Impact Of Host Factors On The Adaptive Immune Response To Aav Gene Therapy

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Impact Of Host Factors On The Adaptive Immune Response To AAV Gene Therapy

Abstract
Impact of host factor on the adaptive immune response to AAV gene therapy

Scott Ashley
James Wilson

Adaptive immune responses to the transgene product remain an active area of concern for the gene therapy field. How host factors can influence the activation of the immune system is an important consideration in the development of gene therapy for different genetic disorders. One factor considered by many to shape the adaptive immune response is an individual’s genotype. Nonsense mutations were thought to result in an absence of tolerance to a replacement protein provided by gene therapy, due to a lack of antigen presentation during T cell development and negative selection. In this work we demonstrated that a class of nonsense mutations, premature termination codons (PTC), found in ornithine transcarbamylase deficiency (OTC) patients do not inhibit antigen presentation of C-terminal epitopes. We further found that these PTC containing OTC genes were able to induce anergy in a model of peripheral tolerance. These results change how we think about the relationship between the genotype and immune response, which indicate that individuals with PTC mutations may be less at risk of an adverse immune response attenuating the effects of gene therapy. We also identified vector factors that influence the adaptive immune response, activation of TLR9 and the tissues targeted for transduction and expression of the transgene. To investigate how inflammatory signaling might impact the outcome of adaptive immune responses; we use the transgenic OT-1 mouse model to interrogate how TLR9, the primary sensing molecule for vector DNA, can activate cytotoxic T cells against the transgene product OTC. These results confirm an important role for TLR9 induced inflammation being necessary for transgene specific T cell activation. To investigate the influence of TLR9 signaling and tissue targets on the humoral response, we used a mouse model of Mucopolysaccharidosis type I (MPS1) disease, and measured antibody generation to the secreted transgene product alpha-L-iduronidase (IDUA). We report that TLR9 signaling is also instrumental for the formation of anti-IDUA antibodies, as is expression of the transgene from the muscle. This work describes a novel process by which tolerance to a peptide located downstream of a PTC can be induced. This insight can help us better define the risks associated with an adaptive immune response based on an individual’s personal mutation. We also defined important vector factors which are important for activation of an adaptive immune response, and this knowledge could be exploited to generate safer gene therapy delivery methods.

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IMPACT OF HOST FACTORS ON THE ADAPTIVE IMMUNE RESPONSE TO AAV GENE THERAPY

Scott N Ashley

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IMPACT OF HOST FACTORS ON THE ADAPTIVE IMMUNE RESPONSE TO AAV GENE THERAPY

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Isabel, who have supported me and offered friendship and respite during periods of difficulty. Also, all of my friends, both new and old have kept me happy and enjoying life over the years I would like to thank them for being there.
ABSTRACT

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Adaptive immune responses to the transgene product remain an active area of concern for the gene therapy field. How host factors can influence the activation of the immune system is an important consideration in the development of gene therapy for different genetic disorders. One factor considered by many to shape the adaptive immune response is an individual’s genotype. Nonsense mutations were thought to result in an absence of tolerance to a replacement protein provided by gene therapy, due to a lack of antigen presentation during T cell development and negative selection. In this work we demonstrated that a class of nonsense mutations, premature termination codons (PTC), found in ornithine transcarbamylase deficiency (OTC) patients do not inhibit antigen presentation of C-terminal epitopes. We further found that these PTC containing OTC genes were able to induce anergy in a model of peripheral tolerance. These results change how we think about the relationship between the genotype and immune response, which indicate that individuals with PTC mutations may be less at risk of an adverse immune response attenuating the effects of gene therapy. We also identified vector factors that influence the adaptive immune response, activation of TLR9 and the tissues targeted for transduction and expression of the transgene. To investigate how inflammatory signaling might impact the outcome of adaptive immune responses; we use vi
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# TABLE OF CONTENTS

ACKNOWLEDGMENT ........................................................................................................ III

ABSTRACT ........................................................................................................................ VI

LIST OF TABLES .............................................................................................................. XI

LIST OF FIGURES .......................................................................................................... XII

CHAPTER 1: INTRODUCTION ......................................................................................... 1

Gene Therapy .................................................................................................................. 1

Viral Vectors and Immunology ....................................................................................... 2

Development of Viral Vectors ......................................................................................... 4

AAV Vector Structure and Biology ............................................................................... 6

AAV Immunogenicity ..................................................................................................... 7

Unfolded Protein Response ........................................................................................... 8

Antigen Presentation ..................................................................................................... 11

Goals of this Dissertation ............................................................................................ 12

CHAPTER 2 .................................................................................................................. 15
TLR9 SIGNALING MEDIATES ADAPTIVE IMMUNITY AGAINST TRANSGENE PRODUCTS FOLLOWING SYSTEMIC AAV GENE THERAPY

Abstract ........................................................................................................................................... 16

Introduction ...................................................................................................................................... 16

Materials and Methods .................................................................................................................... 18
  Animals ........................................................................................................................................ 18
  Vectors .......................................................................................................................................... 19
  Epitope Mapping .......................................................................................................................... 20
  Liver Homogenization .................................................................................................................. 20
  OTC Activity Assay .................................................................................................................... 21
  Biodistribution ............................................................................................................................ 21
  RNA Analysis ............................................................................................................................... 22
  CD8 Stain on Frozen Sections ...................................................................................................... 22
  Ki67 Stain on Paraffin Sections .................................................................................................... 23

Results ............................................................................................................................................... 23

Discussion ......................................................................................................................................... 29

CHAPTER 3: TOLERANCE TO ANTIGEN LOCATED DOWNSTREAM OF NONSENSE MUTATIONS ................................................................................................................................. 42

Abstract ......................................................................................................................................... 43

Introduction ...................................................................................................................................... 44

Materials and Methods .................................................................................................................. 46
CHAPTER 4: INFLUENCE OF VECTOR ON ANTI-TRANSGENE ANTIBODY FORMATION FOLLOWING AAV-MEDIATED GENE THERAPY

Abstract

Introduction

METHODS
ELISPOT ................................................................................................................................................. 84

Results .................................................................................................................................................. 84

Discussion ............................................................................................................................................ 88

CHAPTER 5: DISCUSSION .................................................................................................................. 98

General Summary .................................................................................................................................. 98

Gene Therapy, Immunology and TRL9 ................................................................................................. 98

Tolerance to Epitopes C-Terminal of a PTC ......................................................................................... 104

Anti-Transgene Antibodies .................................................................................................................... 110

BIBLIOGRAPHY ................................................................................................................................. 117

LIST OF TABLES

Table 1  Private mutations selected to give a range of mutation types, including nonsense, missense, and splice variants resulting in exon-skipping. .......................................................... 74

Table 2  Peptide identification using LC/MS with affinity-purified samples expressing GFP or hOTC-C109X-StrepII ........................................................................................................... 78
LIST OF FIGURES

Figure 1 Antigen presentation from transgene product ................................................................. 14

Figure 2 hOTC Immunodominant Epitope Mapping in C57BL/6 mice ........................................... 32

Figure 3 Evaluation of OTC-SIINFEKL Activity in vivo ................................................................. 33

Figure 4 hOTC-SIINFEKL Mitochondria Localization .................................................................. 34

Figure 5 Systemic Inflammation Breaks Tolerance to a Transgene Product .................................... 35

Figure 6 Effect of T cell population on Immune Response ............................................................... 36

Figure 7 Inflammation after T cell to Antigen Exposure ................................................................. 37

Figure 8 T cell Infiltration and Hepatocyte Proliferation ............................................................... 39

Figure 9 Transgene-Specific T cell Infiltration .............................................................................. 40

Figure 10 Extrinsic TLR9 Signaling is Required for a Destructive CTL Response ......................... 41

Figure 11 In vitro antigen presentation of epitopes downstream of reported OTC gene mutations ...... 66

Figure 12 Alternative start sites enable expression of epitopes downstream of nonsense mutations .... 68

Figure 13 Amino acid coverage of C109X and unique amino acid sequences detected in samples expressing hOTCco-C109X-StrepII ................................................................. 69

Figure 14 In vivo activation of endogenous T cells to an epitope downstream of the PTC ................. 70

Figure 15 In vivo tolerance induction to epitopes downstream of the PTC ....................................... 72

Figure 16 PTC near C-terminus inhibits in vivo tolerance induction to epitopes downstream of mutation. 73

Figure 17 hOTC codon sequence and transfection control ............................................................. 75

Figure 18 Impact of intronic sequence on mRNA expression and antigen presentation ................. 77
Figure 19 TLR9 Signaling is required for an anti-transgene antibody response ........................................ 92

Figure 20 TLR9 Signaling is Required for an Anti-Transgene CTL Response ....................................... 93

Figure 21 TLR9 KO B cells are intrinsically capable of mounting an anti-transgene response ................. 94

Figure 22 CpG+ and CpG- sequences have equivalent activity in vitro ................................................. 95

Figure 23 CpG Depletion Does Not Inhibit Anti-IDUA Formation ....................................................... 96

Figure 24 Suppression of Muscle Expression Increase Transgene Activity ........................................... 97

Figure 25 Model of antigen presentation in PTC containing genes ..................................................... 116
Chapter 1: Introduction

Gene Therapy

Gene therapy is a therapeutic approach designed to treat a wide variety of diseases by delivery of genetic material. There are many different methods that fall under this umbrella including, but not limited to, delivery of a gene to replace a defective gene (Nathwani, Reiss et al. 2014), direct editing of the genome to correct an error (Yang, Wang et al. 2016), delivery of a gene to produce a therapeutic protein agent such as an antibody (Limberis, Adam et al. 2013), delivery of RNA or DNA to down regulated expression of an endogenous protein (Fitzgerald, Frank-Kamenetsky et al. 2014). The most straight forward and explored application is the delivery of a gene by a viral vector to correct a monogenetic disorder. This type of gene therapy has its origins in protein replacement, where individuals are given enzyme to replace a defective or missing protein. However, protein replacement can have limits such as cost, constant re-administration, and trafficking of the enzyme to proper target cells. One application of gene therapy is the promise of a single dose cure that would allow the body to constantly replace the enzyme instead of requiring continuous re-infusions. Vector design can also lead to targeted delivery of the gene allowing for the enzyme to be delivered to its proper location. As an early example of this potential we can look to organ transplant, particularly of the liver, where the transplanted organ, containing the corrected gene, produces the missing protein for the rest of the individual’s life (Starzl, Bilheimer et al. 1984). It is for these disorders that gene therapy is making strides in bringing a better
standard of care (Naldini 2015). For many genetic disorders, however only palliative care is available and the promise of gene therapy could enable individuals facing difficult challenges the opportunity to live a longer with an improved quality life.

**Viral Vectors and Immunology**

One theme in the development of gene therapy for monogenic diseases has been the obstacle that a patient’s own immune system can present. Two types of immune responses can occur, one is innate immunity driven by myeloid lineage leukocytes that primarily respond to generic non-self-patterns often found in pathogens including non-nuclear un-methylated DNA, double stranded RNA, or lipopolysaccharide. The other immune response is adaptive and has the ability to target specific diseases by recognizing either small peptide sequences or the three dimensional structure of large macromolecules. Gene therapy attempts to deliver a corrected gene that will express a protein. The body can potentially recognize that protein, the transgene product, as a foreign or “non-self” entity. When this occurs the adaptive immune response develops specifically targeting both the transgene product and the cells that produce it (Mendell, Campbell et al.). Adaptive immune responses that are able to recognize self-antigens from non-self-proteins are divided into two branches, the humoral, comprised of B cells, and the cytotoxic, mediated by T cells. Both these lymphocytes lineages undergo a maturation that will remove T and B cells which recognize self-antigen from the population by mechanisms of clonal deletion in a process known as central tolerance (Kappler, Roehm et al. 1987, Pelanda and Torres 2012). These safeguards however can
be incomplete as self-reactive T and B cell do escape the thymus and bone marrow respectively and can cause a disease state of auto-immunity where the cells activate against self-tissue (Yurasov, Wardemann et al. 2005, Meffre 2011, Pobezinsky, Angelov et al. 2012). Another level of tolerance exists that helps mitigate the potential damage self-reactive T and B cells can inflict. Peripheral tolerance involves many mechanisms to keep naïve self-reactive lymphocytes from becoming fully active, including a) ignorance, where the lymphocyte will have highly diminished contact with its specific antigen mostly occurring in so called immune-privileged regions such as the eye, brain, and testes (Streilein, Takeuchi et al. 1997) b) anergy, a state of non-responsiveness that occurs when antigen is presented to the lymphocyte without the secondary signal provided by co-stimulation necessary to initiate T cell activation (Schwartz 2003) or c) induced by direct suppression of the auto-reactive lymphocytes by regulatory cells (Lim, Hillsamer et al. 2005, von Boehmer 2005). While peripheral tolerance is difficult to overcome it can occur when danger or inflammatory signaling occurs, such as during infection, tissue damage, or cellular stress (Lang, Georgiev et al. 2006, Zhang, Shen et al. 2006, Ferrero-Miliani, Nielsen et al. 2007). These processes are important in understanding the difficulties imposed by the immune system on gene therapy. Many patients that would receive gene therapy do not express the protein at all due to a “null” mutation caused by nonsense mutations, frameshifts, large deletions or chromosomal rearrangements. This would increase the risk of an adverse reaction as no antigen would be presented during central tolerance allowing for a pool of potentially high affinity lymphocytes to exist (Naeher, Daniels et al. 2007). For patients that do express non- or low-functioning
protein it is believed they are at less risk to develop a transgene immune response because lymphocytes should still undergo central tolerance, however escaped cells could still react if enough inflammatory signaling occurs around the time of vector expression. These signals can be caused by the vector itself and potentially by the transgene product or the protein of replacement (Rogers, Martino et al. 2011).

