Cellular Immunity in Recombinant Adeno-Associated Virus Vector Mediated Gene Therapy

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Abstract

Recombinant adeno-associated virus (rAAV) is a promising gene therapy vector that mediates effective in vivo gene delivery. Only recently has the field of gene therapy begun to appreciate that one of the major challenges to the success of rAAV-mediated gene correction is the host immune response. Not only is the rAAV capsid protein a potential target of immunity, the therapeutic protein encoded by the transgene may also be recognized as non-self. The goal of studies reported herein is twofold: (i) to elucidate the mechanisms of antigen presentation relevant to the priming and maintenance of CD8+ T cells specific to the vector capsid and transgene-encoded proteins in murine models; and (ii) to provide insight into the function, kinetics, and fate of these CD8+ T cells, particularly those that infiltrated vector-transduced muscle, in mice and non-human primates.

We have demonstrated that dendritic cells are required to prime CD8+ T cells specific to the vector capsid and the transgene-encoded protein. Priming of capsid-specific CD8+ T cells was not sustained when the antigen was not encoded by the vector-delivered transgene. On the other hand, a transgene antigen-specific CD8+ T-cell response was perpetuated through cross-priming by dendritic cells that continuously acquired the transgene-encoded proteins from vector-transduced muscle.
Transient infiltration of fully functional capsid-specific CD8$^+$ T cells in vector-transduced muscle failed to eliminate transgene-expressing myocytes. This is because transduced myocytes, at least in mice, were unable to present capsid epitopes in the context of class I MHC (major histocompatibility complex) for CD8$^+$ T-cell recognition. However, whether capsid can be processed for class I presentation by vector-transduced target cells in higher order species with diverse MHC haplotypes remains unknown. Thus, to minimize the risks of target-cell killing by capsid-specific CD8$^+$ T cells, we tested a novel approach capable of permanently silencing functional capsid-specific CD8$^+$ T cells in the transduced tissue through sustained expression of class I capsid epitopes from the transgene.

Our investigation of the long-lasting CD8$^+$ T cells specific to the transgene-encoded proteins in non-human primates revealed that they have a phenotype resembling effector cells, but are functionally comparable to memory cells. These CD8$^+$ T cells, therefore, presented potential risks in elimination of transgene-expressing cells.

In summary, we documented in chapter 2 that dendritic cells were required for priming and maintenance of CD8$^+$ T cells specific to the rAAV vector-delivered antigens. Our findings suggest that CD8$^+$ T cells targeting the vector capsid and the transgene-encoded protein have the potential to limit persistent transgene expression. The capsid-specific T-cell response is perhaps manageable, if it is silenced by approaches described in chapter 3 of this dissertation. The transgene antigen-specific CD8$^+$ T cells were fully functional, at
least in non-human primates, as detailed in chapter 4. But their impact on long-term transgene expression is not fully understood. Risks should be further evaluated, especially in clinical trials where significant sequence differences exist between the therapeutic and mutant host genes.
Dedication

To the countless teachers and mentors
who have nurtured and believed in me
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Chapter 1

Introduction

The concept of gene therapy

The advent of recombinant DNA technology heralded a new era of gene-based therapeutics for diseases, an approach now commonly known as gene therapy. It is broadly defined as introduction of healthy copies of genetic material (transgene) into faulty cells for the purpose of correcting a pathological process or outcome. This technology can be used to treat hereditary and acquired diseases. The therapeutic genetic material is not restricted to coding regions of DNA (1), but also non-coding sequences such as microRNA (2-5). Depending on the nature of genetic defects, diseases might be corrected by addition, deletion, and alteration of gene expression. The transgenes can be delivered to target cells using a variety of methods, including naked plasmid DNA, viral vectors, bacterial vectors, yeast vectors, and liposomes (1). The most prevalent approach is to use viral vectors.
Viral vectors can be administered directly to the target tissue of patients, or introduced ex vivo to autologous cells derived from the subject. The latter approach was used in the first gene therapy clinical trial in 1989, where a neomycin-resistant gene was introduced to patient lymphocytes ex vivo using a retrovirus. These manipulated cells, which had the ability to infiltrate tumors, were then reinfused back to the circulation (6). This study demonstrated for the first time the feasibility of transducing human cells with a viral vector and sparked hope for future studies. Shortly thereafter, in 1990, the first clinical trial to treat a genetic disorder, severe combined immune deficiency, was performed in subjects with adenosine deaminase deficiency (7). This trial set the stage for continuous expansion of gene therapy trials in the 1990s. However, later in the decade, an unexpected death caused by an overwhelming immune response to the gene therapy vector derived from an adenovirus (8), and a case reporting retroviral insertional oncogenesis (9), slowed the advancement of gene therapy. Since then, numerous studies have focused on the development of much safer viral vectors without compromising the efficacy of transgene delivery and expression. Gene therapy, therefore, continues to represent a new frontier in disease treatment despite the early setbacks. As of the year 2011, more than 1,500 gene therapy clinical trials have been completed or are currently ongoing (1).

**Recombinant adeno-associated virus (rAAV) vector**

Amongst all the viral vectors for gene therapy, recombinant adeno-associated virus (rAAV) vector has gained favor in recent years. It is derived from adeno-associated virus
(AAV), which was serendipitously discovered in 1965 as a contaminant of adenovirus (Ad) preparations (10, 11). Wild-type AAV is a small, single-stranded DNA virus that infects approximately 80 percent of the human population (12). The genome of AAV is composed of a linear, single-stranded DNA with 2 open reading frames, Rep and Cap, flanked by an inverted terminal repeat (ITR) on each end. The Rep gene encodes for 4 replication proteins and the Cap gene encodes for 3 viral capsid proteins. Virus uptake by infected cells is through clathrin-mediated endocytosis (13, 14). Internalization is followed by acidification of the endosome, facilitating the release of the viral genome. Access to the host cell nucleus by the AAV genome, though not fully understood, requires a help virus, such as adenovirus and herpes simplex virus, to penetrate the nuclear membrane. AAV replication can then begin (15). AAV is capable of infecting both dividing and non-dividing cells and not associated with any human disease.

The safety features and broad tropism for mammalian cells have quickly made AAV an attractive gene therapy vector. The process for packaging recombinant AAV vectors involves replacement of both viral open reading frames with the therapeutic expression cassette that contains the gene of interest, limiting the transgene size to less than 4.7kb. This leaves the viral inverted terminal repeats (ITRs) as the only viral sequences that remain in the rAAV vector genome (16). Importantly, high throughput production of rAAV vectors can be easily achieved in cell culture systems with a negligible amount of contaminating wild-type virions that are capable of continuously synthesizing viral proteins in transduced host cells (17-19).
rAAV vectors can be pseudotyped with 12 different capsid serotypes and more than 120 subtypes (20-28). Improved vectors have been engineered by experimental modifications of the capsid sequence with site-specific mutagenesis (29-31). Other approaches have also been tested to make novel capsids, including DNA shuffling and library selection of AAV evolutionary modifications (32-36), and creation of recombinant hybrid capsids with mixed serotypes (37, 38). Variations in capsid sequence and structure can greatly broaden the host cell range permissive for rAAV vectors. Upon target-cell entry, rAAV vector-encoded transgenes undergo host cell-mediated synthesis of the complementary DNA strand (12). This second-strand synthesis has been demonstrated to be a rate-limiting step for efficient vector transduction (39). Bypassing the second-strand synthesis by packaging a double-stranded transgene as an inverted repeat of self-complementary DNA molecule can greatly accelerate transgene expression and achieve similar levels of protein production as single-stranded vectors at a much lower vector dose (40, 41). The major drawback of self-complementary rAAV vectors (scAAV) is packaging capacity. Only transgenes that are less than 2.1kb can be packaged in the viral capsid as scAAV vectors (40). The newly synthesized, duplex DNA mostly remains as episome that very rarely integrates into the host genome, reducing the chances of insertional mutagenesis (42, 43).
Persistent expression of transgene-encoded proteins delivered by rAAV vectors

Encouraging results, published in the late 1990s, documenting long-term expression of non-self antigens encoded by rAAV-delivered transgenes in mice, provided initial evidence in support of rAAV-mediated gene therapy (44-46). Model antigens that are completely foreign, such as β-galatosidase (β-gal), established high-level expression in transduced skeletal muscle beyond 1.5 years post-vector delivery in the absence of robust cellular immunity (45). Only transient and minimal lymphocyte infiltration was observed in the vector-transduced muscle. Examination of the muscle section stained with haematoxylin and eosin (H&E) revealed very mild lymphocyte infiltrate on day 4 post vector-delivery, but was self-limiting as it became undetectable 2 months later. The cellular infiltration had no effect on the stability of transgene expression. Further analysis of lymphocytes harvested from secondary lymphoid organs showed no activation of cytotoxic T cells targeting the transgene-encoded protein (46). Thus, the initial observation of transgene stability was proposed to have been facilitated by an apparent lack of immunogenicity to the transgene-encoded protein, although the detailed mechanism remains elusive.

Reports of long-term expression were further expanded to transgenes that encode human orthologues with clinical relevance, including erythropoietin (Epo) and β-glucuronidase. Both systemic and intramuscular deliveries of rAAV-Epo vectors to mice resulted in detection of erythropoietin at a level within therapeutic range. Concomitant elevation of hematocrit was demonstrated as a measure of therapeutic outcome (44). Similarly,
persistent cytosolic expression of human β-glucuronidase, a lysosomal storage enzyme deficient in patients with mucopolysaccharidosis VII, was also evident in this small animal model (46). Since then, studies using pre-clinical animal models continued to document stable expression of rAAV-deliveried self and foreign transgenes in tissues as diverse as muscle, liver, lung, central nervous system, and eye (47-53). These reports extended beyond inbred mice into higher order species, such as dogs and non-human primates (47, 54-57). These pre-clinical studies using animal models thus laid groundwork for translation of rAAV vectors-mediated transgene delivery from benchtop to bedside.

One of the most successful clinical trials was to treat a retinal degenerative disorder, Leber’s congenital amaurosis, by retinal infusion of an rAAV vector encoding the RPE65 transgene (58-61). The Phase I trial reported a marked improvement in measurable visual parameters. Partial restoration of visual function following vector treatment was encouraging, and lasted for at least 3 years. Immune responses to the vector capsid and transgene-encoded proteins were minimal, which may have facilitated stable expression of the therapeutic RPE65, in part because the targeted tissue is thought to be protected by immune privilege. More importantly, the photoreceptors affected by the RPE65-gene encoded mutant enzyme were physiologically preserved in the treated patients, providing a basis for the success of gene replacement treatment by replenishing the missing enzyme. This suggested that rAAV-mediated gene therapy must be provided before or soon after disease onset to ensure preservation of appropriate cell targets that may be lost
as the disease progresses. Therefore, similar success may not be easily achieved when irreversible damage occurs to the vital components or structures that are required for functional restoration of the affected tissues.

Indeed, despite a record of success in animal models and a limited number of clinical trials focusing on immune privileged sites, rAAV-mediated gene therapy has met challenges previously unrecognized. As studies continue progressing toward human treatments, host immune responses have become one of the most significant barriers to the success of this viral vector-based therapy. It became apparent that both the transgene-encoded protein and the vector capsid are viable targets for cellular and humoral immune responses. On the one hand, the therapeutic protein encoded by the transgene may represent a neoantigen owing to the type of mutation responsible for the disease phenotype. For example, null mutations and frameshift mutations that introduce a premature stop codon would render patients susceptible to recognition of the transgene-encoded protein as non-self. On the other hand, the viral capsid, though unable to replicate in vector-transduced cells, remains a primary antigen to both AAV-naïve and AAV-experienced hosts. In particular, the latter host is capable of generating a recall response upon re-encounter with the AAV capsid through vector delivery years after the initial exposure to the wild-type virus.

The immune barrier as a major hurdle has been increasingly recognized through clinical trials that delivered rAAV vectors to two major organs, the skeletal muscle and liver.
These organs are of special interest to rAAV-mediated gene therapy because they are easily accessed for vector delivery, highly transducible by rAAV vectors, capable of facilitating stable transgene expression, and considered gene factories that secrete functional proteins into the circulation. Clinical trials using rAAV vectors to deliver transgenes encoding therapeutic proteins that included lipoprotein lipase (62, 63), α-1 antitrypsin (64, 65), coagulation factor IX (66-68), and dystrophin (69) have achieved limited success. These clinical trials failed to reach the most important goal of establishing sustained expression of transgene-encoded proteins at or beyond therapeutic levels.

Reasons for attenuated expression of these transgenes are being investigated yet still remain unclear. Cellular immunity is very likely to have contributed to the loss of transgene expression judging from the fact that i) transgene expression was short lived in a number of clinical trials, and ii) the gradual decline in the measurable level of transgene-encoded proteins was accompanied by a rise of T cells targeting the therapeutic transgene protein and/or the vector capsid (62, 67, 69, 70). Failure to extrapolate findings from experimental animals to clinical settings prompted researchers to investigate mechanisms that enabled transgene stability in the animals. Adaptable of these mechanisms for human treatment may improve the success of rAAV-mediated gene therapy. Several questions regarding the potential immunotoxicity to rAAV vectors have been raised and investigated. Do rAAV vectors blunt T-cell responses entirely? Do vectors elicit T-cell tolerance, anergy, or deletion? Is it possible that T-cell priming
occurred but was actively suppressed? Can vector-transduced cells process rAAV-delivered antigens for MHC (major histocompatibility complex) presentation so that they are recognizable by cytotoxic T cells? Are sufficient innate immune signals elicited upon delivery of rAAV vectors?

Over the years, a number of explanations have been proposed, conflicting findings have been presented, yet many of these questions remain uncertain. Nevertheless, the field of gene therapy has made profound advances in understanding the interaction between the rAAV vector and the host immune response. Numerous confounding factors that could potentially influence the outcome of transgene persistence have been identified. For example, vector doses, types of target tissue, routes of vector administration, host species, capsid serotypes, pre-existing immunity to the vector, and basal levels of inflammation unique to the target tissue. Experimental animals as valuable pre-clinical models provided less complicated settings than humans to address the importance of these factors in the success of gene therapy, and have proven to be indispensable. The remainder of this chapter provides a summary of the current understanding of four major aspects of immune responses relevant to transgene persistence:

1. The innate immunity elicited by rAAV vectors.
2. Presentation of rAAV vector-delivered antigens in the context of MHC by transduced cells
3. Cellular immune responses targeting transgene-encoded proteins
4. Cellular immune responses targeting the vector capsid
Innate immune responses elicited by rAAV vectors

Effective priming of cellular immunity may be regulated by innate immune signals triggered by rAAV vectors. rAAV vectors have been shown in a very limited number of studies to poorly induce innate immune responses (71, 72). This may have a negative impact on priming of effective cellular immune responses because innate signals are critical for antigen presentation, immune cell recruitment to the site of infection, and release of pro-inflammatory cytokines (73). rAAV vectors have been shown to elicit very low levels of inflammatory cytokines and chemokines produced by mouse hepatocytes in vivo, such as IL-6, TNF-α, RANTES, and MIP-1β, when compared with recombinant adenovirus vectors (71). Moreover, production of these cytokines was rather transient and limited to less than 6 hours post vector delivery.

