Cellular immune response to cryptic epitopes during therapeutic gene transfer

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The immune response has been implicated as a critical factor in determining the success or failure of clinical gene therapy trials. Generally, such a response is elicited by the desired transgene product or, in some cases, the delivery system. In the current study, we report the previously uncharacterized finding that a therapeutic cassette currently being used for human investigation displays alternative reading frames (ARFs) that generate unwanted protein products to induce a cytotoxic T lymphocyte (CTL) response. In particular, we tested the hypothesis that antigenic epitopes derived from an ARF in coagulation factor IX (F9) cDNA can induce CTL reactivity, subsequently killing F9-expressing hepatocytes. One peptide (p18) of 3 candidates from an ARF of the F9 transgene induced CD8$^+$ T cell reactivity in mice expressing the human MHC class I molecule B0702. Subsequently, upon systemic administration of adeno-associated virus (AAV) serotype 2 vectors packaged with the F9 transgene (AAV2/F9), a robust CD8$^+$ CTL response was elicited against peptide p18. Of particular importance is that the ARF epitope-specific CTLs eliminated AAV2/F9-transduced hepatocytes but not AAV2/F9 codon-optimized (AAV2/F9-opt)-transduced liver cells in which p18 epitope was deleted. These results demonstrate a previously undiscovered mechanism by which CTL responses can be elicited by cryptic epitopes generated from a therapeutic transgene and have significant implications for all gene therapy modalities. Such unforeseen epitope generation warrants careful analysis of transgene sequences for ARFs to reduce the potential for adverse events arising from immune responses during clinical gene therapy protocols.

factor IX | gene therapy | CTL | AAV

Recombinant adeno-associated virus (rAAV) vectors possess several attractive features, including lack of pathogenicity, broad tissue tropism, and the ability to promote sustained transgene expression, that make them particularly well-suited for clinical gene therapy applications (1). Driven by a rapidly expanding understanding of AAV biology and a wide range of preclinical studies in mice, dogs, and primates, more than 50 clinical trials have been approved for diseases ranging from Parkinson's to Duchenne Muscular Dystrophy. Recombinant AAV vectors do not contain any viral genome elements, with the exception of 145-bp terminal repeats required for packaging, and persistent transgene expression lasting several years has been observed in preclinical animal models. Given the aforementioned observations, it has been perceived generally that despite generating a strong humoral immune response, AAV vector administration does not elicit a cellular immune response to the AAV capsid that could potentially result in the clearance of AAV vector-transduced target cells.

In the first successful clinical trial with an AAV vector, hemophilia B patients were treated by using an AAV serotype 2 (AAV2) vector to deliver coagulation factor IX (F9) cDNA into the liver. Within 2 weeks, therapeutic levels of F9 were detected in the blood (2). However, in 1 patient with an HLA-B0702 allele, circulating F9 levels decreased to baseline several weeks after treatment, concomitant with an elevation of liver enzymes, suggesting damage. Although this observation is consistent with cytotoxic T lymphocyte (CTL)-mediated clearance of AAV vector-transduced liver cells, the peptides from the F9 transgene product did not stimulate a CTL response in vitro. Unexpectedly, a CTL response was induced against the AAV2 capsid, raising potential concerns pertaining to capsid-specific, CTL-mediated elimination of AAV-transduced liver cells (2). The latter observations have prompted researchers to examine the CTL response to AAV capsid in animal models. Indeed, a capsid-specific CTL response can be triggered by using adenoviral vectors to deliver AAV capsid genes or by direct injection of AAV vectors into mice (3, 4). However, these capsid-specific CTLs do not eliminate AAV-transduced target cells in mouse models, contradicting the prior interpretation of the clinical data (5–7). Further, several other findings support the latter notion. For instance, the kinetics of liver cell damage and F9 circulation suggest that the loss of transduced liver cells is likely due to CD8$^+$ CTL reactivity specific for the transgene product, despite no detectable CTL responses against F9 peptides (2). Second, an AAV2 capsid-specific CD8$^+$ CTL response was not induced in 2 subjects with the HLA-B0702 haplotype, even in the presence of high-titer neutralizing antibodies against AAV2 (8). Lastly, AAV2 capsids do not elicit a CTL response in B0702 transgenic mice after muscle injection of a high dose of AAV2 vectors (8). In summary, these studies suggest that alternative mechanisms might be involved in the observed AAV2-transduced liver cell clearance in the patient with the HLA-B0702 allele given AAV2/F9 gene therapy.