**Development of Viral Vectors**

Delivery of therapeutic genetic material into diseased cells is one of the critical aspects of consideration in gene therapy. In the early days of the field, adenoviral vectors were used to package DNA and deliver it to the nucleus of infected cells. These vectors, however, were phased out of use for treatment of genetic disorders, despite positive aspects such as a large gene capacity and high transduction efficiency, since they were found to induce robust immune responses (Bessis, GarciaCozar et al. 0000, Chen, Murphy et al. 0000). Early observations of the enhanced immune responses were found in the transduction of mouse liver with E1 and E3 deleted adenoviral vector expressing LacZ in immune competent CBA mice which cleared both expression of the transgene and genome copies, whereas immune compromised nude mice that lack T cells due to loss of proper thymus development maintained expression throughout the duration of the study (Yang, Nunes et al. 1994). Expression of adenoviral proteins by the vector was found to be a major immunogenic stimulus that resulted in the observed destructive CTL response (Yang, Jooss et al. 1996). These findings resulted in the rational design of the adenoviral vector by removing both the E1 and E4 regions of the genome to produce a less immunogenic
vector capable of long term stable expression in mice (Gao, Yang et al. 1996, Dedieu, Vigne et al. 1997, Wang, Greenburg et al. 1997, Christ, Louis et al. 2000). This vector was eventually taken to the clinic with tragic results when the 18th patient administered the vector, a man with partial ornithine transcarbamylase (OTC) deficiency, had an acute inflammatory system response that resulted in multi-organ failure and death (Raper, Chirmule et al. 2003).

The safety concerns raised in this clinical trial prompted a period of introspection for the field of gene therapy. The field redirected its focus to a different vector system, one based on adenovirus-associated virus (AAV). This virus was originally discovered as a contaminant of adenovirus preps (Atchison, Casto et al. 1966). A primary reason for the use of AAV vectors was the reduced innate immune activation by AAV compared to adenovirus (Zaiss, Liu et al. 2002). With a small viral genome comprised of only two genes Rep, required for replication and Cap encoding for the protein capsid components it was able to be completed gutted with the gene’s removed for the therapeutic transgene (Zhou and Muzyczka 1998). Also during this time a number of new AAV serotypes were discovered broadening the tissue types that could be targeted for transduction including AAV serotype 8 for liver and AAV serotype 9 for lung and the central nervous system (CNS) (Gao, Vandenberghe et al. 2005, Gao, Lu et al. 2006, Limberis and Wilson 2006, Hinderer, Bell et al. 2014, Hinderer, Bell et al. 2014). Initial results reported that transduction of mice liver and muscle mediated tolerance of expression to non-self-antigens (Carter and Samulski 2000). However, despite the promise shown by initial
work, immune responses to AAV mediated gene therapy did arise albeit in a more subdued form than those that plagued adenovirus (Zaiss, Liu et al. 2002).

**AAV Vector Structure and Biology**

AAV, a parvovirus, has become the viral vector of choice for many in the field primarily due to its advantage over other vectors with its lower immunogenicity (Flotte and Carter 1995) compared to that of adenovirus vectors and its lack of integration which has caused problems for retrovirus-based therapies (Hacein-Bey-Abina, Von Kalle et al. 2003). AAV is a simple virus generated from only two genes *Rep* and *Cap* which code for a helicase needed for genome replication (Im and Muzyczka 1990) and for the three subunits of the viral capsid, respectively. The subunits that comprise the AAV capsid are VP1, VP2 and VP3 and give the particle its three dimensional conformation and contain regions of variability which can give different serotypes varying co-receptor affinities and tropism (Zincarelli, Soltys et al. 2008). AAV vectors have had their genomes entirely replaced retaining only the inverted terminal repeats (ITRs) necessary for replication of the construct which form a double stranded hairpin at the ends of the linear vector genome (Bohensky, Lefebvre et al. 1988); the genome therefore has both regions of double and single stranded DNA. The AAV vector enters the cell through endocytosis and traffics through endosomes to the nucleus where it enters and un-coats (Ding, Zhang et al. 2005, Xiao and Samulski 2012). The vector genome is then transcribed leading to expression of the transgene product where leader peptides will guide the therapeutic protein to its intended destination.
AAV Immunogenicity

There are three main components of the AAV vector that have the potential to induce an immune response: a) capsid b) vector genome and c) the transgene product. The capsid is, at best, a foreign protein the body has not yet encountered, however, since AAV is a naturally occurring virus many individuals have had previous exposure and may have antibodies to one or several serotypes (Calcedo, Vandenberghe et al. 2009). Capsid specific T cells have also been found following vector administration in human clinical trials that lead to transduction attenuation (Manno, Pierce et al. 2006). Additionally in our lab we have demonstrated that empty AAV capsid particles devoid of any genome are able to inherently induce an antibody response indicating that the capsid is not only a target of the adaptive immune response, but also inherently immune activating. The vector genome has also been implicated as a major source of immunogenic material (Zhu, Huang et al. 2009, Martino, Suzuki et al. 2011, Rogers, Martino et al. 2011, Rogers, Martino et al. 2014). Innate activation by vector genome is driven primarily through toll-like receptor nine and myeloid differentiation primary response gene 88 (TLR9-MyD88) signaling. Specifically plasmacytoid dendritic cells (pDC) express a high amount of TLR9 in endosomes which may be responsible for the initial inflammatory signaling by secretion of type one interferons. Studies in TLR9 KO mice have implicated its importance in both innate and T cell activation by intramuscular administration of AAV vector (Zhu, Huang et al. 2009, Faust, Bell et al. 2013). While MyD88 signaling seems important to anti-transgene antibody formation, the role of TLR9 is more complicated and it has been shown to have a limited effect on antibody generation despite downstream
signaling protein being vital (Rogers, Suzuki et al. 2015). Finally, the gene of replacement and the gene product may elicit an immune response to the gene therapy. For the transgene product, as mentioned previously, the body may not recognize the protein as non-self and be capable of mounting an adaptive response to it (Figure 1). This is thought to be influenced by the underlying gene mutation, where a null mutation with no expression or non-null with some residual protein may alter the immune response outcome following AAV gene therapy (Cao, Hoffman et al. 2009, Rogers, Martino et al. 2014). It may also be possible for a non-null mutation to be immunogenic in its own right; the generation of a non-functional protein has the potential to cause toxicity or cellular stress which can lead to a pro-inflammatory state setting the stage for adaptive immune activation. (Carrell and Lomas 2002, Zhang, Wang et al. 2011).

**Unfolded Protein Response**

The unfolded protein response (UPR) is an important cell maintenance pathway which is responsible for cell survival in the event of cellular stress caused by an accumulation of improperly folded proteins usually found in the endoplasmic reticulum. This pathway is well defined and mediated by three different branches controlled by a inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATTF6) and protein kinase RNA (PKR)-like ER kinase (PERK) (Ron and Walter 2007). Activation of the UPR can either lead to resolution of the protein folding defect or death of the cell through activation of apoptotic pathways through the pro-apoptotic protein CHOP (Lai, Teodoro et al. 2007). Recent studies have linked the UPR to inflammation through several different
mechanisms. Activation of NF-κB, a key transcriptional regulator in the onset of inflammation, has been proposed as one such mechanism. Oxidative stress or calcium release induced by the UPR has been tentatively linked to NF-κB activation (Nakagawa and Yuan 2000). Another direct link is through PERK-eIF2α attenuation of transcription. PERK senses accumulation of mis-folded protein accumulation when ER chaperone BiP disassociates with the PERK ER domains and binds to the unfolded proteins (Bertolotti, Zhang et al. 2000). This results in PERK auto-phosphorylation which then phosphorylates eIF2α preventing the formation of the 80s ribosome and a non-specific protein synthesis inhibition (Kim, Son et al. 1998). In another study, UV irradiation was used to phosphorylate eIF2α and resulted in activation of NF-κB. The authors concluded that the higher turnover of IκB an inhibitor NF-κB resulted in a depletion of IκB when translation was inhibited allowing NF-κB to migrate to the nucleus(Wu, Tan et al. 2004).

Cross-talk between the UPR and inflammatory pathways also occurs, an example is the liver specific transcription regulator CREBH which can induce the acute phase response (ARP) and is itself induced by pro-inflammatory cytokines (Zhang, Shen et al. 2006). Activation occurs when ER stress induces CREBH to translocate to the Golgi apparatus and becomes cleaved by SP1 and SP2. The functional transcription factor will mediate activation of ARP proteins (Zhang, Shen et al. 2006). The mitochondrion is another cellular compartment that can induce cellular stress under its own mitochondria unfolded protein response (mtUPR). This pathway is less well defined than the traditional UPR found in the endoplasmic reticulum but major signaling pathways have been discovered using a mutated form of mitochondria matrix protein OTC (Zhao, Wang et al. 2002,
Horibe and Hoogenraad 2007). We also know that mitochondrial stress/oxidative stress can result in increases of inflammation in the liver a site of unique mitochondria activity (Satapati, Kucejova et al. 2015).

**Inflammation**

Successful activation of the adaptive immune response requires engagement of the innate immune system to create the necessary conditions for recruitment and priming of T and B cells known as inflammation. This process begins when pattern recognition receptors, such as TLR9, start signaling upon exposure to their binding target. These target molecules are either a molecule unique to a pathogen or, as is the case for TLR9, ones found in a compartment where endogenous molecules of that type are not normally located (Jounai, Kobiyama et al. 2012). These patter recognition proteins can broadly be broken down into two classes those that recognize pathogen associated molecular patterns (PAMP) and ones that binding to markers of damage associated molecular patterns (DAMP). The signaling cascade for TLRs, a class of PAMPs, leads to the freeing of transcription factor NF-kB from its inhibitor molecule, and subsequent migration to the nucleus where it can up-regulate the transcription of pro-inflammatory cytokines, such as type 1 interferons and IL-12 (Tsujimura, Tamura et al. 2004). These cytokines recruit innate immune cells such as neutrophils and macrophages to which can both begin clearance of the disease vector through non-specific cell lysis, and contribute more cytokines to the inflammatory milieu. Professional antigen presenting cells, such as dendritic cells, will also become licensed by inflammatory cytokines and be able to
present both the primary signal of the MHC-antigen complex to T cells, and stimulate co-receptor molecules such as CD28 or inducible co-stimulator (ICOS) (Sallusto and Lanzavecchia 2002). This secondary signaling event is important as without it T cells exposed to their specific MHC-antigen complex alone will undergo the process of anergy, becoming hypo-responsive and quiescent (Lin, Hensley et al. 2007).

**Antigen Presentation**

A major component of the adaptive immune system is its ability to recognize protein sequences conferring individual T cells antigen specificity. The process of antigen presentation has multiple pathways that end with the loading of a linear poly-peptide antigen sequence onto major histocompatibility protein (MHC) which traffics to the cell surface where it can come into contact with a T cell receptor capable of binding to it (Hewitt 2003). It is the amino acid sequence in combination with the MHC that confers specificity of bind to individual T cell receptors, each of which can be unique due to the process of V(D)J recombination. MHC genes are polymorphic in both human and mouse populations, and this diversity can lead to difference in binding strength of it to antigen. The source of these poly-peptides has been a somewhat contentious debate in the field, divided amongst those who think that whole protein during the normal recycling of proteins is degraded by the proteasome and some of the products are further processed by catalytic enzymes before transport through the TAP channel into the ER for MHC loading (Colbert, Farfan-Arribas et al. 2013). Other scientists have described a process where aborted translational event products comprise the majority of peptides trafficked
into the antigen presentation pathway, these products were first observed from early termination and have since been found in a variety of incomplete translation events (Yewdell, Anton et al. 1996, Dolan, Li et al. 2010, Dolan, Li et al. 2011). These competing theories could have an important impact for gene therapy, as without antigen presentation an individual cannot be tolerant to a self-protein and if presentation is fueled primarily be retired full-length protein then people with a mutation that prevents all or partial translation would be likely to be intolerant to the mutated protein.

**Goals of this Dissertation**

In this dissertation I decided to investigate the factors involved in mediating the activation of an adaptive immune response to the transgene product following liver–directed AAV gene therapy. First, I created a model to explore the necessary stimuli needed to initiate a T cell response to a mitochondrial targeted protein OTC, and the resultant adverse events following adaptive immune activation. I hypothesized that TLR9 activation would be necessary and sufficient to engage a T cell response and that this response would attenuate transgene expression by elimination of transduce cells. In Chapter 2 I present my work using a transgenic mouse model to explore the activation and consequence of an anti-transgene CD8⁺ T cell response. Also we studied the influence that genotype has on the ability to present antigen and induce anergy in CD8⁺ T cells. As discussed in Chapter 3, to investigate the antigen presentation from premature termination codon (PTC) containing genes, I conducted a series of *ex vivo* antigen presentation assays to determine the method by which epitopes downstream of a PTC are
presented on MHC class I. Subsequently, in *in vivo* experiments in mice I found that genes containing PTC were still able to induce anergy in CD8+ T cells to epitopes downstream of the mutation. Finally, I sought to understand the factors that drive the activation of a humoral response against a secreted transgene product. In Chapter 4, it is discussed how we determined that TLR9 is necessary for the generation of an anti-transgene antibody response. Furthermore, I found that removal of transgene CpG motifs was insufficient to reduce anti-transgene responses and that CpG islands or other factors such as the tissue location of transgene expression play a vital role in humoral responses.
Figure 1 Antigen presentation from transgene product

A model of immune detection for an AAV vector and transgene product. AAV vector, upon encountering its target cell will enter through endocytosis. Once inside the cell AAV will escape the endosome or be digested as the compartment becomes increasingly acidified. A portion of vector genomes will uncoat in the endosome and bind to TLR9 initiating a signaling cascade resulting in the translocation of transcription factor NF-κB to the nucleus. This will result in the up-regulation of inflammatory genes. Vectors that manage to escape the endosome can enter the nucleus through the nuclear pore. Uncoating will occur inside the nucleus and the released genome can be made into double stranded DNA and transcribed. The resulting transgene product will then as part of the natural turnover of proteins be ubiquitinated and traffic to the proteasome for degradation. Some of the resultant peptide fragments will enter the endoplasmic reticulum (ER) through the TAP1/TAP2 channel. Peptides in the ER will be further trimmed by proteases and then loaded onto the major histocompatibility complex (MHC). The MHC-peptide complex will then traffic to the cell surface and present the antigen to its specific T cell.
Chapter 2

TLR9 signaling mediates adaptive immunity against transgene products following systemic AAV gene therapy

Chapter 2 is adapted from the manuscript Scott N. Ashley, Suryanarayan Somanathan, April Giles, Christine Draper, Deirdre McMenamin, and James M. Wilson. In preparation.
Abstract

Adaptive immune responses to the transgene product remain a concern for the field of gene therapy. Driven by the immune stimulus of vector administration, transgene expression can elicit an adaptive immune response against the therapeutic protein, particularly for recessive diseases in which these antigens are not presented to lymphocytes during central tolerance induction. Here, we show that TLR9 signaling is involved in driving the activation of CD8\(^+\) T cells against mitochondrial-targeted ornithine transcarbamylase (OTC) following systemic AAV vector administration. Using a CD8\(^+\) T cell receptor transgenic mouse model system, we demonstrate that TLR9 signaling extrinsic to T cells is sufficient to induce a robust cytotoxic T cell response against OTC, resulting in transgene expression loss. Overall, our results suggest that inflammation mediated by TLR9 signaling is important for the development of adaptive immune responses to transgene products following AAV gene therapy.

