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Et al.

Class I-restricted T-cell responses to a polymorphic peptide in a gene therapy clinical trial for α-1-antitrypsin deficiency

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Adeno-associated virus (AAV)-mediated gene therapy is currently being pursued as a treatment for the monogenic disorder α-1-antitrypsin (AAT) deficiency. Results from phase I and II studies have shown relatively stable and dose-dependent increases in transgene-derived wild-type AAT after local intramuscular vector administration. In this report we describe the appearance of transgene-specific T-cell responses in two subjects that were part of the phase II trial. The patient with the more robust T-cell response, which was associated with a reduction in transgene expression, was characterized more thoroughly in this study. We learned that the AAT-specific T cells in this patient were cytolytic in phenotype, mapped to a peptide in the endogenous mutant AAT protein that contained a common polymorphism not incorporated into the transgene, and were restricted by a rare HLA class I C alleles present only in this patient. These human studies illustrate the genetic influence of the endogenous gene and HLA haplotype on the outcome of gene therapy.

polymorphism | a-1-antitrypsin | gene therapy | adeno-associated virus | immune response

Recombinant adeno-associated virus (AAV)-mediated gene therapy holds great promise for the treatment of multiple diseases (1–8). Preclinical mouse studies have demonstrated sustained correction with a relative paucity of transgene-specific immunity. Even when present, a potent cytotoxic T-cell (CTL) response was, by itself, insufficient to eliminate transgene expression without an accompanying inflammatory response (9, 10). However, successful transgene expression in small animal models does not always translate to nonhuman primate models and humans. For example, nonhuman primates injected i.v. with an AAV vector expressing GFP elicited a transgene-specific T-cell response that extinguished transgene expression and caused transient transaminitis, which was not seen in mice injected with a similar vector (11). In a hemophilia B trial, an increase in hepatic transaminases with a concomitant decrease in transgene expression was observed in four patients systemically administered with an AAV vector (12, 13). These and other observations have led to the hypothesis that capsid and transgene immunity may compromise the outcomes of AAV gene therapy clinical trials.

AAV gene therapy is currently being pursued as a treatment for the monogenic disorder α-1-antitrypsin (AAT) deficiency. Results from phase I and II studies have shown a dose-dependent increase in wild-type AAT (m-AAT) after local i.m. administration of AAV1 vectors (14–18). In these studies, AAV capsid-specific $CD4^+$ and $CD8^+$ T cells with a cytolytic phenotype were detected in blood following AAV administration. Interestingly, m-AAT transgene expression was stable in most patients and was associated with a regulatory T-cell response characterized by increased $CD4^+$ $CD25^+FOXP3^+$ cells at the site of vector administration (18). However, T-cell responses to the AAT transgene were observed in a more limited number of patients who participated in the phase II clinical trial (17).

Here, we describe and characterize the AAT-specific immune responses found in two trial subjects. Our findings demonstrate the previously underappreciated role of coding sequence polymorphisms and HLA genotypes that in one patient triggered a class I-restricted immune response to the AAT transgene following gene transfer.

Results

The T-Cell Response to AAT Is Multifunctional and Is Mediated by $CD8^+$ T Cells. The phase II clinical trial consisted of nine subjects stratified into three dose cohorts, 6e11, 1.9e12, and 6e12 viral genomes/kg, who were administered AAV1-AAT i.m. Analysis of AAV1 capsid and AAT transgene immunity by IFN-γ enzyme-linked immunospot (ELISPOT) assays indicated that all nine subjects developed AAV1 capsid-specific T cells (17), but only two subjects (305 and 401) developed AAT transgene-specific T cells (Fig. 1 *A* and *B*). None of the subjects had detectable AAV1- or AAT-specific T cells in blood before vector administration,

Significance

The use of adeno-associated virus (AAV)-mediated gene therapy to treat monogenic disorders is currently being tested in phase I, II, and III clinical trials. An immune response to the newly introduced gene product remains a potential problem because of the patient's lack of central and peripheral tolerance to foreign epitopes. In this study, we show the induction of a T-cell response to the therapeutic product in a α-1-antitrypsin (AAT)-deficient subject receiving AAV1-AAT treatment. The response was directed to an AAT epitope upstream of the mutation and was associated with a polymorphism present in the subject but not in the wild-type AAT therapeutic product. Our study highlights the importance of considering polymorphisms in the targeted population when designing a transgene.

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Conflict of interest statement: J.M.W. is an advisor to REGENXBIO, Dimension Therapeutics, and Solid Gene Therapy and is a founder of, holds equity in, and has a sponsored research agreement with REGENXBIO and Dimension Therapeutics; in addition, he is a consultant to several biopharmaceutical companies and is an inventor on patents licensed to various biopharmaceutical companies. T.R.F. is a founder of Applied Genetic Technologies Corporation, is scientist advisor to and has equity in Dimension Therapeutics, and is a scientific advisor to Editas Medicine.

