsid-reactive T-cell response is mainly a memory T-cell response, as confirmed by our finding that removal of the CD45RO$^+$ population (memory T cells) led to the elimination of effector cells in a cytotoxic response assay (Figure 5).

With a single-peptide restimulation approach, we confirmed capsid MHC class I epitopes and showed multifunctionality by flow cytometry in response to activation, and established the functional activity of the cells with CTL assays.

A review of the data demonstrates that a strong binding affinity in some epitopes did not necessarily correlate with the strongest, most frequent cellular response (Table 2). This might be due to the limited number of available spleens with the same haplotype but importantly may also be due to the fact that the binding affinity of an epitope to its cognate MHC class I molecule is not the only parameter for its dominance. Several other parameters, such as the intracellular processing of AAV, the composition of the peptides, as well as yet unidentified parameters may be important as well.[29]

We addressed this by examining another aspect of antigen processing and presentation, the natural presentation of epitopes in the context of MHC class I. We confirmed the natural presentation of our epitopes in nucleofection experiments in APCs as well as in a commercially available ProPresent assay in hepatocytes. Different AAV capsids bind to different cellular receptors and thus show differences in infecting different cell types.[30] In order to minimize influences by this on validating the identified epitopes, we used nucleofection. As stated in Results, this approach represents an artificial system as peptide processing and presentation follow the classical pathway of MHC class I presentation, and we cannot exclude that they differ from peptides derived from cross-presentation. Nucleofection most likely also yielded peptide presentation levels that are higher than that achieved by infection with AAV. This was useful as we used the transfected cells as APCs to expand antigen-specific T cells. By nucleofection, the innate immune system might be triggered via the TLR9 pathway and lead to unspecific activation of the APCs. Relevant triggering of the TLR9 pathway has been reported for AAV gene transfer in mice even at considerably lower DNA doses.[31] We addressed and excluded the effect of possible induction of unspecific killing due to very high DNA doses in our assay by including a GFP control group.

The nucleofection assay results confirmed the epitopes in traditional APCs and the MHC class I presenting pathway. To further confirm cross-presentation in hepatocytes, a ProPresent assay was performed on an AAV-infected human hepatocyte cell line[17] and confirmed one peptide in particular. Both assays have the caveat that only a limited number of HLA types could be tested. Finally, apart from the assays presented in this publication, some epitopes have been confirmed during immune monitoring in gene transfer trials.[9,15] Positive cellular responses were observed in trials after stimulation with peptide pools that contained the epitopes we have identified for the matching haplotypes of the subjects.[15]

In summary, the data presented here suggest that subjects truly naive to wild-type AAV, such as young children, may be more likely to achieve long-term transgene expression upon AAV-mediated gene transfer, since there would be no activation/expansion of memory-derived capsid-specific CD8$^+$ T-cell populations.

At present immune monitoring of capsid T-cell responses of PBMC from subjects enrolled in AAV gene transfer studies is