The CD8$^+$ CTL immune response is crucial for the specific eradication of cells displaying foreign peptides (i.e., virus- or bacteria-infected cells). Epitopes recognized by CTLs likely arise from primary ORFs, particularly via the degradation of newly synthesized proteins. However, recent studies indicate that CTL epitopes can also be encoded by nonprimary ORF sequences and other nontraditional sources in tumor- and virus-infected cells (9). These cryptic epitopes may be generated from a variety of sources at the transcriptional and translational levels, such as alternative reading frames (ARFs), ribosome frame shifts and, perhaps, protein synthesis from aberrant splicing (9–11). In an attempt to explain the clinical results of the recent AAV2/F9 trial, we investigated whether epitopes from the F9 ARF elicited a CTL response to eradicate AAV2-transduced liver cells. We first inserted the well-defined OVA peptide epitope SIINFEKL into the F9 ARF to determine whether transcription and trans-

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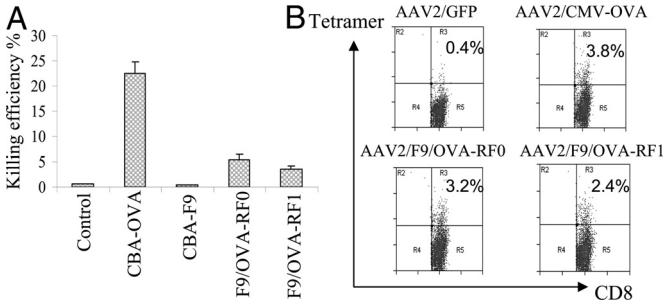

Fig. 1. OVA epitope presentation in the F9 ARF1. (*A*) The activation of spleen cells from OT-1 mice by OVA epitope presentation. Cos/K^b cells were transfected with the indicated constructs. The next day, the cells were incubated with OT-1 spleen cells overnight, and CD69 expression on CD8$^+$ T cells was analyzed by flow cytometry. (*B*) Tetramer staining of OVA-specific CTLs. C57BL/6 mice were administered i.p. 2×10^{11} particles of AAV2 vectors harboring the indicated constructs. After 30 days, mice were killed, and spleen cells were harvested and analyzed for OVA epitope tetramer and CD8 staining by flow cytometry. RF, reading frame.

lation were initiated. Subsequently, we immunized HLA-humanized mice with an AAV2/F9 vector to verify a CTL response to native epitopes from the F9 ARF. The data described here suggest that a cryptic epitope (p18) from the F9 ARF1 induces CTL reactivity that, in turn, eliminates AAV vector-transduced liver cells in an HLA-humanized mouse model.

Results

F9 cDNA Contains ARFs. Initially, Vector NTI (VNTI; Invitrogen) was used to analyze the ORFs of the F9 cDNA sequence. Several ORFs were found from different start codons (Fig. S1); however, an ORF localized to nucleotides 413–604 (+1 frame shift, named ARF1) of the F9 cDNA contained a Kozak sequence with an A at −3 and a G at +4 with respect to the ATG start codon of the ARF1 (12), which potentially translates a 64-aa peptide (Fig. S2). To test whether this F9 ARF1 could initiate synthesis of an immunogenic peptide, the well-defined H-2K^b-restricted OVA$_{257–264}$ peptide (SIINFEKL) was inserted into the F9 ORF or the F9 ARF1. The resulting recombinants were subcloned downstream of the chicken β-actin (CBA) promoter and upstream of a poly(A) sequence (pF9/OVA-RF0 and pF9/OVA-RF1, respectively; Fig. S3). Cos1/K^b cells were transfected with these constructs, and the capacity to stimulate OVA-specific T cell reactivity was assessed. The β-galactosidase (LacZ)-expressing B3Z T hybridoma cell line and spleen cells from OT-1 mice were used to detect presentation of an OVA$_{257–264}$/K^b complex by Cos1/K^b cells (13). LacZ staining of B3Z T cells after coculture with transfected Cos1/K^b cells was most intense for the positive control (pCBA-OVA, 83% ± 12%); however, LacZ$^+$ cells were observed in both F9 constructs pF9/OVA-RF0 and pF9/OVA-RF1 (15% ± 4% vs. 9% ± 3%, respectively; $P < 0.05$; Fig. S4). Additionally, no LacZ activity was detected for Cos1/K^b cells only expressing F9, the negative control pCBA-F9, which lacks the OVA sequence (<1%). OVA-specific T cell activation under various conditions was also observed with spleen-derived OT-1 CD8$^+$ T cells, as measured by up-regulation of CD69 expression (Fig. 1*A*). A total of 5.3% and 3.5% of CD69$^+$CD8$^+$ cells were detected for pF9/OVA-RF0 and pF9/OVA-RF1, respectively, which were lower than that for the positive control (pCBA-OVA, 22.4%). Consistent with T cell stimulation, H-2K^b–OVA complexes were readily detected on the surface of Cos1/K^b transfectants stained with the 25-D1.16 antibody (Fig. S5).