Introduction

AAV vectors have shown potential in clinical trials to treat several recessive diseases by replacing the missing or defective gene (Simonelli, Maguire et al. 2010, Nathwani, Reiss et al. 2014, Jacobson, Cideciyan et al. 2015, Naldini 2015). In many preclinical animal models, adaptive immune responses to the transgene product have been observed; this is particularly true when a “non-self” protein is expressed from the delivered gene, as the recipient may be more likely to develop an immune response to antigens that are not present during lymphocyte development (Fields, Kowalczyk et al. 2000, Ding, Hodges et
However, the adaptive immune response is tightly controlled and exposure to a novel protein often results in tolerance rather than immunity, unless an inflammatory signal is present to initiate an adaptive immune response to the novel antigen (Lang, Georgiev et al. 2006). One potential immunogenic stimulus associated with AAV-mediated gene therapy is the vector genome. The immune system’s major sensing agent for this foreign material is TLR9, located in the endosomal compartment, which recognizes unmethylated CpG DNA and induces pro-inflammatory cytokine signaling that can result in an adaptive response (Hemmi, Takeuchi et al. 2000, Rogers, Martino et al. 2011).

The ability of TLR9 to upregulate pro-inflammatory cytokines in response to AAV vectors has been observed both by ex vivo stimulation of plasmacytoid dendritic cells (pDCs) and by intramuscular (IM) AAV2 vector administration in mice (Zaiss, Liu et al. 2002, Zhu, Huang et al. 2009). Self-complementary AAV genomes containing double-stranded DNA were implicated in more robust TLR9 activation resulting in a diminished induction of tolerance (Martino, Suzuki et al. 2011). Additional work has been done to avoid TLR9-induced inflammatory signaling by CpG depletion or by increasing the CpG methylation state in DNA-based therapies (Reyes-Sandoval and Ertl 2004, Faust, Bell et al. 2013, Shapir, Miari et al. 2015). However, there is evidence that TLR9 binds the phosphodiester backbone of DNA regardless of the nucleotide sequence, and that activation is merely enhanced by CpG motifs (Haas, Metzger et al. 2008).
I sought to investigate the influence of TLR9 on adaptive immune responses following AAV gene therapy using a systemic of administration for delivery to the liver. I found that systemic inflammation mediated by a TLR9 agonist was able to break tolerance to the mitochondrially targeted ornithine transcarbamylase (OTC) protein and cause the destruction of transduced cells. These findings suggest that, while TLR9 activation can be consequential in eliciting a transgene specific adaptive immune response, our ability to curb its influence may be limited.

Materials and Methods

Animals

6-10 weeks of age OT-1 mice on a C57BL/6 background, C57BL/6, and B-cell -/- muM- (stock number 002288) mice were acquired from The Jackson Laboratory (Bar Harbor, ME). 6-8 weeks of age TLR9 KO mice on a C57BL/6 background mice were a kind gift from Dr. Koretsky (University of Pennsylvania, Philadelphia, PA); these mice were created by Dr. Akira (Osaka University, Osaka, Japan) (Hemmi, Takeuchi et al. 2000). All mice were housed and bred under specific pathogen-free conditions in the Translational Research Laboratory Animal Facility at the University of Pennsylvania. Procedures were performed under IACUC approved protocols.
Vectors

AAV2/8sc.TBG.hOTCco (self-complementary codon-optimized human ornithine transcarbamylase transgene under the control of a TBG promoter containing the G4SVPA poly A signal sequence) was produced by the University of Pennsylvania Vector Core. To generate the sc.hOTCco-SIINFEKL transgene cassette, the immunodominant epitope of chicken ovalbumin (Ova257-264 SIINFEKL) was cloned into the sc.hOTCco transgene immediately following the C-terminal residue.

Adoptive Transfer

Mice were administered intravenously via the tail vein 1x10^{11} genome copies (GCs) of AAV8 expressing either sc.hOTCco-SIINFEKL or mutant variants in a total volume of 100 µl PBS. On day 14 post vector administration, CD8^{+} T cells were collected from OT-1 mouse spleens using CD8a^{+} T Cell Isolation Kit (Miltenyi Biotec, San Diego, CA). CD8^{+} T cell purity was >90%, as measured by flow cytometry. Vector treated mice were then given 1x10^{6} CD8^{+} T cells in 100 µl PBS by tail vein injection. Concurrent with adoptive cell transfer and for three days following, mice were given intraperitoneal (IP) a mixture of 20 µg TLR9 ODN 2395 and 20 ug ODN M363 (InvivoGen, San Diego, CA). For labeling T cells, CFSE (eBioscience, San Diego, CA) was diluted in DMSO to a 5 mM stock solution. T cells were resuspended to 5x10^{7} cells/ml. 1 µl of 0.5 mM CSFE was added to each ml of cell suspension and incubated at 37 °C for 10 minutes. Cells were then washed two times with DMEM and two times with PBS, before resuspension in PBS at a concentration of 1x10^{7} cells per ml. Retro-orbital bleeds were performed
weekly throughout the study and samples were submitted to Antech Diagnostics (Irvine, CA) for analysis of transaminase and bilirubin levels.

**Epitope Mapping**

C57Bl/6 mice received IM $5 \times 10^{11}$ GC of AAVrh32.33.CB7.hOTCco followed 12 weeks later by an intramuscular injection of $5 \times 10^{10}$ particles (delivered as two 50 µl injections) of Ad5 vector expressing hOTCco. On day 10 post Ad5 vector immunization, mice were sacrificed and splenocytes were harvested as described above (Section: Adoptive Transfer). An interferon γ (IFN-γ) ELISPOT assay (BD Biosciences, San Jose, CA) was performed according to the manufacturer’s instructions. Cells were seeded at a concentration of $2 \times 10^5$ or $2 \times 10^4$ cells per well along with 2 µg/ml of hOTC peptide pools or pool B containing an overlapping 15-mer peptide library (Mimotopes, Victoria, Australia) spanning the full length of the Ad5 hexon. Splenocytes were incubated at $37^\circ C$ in 5% CO$_2$ for 18 h. Spot forming units (SFUs) were counted using the AID ELISPOT Reader System (Cell Technology, Columbia, MD).

**Liver Homogenization**

Livers previously harvested and frozen at -80°C were ground in liquid nitrogen. 20 mg of ground liver and added 20 µL of 50 mM Tris buffer ph 7.5 (Sigma-Aldrich, St. Louis, MO) per mg liver powder, then homogenize on ice for 5 seconds, 10 times. We repeat homogenization routine once, then centrifuged at 13,200 rpm for 2 min and transfer 20
µL supernatant into another new labeled tube with 180 µL 50 mM Tris buffer ph 7.5, homogenate can be kept on ice until use in activity assay.

**OTC Activity Assay**

We added 50 µl of diluted supernatant to 500 µl reaction buffer consisting of 5mM ornithine (Sigma-Aldrich, St. Louis, MO) in 50 mM Tris buffer pH 7.5. To start the reaction we added 50 ul 4.8mM carbamyl phosphate (Sigma-Aldrich, St. Louis, MO) to reaction mixture and allow reaction to continue for 5 min at 25C. The reaction was stopped by quenching with 500 µl of 5 mM C14 citrulline, 30% trichloroacetic acid (Sigma-Aldrich, St. Louis, MO). Activity was determined by formation of citrulline as a ratio of C14 citrulline as measured by liquid chromatography–mass spectrometry.

**Biodistribution**

Liver samples were frozen on dry ice at the time of necropsy, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Detection and quantification of vector GCs in extracted DNA were performed by real-time PCR, as described previously (Bell, Moscioni et al. 2006). Briefly, genomic DNA was isolated, and vector GCs were quantified using primers/probes designed against the sequence of the TBG promoter. Quantification of GCs from liver was performed on one liver sample from each mouse.
RNA Analysis

Liver samples were frozen in liquid nitrogen at the time of necropsy. RNA was extracted using TRIZOL (Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. 12 µg of RNA was then treated with DNase I (Roche, Basel, Switzerland), according to the manufacturer's protocol. The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to remove DNase prior to cDNA synthesis by reverse transcription using the Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA). Real-time PCR was then performed on cDNA with primers binding to the hOTCco transgene with Power SYBR Master Mix for detection (Life Technologies, Carlsbad, CA).

CD8 Stain on Frozen Sections

Frozen liver sections were fixed in acetone at -20 °C for 7 minutes, air dried, blocked in 1% donkey serum (Jackson Immuno West Grove, PA) in PBS for 20 minutes, and incubated with 1:20 primary rat antibody against CD8 (clone 53-6.7, BD Biosciences) diluted in blocking buffer for 45 minutes. After washing in PBS, sections were stained with secondary donkey antibodies labeled with FITC (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 minutes, washed in PBS, and mounted in Vectashield immunofluorescence stain containing DAPI (Vector Laboratories, Burlingame, CA).
Ki67 Stain on Paraffin Sections

Paraffin sections were deparaffinized through an ethanol and xylene series, boiled in a microwave for 6 minutes in 10 mM citrate buffer, pH 6.0 for antigen retrieval, and treated sequentially with 2% H$_2$O$_2$ (Sigma-Aldrich, St. Louis, MO) for 15 minutes, avidin/biotin blocking reagents (Vector Laboratories, Burlingame, CA) for 15 minutes, and blocking buffer (1% donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS with 0.2% Triton) for 10 minutes. Sections were then incubated for 1 hour with 1:500 primary rabbit serum against Ki67 (ab15580, Abcam, Cambridge, United Kingdom) and 45 minutes with FITC-labeled secondary donkey antibody (Jackson Immunoresearch, West Grove, PA) diluted in blocking buffer. Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing DAPI as a nuclear counterstain.

Results

In previous work, our group investigated the importance of inflammation on gene therapy in the liver using reporter gene, nuclear-targeted beta-galactosidase (nlacZ) (Somanathan, Breous et al. 2010, Breous, Somanathan et al. 2011). Our results suggested that in the presence of a strong inflammatory signal, T cell targeting of a transgene product could result in the loss of transgene expression and destruction of transduced cells. Thus, I probed the ability of TLR9 signaling to initiate a destructive cytotoxic T cell (CTL) response following gene therapy for human OTC (hOTC), a promising candidate for clinical applications. First we attempted to determine if hOTC could be targeted by a
CTL response in C57BL/6 mice. However, even when expressing hOTC using an adenoviral vector, which is known to elicit strong immune responses following IM administration, a CTL response did not develop against hOTC (Figure 2a). This may be due to the high level of OTC protein homology (92%) between human and mouse. We attempted a more stringent regimen by priming the mice with $5 \times 10^{11}$ GC of AAVrh32.33CB7.hOTCco, then administering $5 \times 10^{10}$ particles of Ad5.hOTC with subsequent IP injections of TLR9 agonist to both the primed and an unprimed control group twelve weeks later (Figure 2b). While vector priming and TLR9 agonist administration led to a strong T cell activation against a hOTC epitope we determined that this was most likely the breaking of tolerance of antigen experienced T cells due to the transgene products’ similarity to mOTC. In order to activate naive T cells against hOTC, we cloned a well-characterized antigenic peptide from chicken ovalbumin (Ova257-264 SIINFEKL) to the C-terminus of hOTC. This model system allowed us to use OT-1 mice, which are transgenic for a SIINFEKL-specific T cell receptor (TCR) found on all T cells, to interrogate transgene-specific immune responses to systemic inflammation caused by TLR9 and its outcome on gene therapy.

The initial experiments with hOTCco-SIINFEKL were designed to investigate the behavior of the protein compared to the untagged version. hOTCco-SIINFEKL was packaged in an AAV8 vector with a TBG promoter (AAV8.TBG.hOTCco-SIINFEKL). 6-10 week old male SPF-Ash mice, a model of OTCD where activity is ~5% normal, were injected with either AAV8.TBG.hOTCco-SIINFEKL or the untagged
AAV8.TBG.hOTCco. Mice were evaluated for OTC enzyme activity at day 14 post administration (Figure 3 a) which showed minimal activity compare to control. We also evaluated urine orotate a metabolic marker of OTCD and showed that correction was slower to occur in hOTCco-SIINFEKL treated mice (Figure 3 b). We also investigated the ability of hOTCco-SIINFEKL to properly migrate to the mitochondria by transfecting the human liver cell line HuH7 on slide wells with OTC plasmids including one lacking the leader sequence required to guide the protein. Transfected cells were probed with Mito Tracker CMXRos to visualize the mitochondria and following up take, they were fixed for OTC immunohistochemistry (Figure 4). Staining revealed that hOTC-SIINFEKL translocated to the mitochondria similar to wild type hOTC, whereas without the leader sequence hOTC is dispersed throughout the cytoplasm indicating a disruption of normal biogenesis which could impact antigen presentation. Despite impaired OTC activity due to the addition of the tag, mitochondrial localization remained intact, and we will continue our investigation using hOTCco-SIINFEKL.