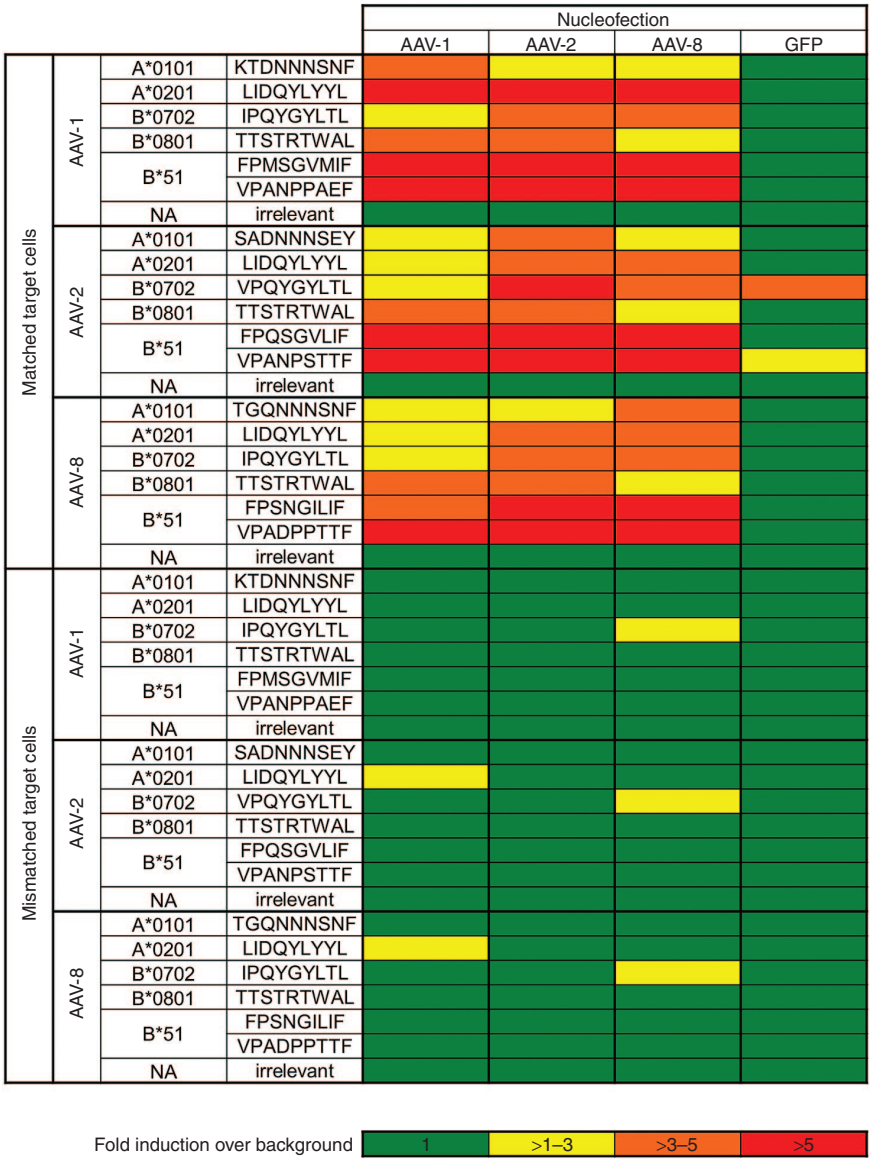


Figure 5 AAV capsid transfected APCs induce the proliferation of effector cells that recognize and kill peptide-loaded target cells. HLA-matched and mismatched target cells were loaded with the peptides we determined for the corresponding AAV capsids (left four columns). Effector cells were generated *in vitro* as described, and cytotoxicity was measured as described. The right four columns show the fold-induction of cytotoxicity in the described CTL assay over background. CTL, cytotoxic T lymphocyte.

typically performed by ELISpot and other functional assays. The ELISpot assay has proven to be most robust and sensitive for monitoring and is employed by most groups, but also requires most cells. Because of lack of more specific knowledge, currently, cells are stimulated with peptide pools spanning the entire capsid and thus require drawing a relatively large volume of blood that limits the frequency of monitored time points. It would be a big advantage to at least reduce the number of peptides that need to be tested. Furthermore, the identification of reactive epitopes for a given antigen allows for more sophisticated methods, for example, the design of HLA-specific and antigen-specific reagents, like MHC class I multimers,[32] which requires comparatively few cells and would allow for closer monitoring where applicable. Even monitoring of clinical trials to the liver with alternate AAV serotypes, for example, AAV3 or AAV9 will profit from these findings as some epitopes are conserved between serotypes and the same reagents could be used (Supplementary Table S2).

More recently, progress in AAV vector capsid engineering[33] positis this approach as a means of increasing transduction efficiency. Ultimately, the data presented here might be used for the design of less immunogenic AAV capsids, in a manner similar to that currently employed with MHC class II epitopes of several therapeutic proteins[34,35] and has been shown to successfully reduce AAV immunogenicity in a murine model.[36] The results from recent liver-directed gene transfer trials strongly support the hypothesis that cellular immune responses occur only after a certain threshold dose is passed. Whether the elimination of a dominant epitope also renders other epitopes in a protein less immunogenic[35] or leads to immunogenicity of subdominant epitopes[37] is still controversial.

At present, various AAV serotypes and engineered capsid mutants are being tested for their capacity as vectors for multiple indications. We believe that for successful gene transfer, not only their transduction efficacy but also their capacity to provoke a cellular immune response must be understood. An optimization

Table 3 Capsid peptide sequences identified from MHC molecules using a database containing sequences for proteins 1–4

Donor	*Sequence*	*Protein*	*Amino acid start/end*	*Expect value*
AAV-2 CAP	SLDRLMNPL	AAV2 capsid	429–437	0.03
	VLPGYKYLG	AAV2 capsid	46–54	0.29
AAV-2 FIX	SLDRLMNPL	AAV2 capsid	429–437	0.0068
	LMNPLIDQ	AAV2 capsid	433–440	0.19
	LNNGSQAVGR	AAV2 capsid	380–389	0.23
	NNHLYKQIS	AAV2 capsid	253–261	0.31
	GTTTIANNL	AAV2 capsid	327–335	0.33

AAV, adeno-associated virus.

of both parameters will enable therapeutic, safe, and sustained gene transfer.