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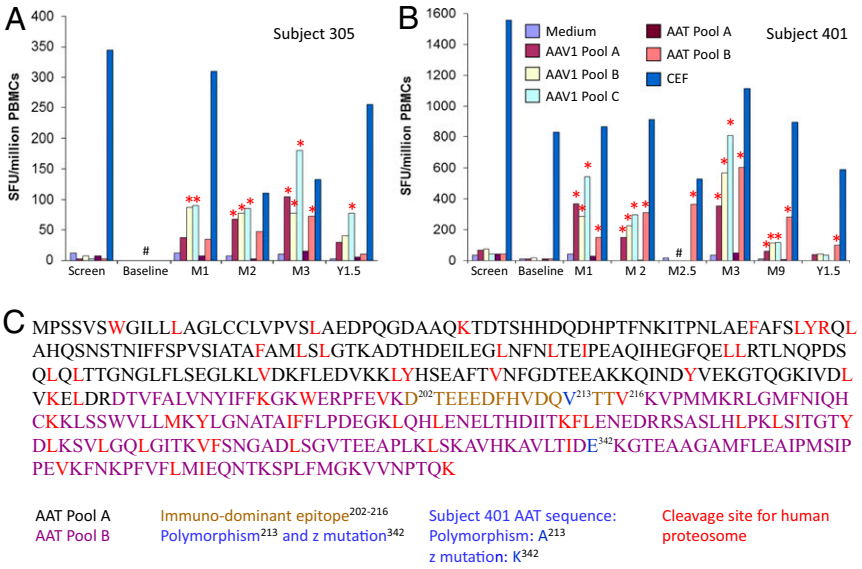

Fig. 1. Time course of immune response and epitope location. The time course of the T-cell response to AAV1 capsid and AAT transgene in PBMCs from subjects 305 (*A*) and 401 (*B*) was analyzed in duplicate by IFN-γ ELISPOT assay using peptide libraries specific for the full-length AAV1 capsid (pools A, B, and C) and the AAT transgene (pools A and B). The CEF peptide pool was used at each time point as a positive stimulation control. M, month; SFU, spot-forming units; Y, year. Asterisks indicate a positive response; the hashtag indicates that no sample analysis was available. (*C*) Full amino acid sequence of the AAT transgene indicating peptides included in pool A (black) and B (purple), the location of immunodominant epitopes (brown), the location of polymorphisms and z-AAT mutations (blue), and C-terminal cleavage sites for human proteasome (red). Superscript numbers indicate the amino acid position of the mature AAT protein.

suggesting that these were primary responses. Nonetheless, we cannot rule out the presence of AAV1- or AAT-specific memory T cells in lymphoid organs before vector administration and the induction of an anamnestic response. Of the two subjects with anti-AAT immunity, subject 305 had a delayed T-cell response to AAT peptide pool B 3 mo after vector administration. However, the low magnitude of the response precluded further analysis of this subject.

In contrast, subject 401, who is homozygous for the z-AAT mutation (Glu342Lys), had a sustained and persistent T-cell response to peptide pool B of AAT. The T-cell response increased soon after vector administration, peaked at 3 mo (Fig. 1*B*), and was detected for more than 1 y. Transgene expression was lower in this subject than in the other two subjects who received the same dose of vector (17), as were vector genomes from muscle biopsies (18). AAT expression before vector administration was 20–30 nM, and the levels were similar to those observed in other subjects within the same cohort (17). Deep sequencing of peripheral blood mononuclear cells (PBMCs) isolated before and 9 mo after gene therapy revealed an expansion of the T-cell repertoire, although the antigen specificity of the expanded T-cell subsets could not be identified by this analysis (*SI Text* and Fig. S1, and Table S1). Epitope mapping with overlapping peptide subpools at 2.5 and 3 mo identified a positive ELISPOT response against peptide DTEEEDFHVDQVTTV (17). This epitope, hereafter called "peptide 46," was located at position 202–216 of the mature protein and was flanked by proteasome cleavage sites identified by *in silico* analysis using the NetChop server (Fig. 1*C*) (19). The epitope is distant from the z-AAT mutation, which is located at position 342 of the AAT protein.

We investigated whether the immune activation of subject 401 to wild-type AAT could be the result of a previously identified Val213Ala polymorphism found in a minority of wild-type AAT alleles and in most z-AAT alleles (20). DNA sequencing revealed that subject 401, who was homozygous for z-AAT, was also homozygous for the Val213Ala polymorphism that resides in the portion of the AAT protein that spans the immunodominant peptide 46 from subject 401 (Fig. 1*C* and Fig. S2). The existence of the polymorphism in the immunodominant peptide suggests but does not prove that it played a role.