To determine whether OVA-specific CD8$^+$ CTL reactivity could also be induced in vivo from the ARF1 of the F9 cDNA, pF9/OVA-RF0 and pF9/OVA-RF1 were used to make AAV2 vectors: AAV2/F9/OVA-RF0 and AAV2/F9/OVA-RF1, respectively. A total of 2×10^{11} particles of AAV2 vectors were injected into C57BL/6 mice i.p. One month after injection, spleen cells were collected and analyzed for induction of OVA-specific CD8$^+$ T cells by staining with an OVA-specific H-2K^b tetramer. Consistent with the in vitro results, the administration of AAV2/F9/OVA-RF1 mounted an OVA-specific CTL response in mice, as did expression from RF0 (Fig. 1*B*). Higher OVA-specific CTLs were demonstrated in mice immunized with the positive control vector AAV2/CMV-OVA [chicken ovalbumin cDNA was driven by the cytomegalovirus early promoter (CMV)] than AAV2/F9/OVA-RF0 (3.8% vs. 3.2%, respectively). Decreased tetramer staining was detected in mice with AAV2/F9/OVA-RF1 (2.4%). In vivo CTL cytotoxicity to OVA peptide-pulsed target cells was also observed in mice injected with the AAV2/F9/OVA RF1 or RF0 vectors (Fig. S6). The positive control induced the strongest in vivo CTL-mediated killing (80%), followed by AAV2/F9/OVA-RF0 (61%) and, finally, AAV2/F9/OVA-RF1 (40%). Collectively, in vitro and in vivo data demonstrate that an F9 cDNA ARF1-derived OVA epitope can effectively elicit a CTL response; however, translation of the F9 ARF1 is likely lower than that of the primary F9 ORF.

A CTL Response Was Elicited from Epitopes of F9 ARF1. The OVA epitope generated from the F9 cDNA ARF1 triggered a CTL response in vitro and in vivo. Next, we determined whether native epitopes derived from the ARF1 of the F9 cDNA induced specific CTL reactivity and clearance of AAV-transduced liver cells. We used different epitope prediction algorithms to define potential peptides from the F9 ARF1 in the human HLA-B0702 allele, the same as the patient with AAV2/F9 gene therapy. The top 3 candidate peptides (p18, p31, and p52; Table 1 and Table S1) were chosen to test the immunogenicity of the predicted ARF1 epitopes. First a peptide-MHC-B0702 competition binding assay was carried out (14). Both peptides p18 and p52 had high B0702 molecule-binding capacity (IC_{50}, 9.1 nM and 4.7 nM, respectively), but low affinity was found for p31 (IC_{50}, 2212 nM; Table 1). To test whether a CTL response is elicited by the 3 candidate peptides in vivo, mice transgenic for human MHC-B0702 (B0702 Tg mice) were used (14). Dendritic cells (DCs) pulsed with a mixture of peptides (p18, p31, and p52) were used

Table 1. Immunogenicity of epitopes from the F9 ARF in B*0702 transgenic mice

Name	Sequence	Length	Position	B*0702 binding IC_{50}, nM	IFN-γ response in vitro*	In vivo CTL killing, %†
p18	APVLRDIDL	9	18	9.1	180 (30)	51 (12.3)
p31	SPVNQQCHF	9	31	2,212	155 (24)	ND
p52	SPVLRLFFL	9	52	4.7	100 (28)	ND
OVA	SIINFEKL	8	263	ND	98 (31)	2 (0.7)

ND, not done.

*IFN-γ ELISPOT assay. Values are expressed as the mean net spots per 10^6 spleen cells (±SEM). IFN-γ spot in the background with only medium was 80 (31) per 1×10^6 cells.

†The values represent the mean in vivo CTL killing (±SEM).