Microarray analysis of RNA isolated from liver of mice immunized with rAAV vectors encoding coagulation factor IX suggested reduced activation of type I IFN-dependent genes compared with recombinant adenovirus vectors (74). Nevertheless, rAAV vectors were able to activate plasmacytoid dendritic cells to induce production of type I IFNs in vivo through the TLR9-MyD88 pathway (75). In knockout mice that lacked either the TLR9 or MyD88 genes, CD8+ T cells targeting rAAV vectors were poorly activated compared with wild-type mice. Transgene expression was prolonged in the TLR9/MyD88 deficient mice. Although the detailed mechanism of how encapsidated transgenes were exposed in the endosome for TLR9 recognition remains elusive,
signaling through this pathway is a demonstrated requirement for activation of CD8\(^+\) T cells to both the transgene-encoded proteins and the vector capsid. Moreover, TLR-9-dependent induction of the innate immune response was enhanced by self-complementary transgenes, suggesting that genome configuration may influence the inflammatory responses to rAAV vectors (76). Enhancement of innate immune responses may facilitate priming of a more robust T-cell response, resulting in transgene clearance. A murine study of liver-targeted rAAV delivery provided direct evidence supporting this hypothesis. Transgene expression in hepatocytes was cleared by CD8\(^+\) T cells through administration of TLR agonists (LPS and CpG) to mice that otherwise persistently expressed the transgene (77, 78)

With robust activation of the innate immune responses, CD8\(^+\) T cells specific to rAAV vector-delivered antigens may present the greatest risk to persistent transgene expression. CD8\(^+\) T cells are specialized in target-cell killing through recognition of antigen-derived class I epitopes that are displayed on the surface of host cells (73). rAAV vector-transduced cells have the potential to process vector-delivered antigens for class I presentation. Therefore, understanding antigen presentation of the vector capsid and the transgene-encoded foreign protein is critical to the evaluation of potentially destructive CD8\(^+\) T cells.
**Cellular presentation of rAAV vector-encoded antigens**

Initial studies suggested that MHC class I antigen presentation of rAAV-encoded proteins is inefficient at priming functional CD8+ T cells. Adoptive transfer of rAAV-pulsed dendritic cells to mice that were subsequently treated with rAAV vectors failed to facilitate transgene clearance (79). This was interpreted as evidence that class I presentation of transgene-encoded antigens by dendritic cells was too inefficient to prime functional CD8+ T cells. Indeed, transgene expression was barely detectable in dendritic cells, consistent with the finding that dendritic cells may not be permissive to transduction, at least with some AAV serotypes (71, 79-81). In contrast, rAd vectors transduced dendritic cells much more efficiently and induced synthesis of transgene-encoded proteins at high levels. Clearance of transgene expression in rAd-treated animals has been associated with effective direct presentation of transgene-encoded epitopes by these dendritic cells (79). Taken together, failure to transduce dendritic cells by some rAAV vector serotypes was proposed as an explanation for lack of CD8+ T-cell immunity to transgene-encoded proteins in some animal studies (82).

Based on the hypothesis that inefficient transduction of dendritic cells limits activation of CD8+ T cells specific for transgene-encoded proteins, it was further proposed that tissue-specific promoters might restrict transgene-expression to non-professional antigen presenting cells and minimize an unwanted CD8+ T-cell response (83). If dendritic cells are unable to synthesize transgene-encoded proteins due to promoter incompatibility, efficient class I presentation of epitopes derived from the transgene-encoded proteins
may be prevented. Consistent with this idea, AAV capsid serotypes that facilitate vector transduction of dendritic cells were also suggested to be less ideal for gene therapy. rAAV vectors with serotype 5 capsid encoding HIV envelope glycoprotein 160 were shown to induce robust HIV-specific T-cell responses compared with other commonly used capsid serotypes (84). This finding was interpreted as the result of enhanced transduction of dendritic cells by rAAV5 vectors. The serotype 8 rAAV vector, in contrast, was considered a much safer candidate for gene delivery as its capsid structure and cell-binding domains do not favor dendritic cell entry, resulting in minimal transduction of dendritic cells and perhaps impaired immunity (85-87).

In chapter 2, we provide evidence that challenges the commonly accepted misconception of the requirement for T-cell priming. We elucidate the mechanism of antigen presentation relevant, not only to the priming, but also to the maintenance of CD8$^+$ T cells specific to the vector capsid and transgene-encoded proteins in murine models. Our findings have the potential to reshape strategies currently adopted by gene therapists to circumvent deleterious T-cell immunity to rAAV-delivered antigens.

**Cellular immune responses targeting transgene-encoded proteins**

*De novo* priming of T-cell responses by transgene-encoded non-self proteins has only been recognized in recent years, in part because early studies in animals treated with rAAV vectors showed stable transgene expression, suggesting a lack of cellular immunity. Few studies have documented a detectable T cell population specific to rAAV-
encoded transgene protein, perhaps because the mouse strain (C57Bl/6) used in the earlier reports was different from the one (Balb/c) used in the studies described below, although the conclusive reason has not been empirically validated.

Two murine studies published in 2007 provided early evidence that functional impairment of these T cells was responsible for ignorance of persistent transgene expression (88, 89). In both studies, transgenes that encoded the HIV gag protein established stable expression in vector-transduced muscle. Gag-specific CD8+ T cells were primed but failed to proliferate in vivo or in vitro upon re-encounter with cognate antigen. When the rAAV-primed animals were challenged with rAd vectors expressing the same transgene, expansion and effector function of gag-specific CD8+ T cells was impaired, suggesting that they were exhausted. In addition, the frequency of gag-specific T cells following the rAd challenge was even lower than the peak frequency primed by the rAd-gag vector alone, suggesting that rAAV vectors not only primed exhausted T cells, but also actively suppressed functional T cells elicited by the rAd vectors.

Administration of adjuvant to promote Toll-like receptor (TLR) signaling did not restore the proliferative capacity of gag-specific CD8+ T cells that expressed markers of exhaustion, such as programmed cell-death 1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4) (89). Only a small proportion of the gag-specific CD8+ T cells were able to secrete multiple cytokines, indicating that a substantial number of the T cells were non-responsive. Similar findings were also reported in non-human primates where
intramuscular delivery of an rAAV8 vector induced functionally impaired T cells that failed to boost (85).

A recent report from our laboratory suggested that a different pathway might explain the failure of the CD8⁺ T cells specific for the transgene-encoded proteins to eliminate vector-transduced cells (90). T cells that infiltrated vector-transduced muscle, where the cognate antigen was continuously synthesized, were susceptible to programmed cell-death. Such an antigen-driven apoptosis was speculated to be associated with inadequate priming of early effector T cells that were susceptible to death signals in the transduced tissues upon infiltration.

In addition to functional impairment and antigen-driven apoptosis, priming of regulatory T cells (Treg) by rAAV vectors is an alternative mechanism to explain transgene tolerance (91). Treg are a subset of CD4⁺ T cells that are specialized for immune suppression. The majority of them are identified by constitutive expression of CD25 and upregulation of the transcription factor Foxp3 (92). Treg regulate CD4⁺ and CD8⁺ T cells through modulation of differentiation and effector functions, a process important to the negative control of excessive immune responses associated with pathogenic infection or cancer (93). Mechanisms of Treg-mediated immune suppression can be categorized into 4 groups, including secretion of immune-suppressive cytokines, regulation through metabolic signaling, releasing cytolytic molecules, and expression of inhibitory ligands/receptors.
Initial evidence suggesting induction of Treg by rAAV vectors was obtained in Treg-deficient mice (94). rAAV vaccination of these mice generated a CD4^+CD25^+ T-cell population characterized by expression of Foxp3 and CTLA-4, a T-cell inhibitory ligand. Additional evidence supported Treg-mediated suppression of CD8^+ T cells specific for transgene-encoded proteins. Adoptive transfer of Treg isolated from rAAV-treated donor mice to recipient mice that were pre-treated with rAd vectors encoding the same transgene inhibited the ability of CD8^+ T cells from the recipient mice to produce IFN-γ in response to transgene-encoded antigens (95).

CD8^+ T cells specific for the transgene-encoded proteins sometimes succeed in terminating transgene expression. Model antigens completely foreign to rAAV vector-treated animals have been documented to be cleared in a variety of species, from inbred mice to non-human primates (96-99). This is in direct contrast to the previous observation that transgene-encoded foreign proteins persist in the same animal models. The real reason that effectively explains this apparent discrepancy remains ambiguous. However, as eluded earlier in this chapter, many confounding factors may have played a role, such as vector doses, types of target tissue, routes of vector administration, and capsid serotypes. Common model antigens were used in these studies, including influenza virus hemagglutinin A (HA) (97), chicken ovalbumin (OVA) (99), enhanced green fluorescent protein (eGFP) (96), and α-galactosidase (α-gal) (98). These antigens were targeted to
different subcellular compartments such as the cytosol (96), plasma membrane (97), and secretory pathway (98, 99).

In the study where the transgene encoding influenza virus hemagglutinin A (HA) was delivered to skeletal muscle of mice by rAAV vectors, membrane expression of HA was not detected at 26 days post vector delivery (97). HA-specific CD4$^+$ and CD8$^+$ T cells were detected in draining lymph nodes soon after vector delivery. Evidence that supported T-cell mediated killing was prolonged transgene expression after administration of CD40L antagonist to block the interaction between T cells and antigen presenting cells. Antigen expression was also preserved by antibody-mediated depletion of CD4$^+$ T cells. This was one of the first reports that documented CD8$^+$ T cell-mediated transgene clearance through recognition of transgene-encoded non-self antigens in the presence of sufficient CD4$^+$ T-cell help.

Subsequently, expression of the secreted antigen chicken ovalbumin (OVA) encoded by rAAV vectors was also shown to be limited by T-cell immunity (99). Consistent with the HA study, clearance of transgene-encoded OVA was CD4$^+$ T-cell dependent. Only circumstantial evidence supported that transgene expression was cleared by CD8$^+$ T cells. Taken together, these 2 studies illustrated potential risks of T-cell mediated transgene clearance, especially when transgenes encode membrane-bound or secreted therapeutic proteins, such as dystrophin and coagulation factor IX.
Both of the studies described above delivered rAAV vectors to skeletal muscle of mice. But transgene clearance was not restricted to this route of vector delivery or this animal species. It has also been shown after systemic delivery of vector to liver of non-human primates (96). In this study, transgene-encoded eGFP expression was restricted to 37 days following vector delivery. Analyses of mononuclear cells harvested from liver, spleen and blood demonstrated that eGFP-specific CD8$^+$ T cells secreted multiple cytokines and degranulated upon antigen re-exposure. CD4$^+$ T cells capable of secreting IFN-γ, TNF-α and IL-2 were also detected, suggesting that sufficient help was provided to the CD8$^+$ T-cells. Importantly, rAAV capsid-specific T cells were not detected in these monkeys, consistent with a mouse study showing loss of cytosolic expression of α-galactosidase in the absence of capsid-specific immune responses (98).

In brief summary, these animal studies emphasized that transgene-encoded foreign proteins are sometimes immunogenic. Further investigation of immune responses against the transgene-encoded therapeutic proteins in humans or genetically related animals is critical to the development of successful gene therapy. In chapter 4, we detailed our findings on T-cell responses to the transgene-encoded protein that is completely foreign to the vector-treated host. We used non-human primates to address this question because this species is evolutionarily closer to humans than mice.

In addition to transgene-encoded proteins, the pre-formed vector capsid is also a viable target for immune recognition. In fact, the T-cell response specific for the capsid should
be accelerated when compared with the transgene-specific response because processing of the capsid is not limited by gene transcription and translation. Moreover, a significant percentage of the human population may have capsid-specific memory T cells primed by natural AAV infection. The memory T cells may be activated by rAAV capsid faster than naïve T cells.

**Cellular immune responses targeting the vector capsid**

A liver-directed rAAV clinical trial provided initial, albeit circumstantial, evidence that capsid-specific T cell responses may be associated with the loss of transgene expression in vector-transduced hepatocytes (67). In this study, a transgene that encoded the coagulation-promoting enzyme, factor IX, was delivered through the hepatic artery of patients with hemophilia B disorder. Among the 8 treated subjects, efficacy of the transgene-encoded factor IX was demonstrated in at least 1 subject treated with the highest vector dose, but therapeutic levels of the transgene-encoded factor IX were limited to 8 weeks. Loss of factor IX expression was temporally correlated with serum elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), suggestive of liver damage.

Evaluation of immune responses was not originally envisioned for this trial, so the protocol was modified to permit immune analysis of a subsequently treated subject. This subject received an intermediate dose of the rAAV vector and had no detectable factor IX expression. Nonetheless, a flare of ALT/AST occurred in the same time frame as the
high-dose subject. Analysis of peripheral blood mononuclear cells revealed a capsid-specific T-cell response that coincided with the kinetic of liver damage. Similar responses were not observed for the transgene-encoded wild-type factor IX, at least in part because the subjects selected for this trial carried mutations with minimal difference to the wild-type gene and thus exhibited some level of tolerance to the transgene product.

Based on the findings from the factor IX clinical trials, it was hypothesized that vector transduction of hepatocytes resulted in MHC class I presentation of rAAV capsid epitopes, a process termed cross-presentation. The hepatocytes displaying the capsid epitopes on the cell surface could then be recognized by capsid-specific cytotoxic CD8+ T cells for destruction. It was proposed that the effectors were re-activated memory T cells primed by prior natural AAV infection. Nearly 90 percent of humans are estimated to have been exposed to AAV and have developed immune memory against AAV capsid proteins (12). Pre-clinical animal studies failed to reveal an impact of capsid-specific T-cell responses on transgene expression, perhaps because those animals were not naturally infected with the virus. Although subsequent studies confirmed the existence of capsid-specific memory T-cell populations in adult humans (100), the memory T-cell hypothesis derived from this single case of rAAV-vector infusion to liver was not confirmed by further experimentation in humans or animal models. Long-term transgene expression can sometimes be established in humans even in the presence of detectable capsid-specific T cells, suggesting that T cell response to the capsid is not always associated with loss of transgene expression (64, 65).
In a phase I clinical trial of patients with alpha-1 antitrypsin (AAT) deficiency, delivery of rAAV vectors encoding a corrected copy of the mutant gene to muscle resulted in long-term transgene expression (65). Expression remained measurable for more than 1 year in 2 subjects despite the presence of capsid-specific CD4+ and CD8+ T cells in blood. Effector T cells were initially detected within 2 weeks of vector delivery and persisted for at least 3 months. Although the level of rAAV-encoded AAT in circulation was sub-therapeutic, the sustained nature of the foreign-transgene expression suggested an absence of immune-mediated elimination of vector-transduced myocytes by capsid-specific T cells. This was further supported by a lack of serum elevated creatine kinase (CK), a marker of muscle death.

In a follow-up phase II trial treating alpha-1 antitrypsin deficiency where the vector dose was increased, long-term transgene expression in muscle and the safety of rAAV delivery was once again demonstrated despite the detection of capsid-specific T-cell responses (101). No clinical symptoms were observed other than a transient creatine kinase elevation indicative of some muscle death. In addition to detectable T cells in the circulation, muscle biopsy revealed mononuclear cell infiltration of the vector-treated muscle. Immunohistochemical staining showed that a great proportion of the infiltrating cells were CD8+CD3+ lymphocytes. It is unknown whether the CD8+ T cells detected in the muscle biopsy were capsid-specific, as T cells targeting a single epitope of the transgene-encoded alpha-1 antitrypsin were also detected in blood.
Another human study delivering the lipoprotein lipase (LPL) gene to skeletal muscle of subjects with a missense mutation in the target gene also documented T-cell responses specific to the vector capsid, but not the transgene-encoded protein (62, 63). Nevertheless, the causal relationship between the capsid-specific response and transient transgene expression was not established as detectable capsid-specific CD8$^+$ T cells were absent in 2 of 4 subjects in the high-dose cohort.