Next, we evaluated the robustness of the AAT immune response in subject 401 by measuring T-cell polyfunctionality (i.e., the ability to secrete or activate multiple immune modulators). Activated $CD8^+$ T cells secreted multiple cytokines/chemokines, e.g., TNF, IFN-γ, IL-2, and macrophage inflammatory protein 1α (MIP-1α), and the degranulation marker CD107a, which is a surrogate for cytotoxic activity (Fig. 2*A*). T-cell responses to peptide 46 were dominated primarily by IFN-γ– and TNF-secreting $CD8^+$ T cells, and more than half of the activated $CD8^+$ T cells were polyfunctional, coexpressing at least three of the five evaluated factors (Fig. 2*B*, pie charts). Further phenotypic characterization of peptide 46-stimulated $CD8^+$ T cells showed an abundance of cells with the effector T-cell (Teff) phenotype ($CD27^-CD45RO^-$) (Fig. 2*B*, density plots). The skewing of the immune response to a Teff phenotype suggests ongoing antigen exposure, as is consistent with the low but persistent m-AAT expression in blood and in muscle biopsies (17, 18).

The activation profiles of $CD4^+$ T cells were more distinct following stimulation with the AAT peptide library, peptide pool B, or peptide 46. $CD4^+$ T-cell response to the AAT peptide library was dominated by the coexpression of IFN-γ and IL-2 in more than half of the $CD4^+$ T cells (Fig. 2*C*). In contrast, the response to pool B was significantly lower and was dominated by cells coexpressing IL-2 and TNF. In both instances, the $CD4^+$ T-cell response was mediated by central memory (Tcm) ($CD27^+CD45RO^+$) and effector memory (Tem) ($CD27^-CD45RO^+$) T cells (Fig. 2*D*). $CD4^+$ T-cell responses were absent in cells stimulated with peptide 46. Overall, these data indicate that the response to AAT immunodominant epitope peptide 46 is dominated by effector $CD8^+$ T cells with a cytotoxic profile.

We also detected a T-cell response to AAV capsid in subject 401, and we compared this response with two other trial subjects (306 and 302) who did not develop AAT immune responses. These studies indicated that capsid T-cell responses are multifunctional and diverse (*SI Text* and Fig. S3).

The Importance of HLA Restriction on the Presentation of the Immunodominant AAT Epitope. The observation that only one trial subject developed a robust T-cell response, although all are

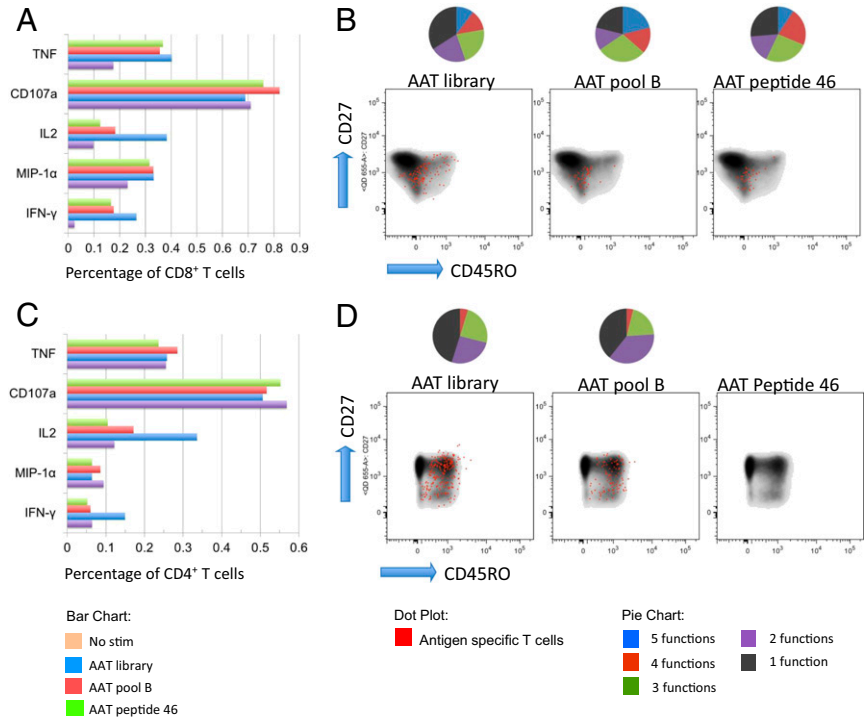

Fig. 2. Functional and phenotypic analysis of the AAT transgene-specific T-cell response in PBMCs from subject 401. (*A* and *C*) Functional profile of cytokine (IFN-γ, TNF, and IL-2), chemokine (MIP-1α), and surface expression of a degranulation factor (CD107a) by $CD8^+$ (*A*) and $CD4^+$ (*C*) T cells from PBMCs obtained at the 2.5-mo time point following stimulation with AAT peptide library, AAT pool B, or AAT immunodominant peptide 46 DTEEEDFHVDQVTTV. (*B* and *D*) Memory phenotype characterization of AAT-specific $CD8^+$ (*B*) and $CD4^+$ (*D*) T cells. Naive cells were defined as $CD27^+$ $CD45RO^-$, Tcm cells were defined as $CD27^+CD45RO^+$, Tem cells were defined as $CD27^-CD45RO^+$, and Teff cells were defined as $CD27^-CD45RO^-$. Pie charts represent the total AAT-specific T-cell response. Each slice indicates the proportion of cells that produce one to five cytokines as indicated by different colors.