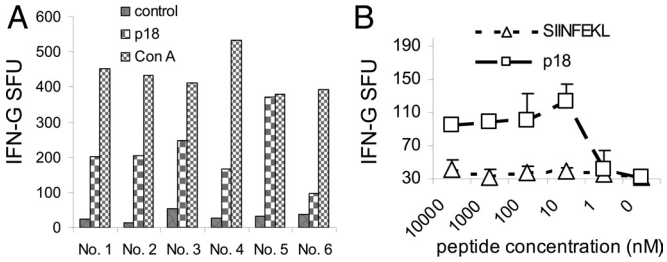

Fig. 2. CTL responses to epitopes from the F9 ARF1. (*A*) IFN-γ ELISPOT assay using spleen cells of B0702 mice immunized with the p18 peptide from the F9 ARF1. Bone marrow-derived DCs were pulsed with the p18 peptide at 10 μg/mL and were injected into B0702 mice 3 times at weekly intervals. Ten days after the last immunization, spleen cells were harvested and analyzed for IFN-γ by an ELISPOT assay. (*B*) Peptide dose–response assay. Spleen cells from B0702 mice immunized with p18 peptide-pulsed DCs or the SIINFEKL control (as described above) were incubated with the indicated concentration of p18 peptide, and the frequency of IFN-γ-secreting T cells was determined by ELISPOT.

to immunize B0702 Tg mice, and an IFN-γ ELISPOT assay was performed to detect peptide-specific CTL reactivity. Ten days after the last DC immunization, spleen cells were harvested and stimulated with individual peptides. As shown in Table 1, based on IFN-γ ELISPOT data, peptide p18 APVLRDIDL induced a stronger CTL response than p31 SPVNQQCHF [180 ± 43 spot-forming units (SFUs) per 1×10^6 cells vs. 155 ± 11 SFUs per 1×10^6 cells, respectively), whereas no significant response was elicited with p52 SPVLRLFFL compared with the control peptide OVA SIINFKEL (100 ± 13 SFUs per 1×10^6 cells vs. 98 ± 10 SFUs per 1×10^6 cells, respectively). A robust p18-specific response was consistently detected in individual B0702 Tg mice vaccinated with p18 but not the control peptide (range, 181–364 SFUs per 1×10^6 cells vs. 23–48 SFUs per 1×10^6 cells, respectively; $P < 0.01$; Fig. 2*A*). The dose–response curve was consistent with data from the peptide-binding assay, in that peptide p18 has a high relative affinity for the MHC B0702 molecule and elicits responses to a peptide concentration even as low as 10 nM (Fig. 2*B*). Intracellular IFN-γ expression by $CD8^+$ T cells was also assessed via flow cytometry. In the presence of the p18 peptide, the frequency of $CD8^+$ T cells expressing IFN-γ was 4-fold higher than the control (Fig. S7).

The results of the peptide immunization study indicated that epitopes from the ARF1 of F9 cDNA, particularly p18, induced a CTL response. To confirm results from the ELISPOT assay described above, we carried out an in vivo CTL assay; strong in vivo CTL cytotoxicity was detected in B0702 Tg mice immunized with p18 peptide-pulsed DCs: 42–69% of p18-specific CTL-mediated killing in vivo was observed (Fig. S8).

An Epitope-Specific CTL Response Is Elicited by AAV2/F9 Gene Delivery. Among 3 test peptides from the F9 ARF1, p18 induced a stronger CTL response than the p31 and p52 peptides. Epitopes generated from gene expression are affected by several factors, such as the level of protein expression, the efficiency of antigen degradation, and processing. To determine whether peptides from the ARF1 of the F9 cDNA could be processed and presented during AAV2-mediated transduction, 2×10^{11} particles of AAV2/ F9 were injected i.p. into B0702 Tg mice. Thirty days after AAV2/ F9 administration, F9 was not detected in the serum; however, a high titer of F9 inhibitor—the antibodies that either inhibit the activity or increase the clearance of a clotting factor IX—was noted, indicating humoral immunity specific to the transgene. Furthermore, consistent with peptide immunization results, a significant increase of IFN-γ-secreting T cells in response to p18 was observed in AAV2/F9-treated B0702 Tg mice (119 ± 45 SFUs per 1×10^6 cells). No significant increase in the frequency of IFN-γ-secreting T cells was detected for p31, p52, or the negative control OVA peptide (46 ± 21 SFUs per 1×10^6 cells, 32 ± 11 SFUs per 1×10^6 cells, and 37 ± 8 SFUs per 1×10^6 cells, respectively; Fig. 3*A*). Consistent with the ELISPOT data, in vivo CTL cytotoxicity for p18-pulsed target cells was also increased, ranging from 27.4% to 42.5% killing (Fig. 3*B*). Our data confirm that an epitope (p18) derived from the F9 ARF protein is processed and presented to form a peptide–HLA-B0702 complex and elicit CTL reactivity.