Conflicting findings regarding the capsid-specific T cells and transgene expression have been reported in a number of animal studies using inbred mice and non-human primates. Mice immunized intramuscularly or intravenously with rAAV vectors generated circulating capsid-specific CD8$^+$ T cells that were visualized by class I MHC tetramers (102, 103). These T cells were functional judging by their ability to co-produce IFN-γ and TNF-α, efficient killing of cognate peptide-loaded syngeneic cells in vivo, and robust proliferation upon re-encounter with antigen. Fully functional capsid-specific T cells did not attenuate long-term expression of the transgene encoded by the rAAV vector, providing initial evidence that these T cells probably do not mediate destruction of transduced cells (102, 103). Corroborative evidence was obtained by pre-treating mice with recombinant adenovirus vectors encoding AAV capsid genes. This approach generated a pool of memory capsid-specific CD8$^+$ T cells before the delivery of rAAV vectors that were serologically distinct but retained a cross-reactive class I capsid epitope.
Levels of transgene expression encoded by the rAAV vector were not compromised in the presence of pre-existing T-cell immunity to the capsid (102, 103).

There is no satisfactory explanation for the lack of target-cell killing by capsid-specific CD8$^+$ T cells. Regulatory T cells that could potentially inhibit the response have been ruled out, as depletion of this T cell subset in mice failed to affect the longevity of the rAAV-encoded transgene expression (102). The inability of tranduced parenchymal cells, such as hepatocytes and myocytes, to present exogenously acquired capsid protein has also been suggested, but remains untested. In one study, where reduced expression of transgene-encoded factor IX was observed in mice pre-immunized with capsid peptide-loaded dendritic cells, partial killing of vector-transduced hepatocytes was restricted to those that co-synthesized the full-length AAV capsid and factor IX (81). The major conclusion from this study was that tranduced hepatocytes were incapable of cross-presenting pre-formed capsid epitopes that could render them targets of capsid-specific CD8$^+$ T cells. However, a subsequent study published by the same group suggested epitopes in pre-formed vector capsid can be processed for class I cross-presentation to CD8$^+$ T cells, if the epitope is highly immunogenic and positioned in different context within the capsid (104). This was achieved by expressing an immunodominant class I epitope SIINFEKL, derived from chicken ovalbumin, as a fusion capsid with genetic alteration. Delivery of this rAAV vector with chimeric capsid to mice vaccinated with SIINFEKL peptide-loaded dendritic cells resulted in diminished transgene expression.
Cross-presentation of a capsid epitope was also demonstrated by in vitro transduction of human cell lines (105). Immortalized human hepatocytes were co-cultured with rAAV vectors to facilitate capsid uptake. A soluble T-cell receptor specific for a class I MHC molecule loaded with a capsid epitope was used to probe for surface display of the epitope by transduced hepatocytes in culture. Although modest, the MHC-peptide complex was indeed detected on the plasma membrane of these hepatocytes and was suggested sufficient to flag the cells for destruction by capsid-specific CD8$^+$ T cells.

While the influence of capsid-specific T-cell responses on long-term gene expression is under investigation, it is prudent to design strategies to minimize the potential risks capsid-specific T cells. At least 2 strategies have been proposed, including therapeutic drug interventions (106) and re-design of the rAAV vectors (29, 30, 32-34). The most widely accepted strategy is a short course of drug-mediated immune suppression that is administered at the same time as rAAV vector delivery. The theory is that temporary blockade or dampening of the immune response in general could delay or even prevent activation and proliferation of capsid-specific T cells until the non-replicative vector capsid is degraded or turned over in transduced cells. Another strategy is to focus on engineering rAAV vectors. For instance, selection of capsid serotypes with low immunogenicity may minimize unwanted capsid-specific T-cell responses. Alternatively, a transgene cassette that facilitates high levels of protein expression at a low vector dose may also limit the T-cell response (41, 57, 107).
Serotype 8 capsid has been proposed to have low immunogenicity compared to other serotypes while retaining liver-tropism. This was supported by evidence from in vitro cell culture systems and in vivo animal studies (86-88, 108). rAAV8 vector did not transduce dendritic cells efficiently in cultured cells, in part because it lacked a heparin-binding domain (108). It has been suggested that dendritic-cell entry is heparin dependent. Thus it is likely that rAAV8 vector may not be effectively presented by professional antigen presenting cells that are critical for CD8\(^+\) T-cell priming. Generation of mutant AAV8 capsids that incorporated the heparin-binding domain through molecular cloning was shown to facilitate dendritic-cell entry (108), which was speculated to increase vector immunogenicity through efficient more class I presentation. The serotype 8 capsid, when delivered to mice as pre-formed proteins, failed to elicit capsid-specific T cells, suggesting that the exogenously acquired capsid may not be efficiently processed for class I presentation (103).

Additionally, high-resolution structural comparison of the serotype 8 capsid with a more immunogenic serotype, designated rh32.33, revealed regions on the capsid surface that may mediate differential receptor binding relevant to effective T cell priming (87). The most significant structural difference was mapped to variable loops close to the β-barrel core that is highly conserved throughout the common capsid serotypes. Hybrid capsids generated by mixing unique domains from different serotypes are being evaluated to produce a chimeric capsid with minimal T-cell priming potential.
Another approach to minimize capsid input without compromising transduction efficiency is to use self-complementary AAV (scAAV) vectors. A much lower dose of scAAV vector can be used to achieve the same level of transgene expression as from a single-stranded rAAV vector. Indeed, a pilot clinical trial showed for the first time that an optimized rAAV8 vector with self-complementary transgene encoding factor IX achieved a higher level of circulatory factor IX at a dose 100-fold lower than that of the single-stranded rAAV vectors (107).

Questions our studies address in the following chapters

In summary, deleterious CD8$^+$ T-cell responses to either the transgene-encoded protein or the vector capsid have been documented in animal models and humans. Whether vector-transduced target cells that were non-professional antigen presenting cells could prime these T cells is unknown. We investigated if transduced myocytes alone were sufficient to elicit CD8$^+$ T-cell responses specific to the vector capsid and the transgene-encoded protein using murine models. Furthermore, we explored the antigen presentation mechanism that maintained CD8$^+$ T cells specific for the transgene-encoded model antigens in the face of prolonged antigen expression. The results and interpretations are detailed in chapter 2.

Activation of CD8$^+$ T cells targeting the vector capsid has been reported in several clinical trials. In some cases, it was associated with loss of therapeutic efficacy. Published

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studies have focused on capsid-specific CD8\(^+\) T cells in blood and secondary lymphoid organs, but not the vector-transduced tissues. In chapter 3, we describe a detailed characterization of T-cell kinetic, function and fate in vector-transduced muscle. Moreover, we report a novel approach to silence these capsid-specific CD8\(^+\) T cells.

Transgene antigen-targeted T-cell responses have also been detected in the absence of persistent transgene expression in numerous rAAV vector-treated animals, and recently in a clinical trial treating Duchenne muscular dystrophy. It remains controversial whether CD8\(^+\) T cells specific for the transgene-encoded protein limit stable transgene expression. We therefore studied the differentiation, function and fate of these T cells in vector-transduced muscle. Non-human primates, which are genetically closer to humans than mice, were used to study whether the transgene antigen-specific CD8\(^+\) T cells are fully functional in muscle and capable of developing memory populations. Findings and in-depth discussions are provided in chapter 4.

In the final chapter, I summarize the findings, integrate the conclusions, and provide general interpretations. I also include future directions based on the results we have obtained to envision future contributions for the development of a much safer rAAV gene therapy vector.
Chapter 2

Continuous CD8$^+$ T-cell priming by dendritic cell cross-presentation of persistent antigen following gene therapy

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Running title: T-cell priming after gene therapy
Introduction

Delivery of recombinant adeno-associated virus (rAAV) gene therapy vectors to muscle can result in long-term expression of transgenes that encode non-self proteins (109). CD8\(^+\) T cells targeting these proteins are maintained at low frequency in spleen and draining lymph nodes of mice (88, 89), but continuously infiltrate transduced muscle where they undergo programmed death (90). Stable CD8\(^+\) T-cell frequencies can only be maintained if those that infiltrate and die in the muscle are replaced. Little is known, however, about mechanisms of class I antigen presentation and CD8\(^+\) T-cell priming after persistent transgene expression is established. Vector transduction and antigen synthesis by dendritic cells is thought to be required for CD8\(^+\) T-cell priming (79, 82, 84, 86). Because transduced dendritic cells have a limited life-span, and rAAV gene therapy vectors don’t replicate or spread to new cells, this mechanism cannot satisfy an apparent requirement for ongoing antigen presentation to CD8\(^+\) T cells. Here we demonstrate that vector-transduced dendritic cells were not involved in CD8\(^+\) T-cell priming. Instead, CD8\(^+\) T cells were primed by dendritic cells that acquired antigen from rAAV-transduced muscle cells.

Materials and Methods

Serotype 1 or 2 rAAV vectors that encode β-galactosidase (β-gal) (rAAV1\(_{β-gal}\) and rAAV2\(_{β-gal}\), respectively) were used to generate CD8\(^+\) T-cell immunity in mice as described previously (90). DNase-resistant rAAV\(_{β-gal}\) particles (5 X 10\(^{10}\)) were
administered to the quadriceps muscle of CB6F1 (H-2^{bkd}) mice. Three weeks later, the mice were irradiated (two doses of 6Gy separated by a 4 hour interval) and reconstituted with parental (H-2^b or H-2^d) bone marrow (110). Chimerism was then verified and cellular immune responses were analyzed by IFN-γ ELISpot assay (Figure 2.1A) (90). ELISA assays were used to quantify intramuscular β-gal levels and serum titers of antibodies specific to β-gal and rAAV capsid.

**Results and Discussion**

Evidence that dendritic cells cross-present antigens encoded by recombinant DNA or viral vaccine vectors was first obtained by examining patterns of CD8^+ T-cell priming in mice transplanted with bone marrow partially mismatched at H-2 class I loci (110-112) (113). We adapted this approach to determine if dendritic cells acquire antigen from rAAV-transduced muscle to continuously prime CD8^+ T cells. As shown in Figure 2.1A, rAAV1_β-gal or rAAV2_β-gal vectors were delivered to the quadriceps muscle of CB6F1 mice 3 weeks before lethal irradiation and reconstitution with parental Balb/c (H-2^d) or C57Bl/6 (H-2^b) bone marrow that was depleted of T lymphocytes to prevent graft-versus-host disease (Figure 2.1B). Transduced myocytes of CB6F1 recipient mice express H-2^b and H-2^d class I molecules, but bone marrow-derived mononuclear cells, including dendritic cells, should express only parental (H-2^b or H-2^d) class I molecules after successful bone marrow reconstitution. This was confirmed 28 days after transfer of Balb/c or C57Bl/6 bone marrow, when greater than 98 percent of mononuclear cells
stained with monoclonal antibodies directed against H-2\textsuperscript{d} or H-2\textsuperscript{b} class I molecules, respectively (Figure 2.1C, left and middle panels). Co-staining with both antibodies was observed after transfer of CB6F1 bone marrow (Figure 2.1C, right panel). Importantly, β-gal expression persisted in muscle through lethal irradiation and bone marrow reconstitution. As an example, β-gal protein was detected in the muscle of CB6F1 mice 63 days after vector delivery, which was 42 days after reconstitution with H-2\textsuperscript{b} bone marrow (Figure 2.1D).

Temporal separation between rAAV vector delivery to muscle and immune system reconstitution by bone marrow transfer (Figure 2.1A) was an important feature of the experiment design. It permitted a direct test of the hypothesis that CD8\textsuperscript{+} T-cell priming occurs continuously in animals with persistent transgene expression and does not depend on antigen-presentation by vector-transduced dendritic cells. CD8\textsuperscript{+} T-cell priming was assessed by IFN-γ ELISpot assay after stimulation of mononuclear cells with H-2L\textsuperscript{d} or H-2K\textsuperscript{b} restricted β-gal epitopes. In this model the H-2L\textsuperscript{d} epitope (β-gal\textsubscript{876-884}) was consistently more dominant than the H-2K\textsuperscript{b} epitope (β-gal\textsubscript{96-103}) (Figure 2.2A), but patterns of CD8\textsuperscript{+} T cell priming and class I restriction could nonetheless be mapped in CB6F1 mice reconstituted with H-2\textsuperscript{d} or H-2\textsuperscript{b} bone marrow. Our observations in mice treated with the rAAV\textsubscript{1}\textsubscript{β-gal} and rAAV\textsubscript{2}\textsubscript{β-gal} vectors were consistent with CD8\textsuperscript{+} T-cell priming by parental dendritic cells and not F1 cells such as myocytes. For instance, a response was detected against the H-2L\textsuperscript{d} restricted β-gal epitope, but not the H-2K\textsuperscript{b} restricted epitope, in F1 mice reconstituted with H-2\textsuperscript{d} bone marrow (Figure 2.2A). The
opposite pattern of epitope recognition was observed in animals that received the H-2\textsuperscript{b} bone marrow (Figure 2.2A). Mice that received autologous (H-2\textsuperscript{bxd}) bone marrow responded to both epitopes as expected (data not shown).

These results indicate that CD8\textsuperscript{+} T cells are primed after persistent antigen production is established with a rAAV vector, providing an explanation for how the response is maintained despite programmed death of effector populations that infiltrate transduced muscle (90). Failure to detect responses against both β-gal epitopes in mice reconstituted with H-2\textsuperscript{b} or H-2\textsuperscript{d} bone marrow excluded the possibility that transduced F1 (H-2\textsuperscript{bxd}) myocytes presented antigen directly to CD8\textsuperscript{+} T cells. For this reason antigen presentation by myocytes that lack the capacity for co-stimulatory signaling cannot explain a defective CD8\textsuperscript{+} T-cell response that failed to eliminate transduced myocytes. CD8\textsuperscript{+} T cells were primed even when vector delivery to muscle preceded reconstitution of the immune system by three weeks. It is very unlikely that parental dendritic cells were transduced with vector particles introduced into muscle 3 weeks earlier. In support of this argument, capsid-specific antibodies present at high titer in serum after vector delivery declined substantially following irradiation and immune reconstitution, suggesting an absence of capsid antigen to regenerate the response (Figure 2.2B, left panel). Antibodies to β-gal were maintained before and after immune reconstitution, reflecting the persistence of antigen expression in muscle (Figure 2.2B, right panel). Moreover, T-cell responses against two well-defined AAV capsid epitopes presented by H-2K\textsuperscript{b} (\textit{cap}702-710) and H-2L\textsuperscript{d} (\textit{cap}372-380) class I molecules were not detected in F1 mice after reconstitution with H-2\textsuperscript{b}.
or H-2\textsuperscript{d} bone marrow (Figure 2.2C). Capsid-specific CD8\textsuperscript{+} T cell responses were readily generated when an H-2\textsuperscript{d} immune system was transferred to F1 mice before vector delivery (Figure 2.2D and E). CD8\textsuperscript{+} T-cell responses were detected against the H-2\textsuperscript{d} but not H-2\textsuperscript{b} restricted β-gal (Figure 2.2E, left panel) and capsid (Figure 2.2E, right panel) epitopes. The opposite pattern of capsid-specific CD8\textsuperscript{+} T-cell reactivity was observed after H-2\textsuperscript{b} bone marrow reconstitution (data not shown). These results are consistent with a requirement for dendritic cells to generate a response to particle-associated capsid proteins.(87, 108)

Intramuscular delivery of rAAV1\textsubscript{β-gal} and rAAV2\textsubscript{β-gal} induced CD8\textsuperscript{+} T-cell responses, even though the latter vector is thought to transduce dendritic cells inefficiently.(81, 108) Indeed, direct transduction of dendritic cells by rAAV vectors was not required for T cell priming once persistent antigen expression was established in this model. The pattern of CD8\textsuperscript{+} T cell priming in bone-marrow reconstituted F1 mice is best explained by dendritic cell cross-presentation of antigen acquired from rAAV1\textsubscript{β-gal} and rAAV2\textsubscript{β-gal} transduced myocytes. Our observations also indicate that use of cell-type specific promoters that permit transgene expression in myocytes, but not dendritic cells, is unlikely to prevent CD8\textsuperscript{+} T-cell priming as proposed.(114) Finally, the model described here may be generally useful to identify the defects in antigen presentation that reinforce priming of an ineffective CD8\textsuperscript{+} T-cell response and may be important to successful gene therapy.
Figure 2.1 Vector treatment and immune reconstitution.