Male C57BL/6 mice 6-8 weeks old were treated with an intravenous (IV) injection of AAV8.TBG. hOTC-SIINFEKL. Fourteen days after vector injection, 1x10^6 SIINFEKL specific T cells were adoptively transferred from OT-1 mice. Concurrent with the adoptive transfer, we gave 40 µg of TLR9 agonist by IP injection (Figure 5 a). As anticipated, we only observed a CTL response (measured by transaminase levels) to the transgene product when both a population of transgene-specific OT-1 T cells and TLR9 inflammatory signals were present; transaminases aspartate transaminase (AST)
and alanine transaminase (ALT) were both elevated, indicating hepatocyte lysis (Figure 5 b). We also observed a ten-fold reduction in vector genome copy number and hOTCco mRNA in the livers of mice treated with both OT-1 T cells and TLR9 agonist, consistent with T cell mediated killing of transduced hepatocytes (Figure 5 c). We next tested whether a smaller T cell population (i.e., more similar in number to an endogenous, protein-specific naïve T cell population) could induce a comparable result (Moon, Chu et al. 2007, Jenkins and Moon 2012). We found that as few as three thousand adoptively transferred cells could cause transaminase elevation (Figure 6 a). We also observed a trend of decreased vector genome copies and reduced transgene mRNA (Figure 6 b), although this was not statistically significant at lower doses. Finally we wanted to determine if exposure of T cells to antigen prior to an inflammatory event would inhibit transgene loss. Inflammatory TLR9 agonist was either administered IP as before concurrent with CD8$^+$ T cells or 21 days post adoptive transfer. Transaminase elevations were muted in the late inflammation event group (Figure 7 a, b), as was transgene loss measured by mRNA or vector genomes though some did occur (Figure 7 c, d). This mRNA loss may have been sufficient to reduce protein expression as in two mice the loss of protein was observed by western blot, however one of the mice did retain normal expression.

In order to directly evaluate T cell mediated killing of transduced cells in the liver, mice were treated with AAV8.TBG.hOTCco-SIINFEKL, OT-1 CD8$^+$ T cells and 40 µg TLR9 agonist as previously described, and sacrificed at 3, 7, and 14 days after
adoptive transfer for histological evaluation of the liver. Liver sections were stained for CD8a to mark CD8+ T cells, and infiltration was quantified by image analysis (Figure 8 a, b). Marked CD8+ T cell infiltration occurred at periportal and pericentral locations beginning on day 3; by day 7 the infiltrates with TLR9 agonist had penetrated deeper into the liver, whereas further penetration was not seen for T cells without the enhanced TLR9 stimulation from administered agonist. In both groups, infiltration was resolved by day 14. Our previous experiment suggested that hepatocyte death occurred following treatment with TLR9 agonist; however terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and caspase-9 immunofluorescence did not show differences between control and experimental groups (data not shown). As an indicator of cell death, we also examined the regrowth of hepatocytes that were predicted to have undergone lysis resulting in loss of AAV genomes (Figure 8 c). Using Ki-67 staining to mark proliferation, we observed hepatocyte division to be spread out evenly across liver regions in the experimental groups, mirroring the degree of CD8+ T cell infiltration. We determined the percent of hepatocytes that were Ki-67 positive by image analysis (Figure 8 d), and found hepatocyte division to be much higher than the normal expected rate of turnover, indicating increased cell death above background (Miyaoka, Ebato et al. 2012, Miyaoka and Miyajima 2013). By determining genome copies at each time point (Figure 8 e), we observed that most loss occurred between three and seven days post adoptive transfer, correlating with the histology results (Figure 8 a, c); together, these data indicate that the majority of T cell infiltration and hepatocyte death occurs during this time span 7 to 14 days.
Next, we examined whether the infiltrating cells were indeed transgene-specific CTLs. We labeled CD8\(^+\) T cells with CFSE fluorescent cell staining dye before delivery into C57BL/6 mice previously administered with AAV8.TBG.hOTCco-SIINFEKL. Seven days after adoptive transfer and TLR9 stimulation, we isolated lymphocytes from the liver or spleen, and stained them with anti-CD8a and SIINFEKL tetramer (Figure 9 b, c). The majority of liver-isolated lymphocytes were tetramer positive, regardless of whether mice received TLR9 agonist (Figure 9 c). In contrast, the majority of spleen isolated T cells were tetramer positive only if the mice received TLR9 agonist. In addition, CFSE staining of tetramer positive splenocytes remained high in mice that did not receive TLR9 agonist (Figure 9 d) suggesting that transgene-specific activation of CD8\(^+\) T cells in the presence of TLR9 stimulation increased proliferation. We then sought to determine if intrinsic TLR9 signaling in transferred T cells alone would trigger an immune response, or if extrinsic signaling was required for the effect. We administered 6-8 week old male C57BL/6 mice or TRL9 \(-/-\) mice with AAV8.TBG.hOTCco-SIINFEKL. Fourteen days later, we adoptively transferred \(1 \times 10^6\) OT-1 CD8\(^+\) T cells into recipient mice that were also given 40 \(\mu\)g TLR9 agonist (Figure 10 a). AST and ALT elevation only occurred in the C57BL/6 group; despite TLR9 signaling being intact in transferred OT-1 T cells, this was insufficient to activate a robust CTL response (Figure 10 b,c). These results indicate that the TLR9 signaling required for initiating T cell responses must be extrinsic to the T cells themselves, perhaps occurring in transduced hepatocytes or antigen-presenting cells.
Discussion

Adaptive immune responses to a transgene product are complex from activation of antigen presenting cells to the balance of a productive to destructive immune response. Thus, when determining the best method for delivering a successful treatment, there are many factors to consider, such as the route of administration and vector genome composition. These components may have a dramatic effect on the likelihood that a given therapy will induce an inflammatory environment, elicit an adaptive anti-transgene response, and lead to an ineffective treatment (Cao, Hoffman et al. 2009, Martino, Suzuki et al. 2011, Rogers, Martino et al. 2014). Here, we show that loss of TLR9 signaling dramatically attenuates the CTL responses against the transgene following IV administration of an AAV vector.

Relevant to our interest in liver-directed gene therapy for metabolic disorders, we examined transgene-specific T cell response to a mitochondrially targeted protein, OTC. Given the previous evidence of minimal immune responses following liver-directed gene transfer with AAV vectors, we anticipated that hepatic OTC expression would not elicit a robust immune response (Lang, Georgiev et al. 2006). The high homology between mouse and human protein complicated matters. In an ideal model, there would be no prior exposure to the transgene product; however, the OTC -/- model is neonatal lethal, and the SPF-Ash OTCD mouse line still expresses residual (around 5% of normal) activity (Wang, Wang et al.). Therefore, we added the immune-dominant epitope Ova257-264 to the C-terminus of hOTC to increase its immunogenicity. While addition of the
SIINFEKL epitope reduced OTC activity of the construct, mitochondrial localization was retained and so therefore we would expect turnover and antigen processing to be similar. Using the OVA epitope allowed us to measure and manipulate the transgene-specific T cell population for more sensitive readouts of immune activation. Using OT-1 transgenic T cells, we demonstrated that a robust response against the transgene product could be mediated by the activation of TLR9. This immune response was characterized by large elevations in transaminases and loss of transgene expression, as measured by hOTC-SIINFEKL mRNA and genome copy levels. We also observed that antigen-specific T cell populations of as low as 3,000 cells could induce transaminase elevation and modestly decrease genome copy number. In addition we showed that inflammation after transgene expression and presumed exposure to CD8+ T cells resulted in an attenuated clearance of the transgene and product, indicating that the most at risk timeframe for a robust immune response is during first contact of antigen to the T cells which will occur when expression begins a few days following vector administration. Further, we observed increased T cell infiltration of predominantly transgene-specific T cells, which corresponded to an increase in hepatocytes entering the cell cycle and subsequent loss of genome copies. Together, these data suggest that as hepatocytes are destroyed, dividing cells replicate to replace them. An alternative hypothesis could be that the cell division is responsible for the loss of the genome copies that are not retained when the nuclear envelope is dissolved and reformed; this possibility is supported by the lack of an increase in TUNEL or caspase 9 staining. As systemic administration of TLR9 agonist induced T cell activation against the transgene product, we were curious as to whether
TLR9 signaling intrinsic to T cells alone would be sufficient to activate them. We used TLR9 KO mice as recipients of the OT-1 T cell transfer. Our results showed that TLR9 signaling extrinsic to T cells was required for activation, such as both B cells and pDCs that respond strongly to TLR9 stimulation (Montoya, Jie et al. 2006, Jiang, Lederman et al. 2007). Therefore, future studies may help to further our knowledge and ability to design less immunogenic AAV vectors.
Figure 2 hOTC Immunodominant Epitope Mapping in C57BL/6 mice.

(A) C57BL/6 mice were given IM 5x10^10 p Ad5 hOTC. Mice (n=3) were sacrificed 28 days later, and IFN-gamma ELISPOT was performed on isolated T cells. (B) One cohort of C57Bl/6 mice was injected IM with 5x10^11 AAVrh32.33 CB7.hOTCco twelve weeks later mice were injected IM with 5x10^10 of Ad5.hOTC vector and TLR9 agonist 40 µg IP concurrent with ad administration and for the three following days N=4. 10 days after vector administration mice were sacrificed for IFN γ ELISPOT.
Male SPF-Ash mice (n=3) ages 6-10 weeks were injected IV with AAV8.TBG.hOTCco or AAV8.TBG.hOTCco-SIINFEKL 1x10^{11} GC. (A) Mice urine was collected and urine orotate was measured. (B) OTC enzyme activity was assayed from liver collected at day 14 necropsy.

Figure 3 Evaluation of OTC-SIINFEKL Activity in vivo.
Huh7 cells were transfected in slide well plates with plasids that contained hOTC transgenes. 48 hours after transfection MitoTracker is added to media to visualize mitochondria (red) cells are fixed thirty minutes later for OTC immunohistochemistry (green) and nuclear DAPI (blue).
Figure 5  Systemic Inflammation Breaks Tolerance to a Transgene Product.

(A) Schematic of experimental design detailing IV administration of AAV8.TBGhOTCco-SIINFEKL and subsequently OT-1 CD8+T cell transfer at 14 days after vector delivery, concurrent with administration of 40 µg TLR9 agonist for three days following adoptive transfer, n=3. (B) Serum AST and (C) Serum ALT levels were monitored every seven days following vector delivery. (D) Genome copies and (E) transgene mRNA from liver were determined by qPCR following termination of the study. These experiments were independently performed three times. Statistical analysis was performed by one-way ANOVA with Bonferroni test comparing to vector only control *P<0.05, **P<0.001, ***P<0.0001.
Figure 6 Effect of T cell population on Immune Response.

6-8 week old male C57Bl/6 mice (n=3) were given IV XX GC of AAV8.TBGhOTCco-SIINFEKL. Twenty-eight days after vector delivery, OT-1 CD8+T cells were transferred to recipient mice concurrent with 40 µg TLR9 agonist administration for three days following adoptive transfer. (A) Serum AST (B) ALT and (C) bilirubin levels were monitored every seven days following vector delivery. (D) Genome copies and (E) transgene mRNA from liver were determined by qPCR following termination of the study. Analysis was performed by one-way ANOVA with Bonferroni test comparing to no transfer control *P<0.05, **P<0.001, ***P<0.0001.
Figure 7 Inflammation after T cell to Antigen Exposure

6-8 week old male C57Bl/6 mice (n=3) were injected IV with AAV8 hOTCco-SIINFEKL. Fourteen days after vector delivery, OT-1 CD8+ T cells were transferred to recipient mice concurrent with 40 µg TLR9 agonist administration that continued for three days following adoptive transfer or with the TLR 9 agonist given 21 post adoptive transfer, control mice were not given agonist. (a,b) Serum AST and ALT levels were monitored every seven days following vector delivery. (c,d) Genome copies and transgene mRNA from liver were determined by qPCR following termination of the study. (e) Western blot probed for OTC, the OTC-SIIFKEKL band migrates above the endogenous OTC.
Figure 8  T cell Infiltration and Hepatocyte Proliferation.

Vector was administered to 6-8 week old male mice (n=4) IV and 14 days later OT-1 CD8⁺T cell were transferred, concurrent with administration of 40 ug TLR9 agonist for three days following adoptive transfer. Mice were sacrificed on days three, seven, and 14 following CD⁺ T cell transfer. (a, b) Liver sections were stained for CD8, and percent area covered by infiltrating T cells was determined using Image J. (c, d) Liver sections were stained for Ki-67 proliferation marker, and percent positive hepatocytes were calculated. (e) AAV vector genome copies were determined by qPCR following termination of each cohort. For image analysis experiments, five images per mouse were analyzed. Analysis was performed by one-way ANOVA with Bonferroni test comparing columns to their day appropriate control *P<0.05, **P<0.001, ***P<0.0001. NS, not significant.
Figure 9 Transgene-Specific T cell Infiltration.