To further confirm the direct killing of AAV2/F9-transduced cells mediated by p18-specific CTLs in vivo, B0702 transgenic mice were immunized with p18-pulsed DCs 3 times weekly. Ten days after the last immunization, 1×10^{11} particles of AAV2/F9 vectors were administered via tail vein injection. F9 expression was detected at the indicated time points after the AAV2/F9 injection. As shown in Fig. 3*C*, the F9 level in the blood was about 50% lower in p18-immunized mice compared with control mice at week 6 ($P < 0.05$). F9 inhibitor was not detected in the blood

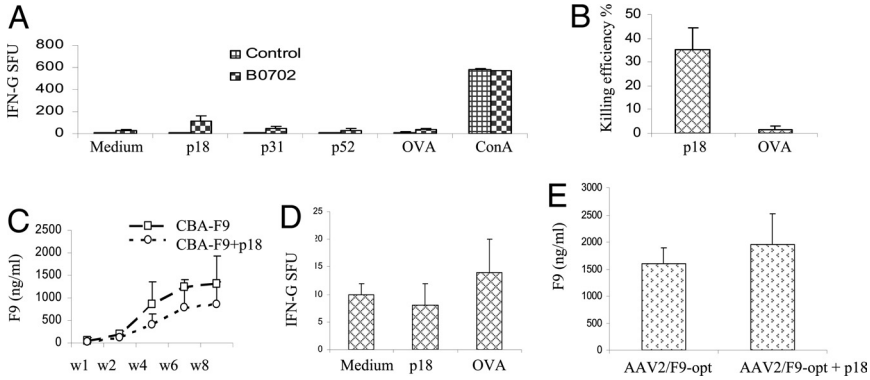

Fig. 3. A CTL response to the F9 ARF epitopes after AAV2/F9 delivery. (*A*) Detection of CTL responses in B0702 mice injected with AAV2/ F9 vectors. A total of 2×10^{11} particles of AAV2/ F9 vectors were injected i.p. into B0702 mice. Thirty days later, spleen cells were harvested, and the frequency of IFN-γ-secreting T cells was measured via ELISPOT. (*B*) In vivo CTL assay (*SI Materials and Methods*) against p18-pulsed target cells in B0702 mice after administration of 2×10^{11} particles of AAV2/F9. (*C*) CTL-mediated killing of AAV2/F9-transduced cells. B0702 mice were immunized with p18-pulsed DCs as described above. Ten days after the last immunization, 1×10^{11} particles of AAV2/F9 were administered i.v. The F9 level in the blood was detected at the indicated time points (*SI Materials and Methods*). (*D*) Detection of CTL responses in B0702 mice injected with AAV2/F9-opt particles. B0702 mice received i.p. 2×10^{11} particles of AAV2/F9-opt vector. One month after the injection, the frequency of IFN-γ-secreting T cells in the spleen was measured via ELISPOT. (*E*) CTL-mediated killing of AAV2/F9-opt-transduced cells in vivo. A total of 1×10^{11} particles of AAV2/F9-opt vector were administered to p18-immunized B0702 mice. Six weeks later, blood was collected, and F9 was quantitated. The data represent the standard derivation from 4 mice.

of any mice, therefore ruling out a possible role of F9 inhibitors in neutralizing F9 protein in the blood. All these results support the hypothesis that p18-specific CTLs mediate the elimination of AAV/F9-transduced cells in vivo.