expected to carry the Val213Ala polymorphism (20), suggests a possible HLA allele restriction. To identify the allele(s) most likely responsible for presenting the immunodominant epitope, we HLA-typed all trial subjects and compared the haplotype of subject 401 with that of the subjects who did not activate T cells (Table 1). We were looking for a rare HLA allele that segregated with the development of AAT-specific T cells. In this analysis, any HLA allele that was found in a subject who did not mount an AAT-specific T-cell response would be precluded from being responsible for presenting the immunodominant epitope in subject 401. Of the HLA alleles present in subject 401 (HLA-A*02/03, HLA-B*35/44, and HLA-C*04/05), HLA-C*04/05 uniquely segregated with the T-cell response in subject 401 (Table 1). Furthermore, we performed a computational HLA class I affinity prediction for 8- to 12-mer peptides spanning the polymorphic site for patient 401's HLA genotype. This analysis revealed a single 8-mer peptide (HVDQVTTV) predicted to bind with high affinity to HLA-C*05:01; however, no peptides were predicted to bind with high affinity to HLA-A or -B alleles (Tables S2 and S3). This result suggests that the polymorphic site generates a high-affinity neoepitope in patients carrying the HLA-C*05:01 allele.

To test HLA restriction of the dominant AAT epitope, we devised a strategy using an ex vivo antigen-presentation model. Individual HLA alleles were transfected into K562 cells, a human myelogenous cell line lacking endogenous HLA expression, and these cells subsequently were used to activate subject 401's AAT-specific T cells (Fig. 3). Next, transiently transfected cells were immune-stained to monitor cell-surface class I expression. As expected, HLA staining was absent in K562 cells transfected with a sham construct, whereas strong cell-surface staining was observed in more than 50% of K562 cells transiently transfected with HLA-A*02, HLA-C*04, or HLA-C*05 (Fig. 3). Next, peptide-loaded transfected cells were used as antigen-presenting cells (APCs) by coculturing with PBMCs from subject 401. HLA-C*05 K562 cells activated subject 401's AAT-specific T cells threefold over unloaded targets (Fig. 3, *Upper Right*). Activation was lower when target cells were transfected with HLA-A*02. Although subject 401 carried two unique HLA-C alleles, C*04-transfected cells did not activate T cells and were similar to control mock-transfected cells.

We observed a similar T-cell response when we used our ex vivo approach for antigen presentation (Fig. 3, *Upper Right*) or when we used professional APCs (Fig. 3, *Lower Left*); as expected, the magnitude of the response was lower with nonprofessional APCs.

Table 1. HLA haplotyping of the subjects enrolled in the clinical trial

	HLA class I		
Subject	A	B	C
301	02, 29	18, 44	07, 16
302	01, 31	18, 57	06, 07
303	11, 30	13, 13	06, 06
304	03, 29	44, 51	15,16
305	02, 02	35, 50	06, 15
401	02, 03	35, 44	04, 05
306	01, 02	07, 07	07, 07
307	02, 03	08, 45	06, 07
308	NA	NA	NA

NA, sample not available for HLA typing. Numbers indicate the two alleles for A, B, and C loci.