MATERIALS AND METHODS

Splenocytes and cell lines

Research on splenic samples was conducted in accordance with procedures approved by The Children's Hospital of Philadelphia Institutional Review Board (Supplementary Materials and Methods and Table S1). The human hepatocyte HHL5 cell line expressing the HLA-B*0702 allele used in the CTL assay was previously described.[17] Positivity for the MHC class I molecule was confirmed by staining with PE-conjugated anti-HLA-B7 antibody (EMD Millipore, Billerica, MA) followed by flow cytometry.

AAV vectors and peptides

AAV vectors were produced in HEK-293 cells using a triple transfection method as previously described[38] and purified with cesium chloride gradient centrifugation methods.[39] For AAV epitope discovery studies, libraries of peptides consisting of 15-mers overlapping by 10 amino acids spanning the entire sequence of different AAV capsids' VP1 proteins were synthesized (Mimotopes, Clayton, Australia); 9-mers used to validate identified epitopes were obtained from Genemed Synthesis[8] (San Antonio, TX). Peptides were arranged into a matrix format of 24 pools containing 12–13 peptides each, such that each peptide was present in two orthogonal pools.

In vitro restimulation of T cells

Human splenocytes as well as all PBMCs (Cellular Technologies, Shaker Heights, OH) were thawed, washed, counted, and resuspended at a concentration of 2×10^6 cells/ml in AIM-V lymphocyte media (Gibco, Life Technologies, Grand Island, NY) containing 3% human serum (Bioreclamation, Baltimore, MD), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). Cells were cultured with AAV empty capsid particles, single peptides, or peptide pools at a final concentration of 10 µg/ml as previously described[9] for up to three rounds of restimulation. Expanded $CD8^+$ T cells were characterized after each round of stimulation by IFN-γ ELISpot, MHC class I pentamer staining, or intracellular cytokine staining.

Human IFN-γ ELISpot

Human IFN-γ secretion in response to a specific antigen was tested using an ELISpot assay (Mabtech, Cincinnati, OH) as previously described.[8,13] Splenocytes were thawed, washed, and counted. Media alone and phorbol 12-myristate 13-acetate (Sigma, St Louis, MO)/ionomycin (Sigma) were used as negative and positive controls, respectively. Controls and antigens were added in triplicate to the plates, antigens at a final concentration of 5–10 µg/ml. The number of spot forming units (SFU) per well was determined with an Immunospot plate reader (Cellular Technologies); results were expressed in SFU/10^6 cells and were considered positive when the SFU/10^6 cell count was ≥3-fold the negative (media) control count and at least 50 SFU/10^6 cells.

Flow cytometry, intracellular cytokine staining, and polyfunctional analysis of T-cell activation markers

Surface staining of splenocytes and restimulated T cells with MHC class I pentamers (Proimmune, Oxford, United Kingdom) was performed as previously described.[9] Polyfunctional analysis of T-cell activation markers was performed as previously described.[40] Briefly, cells were stimulated for 6 hours with peptides and controls in the presence of transport inhibitors. Afterwards, cells were washed and stained for the surface markers CD3, CD4, CD8, CD14, CD16, CD19 and CD107a (BD Biosciences, San Jose, CA or Caltag, Life Technologies, Buckingham, UK), fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) followed by intracellular staining for cytokines using antibodies against IFN-γ, TNF-α, and IL-2 (all BD Biosciences). Acquisition was on a FACS Canto II flow cytometer, and analysis was performed using the FACSDiva (BD Biosciences) and the Flowjo (Treestar, Ashland, OR) software.

CTL assay

CTL assay was performed as previously described.[17] Briefly, lactate dehydrogenase release following CTL-mediated target cell lysis was measured with the CytoTox 96 Non Radioactive Cytotoxicity Assay (Promega, Madison, WI). HHL5 target cells were plated and transduced at an MOI of three hundred thousand with an AAV vector, or loaded with 10 µg/ml of SADNNNSEY or VPQYGYLTL peptide epitopes and incubated for 18 hours at 37 °C in 5% CO_2. After the incubation, plates were washed, and effector cells were added at a range of effector:target ratios, cell lysis was measured after 4 hours of incubation.