6-8 week old male C57Bl/6 mice (n=4) were given AAV8 hOTC-SIINFEKL; 14 days later TLR9 agonist and CFSE-labeled OT-1 CD8$^+$ T cells were administered. Mice were sacrificed seven days following CD8$^+$ T cell transfer, and lymphocytes were isolated from liver and spleen. (a,c) Lymphocytes isolated from spleen or liver by forward and side scatter were stained for CD8. (b) Spleen- and (d) liver-isolated CD8$^+$ T cells were stained with SIINFEKL tetramer. (f, e) Tetramer positive splenocytes were analyzed for CFSE retention.
Figure 10 Extrinsic TLR9 Signaling is Required for a Destructive CTL Response

(a) C57BL/6 or TLR9 KO male mice (n=5) ages 6-8 weeks were administered IV AAV8 expressing hOTCco-SIINFEKL. Fourteen days after vector delivery, OT-1 CD8+ T cells were transferred to recipient mice concurrent with 40 µg TLR9 agonist administration that continued for three days following adoptive transfer. (b-c) Serum (b) AST and (c) ALT levels were monitored every seven days following vector delivery. Genome copies and transgene mRNA expression was determined following termination of the study by the study.
Chapter 3:

Tolerance to Antigens C-Terminal to Premature Termination Codon

Chapter 3 is adapted from the manuscript Scott N. Ashley, Suryanarayan Somanathan, Christian Hinderer, Maxwell Arias, Deirdre McMenamin, Christine Draper, and James M. Wilson. In preparation.
Abstract

Cytotoxic T cell (CTL) responses to the transgene product remain an active area of concern for the gene therapy field. The patient’s underlying genetic mutation may influence the qualitative nature of these potentially destructive T cell responses. Individuals with a mutation that introduces a premature termination codon (PTC) preventing synthesis of full-length peptide have been considered more likely to mount a transgene-specific T cell response due to a lack of endogenous antigen presentation and, therefore, no immune tolerance to C-terminal epitopes. Here, we demonstrate that a human ornithine transcarbamylase (OTC) gene containing various PTC-inducing nonsense mutations is able to generate and present epitopes downstream of the termination codon. Generation of these epitopes occurs primarily from alternative translation start sites downstream of the stop codon. Furthermore, we show that expression of these genes from AAV vectors in C57Bl/6 mice is able to induce peripheral tolerance to epitopes downstream of the PTC. These results suggest that, despite the lack of full-length endogenous protein, patients with PTC-inducing nonsense mutations may still present T cell epitopes downstream to the premature termination that may render the subject tolerant to wild-type transgene products, and therefore less likely to generate a transgene-specific immune response resulting in an overall improved therapeutic outcome.
**Introduction**

OTC deficiency is a rare X-linked metabolic disorder that is considered a therapeutic target for gene therapy (Hudak, Jones et al. 1985, Nguyen and Ferry 2004). Most OTC gene mutations are “private”, meaning that they are unique to a particular individual or family, and thereby result in a patient population with a diverse array of mutations (Yamaguchi, Brailey et al. 2006). While some groups have begun to investigate the influence of genotype on immune responses to the transgene product in other genetic disorders (Cao, Hoffman et al. 2009, Nayak, Doerfler et al. 2014, Rogers, Martino et al. 2014), the impact of these diverse mutations on the outcome of gene therapy for OTC remains unknown. Because OTC localizes to the mitochondria, the development of transgene-specific T cells may be more likely and problematic than an antibody response (Limberis, Figueredo et al. 2007, Mendell, Campbell et al. 2010, Flotte, Trapnell et al. 2011). Whereas a healthy individual could deplete transgene-specific T cells via mechanisms of central and peripheral tolerance (Mendell, Campbell et al. 2010, Flotte, Trapnell et al. 2011), the OTC-deficient patient with mutations that eliminate expression of the protein (or parts of the protein) may be unable to induce tolerance to the wild-type gene, rendering them susceptible to CTL responses upon transgene delivery. Indeed, preclinical evidence in other disease models suggests that certain mutations may limit tolerance induction and predispose patients receiving gene therapy to a T cell mediated transgene response (4-6).
Presentation of self-antigens on the major histocompatibility complex (MHC) is necessary to develop tolerance against endogenous epitopes by deleting or anergizing self-reactive T cells (Kappler, Roehm et al. 1987, Schwartz 2003). Tolerance to self-antigens is mediated by several mechanisms, including the deletion of auto-reactive T cells in the thymus and active suppression or induction of anergy in the periphery, all of which require the presentation of self-antigens in primary lymphoid organs (Fields, Gajewski et al. 1996, Pobezinsky, Angelov et al. 2012). As such, the impact of genetic mutations on antigen presentation has the potential to influence tolerance induction.

Genetic defects that allow the expression of a full-length non-functional protein are thought to result in tolerance to epitopes derived from that gene; in contrast, deletions, frameshifts, and nonsense mutations that prevent full-length protein expression (i.e., null mutations) may impair the ability to develop tolerance and could predispose individuals who receive gene therapy to a T cell-mediated transgene response (Fields, Arruda et al. 2001, Herzog, Mount et al. 2001, Herzog, Fields et al. 2002). Nonsense mutations can also result in truncated non-functional proteins, where tolerance induction may be limited to epitopes upstream of the PTC. In addition, mutations leading to a PTC increase the rate of mRNA degradation due to nonsense-mediated decay (NMD) mechanisms, which may further limit potential antigen presentation and tolerance induction (Lykke-Andersen and Jensen 2015).

An extensive body of work suggests that the majority of antigens presented on the MHC are not derived from the degradation of mature full-length protein but from defective
ribosomal initiation products (Yewdell, Anton et al. 1996, Dolan, Li et al. 2010, Dolan, Li et al. 2011, Croft, Smith et al. 2013). The work of Shastri and colleagues has demonstrated translation of unconventional start sites leads to cryptic peptides capable of MHC I mediated induction of T cells (Starck and Shastri 2011, Starck, Jiang et al. 2012, Starck and Shastri 2016, Vilborg and Steitz 2016). Furthermore, evidence suggests that epitopes can also be generated from the N-terminal region upstream of a nonsense mutation during the first or pioneering run of translation (Apcher, Daskalogianni et al. 2011). Here, we investigate the ability of mice to present antigens and tolerize CD8+ T cells to epitopes downstream of a nonsense mutation. Our findings reveal a previously unknown mechanism of epitope presentation downstream of a PTC. This finding could result in the re-evaluation of how an individual’s genotype could influence the outcome of gene therapy.

Materials and Methods

Animals

OT-1 mice on a C57Bl/6 background were acquired from The Jackson Laboratory (Bar Harbor, ME) and bred at our facility (Animal Facility of Translational Research Laboratories [TRL], University of Pennsylvania, Philadelphia, PA). All mice were housed under specific pathogen-free conditions in the TRL Animal Facility at the University of Pennsylvania. All experimental procedures, including the use of mice, were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.
Vectors

AAV8 vectors expressing a single-stranded, nuclear-targeted form of β-galactosidase (AAV8.ss.nLacZ), or a self-complementary human codon-optimized ornithine transcarbamylase (AAV8.sc.hOTCco) were designed under the control of TBG promoter, with a G4SVPA poly(A) signal sequence. To create the sc.hOTCco-SIINFEKL transgene, the immunodominant epitope of chicken ovalbumin [Ova_{257-264} (SIINFEKL)] was cloned onto the C-terminus directly following the last hOTCco amino acid. Site-directed mutagenesis was used to create the sc.hOTCco-SIINFEKL stop codon mutants: C109X, R141W, and Q154X. An AAV8.sc.hOTCco-SIINFEKL vector was designed containing the TBG enhancer/promoter and an SV40 poly(A) tail. AAV vectors were produced as previously described using an iodixanol step gradient (Lock, Alvira et al. 2010). E1/E3-deleted, replication-deficient, serotype 5 adenoviral vectors (Ad5) expressing hOTCco-SIINFEKL, hOTCco-C109X-SIINFEKL, or cytoplasmic-targeted chicken ovalbumin were produced as previously described (Gao, Yang et al. 1996). All vectors were produced by the Penn Vector Core at the University of Pennsylvania.

Ex-vivo stimulation assay

MC57G cells, a fibrosarcoma cell line expressing both H-2Kb and H-2Kd, were seeded on 6-well plates to achieve 70% confluence after 18 h. Transfection using Lipofectamine
(Life Technologies, Grand Island, NY) was performed according to the manufacturer’s instructions. Cells were transfected with a plasmid containing either hOTCco-SIINFEK- or the hOTCco-SIINFEKL stop codon mutants that were developed using site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. After 24 h post-transfection, 6-well plates were washed once with PBS, cells were mechanically scraped off the plate and into suspension, and washed once more before being resuspended at 2x10^6 cells/ml, seeding 100 μl/well on an enzyme-linked immunospot (IFN-γ ELISPOT) plate (BD Biosciences, San Jose, CA). OT-1 splenocytes were harvested from OT-1 mice as previously described (Limberis, Figueredo et al. 2007) and then seeded 1:1 or 1:10 with the MC57G cells. This combination was then incubated at 37°C in 5% CO₂ for 18 h. The ELISPOT plate was developed according to the manufacturer’s protocol, and spots were counted using the AID ELISPOT Reader System (Cell Technology, Columbia, MD).

**Tolerance induction**

Mice received 1x10^{11} genome copies of AAV8 expressing either sc.hOTCco-SIINFEK- or sc.hOTCco-SIINFEKL stop codon mutants, or an ss.nLacZ control, delivered IV in 100 μl of PBS. On day 14 post vector administration, 1x10^{10} GC of Ad5.TBG.hOTCco-SIINFEKL or 3x10^{9} particles of Ad5.TBG.OVA were injected IV. Mice were sacrificed and splenocytes were harvested on day 27 after vector administration as previously described (Limberis, Figueredo et al. 2007). An interferon γ (IFN-γ) ELISPOT assay (BD Biosciences, San Jose, CA) was performed according to the manufacturer’s
instructions. Cells were seeded at a concentration of 2x10^5 or 2x10^4 cells per well along with 2 µg/ml SIINFEKL peptide or pool B containing an overlapping 15-mer peptide library (Mimotopes, Victoria, Australia) spanning the full length of the Ad5 hexon. Splenocytes were incubated at 37°C in 5% CO_2 for 18 h. Spot forming units (SFUs) were counted using the AID ELISPOT Reader System (Cell Technology, Columbia, MD). Tetramer staining was performed according to the manufacturer’s instructions (MBL International, Woburn, MA). Splenocytes were resuspended to a concentration of 5x10^6 cells/ml. 200 µl of the cell suspension was stained with either 2 µl iTAg Tetramer/PE-H-2 Kb OVA (MBL International, Woburn, MA) and 3 µl anti-mouse CD8α-APC (Clone 53-6.7; eBiosciences, San Diego, CA), or a cocktail containing 2 µl iTAg Tetramer/PE-H-2 Kb OVA, 2 µl CD62L-FITC (Clone MEL-14), 3 µl PD-1-APC (Clone RMPI-30), and 1 µl CD44-PE-Cyanine7 (Clone IM7; eBiosciences, San Diego, CA), then incubated for 30 min in the dark at room temperature. Cells were washed with PBS, resuspended in 500 µl PBS with 1% FBS, and run on an FC 500 series flow cytometer (Beckman Coulter, Brea, CA). Analysis was performed using FlowJo software (Becton Dickinson, Franklin Lakes, NJ)

**Adenoviral vector immunization**

Mice received 5x10^{10} of Ad5 expressing either hOTCco-SIINFEKL, nonsense mutant variant C109X (hOTCco-C109X-SIINFEKL), or an nLacZ control delivered IV in 100 µl PBS. On Day 10 post vector immunization, mice were sacrificed and splenocytes were
harvested as described above. An IFN-γ ELISPOT assay was performed on harvested splenocytes as described in the previous section.

**Peptide Isolation**

Cloning was used to swap out SIINFEKL with a StrepII tag with a SerAla linker (SA-WSHPQFEK) on hOTCco-C109X. Nine, 15 cm plates of HEK 293T cells were transfected per condition using 3 μg polyethylenimine per 1 μg DNA with either hOTCco-C109X-StrepII or eGFP; cells were grown for 24 h. Proteasome inhibitor MG132 (APExBIO, Houston, TX) was added to the cells to reach a concentration of 1 μM. Two h after proteasome addition, cells were harvested using a gravity-flow Strep-Tactin sepharose column (IBA, Goettingen, Germany). Media was removed and cells were washed twice with PBS containing 1 μM proteasome inhibitor MG132 at 1500 rpm; all spins are performed at 4°C. Cells were resuspended in 2 ml of Strep-Tactin wash buffer with 25 μM MG132 and 1X complete protease inhibitor (Sigma Aldrich, St Louis, MO). Cells were lysed by five freeze-thaw cycles in liquid nitrogen, followed by a 37°C water bath. DNA was sheared by passing lysate through 18G needles five times over ice. Lysate was spun down at 4000 rpm for 15 min. Supernatant was collected and spun down at 14,000 rpm for 5 min. StrepII-tagged peptides were collected using 1 ml gravity flow Strep-Tactin sepharose columns (IBA, Goettingen, Germany), as per manufacturer’s instructions. Fractions were analyzed using Novex NuPAGE SDS-PAGE (Invitrogen, Carlsbad, CA), per manufacturer’s instructions and stained with Sypro Ruby Protein stain.
Sample fractions were submitted for mass spectrometry analysis (Bioproximity, Chantilly, VA).

**Protein Denaturation, Digestion, and Desalting**

Samples were sent to Bioproximity (Chantilly, VA) for digestion using the filter-assisted sample preparation method (Wisniewski, Zougman et al. 2009). Briefly, the samples were suspended in 5% sodium deoxycholate SDC, 50 mM Tris-HCl, pH 7.6, 3 mM DTT; they were sonicated briefly and incubated in a ThermoMixer (Eppendorf, Hauppauge, NY) at 60°C with 1000 rpm for 20 min. Samples were centrifuged, and the supernatant was transferred to a 30 kD molecular weight cut-off device (Millipore, Billerica, MA) and centrifuged at 13,000 g for 30 min. The remaining sample was buffer exchanged with 1% SDC in 100 mM Tris-HCl, pH 7.6 and alkylated with 15 mM iodoacetamide. Samples were digested using trypsin at an enzyme to substrate ratio of 1:100, overnight at 37°C in a ThermoMixer at 1000 rpm. Digested peptides were collected by centrifugation. A portion of the digested peptides (about 20 µg), were desalted using reversed-phase stop-and-go extraction tips (Rappsilber, Mann et al. 2007). Peptides were eluted with 80% acetonitrile, 5% ammonium hydroxide and lyophilized for approximately 1 h in a Savant SpeedVac (Thermo Fisher Scientific, Waltham, MA) to remove volatile components.

**Liquid Chromatography-Tandem Mass Spectrometry**

Each digestion mixture was analyzed by ultra-high pressure liquid chromatography (LC) mass spectrometry (MS)/mass spectrometry by Bioproximity (Chantilly, VA). LC was
performed on an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA). Mobile phase A was 97.5% Milli-Q water (Millipore, Billerica, MA), 2% acetonitrile, and 0.5% acetic acid; mobile phase B was 99.5% acetonitrile and 0.5% acetic acid. The 35 min LC gradient ran from 0% B to 25% B over 20 min, and then to 80% B for 3 min. Samples were loaded directly onto the 15 cm x 50 µm I.D. EASY-Spray column (Thermo Fisher Scientific, Waltham, MA), packed with 2 µm PepMap C18 media (Thermo Fisher Scientific, Waltham, MA). The LC system was interfaced to a Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) via nano-electrospray ionization using an EASY-Spray source with an integrated column heater (Thermo Fisher Scientific, Waltham, WA). The column was heated to 50°C, and an electrospray voltage of 2.2 kV was applied. The mass spectrometer was programmed to acquire, by data-dependent acquisition, tandem mass spectra from the top 20 ions in the full scan from 400-1200 m/z. Dynamic exclusion was set to 15 s, singly-charged ions were excluded, isolation width was set to 1.6 Da, full MS resolution was set to 70,000 and MS/MS resolution was set to 17,500. Normalized collision energy was set to 25, automatic gain control to 2e5, max fill MS to 20 ms, max fill MS/MS to 60 ms, and the underfill ratio to 0.1%.