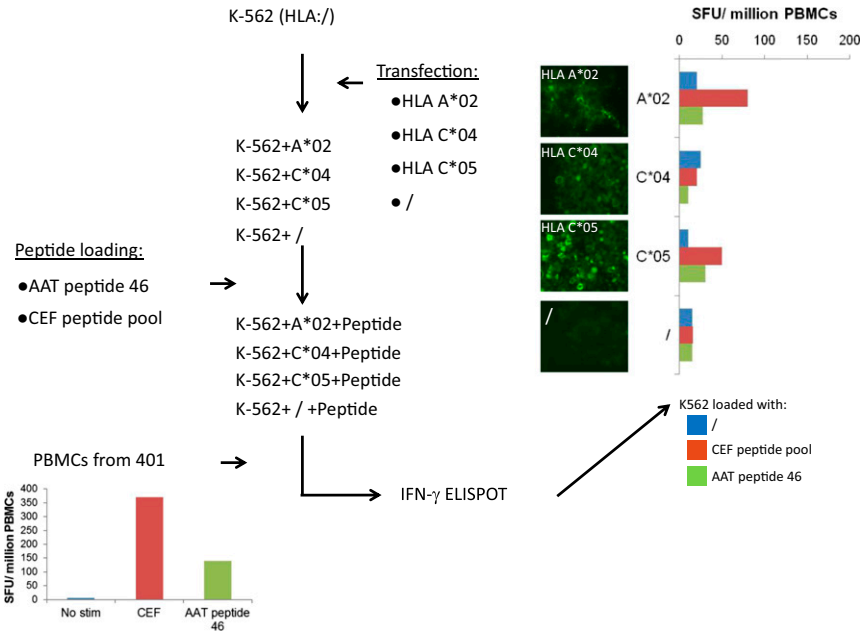

Fig. 3. HLA restriction of the dominant AAT epitope. Flowchart of the experiment and immunostaining for HLA surface expression in human K562 cells transfected with *HLA-A*02, HLA-C*04,* or *HLA-C*05* plasmids. The graph at the upper right shows the IFN-γ ELISPOT response at the 2.5-mo time point of subject 401's PBMCs cocultured with HLA-transfected K562 cells pulsed with AAT immunodominant peptide 46 (DTEEEDFHVDQVTTV) or the CEF peptide pool. The graph at the lower left shows the IFN-γ ELISPOT response using the same PBMCs but cultured directly with the CEF peptide pool and AAT immunodominant peptide.

Discussion

The number of AAV clinical trials is increasing significantly, with vectors being administered for the treatment of a variety of disorders. Although T-cell responses to AAV capsid have been described previously (1, 13–15, 21, 22), the activation of cellular immune responses to the transgene products following AAV gene therapy has been less of a problem. It is hypothesized that patients carrying null mutations are at higher risks of mounting an immune response to the wild-type therapeutic gene product, because they should lack central and peripheral tolerance to all T-cell epitopes. Based on this consideration, patients with null genotypes have been excluded from participating in some ongoing AAV clinical trials. A corollary of this hypothesis is that patients with missense mutations are at a lower risk of mounting T-cell responses to the transgene-derived protein product because they should be tolerant to all epitopes except those involving the mutation.

A detailed analysis of the immune responses elicited in the AAV1-AAT clinical trial clearly demonstrates the development of T cells to the wild-type m-AAT transgene product in a subset of subjects. The subject with the most robust m-AAT T-cell response had lower transgene expression than the other two subjects in the same dose-level group, suggesting that the immune response may have compromised the outcome of the therapy (17). In addition, the strong AAV-specific T-cell response detected in this subject may have played a role in lowering transgene expression, although similar AAV-specific T-cell responses in the high-dose group did not compromise transgene expression (17). Interestingly, the immunodominant epitope in AAT was located outside the z-mutation and occurred in a previously identified polymorphic site.

Why was a T-cell response generated to an epitope spanning a polymorphic residue but not to the site of the z-mutation? Furthermore, why was this T-cell response present only in one subject? We believe that the answers to both questions relate to class I restriction and HLA genotypes. In this regard, the z-AAT mutation occurs in a region not flanked by proteasome cleavage sites, suggesting that it may not be processed efficiently for class I presentation. Furthermore, based on *in silico* analyses, none of the HLA class I alleles present in the research subjects bound effectively to peptides spanning the z-AAT allele. A similar analysis of the AAT immunodominant epitope revealed proximity to proteasome cleavage sites and high-affinity binding to a rare HLA-C allele found only in subject 401. In vitro binding and T-cell activation studies confirmed HLA-C*05 class I restriction of this peptide to the T cells of subject 401.

Several mechanisms could explain the generation of T cells against m-AAT in subject 401. One possibility is that the polymorphism could have diminished class I binding relative to the same peptide from the m-AAT transgene; alternatively, the polymorphism could affect the actual processing of the peptide for class I presentation. Either scenario could result in incomplete deletion of T cells in the thymus to the polymorphic peptide ending with activation and targeting of T cells to transduced cells in the periphery. However, *in silico* analysis failed to reveal differential binding of HLA-C*05 to the polymorphic and wild-type immunodominant peptides, and the Val213Ala change does not appear to affect proteasome cleavage sites. Thus the *in silico* analysis argues against these hypotheses; however, this evidence is not definitive because nonanchor residues in class I presented peptides, although not influencing peptide binding, have been shown to have substantial effects on T-cell responses (23). Another possibility is that i.m. injection of AAV1/m-AAT broke peripheral tolerance to self-reactive T cells by activating local inflammation at the site of m-AAT expression; this possibility is consistent with the presence of persistent inflammation at the injection site (17). In this case the polymorphism is incidental. It is possible that both mechanisms contributed to the final result.

Gene therapy with AAV vectors could potentially activate an immune response to both the transgene and the viral capsid. The interplay of these immune responses may further influence the outcome of the treatment (24). It is interesting that other subjects in this trial developed $CD8^+$ and $CD4^+$ T-cell responses to capsid with a concomitant increase in FoxP3-expressing regulatory T-cell responses in both peripheral and locally infiltrating T cells (18). These subjects also demonstrated an exhausted $CD8^+$ T-cell phenotype in situ, with expression of both PD-1 and PDL-1.