(A) Experimental timeline for vector treatment, bone marrow reconstitution and analysis of cellular immunity. (B) Flow cytometric analysis of parental bone-marrow cells before (top panels) and after (bottom panel) T cell depletion using a cocktail of anti-CD90.2, -CD4 and -CD8 antibodies with rabbit complement. Numbers represent the percentage of CD3 positive cells that co-expressed CD4 or CD8. Plots were gated on live (propidium iodide-negative) cells. (C) Flow cytometric analysis of peripheral mononuclear cells from CB6F1 mice that were reconstituted with bone-marrow cells from C57Bl/6 (left panel), Balb/c (middle panel) or CB6F1 (right panel) mice 4 weeks after lethal irradiation and bone marrow reconstitution at D49 as shown on the experimental timeline. Cells were stained with anti-mouse H-2^d and H-2^b antibodies. Numbers represent the percentage of cells of donor origin. (D) β-galactosidase was quantified in muscle by antigen-capture ELISA 63 days after rAAV1β-gal injection. Asterisk indicates that expression level was below the detection limit. Data represent at least 3 independent experiments.
Figure 2.2 Cellular and humoral immune responses in bone marrow-chimeric mice.

(A) IFN-γ ELISpot analysis of mononuclear cells isolated from draining lymph nodes of bone marrow-chimeric CB6F1 mice previously immunized with rAAV1β-gal (left panel) or rAAV2β-gal (right panel) vectors. Cells isolated from mice reconstituted with H-2b or H-2d bone marrow were stimulated in parallel with β-gal96-103 (H-2Kb-restricted, black bar) and β-gal876-884 (H-2Ld-restricted, grey bar) peptides. Asterisk indicates levels below the detection limit. (B) Antibody capture ELISA measuring serum levels of anti-capsid (left panel) and anti-β-gal (right panel) antibodies in rAAV2β-gal immunized CB6F1 mice before and after bone marrow reconstitution. Open bar represents pre-bone marrow reconstitution at D21 and hatched bar represents post-bone marrow reconstitution at D63. Error bars represent standard error of means. Statistical difference was calculated using
Student’s $t$ test. Asterisk represents $P=.001$ and NS represents $P=.706$, not reached statistical significance. (C) IFN-$\gamma$ ELISpot analysis of mononuclear cells isolated from draining lymph nodes of bone marrow-chimeric CB6F1 mice previously immunized with rAAV2$\beta$-gal vectors. Cell isolated from H-2$^b$-and H-2$^d$-bone marrow chimeras were stimulated with epitopic peptides derived from rAAV2 capsid, including rAAV2 cap$_{702-710}$ (H-2$^Kb$-restricted) and rAAV2 cap$_{372-380}$ (H-2$L^d$-restricted). Asterisk indicates levels below the detection limit. (D) Experimental timeline for testing capsid responses in animals reconstituted with bone marrow before vector treatment. (E) IFN-$\gamma$ ELISpot analysis of mononuclear cells isolated from draining lymph nodes of H-2$^d$-bone marrow-chimeric CB6F1 mice previously immunized with rAAV2$\beta$-gal vectors. Cells were stimulated with 4 epitopic peptides in parallel including $\beta$-gal$_{96-103}$ (H-2$K^b$-restricted), $\beta$-gal$_{878-884}$ (H-2$L^d$-restricted, grey bar), rAAV2cap$_{702-710}$ (H-2$K^b$-restricted, open bar) and rAAV2cap$_{372-380}$ (H-2$L^d$-restricted, hatched bar). Asterisk indicates levels below the detection limit. Data represent at least 3 independent experiments each with cells pooled from 6 mice.
Chapter 3

Transgene-sustained capsid expression silences rAAV-specific T-cell responses

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\textbf{Running title:} Sustained expression silences capsid-specific T-cell responses
**Introduction**

Cellular immunity has the potential to limit the success of gene replacement therapy with recombinant adeno-associated virus (rAAV) vectors. Vector transgene-encoded proteins, which are often foreign, represent one potential target of the cellular immune response. Cytotoxic CD8$^+$ T-cell responses against proteins expressed from the transgene are generated in mice and humans, and at least in the murine model they may be ineffective because they lose function and are susceptible to apoptosis upon infiltration of vector-transduced tissue (88, 89). Capsid-specific CD8$^+$ T cells also expand in mice and some humans treated with the vector even though this protein is not encoded by the transgene. In one human study of rAAV vector delivery to the liver, loss of transgene-encoded factor IX expression was temporally correlated with transient elevation of liver transaminases and the onset of a capsid-specific T-cell response (67). Expansion of capsid-specific CD8$^+$ T cells has also been described after rAAV vector delivery to skeletal muscle of humans, but no obvious impairment of transgene expression was described (64, 65). The impact of capsid-specific CD8$^+$ T cells on the outcome of gene therapy may be variable depending on many factors, including the AAV capsid serotype used for vector transduction, the organ and cell type targeted for gene delivery, and capacity of each host for class I processing and presentation of dominant epitopes derived from the capsid. Because the threat posed by capsid-specific CD8$^+$ T cells is probably transient, but as yet predictable, a brief course of immune suppression is being evaluated to increase success of gene replacement therapy.
Circumstantial evidence from cell culture models also support the concept that AAV capsids are processed for class I MHC presentation in transduced cells. For instance MHC class I molecules loaded with capsid epitopes have been visualized on the surface of a cultured human hepatocyte cell line that was transduced with an rAAV vector (100). Moreover, ubiquitination of AAV capsid proteins and improved transduction efficiencies in the presence of proteasome inhibitors in cell culture models suggests that capsids are available for class I MHC processing (115). Nevertheless, modeling the impact of capsid-specific CD8$^+$ T cells on transgene expression in mice has been difficult even though they generate responses after vector delivery to muscle or liver. CD8$^+$ T cells elicited by the vector capsid were weak in some studies (81). Whether this primary T-cell response was sufficiently fast to recognize transduced target cells before natural turnover of capsid antigens and class I complexes rendered them resistant to recognition is also unknown. Mice, unlike humans, are not naturally infected with AAV and may lack memory capsid-specific immunity that could accelerate and strengthen the impact of CD8$^+$ T cells on transgene expression. For this reason, the influence of capsid-specific CD8$^+$ T cells on transgene expression has only been modeled in mice that were already immune to capsid after priming or adoptive transfer of effector cells elicited by vaccination with recombinant adenovirus vectors or dendritic cells (81, 102, 103). Even with pre-priming of capsid-specific effector cells by vaccination, no obvious loss of transduced cells was observed in the animals. It is likely that the model epitope used in these studies was inefficiently processed from the AAV capsid for class I presentation on murine hepatocytes or myocytes. Nevertheless, the risk of a destructive capsid T-cell
response was illustrated by a novel experimental approach that involved incorporation of a dominant ovalbumin epitope into the AAV capsid (104). Fewer hepatocytes were stably transduced with this vector in the presence of OVA-specific CD8\(^+\) T cells generated by dendritic cell vaccination. This study reinforced the concept that more efficient processing of dominant epitopes from the capsid, perhaps because of altered positioning within the protein, could represent a potential risk to stable transgene expression in humans with pre-existing T-cell immunity.

Transgenes are typically delivered to target tissues or the circulatory system using very large doses of vector capsid. CD8\(^+\) T cells generated by the vector capsid might represent the primary risk to successful gene replacement therapy when memory responses primed by natural infection are weak or are absent in AAV-naïve individuals. In this study we examined the timing, function, and fate of CD8\(^+\) T cells primed only by the vector capsid. CD8\(^+\) T cells primed by the capsid were present in muscle at high frequency within days of vector delivery, and reduced the number of transduced target cells that expressed the capsid epitope. Transduced myocytes were not, however, completely eliminated. Stable low-level expression of a transgene that encoded the capsid epitope was associated with ultimate exhaustion of functional capsid-specific CD8\(^+\) T cells. Our results indicate that capsid-specific T cells elicited by the vector may represent a risk even in settings where pre-existing capsid immunity due to natural infection is weak or absent. The study also suggests a unique strategy to very precisely limit the function of capsid-specific T-cell
responses without generalized immune suppression that is now being considered in human gene therapy trials.

**Materials and Methods**

*Vectors* The serotype 2 recombinant AAV vectors encoding eGFP were obtained from the Vector Core Facility at The Research Institute of Nationwide Children’s Hospital (Columbus, OH). The transgene plasmid used to package the rAAV2(eGFPCap9) vector was engineered by cloning the coding sequence of peptide aa367-aa386 from AAV2 capsid that contains an H-2Dd-restricted epitope (AAV2Cap<sub>372-280</sub>) into the plasmid template, pAAV.CMV.eGFP, between the EcoRV and NotI sites. The plasmid construct expresses the capsid epitope AAV2Cap<sub>372-280</sub> at the C-terminal of the fusion protein, 11 amino acids downstream of the eGFP (Figure 3.1). The pAAV.CMV.eGFP plasmid was a courteous gift from Dr. K. Reed Clark at Nationwide Children’s Hospital. The rAAV2(eGFPCap9) vector was packaged and generously provided by Dr. Brian Kaspar also at Nationwide Children’s Hospital.

*Animals and vaccination* Female Balb/c and C.129S7(B6)-Rag1<sup>tm1Mom</sup>/J mice used in this study were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility at The Research Institute at Nationwide Children’s Hospital. Use and care of the mice were in accordance with the 8<sup>th</sup> edition of the Guide for the Care and Use of Laboratory Animals (116) and approved by the Institutional Animal Care and Use Committee at Nationwide Children’s Hospital. DNase-resistant particles (5 x 10<sup>10</sup>) of
rAAV2(eGFP) and rAAV2(eGFP-Cap9) were directly injected the left quadriceps muscle of 6- to 8-week-old mice.

Flow cytometry Mononuclear cell suspensions generated from spleen and draining lymph nodes were processed by homogenizing tissues with wire mesh and then subjected to sequential centrifugation followed by lysis of red blood cells. Quadriceps muscle was digested in 0.2% type II collagenase (Sigma-Aldrich, St Louis, MO) prior to Percoll gradient centrifugation for enrichment of intramuscular mononuclear cells. Tetramer staining was performed by first incubating single cell suspensions with antibodies to CD3, CD4, B220, F4/80, and CD8 followed by relevant H-2D\textsuperscript{d}-Cap\textsubscript{372-380} and H-2K\textsuperscript{d}-eGFP\textsubscript{200-208} tetramers (National Institutes of Health Tetramer Facility, Atlanta, GA). Live/dead discrimination staining was performed by incubation with propidium iodide immediately before flow analysis using the flow cytometer LSR II (BD Biosciences, San Diego, CA). For apoptosis analysis, Annexin V was mixed with propidium iodide at the last staining step. Flow cytometric data was analyzed with FlowJo software (TreeStar, Ashland, OR).

IFN-\gamma ELISpot assay Flat-bottomed membrane plates (Millipore, Billerica, MA) were coated with anti-mouse IFN-\gamma antibodies (U-CyTech, Utrecht, Netherlands) at 4°C overnight. Mononuclear cells from spleen and draining lymph nodes (1 - 2 x 10\textsuperscript{5} cells/well) were plated in duplicate wells and co-cultured with peptides (2ug/ml) for 48 hours in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum.
and pen/strep. Negative control wells were added with DMSO at a concentration equivalent to those in peptide-stimulated wells. For cells isolated from muscle, irradiated splenocytes from naïve Balb/c mice, as antigen presenting cells, were mixed with intramuscular mononuclear cells (0.5 ~ 1 X 10^5 cells/well) before being co-cultured with relevant peptide epitopes. IFN-γ spot-forming cells were developed and counted using a Cellular Technologies Limited Systems analyzer (CTL, Celveland, OH). Responses were considered positive if there were fewer than 10 spots in the negative control wells (DMSO) and a net increase of at least 10 spots in the relevant peptide-stimulated wells after background subtraction. Responses were reported as numbers of spot-forming units (SFU) per million mononuclear cells after subtracting the background in the DMSO stimulated control wells.

Imaging of intramuscular eGFP fluorescence Mice were anaesthetized with isofluorane and with hairs removed over the injection site prior to imaging. A light-tight chamber equipped with an in vivo optical imaging system (IVIS 200; Xenogen Corporation) and a cooled charge-coupled device camera was used to capture gray-scale reference images as well as eGFP fluorescence. A series of fluorescent images were taken sequentially at incremental excitation wavelengths (480nm and 520nm) to establish autofluorescence for background subtraction. Fluorescence intensity was quantified using Living Image software version 3.0 (Xenogen Corporation) and reported as total photon flux within a region of interest in photons per second per cm^2.
eGFP antigen capture ELISA Immunolon plates were coated with goat anti-eGFP polyclonal antibodies (Rockland, Gilbertsville, PA) overnight at 4oC. Quadriceps muscle were homogenized and centrifuged to collect eGFP-containing supernatants that were added to duplicate wells of the antibody pre-treated plates. Recombinant eGFP (BioVision, San Francisco, CA) was serial diluted with non-transduced muscle homogenate and plated in parallel as a standard. A biotinylated mouse anti-eGFP antibody was then added followed by Neutavidin-horseradish peroxidase (Pierce, Rockford, IL) before plates were developed with tetramethylbenzidine substrate. Absorbance was read at 450nm using a Wallac Victor3 multilabel counter (Perkin Elmer, Waltham, MA). Results were calibrated by subtracting background readings in non-coated wells and presented as micrograms of eGFP per gram of muscle tissue by fitting the values to the standard curve generated with the recombinant eGFP serial diluted at known protein concentrations.