Sorting of memory T cells

Cells were stained with fluorescein isothiocyanate (FITC)-conjugated antihuman CD4 and antihuman CD8 antibodies and APC-conjugated antihuman CD45RO (BD Biosciences) for 20 minutes at 4 °C. Cells were then washed twice and sorted using a BD FACSAria cell sorter by gating on $CD4^+CD8^+$ T cells and sorting $CD45RO^+$ and $CD45RO^-$ cells. Resulting cell fractions were washed twice and resuspended at a concentration of 2×10^6 cells/ml in AIM-V medium (Invitrogen Gibco) supplemented with 3% human serum (Bioreclamation). Cell restimulation and ELISpot testing were performed as described above.

Bioinformatics and statistical analysis

HLA-typing information was used in conjunction with bioinformatics prediction algorithms (SYFPEITHI: http:www.syfpeithi.de/[41] and RANKPEP: http:www.mif.dfci.harvard.edu/Tools/rankpep.html[23]) for the analysis of the reactive 15-mers identified with the IFN-γ ELISpot assay to obtain the 9-mer subsequence predicted to bind to a given HLA allele. The analysis was restricted to common HLA types provided by the algorithms, not allowing all experimentally positive peptides to be matched with a 9-mer in the algorithm. Bioinformatics results were confirmed by synthesizing the predicted 9-mers and testing their reactivity in splenocytes from several donors with the HLA of interest. Statistical analysis was performed using GraphPad (GraphPad Software); P values <0.05 were considered significant.

Expansion of human T cells against naturally presented AAV capsid epitopes via nucleofection

APCs were generated from human splenocytes. Splenocytes were depleted of $CD8^+$ cells using magnetic beads (DYNAL, Invitrogen) before resuspending at a concentration of 5×10^6 cells/ml in PBS/0.5% BSA. Cells were then transfected by following the B-cell nucleofection kit protocol using an Amaxa Nucleofector II (Lonza, Basel, Switzerland) with 5 µg of plasmids encoding AAV1, AAV2, AAV8 capsids, or GFP as a control. Successful transfection was determined by flow cytometry analysis of the cells treated with the control GFP plasmid. Following transfection, APCs were resuspended in AIM-V/3% human serum, added to freshly thawed splenocytes, and expansion was carried out in the same manner as described above. After two subsequent restimulations, cells were harvested for use in CTL assays.

ProPresent assay performed by ProImmune

5×10^7 HHL5-B7 hepatocytes were transduced with AAV2 at an MOI of 100k for 48 hours at 37 °C in 5% CO_2 in serum-free DMEM (Sigma). Afterwards, cells were washed twice with DPBS (Sigma) and harvested with a cell scraper.

In an initial experiment, cell transduction levels after 48 hours were confirmed using AAV2-GFP by flow cytometry. In addition, capsid peptide presentation was confirmed using a soluble T-cell receptor[17]which detects VPQYGYLTL from the AAV2 capsid in the context of a B0702 MHC class I molecule.

We provided protein sequences 1–4 to Proimmune for database testing. Protein 1 was the AAV2 capsid sequence, protein 2 the FIX sequence, and proteins 3 and 4 highly synthetic peptide sequences, created based on two possible alternate open-reading frame sequences of the FIX transgene (Supplementary Data 2).

A peptide expect value score of 0.05 is the default significance threshold. The lower the expect value, the more significant the score.

CONFLICT OF INTEREST

F.M. and K.A.H. consulted for companies that are developing AAV-based therapeutics. K.A.H. is Co-Founder, President, Chief Scientific Officer, and holds equity in a company, Spark Therapeutics, that is developing AAV-based therapeutics. D.J.H. is an employee of Spark Therapeutics. D.J.H., F.M., K.A.H., and G.C.P. hold patents related to AAV gene therapy. All other authors declare no competing financial interest.

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D.J.H. and E.B.-T. contributed to design and execution of the experiments. G.M.P., G.C.P., L.I., R.M.C., and F.M. contributed to design and execution of experiments. S.C.E. contributed to execution of experiments. H.E. contributed to experimental design. K.A.H. and E.B.-T. directed experimental design, conducted data analysis and interpretation, and drafted the manuscript.

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