**Statistical analysis**

All statistics were performed using Prism 5 (GraphPad, La Jolla, CA). One-way ANOVA analysis with Dunnett's Test was performed on each experimental replicate in the study. Data are shown from a representative experiment for each study. For all
figures, significance is depicted as *$p<0.05$, **$p<0.001$, ***$p<0.0001$. The number of animals per group, biological replicates and experimental replicates are defined in the figure legends.
Results

Antigen presentation of epitopes downstream of a nonsense mutation

We set out to investigate the impact that mutations in hOTC cDNA could have on MHC class I antigen presentation. We designed a panel of hOTC constructs (Figure 11 a) into which we inserted an array of mutations previously reported in patients (Table 1) (Grompe, Muzny et al. 1989, Carstens, Fenton et al. 1991, Oppliger Leibundgut, Wermuth et al. 1996, Oppliger Leibundgut, Wermuth et al. 1996, Matsuda and Tanase 1997, Calvas, Segues et al. 1998). The codons selected for translation of the hOTC protein in these cDNA constructs were optimized for efficient translation using standard algorithms (Wang, Morizono et al.). Redundancy in the genetic code allows for several codons to translate into same amino acid. We used the more preferred human codon when multiple codons existed for a single amino acid, by selectively replacing the rare codons, this should result in an increase in the expression without changing the amino acid sequence (Mauro and Chappell 2014). An immunodominant H2-K\(^b\) epitope from chicken ovalbumin (amino acid residues 257-264; SIINFEKL) was inserted at the C-terminus of each construct (Figure 11 a). To facilitate the presentation of the SIINFEKL epitope on MHC class I molecules, we transfected mouse-derived MC57G fibroblast cells with each of the plasmid constructs. Then we performed IFN-\(\gamma\) ELISPOT assays to determine if the transfected cells were able to stimulate splenocytes derived from OT-1 mice, which express a transgenic T cell receptor that recognizes SIINFEKL presented in MHC class I molecules (Figure 11). Our results indicate that all missense mutations
(R26Q, L88N, P225L, T264A, and H302Y) showed comparable antigen presentation and T cell activation to wild-type hOTCco-SIINFEKL which was expected since this category of mutation should not interfere with translation and presentation of the C terminal SIINFEKL epitope. Interestingly, the three nonsense mutants (C109X, R141X, and E154X) also generated robust T cell activation indicative of antigen presentation (Figure 11 b). This finding indicates that, despite the presence of a PTC, the downstream SIINFEKL epitope was still translated and presented.

To determine whether codon optimization impacted the ability to present C-terminal epitopes in this system, we compared codon-optimized with non-codon-optimized human sequences (Figure 17 a). T cell activation was still observed in wild-type codon-optimized sequences as well as those containing nonsense and frameshift mutations upstream of the SIINFEKL epitope (Figure 17 a). In non-Lipofectamine transfection controls, T cell activation was ablated, demonstrating that transfection of our plasmid constructs, and not residual protein contamination within the plasmid preps, was responsible for generating the observed anti-SIINFEKL immune response (Figure 17 b).

**Translation initiation via alternative start sites generates epitopes downstream of a nonsense mutation**

We considered two general mechanisms by which epitopes downstream of a nonsense mutation could be presented on MHC class I molecules: a) read through of the stop mutation and b) initiation of translation downstream of the stop mutation. To investigate these two mechanisms, we designed an expanded panel of hOTCco-
SIINFEKL stop codon mutants including the original C109X, R141X, and E154X nonsense mutants as well as three new constructs containing: three consecutive nonsense mutations (CPL-109-111X), a +1 frameshift mutation (P110+1), and a nonsense mutation near the C-terminus (K353X) (Figure 12a). Interestingly, the triple-stop CPL-109-111X and frameshift P110+1 mutants did not inhibit antigen presentation, which would have been expected if the driving mechanism were stop codon read through (Figure 12b). On the other hand, the K353X mutation, which is very close to the C-terminus, did result in a substantial reduction in antigen presentation, suggesting that an alternative start site upstream of residue 353 may be responsible for epitope expression. Unlike endogenous genome sequences, our OTC construct lacks an intron. Since an intronic sequence is required for eukaryotic NMD (Chang, Imam et al. 2007, Isken, Kim et al. 2008, Lykke-Andersen and Jensen 2015), we created constructs that inserted a mouse intron at amino acid 180 (Figure 18a) and observed mRNA levels using RT-PCR of total RNA harvested from transfected 293 cells (Figure 18b). We also performed an antigen presentation assay using the intron-containing constructs (Figure 18c).

Despite a non-significant trend of reduced RNA levels in those intron-containing constructs with a PTC (Figure 18b), significant levels of SIINFEKL-specific T cell activation were still observed for both wild-type and mutant intron-containing constructs (Figure 18c). Thus, NMD did not appear to inhibit the C-terminal translation and MHC loading of the SIINFEKL epitope. We cannot rule out NMD reducing hOTC mRNA
levels from endogenous transcripts to a point that would eliminate the impact of read-
through or downstream translation.

To assess the location of the alternative start site, we generated a new series of
constructs by mutating each of the in-frame methionine codons located downstream of
C109X into a stop codon, while retaining the C109X mutation. Except for the construct
containing the most downstream mutation (M338X), antigen presentation was observed
in all other methionine-to-stop mutation constructs (Figure 12 c). This finding indicates that the alternative start site is between residues M335 and M338. As no strong alternative start sites are predicted here, it is possible that the final methionine at residue 338 or an unconventional in-frame start site in this region initiates protein synthesis residues. However, we found that T cell
activation still occurs if we mutate individual methionine residues (data not shown) or all
methionine residues (Figure 12 d) to arginine, indicating that at least one of the driving alternative start site is non-canonical. While these studies do not precisely map the most distal start site capable of initiating translation it appears distinct from, but in close proximity to, the most distant methionine.

To directly confirm expression of protein sequences downstream of C109X, we
replaced the SIINFEKL tag with a strep-tag II and affinity purified hOTC fusion protein
from plasmid transfected 293 cells. Tandem LCMS of tryptic digests of these samples
identified multiple hOTC derived peptides from hOTCco-C109X-StrepII transfected cells and no hOTCco-C109X-StrepII derived peptides from GFP-transfected cells (Table 2). All hOTC derived peptides detected by LC/MS were distal to the C109X mutation (Figure 13); total coverage of the gene downstream of the stop codon was 63.

**Epitopes downstream of a stop codon can activate immune responses in vivo**

We hypothesize that, with the help of appropriate inflammatory signals, antigen presentation from our PTC mutant constructs could also induce a CD8+ T cell response to the C-terminal SIINFEKL epitope *in vivo*. Other groups have shown that viral vectors can be used to investigate expression of epitopes via read through, and we adopted a similar approach (Goodenough, Robinson et al. 2014). We packaged into an adenoviral vector our most immunogenic PTC mutant (hOTCco-C109X-SIINFEKL), and immunized IM C57Bl/6 mice. Our results demonstrate a T cell response to SIINFEKL in the context of the C109X stop codon that was as robust as that observed for the wild-type Ad5.hOTCco-SIINFEKL positive control (Figure 14). No T cell activation to SIINFEKL was observed in mice immunized with Ad5.nLacZ, the negative control.

**Expression of an epitope downstream of the PTC induces peripheral tolerance in vivo**

A potentially interesting extension of our observations is that expression of epitopes in genes containing a PTC could induce peripheral or central tolerance if presented to T cells in the appropriate stage of their maturation cycle. Previous research has shown that peripheral tolerance to a foreign protein or epitope can be achieved by
expressing it in the liver using an AAV vector (Lin, Hensley et al. 2007). The in vivo model system we used to study the induction of AAV mediated tolerance is as follows: AAV vector expressing wild type or one of three hOTCco-SIINFEKL mutants was injected IV into mice; 14 days later the animals were infused IV with an Ad vector expressing hOTCco-SIINFEKL) (Figure 15 a). As expected, administration of the AAV8.TBG. hOTCco-SIINFEKL vectors alone (wild type or mutants) did not elicit T cell responses to the SIINFEKL or Ad5 epitopes. Administration of the Ad5 vector without antecedent AAV vector administration did elicit robust T cell responses to both SIINFKL and Ad5 hexon confirming the potential immunogenicity of the Ad vector. Exposure of mice to AAV vectors expressing wild type and mutant hOTCco alleles (C109X, R141X and E154X) eliminated or statically significantly reduced the Ad induced response to SIINFEKL without affecting the response to Ad hexon in much the same way that the AAV vector expressing wild type hOTCco did. This indicates that sufficient expression of the SIINFEKL epitope occurs downstream of the PTC mutation to allow for induction of tolerance to it.

Tolerance from AAV administration and transgene expression in the liver has been attributed to one of several mechanisms such as anergy or suppression driven by CD25+CD4+ regulatory T cells (Dobrzynski, Fitzgerald et al. 2006). For regulatory T cells to play a role, there needs to be shared CD4+ epitopes between the tolerizing AAV vector and the immunizing vector; the only shared sequence capable of eliciting a CD4+ T cell response in the system described above is hOTCco. We eliminated the shared
hOTCco sequence while retaining the shared CTL epitope SIINFEKL by immunizing mice with an Ad5 vector containing cytoplasmic-targeted chicken ovalbumin, instead of the Ad5.hOTC-SIINFEKL vector, after exposure to the tolerance inducing AAV8.hOTCco-SIINFEKL vector. SIINFEKL would be the only overlapping peptide sequence expressed by both the AAV and Ad5 vectors, and therefore would be insufficient to generate a CD25^+CD4^+ T cell response specific for hOTC. Interestingly, we continued to observe the reduction of T cell activation in groups receiving AAV8.hOTCco-SIINFEKL wild-type or mutant vectors compared to AAV8.hOTCco, AAV8.nLacZ, or no AAV8 vector controls when the immunizing vector was Ad expressing ovalbumin (Figure 15 b). This suggests that anergy plays a role in the induction of tolerance to epitopes downstream of a PTC although a component of Treg induced suppression cannot be ruled-out. The observation of AAV induced tolerance to an antigen downstream of a PTC, as demonstrated by a reduction of Ad5 induced IFNγ ELISPOT, was confirmed using tetramer staining for SIINFEKL specific T cells (Figure 15 c). We repeated the previous experiment this time including two new AAV vectors based on previously used mutant constructs M338X and K353X both which lacked the ability to stimulate T cell in antigen presentation assays. (Figure 16). While neither allowed for as robust response as the control no AAV administration group, the responses were significantly higher than hOTC-SIINFEKL and equivalent to the non-specific control OTC. Based on these results, it appears that alternative start site expression can result in tolerance to epitopes downstream of a PTC via a mechanism that likely involves
anergy although other redundant pathways such as regulatory T cell activation could contribute.

Discussion

Antigen presentation has a profound impact on shaping immune tolerance, where location, cell type, and the cytokine milieu can all vary the outcome (Thompson and Thomas 2002, Lee, Epardaud et al. 2007, Hadeiba, Lahl et al. 2012, Riedhammer and Weissert 2015). A point of controversy in the field is the question of which antigen source has the most impact on antigen presentation and tolerance (Yewdell, Anton et al. 1996, Dolan, Li et al. 2010, Dolan, Li et al. 2011, Croft, Smith et al. 2013): full-length proteins that are degraded by the proteasome (Colbert, Farfan-Arribas et al. 2013), or defective ribosomal initiation products that were initially discovered in viral presentation pathways, and later observed in mammalian translation (de Verteuil, Granados et al. 2012). In fact, Shastri and colleagues suggested that non-conventional translation leading to expression cryptic peptides contributes substantially to the repertoire of peptide loaded MHC molecules (Starck and Shastri 2011, Starck, Jiang et al. 2012, Starck and Shastri 2016, Vilborg and Steitz 2016). Transcription of substantial portions of the genome not thought to be involved in traditional translation could expand the scope and complexity of the MHC I/peptide repertoire. Previously, the field of gene therapy assumed that the complete absence of full-length protein expression due to null mutations results in a loss of tolerance via lack of antigen presentation (Mendell, Campbell et al. 2010). However, studies have documented differences in immune responses following gene therapy
between true nulls that lack the gene entirely, compared with expected nulls caused by nonsense mutations (Cao, Hoffman et al. 2009). As nonsense mutations may still lead to truncated, non-functional proteins, this finding was hypothesized to result from epitopes upstream of the nonsense mutation, which has been supported by recent work (Apcher, Daskalogianni et al. 2011).

Interestingly, in the present study, we show that nonsense mutations in the OTC gene do not prevent expression and presentation of downstream peptides that could result in a more immune-tolerant phenotype in the OTC-targeted gene therapy setting. First, we demonstrated that a SIINFEKL epitope downstream of nonsense mutations was still presented at a high enough efficiency in vivo to stimulate a SIINFEKL-specific T cell response. We hypothesized that this finding could be due to two potential mechanisms: a) read-through of the stop codon by the incorporation of cognate tRNA, which could allow for the generation of the downstream epitopes (Goodenough, Robinson et al. 2014) or b) the presence of alternative start sites that could allow for translation initiation downstream of the stop codon and synthesis of the SIINFEKL antigen (Kochetov 2008, Bazykin and Kochetov 2011). Using a second panel of hOTCco-SIINFEKL mutants, we determined that a high degree of antigen presentation and T cell activation still occurred in the presence of both a triple-stop mutant (i.e. CPL109-111X), as well as a construct with a frameshift mutation (i.e. P110 +1). As stop codon read-through would have resulted in reduced or ablated expression of epitopes downstream of the triple-stop and frameshift mutations, respectively, our results suggested that alternative start sites were
responsible for driving C-terminal antigen presentation in this model. Furthermore, the Met-to-stop mutants in this system suggested the presence of multiple start sites that were able to continue driving translation downstream of the PTC. By mutating all methionine residues in the sequence, we also demonstrated that at least one non-canonical start appears to be involved. The fact that T cell activation was lost when a PTC mutation was introduced very close to the C-terminus of the protein indicates that there we no viable alternative translation initiation sites downstream of this mutation. With the use of two viral vector systems in a mammalian model of OTC deficiency (i.e., AAV to induce tolerance and Ad to activate immunity), we were able to confirm the induction of tolerance to epitopes downstream of nonsense mutations in the OTC gene as the result of anergy although other mechanisms of tolerance such as suppression by Tregs could not be ruled-out. In addition we reconfirmed that position of the mutation that seems to be important in allowing for antigen presentation as PTC closer to the c-terminus inhibited both antigen presentation and tolerance induction.