Results

The goal of this study is to investigate the impact of AAV capsid-specific CD8+ T cells on transgene expression in rAAV-transduced mouse muscle. As expected, intramuscular delivery of rAAV vectors encoding the model antigen enhanced green fluorescent protein (eGFP) established life-long expression of the transgene in muscle. To determine the kinetics of the capsid-specific CD8+ T-cell response in transduced muscle, effector cells were visualized using a MHC class I tetramer complexed with an H-2d-restricted dominant epitope that is highly conserved in capsid serotypes commonly used for gene
therapy. Delivery of the rAAV2(eGFP) vector to quadriceps muscle of Balb/c mice primed capsid-specific CD8$^+$ T cells that were highly enriched in transduced muscle within 5 days, the earliest time point tested. Visualization of the capsid-specific CD8$^+$ T cells by tetramer documented a frequency 1000-fold higher in muscle versus spleen and draining lymph nodes (Figure 3.2, A and B). At day 21, capsid-specific T cells reached a peak frequency of 30 percent relative to the total CD8$^+$ T cell pool in transduced-muscle and thereafter quickly contracted (Figure 3.2, A and B). Massive infiltration by the capsid-specific CD8$^+$ T cells that failed to eliminate vector-transduced muscle suggested possible functional impairment. Capsid-specific T cells isolated from transduced muscle at a series of time points were capable of secreting IFN-γ in direct \textit{ex vivo} ELISpot assays (Figure 3.2C). Moreover, the number of IFN-γ-secreting CD8$^+$ T cells correlated well with the T-cell frequency measured by the tetramer analysis for all the time points tested (Figure 3.2B and C), indicating that the function was sustained in the majority of these effector cells.

The frequency of capsid-specific T cells that bound Annexin V, an apoptosis marker, also remained static suggesting that most capsid-specific T cells in muscle were not prone to apoptosis (Figure 3.3A). Accumulation of functional capsid-specific T cells that were not susceptible to programmed death in rAAV transduced-muscle was unexpected, as they did not interfere with long-term transgene expression in muscle. The functional integrity and accelerated kinetics of the capsid-specific CD8$^+$ T cells was in sharp contrast to the eGFP-specific CD8$^+$ T cells that bound annexin V and have been documented to lose
function upon infiltration of transduced-muscle (Figure 3.3B). Together these data indicate that functional capsid-specific CD8\(^+\) T cells infiltrate muscle rapidly after vector treatment and thus have the potential to eliminate transduced cells.

The CD8\(^+\) T cells detected in this study were generated by the pre-formed capsid without pre-vaccination with recombinant adenoviruses or dendritic cells. Because the capsid epitope selected for study is probably not efficiently processed from the exogenously acquired capsid protein in myocytes (104), we assessed the ability of CD8\(^+\) T cells to eliminate transduced muscle cells by incorporating a dominant epitope into the vector transgene. Prior to vector encapsidation, the capsid and eGFP epitopes encoded by the transgene plasmid were tested for proper processing and class I presentation. Mice were immunized intramuscularly with the eGFPCap9 transgene plasmid. CD8\(^+\) T cells specific for the eGFP and Cap9 epitopes were detected in spleen by tetramer visualization (Figure 3.4A) and IFN-\(\gamma\) ELISpot assay (Figure 3.4B), suggesting that both epitopes encoded by the DNA plasmid can be processed for CD8\(^+\) T-cell recognition. This capsid-modified transgene was incorporated into a vector, designated rAAV2(eGFPCap9), and delivered to the quadriceps muscle of mice. Importantly, there was no difference in eGFP fluorescence intensity in immune deficient mice that received the rAAV2(eGFP) and rAAV2(eGFPCap9) vectors, indicating that incorporation of the capsid epitope coding sequence into the transgene of the latter vector did not alter transgene expression. Importantly, delivery of these two vectors to the quadriceps muscle of immune competent Balb/c mice resulted in very different levels of transgene expression (Figure 3.5A and B).
Treatment with the rAAV2(eGFP) vector resulted in an exponential increase in eGFP fluorescence intensity during the first 7 weeks before a plateau was reached (Figure 3.5A and B). This was in sharp contrast to the rAAV2(eGFPCap9) vector-treated mice where eGFP expression was severely attenuated as early as week 2, and thereafter remained static (Figure 3.5A and B). The difference in the kinetic of eGFP expression between the two groups of mice was also reflected in protein levels at week 12 after the eGFP fluorescent signal stabilized in both groups of mice. The total amount of eGFP isolated from the transduced muscle of the rAAV2(eGFPCap9)-treated mice was 10-fold less than that of the rAAV2(eGFP)-treated mice (Figure 3.5C). Nonetheless, eGFP was never cleared from the rAAV2(eGFPCap9)-transduced muscle and was sustained at a low level.

Survival of some transduced myocytes despite early infiltration of muscle by functional capsid-specific T cells prompted us to re-evaluate the fate of the response when the capsid epitope was continuously synthesized in the target tissue. The percentage of Annexin V-positive capsid-specific CD8\(^+\) T cells increased over the period of 12 weeks and eventually reached levels comparable to the eGFP-specific T-cell populations (Figure 3.6A, right panels). This apoptotic profile closely resembled the antigen-driven apoptosis of the eGFP-specific T cells (Figure 3.3B), but was in sharp contrast to the intramuscular capsid-specific T cells that remained mostly Annexin V negative in the rAAV2(eGFP)-treated mice. Rapid contraction of the capsid-specific CD8\(^+\) T cells in rAAV2(eGFP)-transduced muscle was not observed in the muscle that continuously synthesized the Cap9 epitope (Figure 3.6A). Most importantly, the capsid-specific T cells in the muscle
of the rAAV2(eGFPCap9) mice gradually lost function. Ninety percent of the capsid-specific CD8\(^+\) T cells lost the ability to secret IFN-\(\gamma\) upon re-encounter with antigen at day 84 when the frequency of T cells remained elevated (Figure 3.6B). This is consistent with our previous findings of functional impairment specific to the transgene antigen-specific CD8\(^+\) T cells. In summary, antigen-driven anergy and apoptosis in rAAV vector-transduced muscle was specifically induced by continuous expression of the cognate epitope from the transgene.

**Discussion**

Here we investigated the function, kinetic, and fate of AAV capsid-specific T cells that infiltrated vector-treated muscle of mice. De novo priming of CD8\(^+\) T cells by capsid proteins of the vector particles was unexpectedly rapid. Within 5 days of vector delivery, capsid-specific CD8\(^+\) T cells were detected in muscle. Nearly 30 percent of the intramuscular CD8\(^+\) T cells were specific for a single capsid epitope at the peak of the response. Importantly, the intramuscular capsid-specific CD8\(^+\) T cells retained effector functions and were not susceptible to programmed cell death as judged by Annexin V binding. Nevertheless, these T cells can be induced to become functionally impaired through muscle expression of the capsid epitopes encoded by the transgene. Chronic exposure to the cognate antigen induced the intramuscular capsid-specific CD8\(^+\) T cells to undergo programmed cell death.
Capsid-specific CD8\(^+\) T cells that have the potential to eliminate AAV-vector transduced cells could interfere with the success of human gene therapy. However, conditions that favor cytotoxic destruction of transduced cells are undefined because the influence of capsid-specific CD8\(^+\) T cell immunity has been difficult to model in animals. Capsid-specific CD8\(^+\) T cells have been detected in mice treated with AAV vectors, but the response had no obvious impact on sustained gene expression observed in these animals (102, 103). Frequencies of the capsid-specific CD8\(^+\) T cells have been reported to be markedly low in mice that were not pre-primed with rAd vectors encoding AAV capsid proteins or epitope-pulsed dendritic cells (81). Moreover, it has been suggested that the quality of these capsid-specific T cells was fundamentally different from the memory T cells generated in humans after natural AAV infection. Additionally, weak immunogenicity of the rAAV capsid in mice has been proposed in studies where partial attenuation of the transgene expression encoded by rAAV was only achieved in mice with pre-existing capsid-specific T cells through repetitive vaccination by capsid-loaded dendritic cells (81). Few of these studies examined capsid-specific T cell responses in the transduced tissue. High frequencies of capsid-specific T cells in skeletal muscle at day 5 after vector delivery, the earliest time point tested, indicate that the timing and intensity of the response is probably adequate to remove transduced cells if dominant epitopes are processed for cross-presentation. Moreover the cells were fully functional as assessed by production of cytokines like IFN-\(\gamma\). Given the very rapid infiltration of functional primary capsid-specific CD8\(^+\) T cells into muscle, activation of memory CD8\(^+\) T cells primed by prior natural infection may not be necessary to eliminate transduced myocytes.
Indeed, our data suggest that memory capsid-specific CD8\(^+\) T cells might only be a factor in elimination of transduced myocytes if cross-presentation of dominant epitopes in the mice was very transient, for a period of 4 days or less after vector delivery. It is more likely that the capsid epitopes recognized by inbred mice are not properly processed from capid proteins for cross-presentation. A chimeric capsid engineered to contain a dominant class I CD8 model epitope has been suggested to facilitate cross-presentation by rAAV-transduced hepatocytes (104). Reduction of transgene expression in hepatocytes that were transduced by rAAV vectors with the chimeric capsid was only observed in mice pre-treated with dendritic cell vaccination. Therefore, elimination of transduced parenchymal cells by capsid-specific CD8\(^+\) T cells was considered possible if cross-presentation of immunopotent capsid epitopes could occur.

It has been suggested that transient immune suppression might prevent capsid-specific CD8\(^+\) T-cell responses (100, 106). Immune suppression would have to continue until (i) dominant epitope(s) are cleared from transduced cells or (ii) capsid-specific T cells contract to the point that they are no longer a threat. At present there are no estimates of the duration of capsid epitope cross-presentation by target cells in transduced tissues. Our study provides the first detailed kinetic analysis of CD8\(^+\) T cell expansion and contraction in a transduced tissue. In the absence of data on the “wash-out” period for class I-capsid complexes on transduced cells, immune suppression would have to start at the same time as vector administration and continue for at least 12 weeks until the response contracted to eliminate the risk to sustained gene expression.
Here we investigated an alternative to immune suppression for silencing of capsid-specific T cells. The approach was based on our earlier observation that CD8$^+$ T cells primed by the vector transgene lose function and undergo apoptosis upon infiltration of transduced muscle. These CD8$^+$ T cells have no apparent impact on long-term persistent expression of the transgene in muscle. We reasoned that expression of a capsid epitope from the vector transgene might also result in long-term exhaustion and apoptosis of cognate CD8$^+$ T cells in muscle. This approach may have significant clinical implications as a considerable proportion of human population has been suggested to possess capsid-specific memory CD8$^+$ T cells that are considered fully functional and follow an accelerated expansion upon re-encounter with therapeutic recombinant AAV vectors. If pre-treatment with rAAV vectors encoding capsid genes can similarly silence pre-existing capsid-specific CD8$^+$ T cells, this targeted T-cell suppression would have a significant advantage over temporary drug-related immune suppression, because it is antigen-specific, less toxic, and restricted to transduced tissues. Potential B-cell epitopes can be removed to minimize priming of neutralizing antibodies that may block subsequent delivery of therapeutic rAAV vectors with the same capsid serotype. Evaluation of this potential immune-suppression intervention in animal models evolutionarily close to humans may be necessary to further elaborate this idea.
Figure 3.1 Schematic diagram of plasmid constructs.

See Materials and Methods for details. ITR, inverted terminal repeats; eGFP, enhanced green fluorescent protein.
Figure 3.2 Kinetic and functional analysis of capsid-specific CD8\(^+\) T cells

(A and B) The percentage of CD8\(^+\) T cells that stained positive for the AAV2Cap\(_{372-380}\) tetramer was determined by flow cytometric analysis of lymphocytes isolated from spleen, draining lymph nodes (LN), and injected (Inj.) muscle of rAAV2(eGFP)-treated mice. The number within each dot plots represents the tetramer frequency of CD8\(^+\) T cells. Dot plots were gated on FSC/SSC appropriate for live lymphocytes that were CD4\(^-\) B220\(^-\) F4/80\(^-\) CD3\(^+\) (C) Direct ex vivo ELISpot analysis of mononuclear cells (MNC) that secreted IFN-\(\gamma\) in response to the AAV2Cap\(_{372-380}\) peptide stimulation. Results were presented as spot-forming units (SFU) per million MNC isolated from spleen, draining LNs, and injected muscle. All data represent at least 2 independent experiments each with tissues pooled from 6 mice.
Figure 3.3 Apoptosis analysis of intramuscular CD8\(^+\) T cells

AAV2Cap\(_{372-380}\) (A) and eGFP\(_{200-208}\) (B) tetramer-specific CD8\(^+\) T cells isolated from spleen and injected muscle of rAAV2(eGFP)-treated mice were analyzed for Annexin V binding using flow cytometric analysis. Numbers represent the percentage of tetramer-positive CD8\(^+\) T cells stained negative (top left quadrant) and positive (top right quadrant) for Annexin V. Dot plots were gated on FSC/SSC appropriate for live lymphocytes that were CD4\(^-\)B220\(^-\)F4/80\(^-\)CD3\(^+\). All data represent 3 independent experiments each with tissues pooled from 6 mice.
Figure 3.4 Identification of CD8$^+$ T cells in mice immunized with plasmid DNA.

(A) Splenocytes of naïve (left panels) and vaccinated mice (right panels) that received the eGFP-Cap9 plasmid 30 days prior to the analysis were stained with the eGFP$_{200-208}$ (top panels) and AAV2Cap$_{372-380}$ (bottom panels) tetramers. Numbers represent the frequency of tetramer-positive CD8$^+$ T cells. Dot plots were gated on FSC/SSC appropriate for live lymphocytes that were CD4$^-$B220$^-$F4/80$^-$CD3$^+$. (B) Direct ex vivo IFN-γ ELISpot analysis of splenocytes isolated from eGFP-Cap9 plasmid-immunized mice after stimulation with the eGFP$_{200-208}$ and AAV2Cap$_{372-380}$ peptides. Data is representative of a group of 3 mice that were individually analyzed.
Figure 3.5 Patterns of eGFP expression in muscle.

(A) Quantification of eGFP fluorescence in the transduced muscle of immunocompetent (wt) or immunedeficient (RAG⁻/⁻) Balb/c mice immunized with rAAV2(eGFP) and rAAV2(eGFPcap9) vectors using an in vivo optical imaging system (Xenogen). The intensity of the eGFP fluorescence was presented as numbers of photons/sec/cm² (total flux) that was averaged from groups of 5 mice. (B) The amount of eGFP in the transduced muscle of vector-treated immunocompetent Balb/c mice was quantified by antigen-capture ELISA at week 12 post vector delivery. All data represents 2 independent experiments, each with at least 5 mice/group.
### Figure 3.6 Characterization of capsid-specific CD8$^+$ T cells in muscle

(A) AAV2Cap$^{372-380}$ tetramer staining and Annexin V binding of lymphocytes isolated from vector-transduced muscle of mice treated with the rAAV2(eGFP) or the rAAV2(eGFPCap9) vector. Numbers above tetramer gates represent frequency of tetramer-positive CD8$^+$ T cells. Numbers within Annexin V-staining plots represent the percentage of tetramer-positive CD8$^+$ T cells that were negative (top left quadrant) and positive (top right quadrant) for Annexin V. Dot plots were gated on FSC/SSC appropriate for live lymphocytes that were CD4$^-$B220$^-$F4/80$^-$CD3$^+$. (B) Direct ex vivo IFN-$\gamma$ ELISpot analysis of AAV2Cap$^{372-380}$-specific CD8$^+$ T cells isolated from the transduced muscle of mice immunized with rAAV2(eGFP) and rAAV2(eGFPCap9) vectors. All data represents 2 independent experiments, each with tissues pooled from 6 mice.
Chapter 4

Fully functional CD8$^+$ T cells targeting transgene-encoded proteins with a permanent effector phenotype following rAAV treatment

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Running title: Function and differentiation of T cells in non-human primates
Introduction

Successful gene transfer and stable gene expression may be influenced by host immunity. Foreign (non-self) proteins encoded by rAAV vector-encapsidated transgenes provoke immune responses when considered foreign to vector-transduced hosts. Cellular and humoral immune responses specific for the transgene-encoded proteins have been detected in animal models and humans (69, 88-90). Nevertheless, the influence of a cellular immune response on long-term transgene expression is not yet well defined. Some transgene-encoded antigens persist, at least in mice, despite induction of T cells (88, 89). Failure to eliminate transduced cells by antigen-specific T cells that infiltrate antigen-positive tissues is not fully understood. Several muscle-targeted studies in mice have described partially defective T-cell responses with a panel of rAAV vectors using capsid serotypes (1 through 9) that were most commonly used in gene therapy studies (88, 89). In these studies, CD8+ T cells specific for the transgene-encoded proteins were suggested to be partially exhausted by functional and phenotypic measures. These T cells were unable to secrete multiple cytokines (IFN-γ, TNF-α and IL-2). They failed to expand upon secondary exposure to the cognate antigen encoded by a different vaccination modality (rAd, MVA, plasmid DNA). Phenotypic analysis of the functionally defective T cells suggested a lack of memory formation. Importantly, the T cells underwent antigen-driven apoptosis in the vector-tranduced antigen-positive muscle (90).