We conclude that the apparent reduction in transgene expression that developed in subject 401 in this trial was the result of a class I-restricted T-cell response to a peptide in AAT. This T-cell response and its consequence were limited to a single patient who happened to have a rare HLA-C allele that efficiently presented a peptide from the transgene-derived protein. We suspect that a polymorphism in the immunodominant peptide of both z-AAT alleles of subject 401 resulted in a lack of central tolerance that, combined with vector-induced inflammation, resulted in a robust T-cell response to vector-transduced cells. This study shows that HLA genotype and polymorphism play a substantial role in gene therapy and should be considered in the investigation of transgene expression in clinical trials. We speculate that class I restriction may influence the substantial patient-to-patient variability that has been seen in some of the AAV gene therapy hemophilia trials.

Methods

Clinical Trial. This study was conducted under the Food and Drug Administration's Investigational New Drug program with approval from the institutional review boards and biosafety committees of the University of Massachusetts Medical School and Cincinnati Children's Hospital. All procedures and protocols were in accordance with the tenets of the declaration of Helsinki. This study is registered with ClinicalTrials.gov (NCT01054339). All subjects provided written informed consent.

IFN-γ ELISPOT Assay. We analyzed IFN-γ released in PBMCs using previously described methods (1). PBMCs were stimulated in duplicate with medium alone, AAV1, or AAT peptide libraries. The CEF peptide pool [comprising 32 MHC class I-restricted T-cell epitopes (8–12 amino acids in length) that contain sequences derived from the human cytomegalovirus, Epstein–Barr virus, and influenza virus] (Mabtech) served as a positive control, as previously described (25). Positive ELISPOT counts were defined as >55 spot-forming units per million lymphocytes, i.e., values three times over background (25).

Peptide Libraries. We synthesized a peptide library tailored for the full lengths of the AAT and the AAV1 capsid as 15-mers with 10-aa overlap (Mimotopes). The AAT peptide library was divided into two pools, A and B, and the AAV virion peptide 1 (VP1) peptide library was divided into three pools, A, B, and C, as previously described (14).

Intracellular Cytokine Staining. PBMCs were stimulated with the AAV1 or AAT peptide libraries or with individual immunodominant AAT peptide 46 (DTEEEDFHVDQVTTV). Reactive T cells were analyzed functionally and phenotypically using previously described techniques (1). T-cell polyfunctionality was assessed by simultaneous detection of secreted TNF, CD107a, IL-2, MIP-1α, and IFN-γ. Cellular phenotypes were evaluated by the expression of the surface markers(CD3, CD4, CD8, CD27, and CD45RO. Naive T cells were defined as $CD27^+CD45RO^-$, Tcm cells were defined as $CD27^+CD45RO^+$, Tem cells were defined as $CD27^-CD45RO^+$, and Teff cells were defined as $CD27^-CD45RO^-$.

T-Cell Receptor β Chain Deep-Sequencing Analysis. Deep-sequencing analysis of the T-cell receptor β (TCRB) chain was performed using patient PBMCs to detect all possible rearranged genomic sequences and to determine T-cell receptor variable region b (TCR-Vb) use for each clone (Adaptive Biotechnologies) as described earlier (18).

HLA Haplotypes Analysis. HLA analysis was performed by LabCorp in eight of nine subjects enrolled in the trial. Samples for HLA typing were not available from subject 308.

AAT Polymorphism Analysis. DNA was extracted from eight 10-μm paraffin-embedded muscle tissue sections using a QIAamp DNA formalin-fixed, paraffin-embedded tissue kit per the manufacturer's instructions (Qiagen). A 545-bp product was amplified and sequenced (Genewiz) using primers (forward: ttggatggtcagtttcagca and reverse: agagggctgaggagggtgaa) flanking exon 3 (26).

Cell Lines and Transfection. K562 cells were a gift from Carl June, University of Pennsylvania, Philadelphia, PA. Cells were transiently transfected using Lipofectamine reagent (Invitrogen). We used indirect immunofluorescence to image transiently transfected K562 cells expressing individual human HLA using an anti-human MHC class I antibody. Images were collected using a Nikon inverted microscope.

Computational HLA Class I Affinity Analysis. To identify peptides predicted to bind MHC class I with high affinity, a sliding window of 8- to-12-mer peptides centered on the amino acid of interest were generated and ranked for binding using the Immune Epitope Database and Analysis Resource (27). Peptides with an IC_{50} corresponding to a percentile rank of less than one against a standardized database were classified as high affinity.

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Supporting Information

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Changes in T-Cell Repertoire Following Vector Administration

The functional analysis of T cells, which is limited to measurements of cytokine secretion or activation, does not reveal the complexity of the host immune response following vector administration. To understand the breadth of the T-cell response, we longitudinally probed the repertoire of T-cell receptor (TCR) diversity in subject 401. High-throughput sequencing of the TCR β-chain complementary determining region 3 was performed on PBMCs isolated before and 9 mo following vector administration. Briefly, we characterized TCR diversity from DNA isolated from the equivalent number of cells using next-generation sequencing (Adaptive Biotechnologies). We analyzed a total of 663,609 productive sequences from PBMCs isolated before vector administration and 998,208 productive sequences from PBMCs isolated 9 mo after vector administration. At both time points roughly 3.8% of the total sequences were unique clones (Table S1). However, we identified an increased number of productive sequences and unique clones from postinjection samples. This finding indicates an expansion in peripheral blood T-cell clones. For instance, the number of unique clones in peripheral blood increased by 36%, from 25,026 preinjection to 39,207 at 9 mo following vector administration (Table S1). This difference may reflect normal temporal variations in the peripheral blood TCR repertoire or may suggest cellular immune responses to persistent transgene expression. In support of an ongoing adaptive immune response, a VB7-9 bias was observed in samples collected at 9 mo.