Together, these findings suggest that the lack of immune tolerance to a variety of mutations that prevent protein expression could be overcome by the presence of an alternative translational start site allowing for expression in primary lymphoid organs and resultant central tolerance. An important practical implication of this work relates to the relevance of the patient’s genotype in assessing the risk of pathologic adaptive immune responses in the setting of in vivo gene therapy. Patients with PTC mutations may not be as susceptible to CTL responses as are those patients with large deletions. We do not
believe that there exists enough evidence to exclude patients with PTC mutations from participating in gene therapy trial due to the theoretical risk of T cell mediated immunity.
A

hOTC

R28Q  L89N  C109X  R141X  E154X  P225L  T264A  H302Y  SIINFELK

B

SFU per 1x10^6 Splenocytes

Cells  GFP  WT  R28Q  L89N  C109X  R141X  E154X  P225L  T264A  H302Y  Exon 7 Skp  SIINFELK Peptide  PMA/IONO

**  NS  NS  **  NS  ***  **  ***  NS  ***  ***
Figure 11. In vitro antigen presentation of epitopes downstream of reported OTC gene mutations.

(A) Depicted is a schematic of the hOTC open reading frame. Mutant hOTCco-SIINFEKL constructs were generated using site-directed mutagenesis, such that each mutation would lie upstream of the inserted Ova\textsubscript{257-264} (SIINFEKL) epitope. Mutations were selected from previously reported human occurrences (Supplemental Table I). Nonsense mutations are shown in bold. (B) Antigen presentation of mutant hOTCco-SIINFEKL constructs in vitro. MC57G mouse fibroblast cells expressing MHC H-2Kb were transfected with plasmid containing wild-type or mutant hOTC-SIINFEKL constructs in triplicate. Cells were used to stimulate splenocytes from OT-1 mice in an IFN-\(\gamma\) ELISPOT assay. Presented is the activation of IFN-\(\gamma\)-expressing T cells in SFU/1x10\(^6\) splenocytes for various constructs as well as cells only (“Cells”), cells transfected with a GFP expressing construct (“GFP”) and cells activated with a combination of phorbol 12-myristate 13-acetate (PMA) and inomycin (IONO). Equivalent results were obtained from two independent experiments with three replicates per experiment. ***\(p<0.0001\). WT, wild type hOTCco construct. NS: non-significant.
Figure 12  Alternative start sites enable expression of epitopes downstream of nonsense mutations.

(A) Diagram showing within the hOTC open reading frame the location of new mutations designed to elucidate the mechanism of C-terminal expression.  (B – D) MC57G mouse fibroblasts were transfected in triplicate with plasmids containing a control transgene (“GFP”), hOTCco-SIINFEKL wild-type, or the hOTCco-SIINFEKL mutant plasmids. Cells were then cultured with stimulated OT-1 splenocytes in an IFN-γ ELISPOT assay. An additional control was only cells (“Cells”). Presented is the activation of IFN-γ expressing T cells in SFU/1x10^6 splenocytes for various constructs as well as the Cells and GFP controls. This experiment was independently performed five times (B) hOTCco-SIINFEKL constructs included CPL109-111X, P110+1, and K353X mutants.  (C) Antigen presentation assays for a series of Met-to-stop substitutions were performed to locate the alternative start site. Each construct includes a single Met-to-stop mutation in a hOTCco gene that has a C109X mutation. (D) Antigen presentation using a construct with all methionines downstream of C109X substituted to arginine to determine the alternative start site. These experiments were independently performed twice. **p<0.001, ***p<0.0001 in comparison with cells only and GFP negative controls.
Figure 13  Amino acid coverage of C109X and unique amino acid sequences detected in samples expressing hOTCco-C109X-StrepII.

HEK 293T cells were transfected with either hOTCco-C109X-StrepII or control eGFP plasmid constructs. Cells were grown for 24 h post transfection, and peptides were isolated by column chromatography. Eluted peptides were submitted to mass spectrometry analysis. Detected peptides that localized to OTC are highlighted in alternating black and underlined dark grey. Light grey amino acids were not isolated within a peptide.
**Figure 14** In vivo activation of endogenous T cells to an epitope downstream of the PTC.

C57Bl/6 mice (n = 3) were immunized IV with 5x10^10 particles of Ad5 vector containing either wild-type hOTCco-SIINFEKL, hOTCco-C109X-SIINFEKL, or nLacZ. Day 10 after immunization, mice were sacrificed and splenocytes were harvested for an IFN-γ ELISPOT assay. Cells were stimulated with either media, SIINFEKL peptide, or Ad5 hexon. Presented is the activation of IFN-γ-expressing T cells in SFU/1x10^6 splenocytes. NS, not significant in comparison with Ad5.nLacZ control.
Figure 15  In vivo tolerance induction to epitopes downstream of the PTC.

(A – C) C57Bl/6 mice (n = 3/group) were administered IV with AAV8 vectors expressing different hOTCco genes or a no-AAV8? vector control. At day 14, mice were immunized with either (A) Ad5.hOTCco-SIINFEKL or (B, C) Ad5.OVA, which naturally contains the SIINFEKL epitope. At day 27, mice were sacrificed and splenocytes were harvested for either (A, B) IFN-γ ELISPOT assays or (C) CD8 tetramer staining. (A, B) cells were stimulated with media (black), SIINFEKL (white), or Ad5 Hexon (grey). (C) the lymphocyte population was gated by forward and side scatter, and then gated by CD8 staining. The percent of SIINFEKL-specific T cells within the CD8+ population was determined by tetramer staining. Presented for panels A and B is the activation of IFN-γ expressing T cells in SFU/1x10^6 splenocytes. (C) is the percent of CD8+ T cells that are tetramer positive. These experiments were independently performed twice. *p<0.05, **p<0.001, ***p<0.0001 in comparison with no-AAV vector negative control.
Figure 16  PTC near C-terminus inhibits in vivo tolerance induction to epitopes downstream of mutation.

C57Bl/6 mice (n = 4) were administered IV with AAV8 vectors expressing different hOTCco genes or a no-AAV vector control. At day 14, mice were immunized with Ad5.OVA, which naturally contains the SIINFEKL epitope. At day 27, mice were sacrificed and splenocytes were harvested for IFN-γ ELISPOT assay. Cells were stimulated with media (black), SIINFEKL (white), or Ad5 Hexon (grey). Presented for is the activation of IFN-g expressing T cells in SFU/1x10⁶ splenocytes. These experiments were *p<0.05, **p<0.001, ***p<0.0001 in comparison with OTC-SIINFEKL vector positive control. NS: non-significant. SFU: spot forming units
Table 1  Private mutations selected to give a range of mutation types, including nonsense, missense, and splice variants resulting in exon-skipping.

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Exon/Intron</th>
<th>Gender</th>
<th>Age</th>
<th>OTC Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense</td>
<td>R26Q</td>
<td>CGG&gt;CAG</td>
<td>Exon1</td>
<td>Male</td>
<td>4 days</td>
<td>0%</td>
<td>Grompe et al., 1989, PNAS</td>
</tr>
<tr>
<td>Reduced activity/late onset</td>
<td>L88N</td>
<td>A&gt;T</td>
<td>Exon3</td>
<td>Male</td>
<td>4 months</td>
<td>23%</td>
<td>Oppliger et al., 1996, Hum Mutat</td>
</tr>
<tr>
<td>Nonsense</td>
<td>C109X</td>
<td>T&gt;A</td>
<td>Exon4</td>
<td>Male</td>
<td>3 days</td>
<td>ND</td>
<td>Calvas et al., 1998, Hum Mutat</td>
</tr>
<tr>
<td>Nonsense</td>
<td>R141X</td>
<td>CGA&gt;TGA</td>
<td>Exon5</td>
<td>Male</td>
<td>Neonatal</td>
<td>0%</td>
<td>Oppliger et al., 1996, Hum Mutat</td>
</tr>
<tr>
<td>Nonsense</td>
<td>Q154X</td>
<td>GAA&gt;TAA</td>
<td>Exon6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Grompe et al., 1989, PNAS</td>
</tr>
<tr>
<td>Reduced activity/late onset</td>
<td>T264A</td>
<td>ACT&gt;GCT</td>
<td>Exon8</td>
<td>Male</td>
<td>5 months</td>
<td>22%</td>
<td>Matsuda and Tanake, 1997, Am J Med Genet</td>
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<tr>
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<td>CAC&gt;TAC</td>
<td>Exon9</td>
<td>Male</td>
<td>Neonatal</td>
<td>0%</td>
<td>Oppliger et al., 1996, Hum Mutat</td>
</tr>
</tbody>
</table>

ND, not determined
Figure 17 hOTC codon sequence and transfection control

(A) MC57G mouse fibroblasts were not transfected (“Cells”) or transfected with plasmids containing a control transgene (“GFP”), codon optimized wild-type amino acid sequence hOTCco-SIINFEKL, codon optimized mutant amino acid sequence hOTCco-SIINFEKL, native codon wild-type amino acid sequence hOTC-SIINFEKL, or native codon mutant amino acid sequence hOTC-SIINFEKL plasmids. Cells were then cultured with stimulated OT-1 splenocytes in an IFN-γELISPOT assay (B) MC57G mouse fibroblasts were either not transfected or transfected with plasmids containing a control transgene (GFP), wild-type hOTCco-SIINFEKL, or mutant hOTCco-SIINFEKL plasmids with or without Lipofectamine. Cells were then cultured with stimulated OT-1 splenocytes in an IFN-γELISPOT assay. Presented is the activation of IFN-γ expressing T cells in SFU/1x10⁶ splenocytes for various constructs as well as the Cells and GFP controls. Experiments were independently performed twice and in each experiment the individual groups were evaluated in triplicate. ***p<0.0001 in comparison with cells only and GFP negative controls. SFU: spot forming units.
Figure 18  Impact of intronic sequence on mRNA expression and antigen presentation.

(A) Diagram showing the location of the intron that is 78 bp downstream of E154X. (B) RT-qPCR analysis was conducted in 293 cells transfected with wild-type or mutant plasmid constructs and is presented as OTC cDNA’s per 500 ng RNA. (C) MC57G mouse fibroblasts were transfected in triplicate with plasmids containing wild-type hOTCco-SIINFEK-Intron, or mutant hOTCco-SIINFEKL intron constructs. Cells were then cultured with stimulated OT-1 splenocytes in an IFN-γ ELISPOT assay. Presented is the activation of IFN-g expressing T cells in SFU/1x10^6 splenocytes for various constructs as well as the Cells and GFP controls. Both experiments were independently performed twice, n=3 mice / group. ***p<0.0001 in comparison with cells only negative control. NS, not significant.
Table 2  Peptide identification using LC/MS with affinity-purified samples expressing GFP or hOTC-C109X-StrepII.

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Sample</th>
<th>OTC Peptides Identified</th>
<th>Reads(^a)</th>
<th>E-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xtandem!</td>
<td>C109X</td>
<td>9</td>
<td>54</td>
<td>-394</td>
</tr>
<tr>
<td>Xtandem!</td>
<td>GFP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Number of total reads in the sample that align to the OTC sequence.
Chapter 4

Influence of Vector on Anti-Transgene Antibody Formation Following AAV-Mediated Gene Therapy
Abstract

Antibody formation to secreted transgene products remains as a potential barrier to many gene therapies. Driven by exposure of naïve B cells to a transgene product and the immune stimulus of vector administration, expression can result in an adaptive immune response and antibody formation against the therapeutic protein resulting in attenuation of efficacy. Here, we show that TLR9 signaling and the expression location profile is involved in driving the activation of B cells against secreted protein alpha-L-iduronidase (IDUA), following AAV administration. This TLR9-dependent response occurs independently of transgene CpG content, however it is strongly influenced by the location of transgene expression or lack therefore of. Overall, our results suggest that TLR9 signaling is important for the development of adaptive immune responses to transgene products following AAV gene therapy, and that elimination of CpG motifs in the vector genome may not be sufficient to prevent TLR9 activation.

Introduction

Adeno-associated virus (AAV) vectors have been investigated in many diseases where cross correction of transgene product expressed into the blood is vital for therapeutic efficacy. In many of these preclinical models, antibody formation to the transgene product has been observed. However, the adaptive immune response is tightly controlled; exposure to a novel protein often results in tolerance rather than immunity, unless an inflammatory signal is present to initiate an adaptive immune response to the novel antigen (Lang, Georgiev et al. 2006). One potential immunogenic stimulus
associated with AAV-mediated gene therapy is the vector genome. The immune system’s major sensing agent for this foreign material is TLR9, located in the endosomal compartment, which recognizes unmethylated CpG DNA and induces pro-inflammatory reactions that can result in an adaptive response (Hemmi, Takeuchi et al. 2000, Rogers, Martino et al. 2011). Additional work has been done to avoid TLR9-induced inflammatory signaling by CpG depletion or by increasing the CpG methylation state in DNA-based therapies (Reyes-Sandoval and Ertl 2004, Faust, Bell et al. 2013, Shapir, Miari et al. 2015). However, there is evidence that TLR9 binds the phosphodiester backbone of DNA regardless of the nucleotide sequence, and that activation is merely enhanced by CpG motifs (Haas, Metzger et al. 2008).