Whether T-cell immunity to transgene-encoded proteins delivered by rAAV is also defective in primates, including humans, is unknown but critical to the design of
successful gene therapy. We have previously documented an unwanted T-cell response targeting non-self epitopes encoded by the rAAV-delivered therapeutic transgene in patients with Duchenne muscular dystrophy (69). However, there is very limited information on transgene expression and T-cell immunity in non-human primates. Long-term expression of human genes (factor IX and erythropoietin) in monkey species has been reported (41, 57, 117). Human proteins were typically at least 95 percent identical to monkey orthologues, so they may have presented a limited opportunity for immune recognition. It remains controversial whether T-cell immunity to transgene-encoded proteins that are clearly foreign influences persistence expression of the transgene. CD8\(^+\) T cells specific for model antigens such as eGFP were detected in monkeys after vector delivery to liver (83, 96). Concurrent reduction of eGFP expression in transduced hepatocytes was also reported (96), but some appeared to persist at least for a few weeks (83). One recent report described defects in T-cell immunity after delivery of a transgene encoding the HIV-gag protein using an rAAV8 vector (85). The T cell function and response to challenge with the antigen was similar to those described in mice.

In this study, we characterized the maturation and function of CD8\(^+\) T cells targeting transgene-encoded model antigen, eGFP. rAAV2 vectors encoding the transgene were delivered to the skeletal muscle of rhesus macaques. Animals were subsequently challenged with a heterologous recombinant adenovirus (rAd) vector encoding the same transgene antigen to evaluate the ability of T cells to generate recall responses 2.5 years after exposure to the rAAV vector. We showed that rAAV2-primed eGFP-specific CD8\(^+\)
T cells were fully functional judging by their ability to secrete multiple cytokines, undergo cytotoxic degranulation, and mount robust recall responses. Most importantly, we have documented that these T cells were permanently arrested as functional effector cells without acquiring classical memory phenotypes. Interestingly, muscle-infiltrating T cells remained functional effectors following the rAd-vector boost without undergoing antigen-driven apoptosis as previously observed in mice.

Materials and Methods

Vectors Replication-dependent serotype 2 rAAV vectors that packaged a transgene encoding a secreted eGFP were manufactured by The Research Institute at Nationwide Children’s Hospital Vector Core Facility (Columbus, OH). The transgene plasmid was constructed by cloning the coding region of full-length eGFP into multiple clone sites of the pSecTag2 plasmid (Invitrogen). eGFP expression was driven by a cytomegalovirus promoter, and the protein had an N-terminal secretion signal from the V-J2-C region of mouse Ig κ-chain. The E1/E3-deleted serotype 5 rAd vector that expressed a cytosolic-retained eGFP was purchased from Vector Biolabs (Philadelphia, PA).

Animals, immunization, mononuclear cell isolation from blood and muscle Use and husbandry of the rhesus macaques (Indian origin and captive bred) enrolled in this study were approved by The Institutional Animal Care and Use Committee of The Research Institute at Nationwide Children’s Hospital. DNase-resistant particles (5 X10^{12}) of rAAV2eGFP or vector genomes (1 X10^{11}) of rAd5eGFP were injected into the left
quadriceps muscle of young adult rhesus macaques in 500ul of sterile saline. Blood samples were taken according to the approved protocol via venipuncture of the femoral vein. Peripheral blood mononuclear cells were isolated by Ficoll (GE Healthcare) gradient centrifugation from whole blood collection in EDTA-containing Vacutainer tubes. At the time of necropsy, mononuclear cells were isolated from vector-injected and contralateral-control muscle that were collagenase II (0.2% in PBS) digested (Sigma-Aldrich, St Louis, MO) and passed through wire mesh. Single-cell suspensions were collected by Percoll (GE Healthcare) gradient centrifugation and removal of red blood cells with ACK solution (Gibco, Invitrogen).

**IFN-γ ELISpot analysis** Mononuclear cells isolated from blood (2 X10⁵ cells/well) or muscle (1 X10⁵ cells/well) were suspended in 200ul of RPMI 1640 media (supplemented with 10% fetal calf serum, penicillin, and streptomycin) and cultured in duplicate wells of 96-well flat-bottomed membrane plates that were (Millipore, Billerica, MA) previously coated with anti-IFN-γ antibodies (U-CyTech, Utrecht, Netherland). Syngeneic splenocytes (1 X10⁵ cells/well) pulsed with single peptides (2ug/ml) or peptide pools (40ug/ml/peptide) were irradiated and used as antigen-presenting cells to be co-cultured with muscle-extracted mononuclear cells. For peripheral blood mononuclear cells, single peptides or peptide pools were added directly to the wells with additional antigen presenting cells. Negative control wells had no stimulating peptides, but DMSO. After the plates were incubated at 37°C with 7% CO₂ for 48 hours, they were developed for IFN-γ spot formation and subsequently counted using a Cellular Technologies Limited
Systems analyzer (CTL, Cleveland, OH). Positive response was considered as more than 10 spots over values from the DMSO negative control wells, which had an average of less than 5 spots.

*Flow cytometry* Mononuclear cells were first stained with antibodies to CD3 (BD Biosciences, San Jose, CA), CD8 (BD), dump markers, and phenotypic markers. Dump markers, all purchased from BD, included CD4, CD56, CD11c, CD14 and CD20. Phenotypic markers included CD127 (Beckman Coulter), biotin-PD-1 (A kind gift from Dr. Gordon Freeman at Harvard Medical School), CCR7 (BD), and CD45RA (BD). Then cells were stained with streptavidin-Qdot605 (Invitrogen, Carlsbad, CA), followed by incubation with the eGFP\textsubscript{A08} tetramer (provided by the National Institutes of Health Tetramer Facility, Atlanta, GA). The final staining step was to incubate the cells with a vital dye (Invitrogen, Carlsbad, CA) for live/dead discrimination. All incubations were performed at 4°C for 30 minutes. For apoptosis analysis, cells were co-stained with 1µg/ml propidium iodide (BD) and Annexin V (BD) for 10 minutes at room temperature following the tetramer staining. Data was collected with an LSR II flow cytometer (BD) and analyzed using FlowJo software (TreeStar, Ashland, OR).

*Intracellular cytokine staining and CD107a degranulation assay* Mononuclear cells (1 X10\textsuperscript{6} cells/ml) were incubated with the eGFP\textsubscript{A08} peptide (2mg/ml) or the eGFP peptide pool in the presence of anti-CD107a antibody (BD), Golgi Stop (BD), and Golgi Plug (BD) for 10 hours at 37°C. Peptide-stimulated cells were then surface stained with
antibodies to CD3 (BD), CD8 (BD), and CD4 (BD). Cell were then fixed and permeabilized before staining for intracellular cytokines including IFN-γ, TNF-α, and IL-2. A final step of a vital dye, LIVE/DEAD Fixable Blue (BD), was used before the cells were analyzed by a LSR II flow cytometer.

Results

Previous studies suggested that the capsid serotype of recombinant AAV vectors can influence priming of immune responses against the transgene-encoded non-self protein. Most serotypes, including AAV2, generated weak CD8⁺ T-cell responses against the transgene-encoded foreign proteins after intramuscular delivery of the vector to rodents or non-human primates. The response failed to boost when the animal was challenged with rAd vectors encoding the same transgene protein (Ertl 2007, Wilson 2007, Wilson 2009). Studies in mice indicated that these CD8⁺ T cells had an exhausted phenotype and failed to develop into memory populations. However, phenotypic characterization of these CD8⁺ T cells and their memory formation in an animal model evolutionarily closer to humans than mice has been lacking. The goal of this study was to determine if CD8⁺ T cells specific for the transgene-encoded proteins follow a typical differentiation pathway resulting in functional memory populations in non-human primates. A serotype 2 rAAV vector encapsidating a transgene encoding a non-self model antigen, eGFP, was delivered to the quadriceps muscle of 2 rhesus macaques (Macaca mulatta) that were subsequently boosted with an rAd5(eGFP) vector 30 months later. For comparison, 2 other macaques were vaccinated via the same route but with the rAd5(eGFP) vector only. The expansion
and contraction of eGFP-specific CD8$^+$ T cells were followed in both groups of macaques by IFN-γ ELISpot assay using peripheral blood mononuclear cells stimulated with a peptide pool spanning the entire coding region of eGFP. The eGFP-specific CD8$^+$ T-cell response was recalled after the rAd5 vector boost (Figure 4.1A and B). Peak T-cell frequencies against eGFP as measured by the ELISpot assay were roughly equivalent after sequential administration of the rAAV2 and rAd5 vectors to monkeys E091 and 21741, confirming an earlier observation with a similar set of vectors (Figure 4.1A and B) (85). A robust primary eGFP-specific T cell response was observed after delivery of either the rAAV2 or rAd5 vector to naïve monkeys, but the response was accelerated by at least 3 weeks with administration of the rAd5 vector alone (Figure 4.1).

Characterization of the response in all 4 animals at 1 week after the rAd5 administration revealed expansion of eGFP-specific CD4$^+$ helper T cells, but the CD8$^+$ cytotoxic subset nonetheless dominated the responding T-cell population (Figure 4.2A). To compare the impact of the primary exposure to the rAAV2 and rAd5 vectors on CD8$^+$ T-cell expansion, function, and differentiation, we identified a single dominant eGFP epitope, designated eGFP$_{A08}$, presented by the Mamu-A0801 MHC class I molecule shared by all 4 animals. Comparable numbers of IFN-γ spot-forming units (SFU) were measured in blood after stimulation of mononuclear cells with the eGFP peptide pool or the dominant eGFP$_{A08}$ peptide (Figure 4.2B). Removal of the peptide containing the eGFP$_{A08}$ peptide from the eGFP pool reduced spot-forming units by half in 3 of the 4 animals (E091, 21741, and 05D032) (Figure 4.2B).
This single highly dominant class I eGFP epitope facilitated a detailed study of the CD8+ T-cell priming and boosting after the administration of the two vectors. The frequency of eGFP$_{A08}$-specific T cells primed by the rAAV2 vector increased by 10-fold within 1 week following the rAd5 boost as quantified by an eGFP$_{A08}$-complexed class I tetramer (Figure 4.2C). Importantly, the post-boost frequency in the rAAV2-primed animals was much higher (>5-fold) than what was generated in animals receiving the rAd5 vector alone (Figure 4.2C). This boostable CD8+ T-cell response specific for the rAAV transgene-encoded protein differed from previous studies in mice and non-human primates where the T-cell response was attenuated following the rAd5 boost.

The robust recall response observed in the rAAV2-primed animals suggested that the eGFP-specific CD8+ T cells may not be functionally compromised as previously reported (88, 102). Peripheral blood mononuclear cells isolated from both groups of animals were stimulated with the eGFP$_{A08}$ peptide. Almost all the IFN-γ secreting T cells primed by the rAAV2(eGFP) vector co-produced TNF-α when the response became first detectable at week 4 (Figure 4.3A, left panels). The dual-cytokine producers also upregulated surface expression of CD107a, a marker of degranulation (Figure 4.3A, left panels), reinforcing the conclusion that these T cells are functional. Following the rAd5 boost, majority of the eGFP-specific CD8+ T cells were triple-positive for IFN-γ, TNF-α, and CD107a. The proportion of the triple-positive T cells relative to the total T cells that are positive for at least one marker increased during the 24 weeks of follow-up (Figure 4.3B, top panels).
Animals of the rAd5-alone group showed equivalent numbers of IFN-γ/TNF-α dual-producers and IFN-γ single-producers as the rAAV2-primed ones. Most importantly, at 24 weeks post rAd5 delivery, the proportion of triple-positive eGFP<sub>A08</sub>-specific T cells in the rAAV2-primed animals remained dominant, whereas the multifunctional T cells in rAd5-primed animals have already contracted (Figure 4.3B). This suggested that the rAAV2-primed polyfunctional eGFP-specific CD8<sup>+</sup> T cells were maintained longer than the rAd5-primed counterparts.

Multifunctional CD4<sup>+</sup> helper T cells that secreted not only IFN-γ, but also IL-2 and TNF-α were also detected by stimulation of peripheral blood mononuclear cells from both groups of animals with the eGFP peptide pool in an intracellular cytokine staining assay. Representative data from 1 week post rAd5 treatment is shown in Figure 4.4. Both groups of animals had similar proportions of multi-cytokine secreting CD4<sup>+</sup> T cells, suggesting that the T-cell help was perhaps sufficient in animals primed with the rAAV2 vector.

Differentiation of eGFP-specific T cells was compared after priming with the rAAV2 and rAd5 vectors. eGFP<sub>A08</sub> tetramer-positive CD8<sup>+</sup> T cells were analyzed for surface expression of a panel of cellular markers. The combination of programmed cell death-1 (PD-1) and CD127 (IL-7 receptor α-subunit) has been used to make the distinction between effector cells, either functional or exhausted, and memory cells in a number of models studying chronic viral infections. PD-1 was highly expressed by virtually all the rAAV2-primed eGFP<sub>A08</sub> tetramer-positive T cells, and the PD-1 up-regulation was
sustained through 8 months of follow-up (Figure 4.5A and B). This suggested that rAAV2-primed eGFP-specific T cells may be exhausted or recently activated effector cells. A substantial proportion the PD-1 up-regulated tetramer-positive cells were CD127 negative, indicating the inability of rAAV2-primed T cells to self-renew. Remarkably, the dominating PD-1+/CD127− phenotype remained unaltered following the rAd5-boost (Figure 4.5A and B), suggesting that the rAAV2-primed eGFP-specific CD8+ T cells were permanently programmed as effector cells without developing into memory populations. In contrast, the vast majority of the eGFP A08 tetramer-positive T cells from the rAd5-alone group quickly underwent a phenotypic transition from being PD-1+/CD127−, soon after vector delivery, to an opposite pattern of PD-1−/CD127+ at 6 months post rAd5 delivery (Figure 4.5C and D). The phenotypic transitional phase, depicted as a gradual loss of PD-1 expression and concomitant up-regulation of CD127, was most obvious at week 8 (Figure 4.3C and D, middle panels).