Mutation of the ARF1 Sequence Abrogates p18-Specific CTL Reactivity. Mutation of the ARF sequence in a manner which does not change the F9 protein sequence based on codon degeneration may reduce the immunogenicity of ARF1. In other work, we have generated a codon-optimized F9 construct (F9-opt), which induces higher F9 production in mice after AAV delivery compared with the wild-type F9 cDNA (15). Alignment of F9-opt and conventional F9 sequence demonstrated low homology for p18 peptide at both nucleotide and amino acid sequences (Table S2). To determine whether a CTL response against p18 was elicited from the F9-opt construct, we injected 2×10^{11} particles of AAV2/ F9-opt into B0702 mice i.p. As shown in Fig. 3*D*, no p18-specific CTLs were detected ($P > 0.05$ vs. control peptide). Additionally, no in vivo p18-specific CTL-mediated killing was observed. To examine whether p18-specific CTLs eliminated AAV2/F9-opt-transduced cells in vivo, 1×10^{11} particles of AAV2/F9-opt were administered i.v. to B0702 mice preimmunized with the p18 peptide. Six weeks after the AAV2/ F9-opt injection, the blood was analyzed for F9. No difference in F9 levels was observed between p18 peptide-immunized and nonimmunized mice ($P > 0.05$; Fig. 3*E*). These results further verified p18 peptide immunogenicity by demonstrating that mutation of the ARF-derived epitope, without changing the F9 protein sequence, decreases the immunogenicity of the delivered transgene.

Discussion

Gene therapy has successfully been implemented in clinical trials using vectors derived from naked DNA and viral vectors for diseases ranging from genetic defects to cancer. However, one of the major barriers to achieving stable gene expression is the development of innate and adaptive immune responses to the delivery vector and the therapeutic transgene (16, 17). Delivery with naked DNA or viral vectors, like adenovirus (Ad) vector, can trigger an innate immune response in addition to an immune response against the therapeutic product. Also, adaptive immune responses have been documented against viral vector capsids, such as Ad and AAV vectors.

A CTL response to the AAV2 capsid can be elicited through classical MHC-I antigen presentation or cross-presentation (3, 4). Sabatino et al. (3) used an Ad vector to deliver an AAV2 capsid transgene into mouse muscle, in which a capsid-specific CTL response was demonstrated. Also, several groups have demonstrated that immunizing mice by direct injection of AAV2 vectors into skeletal muscle or liver, or with AAV2-pulsed DCs, elicits a similar capsid-specific CTL response (4–7). A recent adverse event in a hemophilia gene therapy clinical trial has been attributed to a capsid-specific CTL response, resulting in the loss of AAV-transduced hepatocytes (2). However, our lab and others have demonstrated that capsid-specific CTLs are not able to clear AAV-transduced target cells in mice, regardless of the type of transgene, promoter, or route of vector administration (5–7). In addition, despite the lack of observation of a CTL response to the transgene (in the aforementioned clinical study), no direct evidence supporting the notion that the therapeutic failure was induced by AAV2 capsid-specific CTLs is available.

In the current study, we present an alternative mechanism for eliciting a CTL response against a therapeutic transgene through generation of cryptic epitopes. In particular, we demonstrate that the F9 cDNA contains ARFs, and the epitope from an ARF (ARF1) can be properly processed and presented on the cell surface to induce a CTL response with peptide immunization or after AAV2/F9 administration. These p18-specific CTLs had the capacity to inhibit transgene expression from AAV2/ F9-transduced target cells in B0702 transgenic mice.

Although most $CD8^+$ T cell epitopes originate from the degradation of newly synthesized protein, and such defective ribosomal products consist of polypeptides resulting from premature termination and misfolding nonfunction, studies have shown that epitopes can also be generated from cryptic translation products (9–11). The majority of cryptic epitopes were identified in tumor cells or after virus infection. Some cryptic epitopes from virus have been used as a vaccine using gene therapy delivery in preclinical studies (10, 11). In this work, we demonstrate that a cryptic epitope p18 from the F9 ARF1 induces CTL reactivity that, in turn, eliminates transgene-expressing liver cells. These results suggest that a CTL response specific for a F9 cryptic epitope offers an alternative explanation for the liver cell cytotoxicity observed in a single patient during the recent AAV/F9 clinical trial (2).

The mechanism for expressing cryptic epitopes generated from cDNA during gene vector delivery remains to be elucidated. One possible explanation is that the endogenous genetic loci contain several *cis* elements to regulate gene expression, including specific promoter/enhancer, 5′ or 3′ untranslated regions, and introns that are not present in synthetic cDNA gene cassette. However, in the case of gene therapy, the therapeutic constructs typically contain cDNAs driven by chimeric (apolipoprotein E enhancer and antitrypsin promoter) or constitutive promoters (CBA, CMV) embedded between *cis*-acting viral elements (AAT TR, HIV LTR, Ad ITR). Such constructs may raise the possibility of cryptic epitope expression resulting in the generation of a CTL response. Considering AAV vectors, the inverted terminal repeats have been shown previously to contain non-TATA-like promoter activity, in addition to chimeric liver-specific expression, adding another layer of complexity to transgene expression in this context (18, 19). As a result, an otherwise normal cDNA cassette functions in the context of unrelated *cis* elements in the milieu of disease organ.