Among the clonotypes, we found the unique sequence CASSWDSYEQYF at a frequency of 0.63% (Fig. S1), representing a substantial expansion of this particular clone in peripheral blood following vector administration. Similar analysis performed at the site of vector administration might demonstrate T-cell trafficking to the source of antigen expression. However, muscle biopsy was not available from subject 401. Interestingly, a similar analysis of the T-cell repertoire in another subject enrolled in the trial (subject 301) demonstrated the presence of unique high-frequency clones in both blood and muscle following vector administration (17). Based on this finding, we expected clone CASSWDSYEQYF identified in the blood from subject 401 to be present in muscle also.

The T-Cell Response to AAV1 Is Multifunctional and Diverse

We compared the intracellular staining analyses of AAV1 capsid-specific T-cell responses from subject 401 and from two other trial subjects (302 and 306) (17) and found that AAV1-specific responses, although varied in magnitude, had a similar cytokine profile among subjects. In subject 401, $CD8^+$ T cells predominantly expressed IFN-γ, TNF, IL-2, and MIP-1α (Fig. S3*A*) with 14% of AAV1-specfic $CD8^+$ T cells coexpressing at least three factors (Fig. S3*B*, pie chart). Although the $CD8^+$ T-cell response to AAV1 was similar among the three subjects, there were differences in the functional profile. The main differences in subjects 306 and 302 were the increased numbers of $CD8^+$ T cells coexpressing three factors (80% and 52%, respectively) and the activation of CD107a (Fig. S3*A*). Interestingly, we could not detect the expression of CD107a among the AAV1-specific T cells from subject 401. Despite these functional differences, the phenotypic profile of the T cells was similar across subjects with an abundance of Tcm and Teff cells (Fig. S3*B*).

$CD4^+$ T-cell responses were similar between subjects 401 and 306, with up-regulation of IFN-γ, IL-2, and TNF (Fig. S3*C*). Fifty-nine percent and 80% of $CD4^+$ T cells from subjects 401 and 306, respectively, coexpressed at least two factors (Fig. S3*D*, pie charts). The phenotypes of activated $CD4^+$ Tcm and Teff cells were similar in these subjects (Fig. S3*D*). AAV1-specfic $CD4^+$ T cells were not activated in subject 302 (Fig. S3*C*).

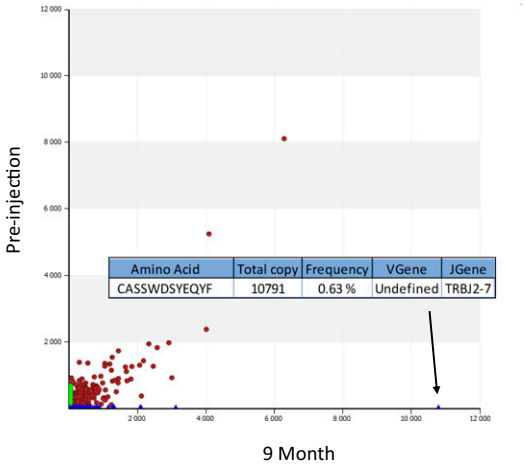

Fig. S1. TCRB-chain deep-sequencing analysis of the number of T-cell clones in blood before (*y* axis) and after (*x* axis) gene transfer in subject 401. Green triangles indicate T-cell clones present before injection. Blue triangles indicate T-cell clones present after injection. Red circles indicate T-cell clones present both before and after injection.

A Transgene *AAT* GTG GAC CAG G**T**G ACC ACC GTG

Subject 401 *AAT* Exon III GTG GAC CAG G**C**G ACC ACC GTG

B Transgene AAT KWERPFEVKDTEEEDFHVDQ**V**TTVKVPMMKRLG

Subject 401 AAT KWERPFEVKDTEEEDFHVDQ**A**TTVKVPMMKRLG

Epitope ^{202}DTEEEDFHVDQ**V**TTV^{216}

Fig. S2. AAT polymorphism. DNA (*A*) and amino acid (*B*) sequence alignment from the AAT transgene and from the AAT region of subject 401 in which the polymorphism was detected. The amino acid sequence of immunodominant epitope peptide 46 is aligned to transgene and subject 401 AAT sequences. The enlarged letters indicate the specific base and amino acid responsible for the polymorphism and the z-mutation.