Expression of CCR7, a marker of central memory cells, was subsequently compared between T cells primed by the rAAV2 and rAd5 vectors, respectively. A considerable proportion of rAAV2-primed CD8+ T cells were consistently CCR7 negative before and after the rAd5 boost (Figure 4.6A), in agreement with the PD-1+/CD127− effector phenotype (Figure 4.5A and B). Majority of the eGFP A08 tetramer-positive T cells in the rAd5-alone group were instead CCR7 positive, consistent with a central-memory phenotype (Figure 4.6B). The overall phenotypic discrepancy depicted by the 3 markers
between the rAAV2 and rAd5 primed eGFP-specific T cells suggested a vector-specific divergence in T cell differentiation and memory development.

rAAV2-primed eGFP<sub>A08</sub> tetramer-positive CD8<sup>+</sup> T cells were not only detected in the peripheral blood, but also in the vector-transduced muscle (Figure 4.7A, left panels). Six months post rAd-boost, the frequency of intramuscular eGFP<sub>A08</sub> tetramer-positive T cells was 100-fold higher than the frequency detected in the peripheral blood at the same time. No eGFP<sub>A08</sub> tetramer-positive T cells were detected in the vector-injected muscle of animals treated with the rAd5 vector alone (data not shown), suggesting that antigen-specific recruitment of the eGFP-specific T cells was prolonged in the rAAV2-treated muscle. Over 90 percent of the intramuscular eGFP<sub>A08</sub> tetramer-positive T cells were PD-1<sup>+</sup>/CD127<sup>-</sup>, consistent with the phenotype observed in the peripheral blood (Figure 4.7B). Most importantly, the muscle-infiltrating T cells were stained negative for Annexin V, indicating that they were not prone to apoptosis (Figure 4.7A, right panels). In addition, these T cells remained functional in muscle as they secreted IFN-γ upon re-encounter with the cognate antigen in direct ex vivo IFN-γ ELISpot assay (Figure 4.7C).

Discussion
In this study, we characterized the function and maturation of CD8<sup>+</sup> T cells primed by a non-self protein (eGFP) encoded by an rAAV vector. Two conclusions were evident from this study. First, an rAAV vector with a serotype 2 capsid primed eGFP-specific CD8<sup>+</sup> T cells that were polyfunctional and had characteristics of memory, including a sharp and
sustained increase in frequency after boosting with a heterologous viral vector. Second, the CD8$^+$ T cells primed by the rAAV vector failed to acquire a memory phenotype, even after recall with the rAd5 vector 2.5 years later. Long-term maintenance of the eGFP-specific effector T cells in blood and muscle was apparently independent of IL-7, a cytokine required for memory T-cell survival in the absence of antigen.

High frequencies of CD8$^+$ T cells were present in blood at week 3 after delivery of the rAAV2 vector to muscle, and remained detectable through 2.5 years of follow-up. Moreover these circulating eGFP-specific CD8$^+$ T cells were capable of cytotoxic degranulation and production of multiple cytokines such as IFN-$\gamma$, TNF-$\alpha$, and IL-2. The robust expansion of functional CD8$^+$ T cells after delivery of eGFP to the same muscle using a recombinant adenovirus vector reinforces the conclusion that the response first primed by the rAAV vector was not impaired. Polyfunctional CD8$^+$ T cells were detected within 1 week of rAd5 vector delivery to naïve and eGFP-immune animals, regardless of prior exposure to the rAAV2(eGFP) vector. Importantly, the peak frequency of eGFP-specific CD8$^+$ T cells was much higher in animals exposed 2.5 years earlier to the rAAV2 vector and there was a notable lack of contraction even 6 months later when the experiment was terminated.

Induction of strong and sustained CD8$^+$ T-cell responses specific for the rAAV-encoded eGFP was unexpected. Functionally impaired CD8$^+$ T cells that proliferated poorly upon re-encounter with the transgene-encoded cognate antigen has been demonstrated in mice
in the face of persistent antigen expression. This functional exhaustion could not be rescued by adjuvanted rAAV-treatment, regulatory T-cell depletion, or PD-1 signaling blockade, but was reversible once T cells were temporarily removed from the cognate antigen upon transfer into antigen-free immunodeficient mice. These results suggested antigen-driven T cell exhaustion, at least in the murine model, and further demonstrated the inability of the T cells to be boosted by either plasmid DNA or heterologous viral vector (MVA and rAd).

Among all the capsid serotypes tested, only serotype 8 has been assessed for T-cell functions in non-human primates. Trangene antigen-specific CD8\(^+\) T cells were also functionally impaired, similar to what has been observed in mice. The inability of these T cells to generate recall responses was interpreted as failure for the serotype 8 rAAV vector to transduce dendritic cells, resulting in inefficient class I presentation of the trangene-encoded antigen. Both serotype 2 and 8 rAAV vectors transduce dendritic cells poorly. The rAAV2 vector that we used, however, primed fully functional eGFP-specific CD8\(^+\) T cells that mounted robust recall responses. Differences in the observed T-cell function when compared to reports by others may be species-dependent. Difference in the subcellular compartments to which the transgene-encoded antigens were targeted may also contribute to the functional discrepancy, as we used secreted eGFP whereas other studies used cytosolic-retained HIV-gag. Significant frequencies of polyfunctional CD4\(^+\) helper T cells detected in our study were absent in other studies. In addition, differences inherent to the capsid serotypes may influence T-cell priming. First, serotype 8 and 2
were phylogenetically classified into two distinct clades, and were isolated from rhesus macaques and humans, respectively. Second, capsid structures may affect cellular tropism that could have impact on antigen presentation. For instance, the rAAV2 capsid contains a heparin-binding domain that is missing from the rAAV8 capsid. This domain has been suggested to facilitate dendritic cells entry.

Failure of the rAAV2-primed CD8\(^+\) T cells to acquire a memory phenotype even after an rAd5 boost was unexpected, given the strong expansion and longevity of the T cells in the circulation. This was in direct contrast to the CD8\(^+\) T cells primed by the rAd5 vector with the development a classical memory phenotype, judged by the low expression of PD-1 and high expression of CCR7 and CD127. Inadequate CD4\(^+\) T-cell help cannot explain the difference in CD8\(^+\) T-cell memory formation as patterns of cytokine production were similar in both groups of animals. The observation that CD8\(^+\) T cells retained all key effector functions despite constitutive expression of PD-1 in the absence of CD127 suggested that the T cells were long-term effector cells. Here, PD-1 was a marker of activation rather than exhaustion. eGFP-specific CD8\(^+\) T cells primed by the rAd5 vector alone were capable of differentiating into a classical memory population, but this developmental pathway was arrested at the effector phase by pre-treating monkeys with rAAV vector. Taken together, our study phenotypically characterized rAAV-primed T cells in non-human primates for the first time. Although the rAAV2 vector does not seem to negatively affect T-cell functions when compared with the rAd5 vector, the consequential arrest in T-cell maturation and memory formation was evident and rAAV-
dependent. The mechanism responsible for the difference in T-cell function observed in mice and monkeys remains elusive. Technical details such as cellular locations of the transgene-encoded proteins, vector doses, and tissue tropisms may all influence T-cell priming and maintenance. Nevertheless, the most possible explanation may nonetheless be species related. Our findings generated in non-human primates, which are evolutionarily closer to humans than mice, may be useful in further improvements of rAAV-mediated gene therapy.

How fully functional CD8$^+$ T cells with an effector phenotype were sustained at a high frequency after a successful boost with a heterologous vector is unknown. Failure to up-regulate CD127, which is critical for IL-7-driven survival of memory CD8$^+$ T cells, suggests that there is an alternative mechanism to support long-term survival of these effector cells in blood and muscle. It is possible that persistent low-level antigen production in muscle, or possibly draining lymph nodes, may stimulate CD8$^+$ T-cell proliferation. We did not detect eGFP in the serum or muscle of rAAV2-treated animals by both protein ELISA and fluorescent imaging at the termination of the study (data not shown). However, high frequencies of eGFP-specific T cells in the transduced muscle 6 months after the rAd5 boost in the animals pre-treated with the rAAV2 vector suggested that eGFP may still present in the muscle to facilitate antigen-specific T-cell infiltration. Inability to detect eGFP expression at 6 weeks post rAd5 delivery may be due to the low sensitivity of protein quantification assays that we used. Nevertheless, the amount of eGFP antigen must be sufficient to promote CD8$^+$ T-cell survival but low enough to
avoid exhaustion. High levels of viral antigens have been shown in several studies to drive T-cell exhaustion following chronic viral infection (LCMV, HIV, HCV). On the other hand, the importance of low-level antigens for sustaining protective immunity, where effector T cells can response rapidly to a secondary viral challenge, has been also suggested for some chronic viral infections. For instance, in persistent murine γ-herpesvirus infection, continuous antigen presentation by dendritic cells is critical for the maintenance of CD8\(^+\) T-cell function critical for the control of viral infection. In addition, persistent antigens could not only maintain T-cell population for long-term survival, they were also critical in orchestrating T-cell migration and tissue infiltration, for instance, antigen-driven T-cell accumulation in lung after influenza virus infection. In our study, CD8\(^+\) T cells that infiltrated vector-transduced muscle were functional and resistant to apoptosis. This apoptosis profile was different from what we previously observed in mice, where eGFP-specific T cells were highly susceptible to apoptosis upon re-encounter with high levels of eGFP in muscle. Over-expression of eGFP in the muscle may explain this antigen-driven death, supported by the finding that functional T cells with the same specificity were apoptosis-resistant in tissues where antigen levels were low (draining lymph nodes) or absent (spleen).

In summary, this study reported phenotypic arrest of the rAAV vector-primed CD8\(^+\) T cells that were specific for the transgene-encoded proteins in non-human primates. Despite the absence of a conventional memory phenotype, the long-term effector T cells generated robust recall responses upon re-encounter with the antigen. This finding may
be particularly useful to the evaluation of T-cell immunity targeting rAAV-encoded therapeutic proteins in rAAV-mediated gene therapy clinical trials.
Figure 4.1 Kinetic of eGFP-specific CD8⁺ T cells.

The number of IFN-γ-secreting cells (SFU) per million peripheral blood mononuclear cells in rhesus macaques E091 (A), 21741 (B), 05D361 (C) and 05D032 (D) was measured by ex vivo ELISpot assay. Cells were stimulated with a synthetic peptide pool spanning eGFP at indicated weeks following respective vector delivery. Rhesus E091 (A) and 21741 (B) were primed with the rAAV2(eGFP) vector at week 0 and boosted with the rAd5(eGFP) vector at week 121 and 114, respectively. Rhesus 05D361 (C) and 05D032 (D) received the rAd5 vector alone at week 0. Each bar is a representative of 4 independent measurements.
**Figure 4.2 Characterization of T-cell subsets specific to eGFP.**

(A) Peripheral blood mononuclear cells isolated at 8 weeks post rAd5 delivery from rAAV2(eGFP)-primed (left panels) and rAAV2(eGFP)-naïve (right panels) rhesus macaques were stimulated in vitro with the eGFP synthetic peptide pool. The percentage of IFN-γ-secreting CD3⁺ cells that belonged to the CD4⁺/CD8⁻ or CD4⁻/CD8⁺ cellular subset was determined by intracellular cytokine staining. Numbers in the top left quadrants indicate the percentage of CD4⁺/CD8⁻ cells. Numbers in the bottom right quadrants indicate the percentage of CD4⁻/CD8⁺ cells. Dot plots shown were gated on FSC/SSC appropriated for lymphocytes and LIVE/DEAD blue⁻/CD3⁺/IFN-γ⁺ cells. Each
plot is a representative of 3 independent measurements. (B) The number of IFN-γ-secreting cells (SFU) per million peripheral blood mononuclear cells in all 4 rhesus macaques at 8 weeks post rAd5 delivery was measured by ex vivo ELISpot assay. Cells were stimulated with the eGFP peptide pool, the peptide eGFP_{A08}, or the eGFP pool without the eGFP_{A08} peptide. Each bar is a representative of 4 independent measurements. (C) The percentage of the eGFP_{A08} tetramer-positive CD8^+ T cells in the blood of 4 monkeys were compared before and 1-week after the rAd5(eGFP) vector delivery. The CD8^+ cells were gated on FSC/SSC appropriate for lymphocytes and LIVE/DEAD blue/dump/CD3^+ cells. For animal E091 and 21741, the pre-rAd5(eGFP) time point was 121 and 114 weeks post rAAV2(eGFP), respectively. For animal 05D032 and 05D361 that were rAAV2(eGFP)-naive, the pre-rAd5(eGFP) time point was week 0. Asterisk (*) indicates statistics performed using Student t test with P<0.01. Each bar is a representative of 3 independent measurements.
Figure 4.3 Functional analysis of eGFP-specific CD8\(^+\) T cells in blood.

Peripheral blood mononuclear cells isolated at the indicated time points from rAAV2(eGFP)-immune (A) and rAAV2(eGFP)-naïve (C) rhesus macaques were stimulated in vitro with the eGFP\(_{A08}\) peptide followed by intracellular cytokine staining. The percentage of CD8\(^+\)/CD4\(^-\) cells that stained positive for different combinations of IFN-\(\gamma\), TNF-\(\alpha\), and CD107a is indicated in respective quadrants. Dot plots shown were gated on FSC/SSC appropriate for lymphocytes and LIVE/DEAD blue/ CD3\(^+\) cells. Co-expression of IFN-\(\gamma\), TNF-\(\alpha\), and CD107a by the eGFP\(_{A08}\)-specific CD8\(^+\) cells is summarized and presented in pie charts as single (green)-, double (purple)-, or triple (orange)-producers (B).
Figure 4.4 Functional analysis of eGFP-specific CD4⁺ T cells in blood.

Peripheral blood mononuclear cells isolated at 1-week post rAd5(eGFP) delivery from monkey E091 (A), 21741 (B), 05D361 (C) and 05D032 (D) were stimulated in vitro with the eGFP peptide pool followed by intracellular cytokine staining. The percentage of CD4⁺/CD8⁻ cells that stained positive for different combinations of IFN-γ, TNF-α, and IL-2 was indicated in respective quadrants. Dot plots shown were gated on FSC/SSC appropriate for lymphocytes and LIVE/DEAD blue⁻/ CD3⁺ cells.
Figure 4.5 Kinetic and phenotypic analyses of CD8\(^+\) T cells

Peripheral blood mononuclear cells isolated at indicated time points from rAAV2(eGFP)-immunized animals E091 (A) and 21741 (B) or rAAV2(eGFP)-naïve animals 05D032 (C) and 05D361 (D) were stained with the eGFP\(_{A08}\) tetramer (top panels). Tetramer positive cells were further analyzed for surface expression of CD127 and PD-1. Dot plots shown in the top row of panels for each monkey were gated on FSC/SSC appropriate for lymphocytes and LIVE/DEAD blue/dump/CD3\(^+\) cells, and the number within each panel indicates the percentage of CD8\(^+\) cells that were tetramer positive. Dot plots shown in the bottom row of panels for each monkey were gated on tetramer\(^+\)/CD8\(^+\) cells, and the number within each panel indicates the percentage of tetramer positive cells that were either CD127\(^-\)/PD-1\(^-\) (top left quadrant) or CD127\(^+\)/PD-1\(^+\) (bottom right quadrant).
Figure 4.6 Additional phenotypic analysis of CD8\(^+\) T cells.