There are many strategies to eliminate epitopes generated from ARFs to avoid unwanted immune responses without compromising the expression and function of the transgene product. For example, the ARF start codon can be silently mutated or a stop codon introduced upstream of the cryptic epitope. In this study, mutation of the ARF sequence based on codon degeneration of the transgene provides another approach to avoid CTL reactivity elicited from the ARF.

We have only analyzed epitope p18 presented by the HLA-B0702 haplotype to induce a CTL response; however, cryptic epitopes arise because of both transcriptional and translation events, such as non-AUG translation initiation (20). Therefore, it is likely that the repertoire of cryptic epitopes from F9 cDNA is much broader (Fig. S1), also involving other MHC molecules. Although these cryptic epitopes may not be common, the finding that a CTL response can be elicited by cryptic epitopes from a clinically approved transgene has significant implications for both viral and nonviral gene therapy approaches. Unforeseen epitope generation should be considered when the immune response to a transgene product or vector does not explain the clinical manifestations. Additionally, careful analysis of transgene sequences should be performed, as in this work, to reduce the potential immune consequences to cryptic epitopes for gene therapy applications.

Materials and Methods

Cells and Virus. B3Z and Cos1/K^b cells were generously provided by N. Shastri (University of California, Berkeley, CA). The B3Z cell is a $CD8^+$ T cell hybridoma that expresses LacZ in response to SIINFEKL peptide (OVA-immunodominant peptide) stimulation in the context of H-$2K^b$ (13). Cos1/K^b cells express the mouse H-$2K^b$ gene and are used as antigen-presenting cells to present the OVA–K^b complex on the cell surface.

AAV virus production was described previously (21). The virus titer was determined by Southern dot blotting.

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. B0702 transgenic mice contain the human HLA-B0702 allele (14). The OT-1 mouse is transgenic for a T cell receptor that recognizes the SIINFEKL peptide of OVA bound by H-2K^b (Taconic Farms). All mice were maintained in a specific pathogen-free facility at the University of North Carolina, Chapel Hill. The University of North Carolina Institutional Animal Care and Use Committee approved all procedures.

Construction of Recombinant Plasmids. PCR was used to introduce the OVA epitope SIINFEKL DNA sequence (agtataatcaactttgaaaaactg) into the ORF and ARF1 of F9 cDNA (defined as RF0: F9ORF0, RF1: F9ORF+1). Different pairs of primers (Table S3) were used to amplify F9 by using pCBA-F9 as a template. The F9 PCR products were purified and digested with EcoRI and ClaI, and then cloned into pCBA-F9 digested by EcoRI and ClaI to generate pCBA-F9/OVA-RF0 and pCBA-F9/OVA-RF1. All constructs were verified by sequencing.

Peptides. Peptides p18 (F9 ARF1 18–26), p31 (F9 ARF1 31–39), p52 (F9 ARF1 52–60), and SIINFEKL (OVA 257–264) were synthesized in the University of North Carolina, Chapel Hill Microprotein Sequencing & Peptide Synthesis Facility and were >98% pure. Peptides were dissolved in DMSO at a concentration of 20 mg/mL and stored at −20 °C.

Immunization. For immunization, DCs were generated as described previously (5). Mature DCs (1×10^6 per milliliter) were pulsed with a mixture of peptides (p18, p31, and p52) or only with p18 at 10 μg/mL for each peptide for 2 h at 37 °C. A total of 5×10^5 DCs were then injected into B0702 mice via tail vein 3 times at weekly intervals (22). Harvested spleen cells served for the in vitro ELISPOT assay.

For AAV2 immunization, 2×10^{11} particles of AAV2/F9-OVA or AAV2/CBA-F9 were i.p. injected into C57BL/6 or B0702 mice, respectively. Thirty days after the injection, in vitro ELISPOT or tetramer staining for spleen cells or in vivo CTL assays were carried out (*SI Materials and Methods*).

Statistical Analysis. The Student's *t* test was used to perform all statistical analyses.

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