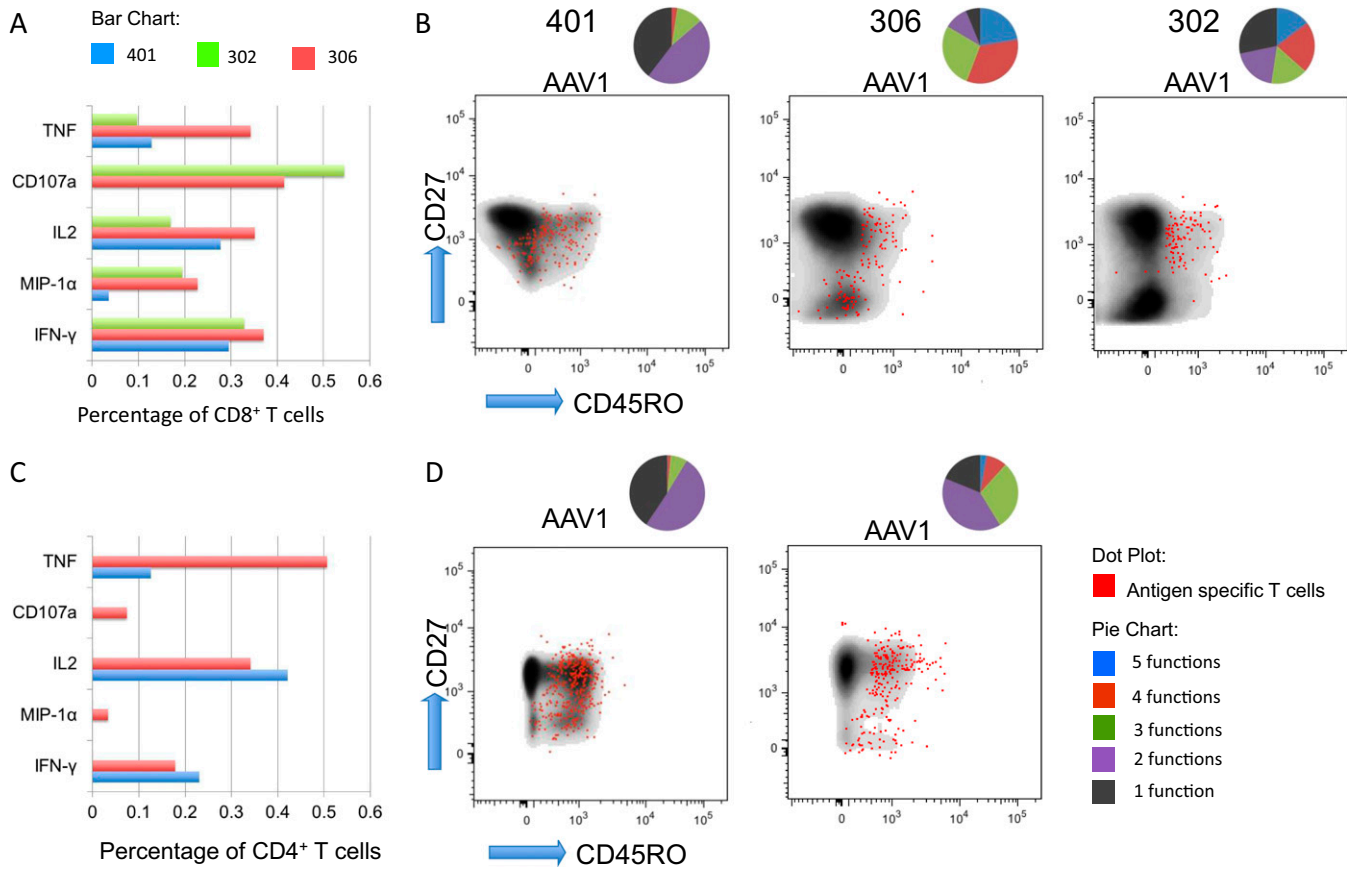

Fig. S3. Functional and phenotypic analysis of the AAV1 capsid-specific T-cell response in PBMCs from subjects 401, 302, and 306. (*A* and *C*) Functional profile of cytokine (IFN-γ, TNF, IL-2), chemokine (MIP-1α), and surface expression of degranulation factor (CD107a) by CD8$^+$ (*A*) and CD4$^+$ (*C*) T cells from PBMCs obtained 2.5 mo following stimulation with the AAV1 peptide library. (*B* and *D*) Memory phenotype characterization of AAV1-specific CD8$^+$ (*B*) and CD4$^+$ (*D*) T cells. Naive cells were defined as CD27$^+$CD45RO$^-$, Tcm cells as CD27$^+$CD45RO$^+$, Tem cells as CD27$^-$CD45RO$^+$, and Teff cells as CD27$^-$CD45RO$^-$. Pie charts represent the total AAV-specific T-cell response; each slice indicates the proportion of cells that produce one to five cytokines, as indicated by different colors.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)