Peripheral blood mononuclear cells isolated from rAAV2(eGFP)-immuned animals (A) and rAAV2(eGFP)-naïve animals (B) at indicated time points post vector delivery were stained with the eGFP\(_{A08}\) tetramer. Tetramer positive cells were further analyzed for surface expression of CCR7 and CD45RA. Dot plots shown were gated on FSC/SSC appropriate for lymphocytes and LIVE/DEAD blue/dump/CD3\(^+\)/CD8\(^+\)/tetramer\(^+\) cells. The number within each panel indicates the percentage tetramer-positive cells that were CD45RA\(^+\)CCR7\(^-\) (top left quadrant) or CD45RA\(^-\)CCR7\(^+\) (top right quadrant).
Figure 4.7 Functional and phenotypic analyses of T cells in muscle.

Mononuclear cells isolated from rAAV2(eGFP)-transduced muscle at 24 weeks post rAd5(eGFP) were stained with the eGFP<sub>A08</sub> tetramer and Annexin V (A). Dot plots shown were gated on FSC/SSC appropriate for lymphocytes and LIVE/DEAD blue<sup>−</sup>/dump<sup>−</sup>/CD3<sup>+</sup> cells. The number indicated in the left panels of (A) is the percentage of CD8<sup>+</sup> cells that were tetramer positive. The number in the right panels of (A) are tetramer positive cells that were either Annexin V negative (top left) or positive (top right). The eGFP<sub>A08</sub> tetramer-positive cells were further analyzed for expression of CD127 and PD-1 (B). The number in each quadrant indicates the percentage of cells that differentially expressed CD127 and PD-1. The number of IFN-γ-secreting cells isolated from the muscle in response to the eGFP<sub>A08</sub> peptide stimulation was measure by ELISpot assay (C). Results were presented as spot-forming units (SPU) per 10<sup>5</sup> CD8<sup>+</sup> cells.
Chapter 5

General discussion

The goal of our studies

Gene therapy is one of the fastest growing fields for the treatment of genetic disorders. Reports of successful gene therapy in humans have steadily increased over the past 20 years (1). The first licensing application for a gene therapy product (Glybera®) to treat lipoprotein lipase deficiency was filed recently by Amsterdam Molecular Therapeutics (AMT) and is under review by the European Medicines Agency (EMA)(118). Although other gene delivery vehicles are being evaluated, the most promising results have been achieved using recombinant adeno-associated virus (rAAV) vectors. rAAV vectors have demonstrated unparalleled safety profile and therapeutic efficacy in a wide range of animal models. This facilitated clinical translation and has prospects for achieving long-term disease correction in humans.

Despite a record of success in many animal models and some initial human trials, the ultimate goal of gene therapy, which is to achieve perpetual transgene expression at or above therapeutic levels, is still limited by a major obstacle, the host immune response.
Of all the immune effector mechanisms, cytotoxic CD8$^+$ T cells represent the greatest risk for elimination of transgene-expressing cells. The goal of our studies reported here was to better define the influence of T-lymphocyte responses to the outcome of gene therapy, and to identify strategies to attenuate the response if needed. We focused on mechanisms of antigen presentation relevant to CD8$^+$ T-cell priming following rAAV treatment and the function, differentiation, and fate of these T cells in the vector-transduced tissue.

**Results for antigen presentation with interpretation and implications**

Efficient antigen presentation is requisite to CD8$^+$ T-cell priming. Whether primed effector cells remain fully functional to kill vector transduced targets and form memory populations largely depends on the quality of antigen presentation. Using a bone marrow-chimeric mouse model, we documented that priming of CD8$^+$ T cells specific for the vector capsid and the transgene-encoded proteins required dendritic cells. Vector-transduced myocytes were incapable of priming CD8$^+$ T cells in the absence of dendritic cells. Such CD8$^+$ T-cell priming is not restricted to the early phase of rAAV-vector delivery to skeletal muscle when cell-free infectious vector particles are still available to directly transduce dendritic cells. It is rather an ongoing process that remains active for as long as the vector-encoded antigen persists, even long after all rAAV vector particles capable of cell transduction are no longer available.
The vector capsid, which is not encoded by the transgene, should be degraded and washed out of the vector-treated host soon after vector delivery. Thus, antigen for priming of capsid-specific CD8\(^+\) T cells is available only transiently. In contrast, maintenance of T cells that are specific for the transgene-encoded proteins through continuous priming is conceivable if transgene expression persists. Using the chimeric-mouse model where partially MHC-mismatched myocytes and dendritic cells present unique epitopes to different T-cell populations, we obtained evidence of antigen cross-presentation. We further defined the cellular requirements for the long-term maintenance of the transgene antigen-specific CD8\(^+\) T cells. rAAV vector-transduced parenchymal cells like myocytes are necessary but insufficient for T-cell priming. Dendritic cells passively acquire transgene-encoded proteins from transduced myocytes and cross-present the antigen to CD8\(^+\) T cells.

Our findings have important clinical implications as they argued against the possibility that target tissue-specific promoters would prevent T-cell immunity specific to the transgene-encoded proteins. We reason that using promoters that restrict transgene expression to vector-targeted muscle or liver cells cannot prevent priming of CD8\(^+\) T cells specific for transgene-encoded proteins. Consistent with this view, we propose that delivery of transgenes with selected rAAV capsid serotypes that do not facilitate dendritic cell entry is also unlikely to minimize the risks of T-cell priming. Dendritic cell-mediated cross-presentation is a robust mechanism to acquire exogenous antigens that cannot be synthesized by professional antigen presenting cells to ensure efficient CD8\(^+\) T-cell
priming. In fact, it is the main mechanism to prime fully functional CD8+ T cells to eliminate cancer cells and control many intracellular infectious pathogens that do not infect dendritic cells. Therefore, approaches to prevent expression of rAAV vector-encoded transgene by dendritic cells are insufficient to impede the generation of potentially destructive CD8+ T cells.

Whether our findings from muscle-targeted transgene delivery can be extrapolated to other tissues, such as liver, requires further investigation. It is unlikely, however, that hepatocytes would differ from myocytes in the transfer of antigens to dendritic cells to facilitate cross-presentation. Many hepatotropic viruses prime CD8+ T cells very efficiently through dendritic cell-mediated cross-presentation, suggesting that our results generated from muscle can be applied to liver or other non-professional antigen presenting cells as targets for gene expression.

Whether the transgene antigen-specific CD8+ T cells cross-primed by dendritic cells are fully functional is unclear. We have shown that CD8+ T cells primed by untransduced dendritic cells produced IFN-γ in response to the transgene-encoded antigen, suggesting that at least they are partially functional. We were unable to perform more detailed functional analysis on these CD8+ T cells due to limited cell yields from the lethally irradiated mice. However, in chapter 4, we reported identification of eGFP-specific CD8+ T cells that remained as fully functional effector cells in non-human primates 2.5 years after the animals were treated with the rAAV-eGFP vector. This piece of evidence
suggests that these CD8$^+$ T cells that are fully functional may have been maintained by dendritic cell cross-presentation.

**Results for capsid-specific T cells with interpretation and implications**

Upon identification of the antigen presenting cells that prime and possibly maintain CD8$^+$ T cells, we next assessed the quality of these T cells in the transduced tissue where they are most likely to impede stable expression of transgene-encoded proteins by elimination of transduced cells. We first evaluated the function, kinetic, and fate of the capsid-specific CD8$^+$ T cells in mice. Then we compared them with the CD8$^+$ T cells targeting the transgene-encoded proteins in the same host. We found that capsid-specific CD8$^+$ T cells were primed in less than 5 days after rAAV vector delivery, much faster than the T cells specific for the transgene-encoded proteins. Muscle infiltration of the capsid-specific CD8$^+$ T cells was 2 days earlier than the transgene antigen-specific T cells. Most importantly, these capsid-specific CD8$^+$ T cells remained fully functional in the vector-transduced muscle. This was in stark contrast to the transgene antigen-specific CD8$^+$ T cells that became exhausted and underwent programmed cell death upon muscle infiltration.

Critical to these findings, transgene expression persisted despite infiltration of capsid-specific CD8$^+$ T cells that were fully functional into muscle. Our results suggest that capsid-specific CD8$^+$ T cells, at least in mice, are not a threat to long-term transgene expression. This is perhaps because myocytes of inbred mice are incapable of processing
exogenously acquired pre-formed vector capsid for class I cross-presentation. Whether transduced muscle of animal species other than inbred mice could cross-present capsid for CD8⁺ T-cell recognition is unknown. Given that fully functional capsid-specific CD8⁺ T cells have the potential to kill vector-transduced myocytes, we developed a strategy to silence the CD8⁺ T cells that infiltrated vector-transduced muscle. This novel approach was inspired by an observation previously made in our lab showing that functional exhaustion and antigen-driven apoptosis of transgene antigen-specific CD8⁺ T cells facilitated long-term transgene expression in muscle. Expression of a class I capsid epitope from the rAAV vector-encoded transgene effectively induced apoptosis and loss of function by otherwise fully functional capsid-specific CD8⁺ T cells in muscle.

Adaptation of our approach to clinical trials by pre-anergizing capsid-specific CD8⁺ T cells through transgene expression of class I capsid epitopes might be a better strategy than general immunesuppression by drugs. Our approach is safer, less toxic, antigen-specific, and maybe more efficient when compared with commonly accepted strategies in the gene therapy field, such as induction of transient immunesuppression and using AAV capsid serotypes that do not transduce dendritic cells. Moreover, our strategy has the potential to silence any functional capsid-specific CD8⁺ T cells, including both memory and effector populations.

One important aspect of our capsid study is that CD8⁺ T cells were evaluated in muscle of AAV-naïve animals. Nearly all the published studies that attempted to assess capsid-
specific T-cell immunity used animals pre-treated with antigen-loaded dendritic cells or effector/memory CD8\(^+\) T cells. Unlike these studies, we addressed the T-cell responses without pre-existing immunity to either the AAV capsid or the transgene-encoded protein. We demonstrated that, even in naïve hosts, rAAV vectors primed fully functional capsid-specific CD8\(^+\) T cells, as well as transgene antigen-specific T cells. This suggests that both population of CD8\(^+\) T cells have the potential to eliminate transduced cells regardless of pre-existing immunity. Therefore, \textit{de novo} priming of capsid-specific CD8\(^+\) T cells upon rAAV vector treatment and their role in limiting stable transgene expression in humans should not be understated.

**Interpretation of data and implications for transgene antigen-specific T cells**

Regardless of whether capsid-specific CD8\(^+\) T cells can engage vector-transduced cells for destruction, it is very likely that we can functionally blunt these T cells, perhaps reducing the threat to successful gene therapy. We therefore further investigated CD8\(^+\) T cells specific for the transgene-encoded proteins. We assessed their function, kinetics, differentiation, and ability to generate recall responses in non-human primates, a species evolutionarily closer to humans than mice. Transgene antigen-specific CD8\(^+\) T cells were fully functional as judged by their ability to secrete multiple cytokines and degranulate to release cytolytic enzymes upon re-encounter with cognate antigens. These CD8\(^+\) T cells generated robust recall responses when challenged with a heterologous viral vector encoding the same transgene 2.5 years later, reinforcing the conclusion that the transgene antigen-specific CD8\(^+\) T cells primed by rAAV vectors remained fully functional. We
provided a third piece of evidence supporting this conclusion by showing that CD4\(^+\) T cells targeting the transgene-encoded proteins provided sufficient help to the CD8\(^+\) T cells by secreting multiple cytokines, such as IFN-\(\gamma\), TNF-\(\alpha\), and IL-2. It was unexpected that transgene antigen-specific CD8\(^+\) T cells that were capable of generating a robust recall response never differentiated into a classical memory population. They failed to express phenotypic markers typical of memory T cells, such as CD127 (IL-7 receptor \(\alpha\)) to maintain long-term survival and CCR7 (chemokine receptor) for lymphoid organ homing. Instead, the transgene antigen-specific CD8\(^+\) T cells phenotypically resembled effector cells.

The mechanism that maintained these T cells as long-term functional effectors is unknown. It is possible that survival of these T cells was through chronic exposure to cognate antigens. The level of this antigen may be very low, as continuous stimulation of T cells with high levels of cognate antigens usually results in exhaustion. In support of this speculation, we were unable to detect transgene encoded protein in the circulation 2.5 years after rAAV delivery. In addition, the cytosolic version of the same transgene delivered by the boosting rAd vector was also undetectable, suggesting that even if the transgene was present and reasonably localized (cytoplasm of transduced myocytes), it is very likely at a level difficult to quantify.

Our findings summarized above focused on characterization of the killer CD8\(^+\) T cells in the vector-transduced tissue, which is directly relevant to the longevity of the target cells.
that are responsible for synthesizing transgene-encoding proteins. A number of studies have evaluated CD8\(^+\) T cells specific for the transgene-encoded proteins following rAAV treatment. However, majority of these studies assessed T cells in the circulation or secondary lymphoid organs such as spleen and draining lymph nodes that may not faithfully reflect the inflammatory response in the transduced tissues. It is critical to address CD8\(^+\) T-cell responses in vector-treated tissues, because the microenvironment, cytokine availability, and immune-activating and immune-inhibitory signals in the transduced tissues are very likely to be different from other compartments. Consequently, the function and fate of CD8\(^+\) T cells targeting rAAV vector-delivered antigens may be tissue-dependent. The studies described here, to the best of our knowledge, are unique in their analysis of CD8\(^+\) T cells that infiltrate transduced tissues.

In our animal models, we selected transgene-encoded proteins that were non-self. This may not always translate to gene therapy studies where the risk of T-cell immunity may be minimized by the limited number of sequence differences between the defective self-gene and the therapeutic transgene. For instance, the disease phenotype may be caused by missense mutations or limited frame-shifts. Nonetheless, our studies may be useful to models outcomes of clinical trials involving significant gene deletion and truncation. Moreover, there was no basal level of chronic inflammation in the vector-targeted tissues in our models, which is different from some of the muscle-directed gene therapies treating diseases like Duchenne muscular dystrophy. How the microenvironment of a chronically inflamed muscle contributes to transgene expression and T-cell function
remains to be determined. In addition, the vector-tranduced tissues, such as eye and brain, may not be susceptible to immune-mediated transgene clearance in some clinical trials because these sites are considered immune privileged. Nevertheless, findings from our studies can be used to evaluate immune-mediated destruction of vector-transduced cells in the treatment where T-cell immunity has the potential to heavily influence the success of gene therapy.

All of our studies used rAAV vectors with a serotype 2 capsid that is considered weakly immunogenic, and yet robust CD8$^+$ T cells were still generated to both the vector capsid and transgene-encoded proteins. A commonly held view regarding the weak immunogenicity of this capsid serotype is its inability to generate effective innate immune responses. Based on our functional results of both the transgene antigen-specific CD8$^+$ T cells and the capsid-specific CD8$^+$ T cells, we speculate that the innate immune signals may not be sufficiently attenuated to impair T-cell immunity.

**Future directions**

We plan to further evaluate whether pre-existing memory capsid-specific T cells can be silenced by pre-treating animals with rAAV vectors encoding class I capsid epitopes in the transgene prior to the administration of rAAV vectors encoding the therapeutic transgene. We reason that if fully functional capsid-specific CD8$^+$ T cells can be silenced, memory T cells elicited by natural AAV infection may be regulated similarly. This approach would have important clinical applications to abrogate potentially destructive
memory T cells in 80 percent of the human population that is immune to AAV. In addition, we strive to continue studying the mechanism that induces programmed cell death to the T cells that infiltrated vector-transduced mouse muscle. Such a mechanism may be used to permanently anergize all unwanted T cells that impede long-term transgene expression following rAAV vector treatment.
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