The involvement of TLR9 signaling following intramuscular (IM) AAV administration has been found to be important in the generation of both anti-transgene antibodies and AAV neutralizing antibodies (Zhu, Huang et al. 2009, Rogers, Suzuki et al. 2015). TLR9 signaling was shown to have a more limited role in the production of anti-transgene antibodies following intravenous (IV) vector administration, with myeloid differentiation primary response gene 88 (MyD88) being required to generate this immune response (Rogers, Suzuki et al. 2015). A tolerizing phenomenon has been observed following hepatic gene transfer using AAV vectors expressing the serine protease factor IX, leading to reduced or ablated anti-transgene antibodies (Cao, Dobrzynski et al. 2007), and even elimination of existing transgene antibodies in large animal studies (Finn, Ozelo et al. 2010, Crudele, Finn et al. 2015). However, our experience with different monogenetic
disorders indicates that this phenomenon is transgene-dependent (Hinderer, Bell et al. 2015). AAV-mediated hepatic gene therapy for mucopolysaccharidosis I (MPS I) in a dog model provoked an antibody response to the transgene product, alpha-L iduronidase (IDUA), demonstrating that hepatic expression alone is insufficient to induce tolerance (Hinderer, Bell et al. 2015).

In this study, we investigated the influence of TLR9 and transgene expression on antibody formation following AAV gene therapy using different routes of administration, primarily focusing on systemic delivery. We found a role for TLR9 in anti-transgene antibody formation against human IDUA (hIDUA) following IV vector administration; this response was also modified by the promoter. Unexpectedly, CpG depletion of the transgene did not influence the immune response to IDUA. Further, we discovered a link between generation of anti-IDUA antibodies and tissue restricted expression. These findings suggest that, while TLR9 activation can be consequential in eliciting a transgene specific adaptive immune response, our ability to curb its influence may be limited.

Methods

Vectors

AAV9 hIDUAco (human codon-optimized IDUA under the control of a CMV, CB7, or EF1alpha enhancer/promoter containing SV40 poly A) was produced along with other AAV vectors using an iodixanol step gradient (Lock, Alvira et al. 2010).
Mouse Anti hIDUA Detection Assay

Clear, flat-bottom polystyrene high-binding plates were coated with 50 μl/well of 0.58 mg/kg Aldurazyme (Biomarin, San Rafael, CA) diluted 1:100 in pH 5.8 PBS. Plates were washed five times using 0.05% PBS-Tween solution and then blocked for two hours with 2% BSA (GE Healthcare, Little Chalfont, UK) diluted in pH 5.8 Dulbecco’s PBS (Thermo Fisher Scientific, Waltham, MA). Serum was diluted 1:1,000 in Dulbecco’s PBS (Thermo Fisher Scientific, Waltham, MA); 50μL of diluted sample was plated and incubated at room temperature for two hours. Positive serum for the standard was diluted 1:10 and then diluted four fold for a series of seven dilutions and a negative control well. Plates were washed as previously described. Streptavidin-conjugated goat anti-mouse IgG antibody (BD Biosciences, San Jose, CA) was diluted 1:10,000 in 2% BSA (GE Healthcare, Little Chalfont, UK), and 100μl of diluted goat anti-mouse IgG antibody was added per well. Plates were incubated for 1 hour and washed as previously described. 150μl of TMB solution was added per well. Plates were read up to 20 minutes after adding stop read value to 450 nm.

IDUA Activity Assay

4-MU substrate (Glycosynth, Winwick Quay, Warrington, UK) was diluted 1:25 in IDUA assay buffer (0.1 M sodium acetate, 0.15 M NaCl, 0.05% TritonX, pH 3.8). In 96-well flat bottom tissue culture plates, 95 ul di-H2O, 100 ul diluted 4-MU substrate, and 5 μl serum sample was added to wells in duplicate. Plates were sealed, gently shaken, and incubated at 37 °C for 2 hours. 180 μl IDUA stop buffer (0.29 M glycine, 0.18 M sodium
citrate, pH 10.9) was added per well. 20 µl sample was removed to black well plates (Corning, Corning New York) for fluorescence readings (365 nm excitation and 450 nm emission).

**ELISPOT**

Mice were administered IM 2x10^{11} GC of AAV9 expressing either hIDUA or a CpG-depleted variant in 50 µl PBS. On day 14 after vector administration, mice were sacrificed and splenocytes were harvested, as previously described (Limberis, Figueredo et al. 2007). Interferon γ ELISPOT assays were performed, according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Cells were seeded at a concentration of 2x10^5 or 2x10^4 cells/well along with 2 µg/ml peptide pool A, B, or C of an overlapping 15-mer peptide library spanning the full-length hIDUA (Mimotopes, Victoria, Australia). Splenocytes were incubated at 37 °C in 5% CO₂ for 18 hours. Spots were counted using the AID ELISPOT Reader System (Cell Technology, Columbia, MD).

**Results**

Previously we demonstrated that TLR9 signaling is capable of triggering a strong CTL response to transgene product following AAV-mediated gene therapy. We next investigated its ability to modulate a humoral response. C57BL/6 or TLR9 KO mice were given IV AAV9.hIDUAco under the control of one of three promoters: the universal promoters CMV or CB7, or the liver-specific promoter TBG (Bell, Wang et al. 2011). We determined transgene product expression by measuring hIDUA activity in serum,
which inversely correlated with the presence of antibodies for all three groups. AAV9 vectors with either universal promoter (CMV or CB7) triggered the production of a robust anti-hIDUA antibody that reduced serum activity (Figure 19 a, b); in contrast, TBG-driven expression resulted in low anti-hIDUA antibody levels and a high, consistent serum activity level (Figure 19 c). However, in the TLR9 KO mice, vectors with the universal promoters CMV or CB7 had dramatically reduced antibody generation to hIDUA, indicating that TLR9 signaling is required for the anti-transgene response (Figure 19 a, b).

To further investigate the role of TLR9 signaling in IDUA-specific responses, we administered $1 \times 10^{11}$ GC AAV9.hIDUAco IV or IM under the control of either the CMV or the CB7 promoters to either C57BL/6 or TLR9 KO mice. After ten days, we sacrificed the mice and performed an IFN-γ ELISPOT on splenocytes. As we anticipated, T cells from TLR9 KO mice stimulated with hIDUA pools did not respond as robustly as those from C57BL/6 mice across all treatment regimens (Figure 20 a, b). Interestingly, the largest response occurred in mice given AAV9.CB7.hIDUAco IV compared to IM; CMV-containing vectors had an overall lower T cell response (Figure 20 a, b) corresponding with an overall lower peak activity. TLR9 signaling is thought to be important for proper B-cell development (Hua and Hou 2013). To confirm that the observed effect was due to loss of TLR9 signaling upon administration of vector and not an intrinsic defect in the B cells, we adoptively transferred $1.5 \times 10^7$ B cells from C57BL/6 mice or TLR9 KO mice into muMt^− mice that lack mature B cells. One week
later, we administered $1 \times 10^{11}$ GC AAV9 CMV.hIDUAco IV and monitored serum for transgene activity and transgene-specific antibody expression (Figure 21 a). We found that B cells from both groups were capable of producing anti-transgene antibodies and ablating serum transgene activity levels, indicating that the absence of TLR9 signaling in B cells did not inhibit these immune responses (Figure 21 b).

We next assessed whether modulating the CpG content would influence the antibody response to hIDUA, as we found previously removal of CpG content can reduce T cell responses in the muscle to a reporter transgene (Faust, Bell et al. 2013). We utilized an AAV vector with the hIDUAco transgene under the control of an EF1-α promoter depleted of CpGs. We designed a second transgene construct in which the hIDUA transgene was depleted of CpG content while retaining the amino acid sequence; we confirmed that the activity of the construct was not changed due to codon effects using an in vitro activity assay (Figure 22 a). We administered mice with vectors AAV9 EF1-α.hIDUAco or EF1-α.hIDUA,CpG- either IV or IM, and assayed IDUA activity and antibodies from serum for each cohort (Figure 23 a, b). IDUA activity peaked at day 7 and remained steady throughout the study in both groups (Figure 23 a, b). Anti-IDUA antibodies rose steadily throughout the experiment in both groups, but did not inhibit IDUA activity or reach levels as high as those measured when AAV9.CMV. hIDUAco or AAV9.CB7.hIDUAco was administered (Figure 23 a, b; Figure 19 a, b). To confirm that CpG depletion did not impact the cytotoxic immune response to IDUA, which would otherwise reduce transgene activity, we examined CTL activation.
mice with either $1 \times 10^{11}$ AAV9.EF1-α.hIDUAco or AAV9. EF1-α.hIDUA.CpG- IM vector, and ten days later performed IFN-γ ELISPOT on splenocytes. T cell responses to IDUA were detectable following IM or IV vector administration; however, we did not observe a difference between groups in the magnitude of the response, indicating effective T cell activation regardless of transgene CpG content (Figure 23 c).

Finally we examined the differences in antibody formation and transgene expression mediated by promoter restriction. As shown earlier, CMV expression results in both low serum activity and high antibody titer as opposed to TBG driven expression. When AAV9.CMV.hDUA and AAV9.CB7.hDUA are mixed 1:1 and administered serum activity peaks and then reduces by 2 fold corresponding with a rise in antibody titer that is similar to mice injected only with CMV containing vectors (Figure 24 a). There is evidence in mice that muscle cells express MHC II under inflammatory conditions and allow for priming of CD4+ T cells, which is a crucial step for the ultimate activation of B cells (Keller, Fokken et al. 2011, Faust, Bell et al. 2013). To reduce muscle expression we cloned four miR206 sequences, that target mRNA in skeletal muscle for degradation, into the poly A section of our CB7.hIDUA which has both initial high expression followed by a robust antibody increase and loss of serum expression (Greig, Peng et al. 2014). Mice injected with the AAV9.CB7.hDUA.4xmiR206 maintained higher expression and lower antibody titer until the last time point of the study where it converged with control group; despite this the activity did not decrease (Figure 24 b).
Discussion

Here we considered the influence of TLR9 and other host factors on humoral responses. For these experiments, we could not use OTC as the transgene, as it would likely be sequestered in the mitochondria and not come into contact with B cells. Instead, we used a vector expressing hIDUA, a secreted transgene that caused antibodies to form against it in our previous studies in both MPS1 -/- and C57BL/6 mice. Indeed, we saw differences in the relative titers of anti-hIDUA antibodies between constructs with expression driven by the universal promoters CMV and CB7 compared to the liver-specific TBG promoter. Other groups previously reported similar results when using a muscle-specific promoter (Doerfler, Todd et al. 2016). Importantly, we demonstrated that a lack of TLR9 signaling dramatically reduced the anti-IDUA antibody response in mice that received either CMV or CB7 hIDUA vector, indicating that its signaling is critical for developing a robust humoral response following global expression.

We next assessed the impact of TLR9 signaling, promoter selection, and route of administration on CTL responses. We compared C57BL/6 or TLR9 KO mice that received AAV9.CMV.hIDUA or AAV9.CB7.hIDUA IV or IM. We found that splenocytes isolated from TLR9 KO mice did not respond to stimulation with hIDUA peptide pools compared to C57BL/6 mice across all experimental conditions. Interestingly, the largest response occurred in mice that received AAV9.CB7.hIDUA IV compared to IM; CMV-containing vectors had an overall lower T cell response. This could indicate that expression level is tied to the strength of immune activation, where the
high activity of CB7 and higher expression of the transgene product could enable better priming of APCs. To further probe this observation, we reduced the CpG content of transgene, as we have done previously (Faust, Bell et al. 2013). We used a universal promoter, EF1-α, as this was previously CpG depleted (Faust, Bell et al. 2013). When given the vectors IV, groups that had retained our codon-optimized hIDUA sequence did not generate a higher antibody titer than those receiving CpG-depleted hIDUA. Surprising, both maintained high levels of serum IDUA activity. The antibody titers were lower in mice that received vectors driven by EF1-α compared to CMV or CB7, suggesting that there is a threshold for transgene-specific antibody levels to negatively affect activity and that some inherent differences between CB7, CMV and EF1-α account for the gap in transgene-specific antibody and serum levels. We next confirmed that CpG depletion did not impact transgene-specific immune responses, as measured by IFN-γ ELISPOT. Although this result was consistent with our antibody data, it did contradict previous results in studies where we modified the CpG content of nuclear-targeted LacZ (Faust, Bell et al. 2013). This difference could be explained by a number of factors, including the vector (in our previous work we used the more immunogenic AAVrh32.33 (Mays, Vandenberghe et al. 2009)) or enhancement of the CpG content of the LacZ control transgene sequence (the depletion could have caused a decrease in pro-inflammatory cytokines that was not capable of inhibiting larger responses to the transgene product).
Lastly, we investigated one aspect of how the site of expression can affect enzyme activity and potentially therapeutic efficacy. To accomplish this we mixed hIDUA expressing AAV9 vectors driven by either CMV or TBG promoters. We demonstrated that the dominant effect of CMV expression is high antibody titer capable of overcoming a presumed tolerogenic effect that liver driven AAV expression can induce (Cao, Dobrzynski et al. 2007, Hinderer, Bell et al. 2015, Doerfler, Todd et al. 2016). It has suggested that one organ group where expression could positively influence antibody generation is skeletal which is capable of presenting on MHC II potentially priming CD4+ T cells for B cell activation (Keller, Fokken et al. 2011, Faust, Bell et al. 2013, Doerfler, Todd et al. 2016). By eliminating muscle expression using miR206 sites in a CB7.hIDUA construct we were able to observe stable expression of the transgene product and a slower raise in anti-transgene antibodies. An interesting observation was both CB7.hIDAU.miR206 and EF1α driven constructs reached an antibody titer elevated to levels that in CMV or CB7 containing vectors serum activity was dramatically reduced, yet in CB7.hIDAU.miR206 and EF1α they were able to maintain higher activity indicating that serum activity loss could be caused by factors beyond antibody titer.

Together, these results demonstrate the importance of TLR9 in developing a robust humoral response to a secreted transgene product and highlight an important but complicated role for promoters. While CpG depletion did not improve the outcome of AAV9 vector administration, this informed us that TLR9 signaling might be independent of CpG influence on our vector genomes. This could be due to the natural DNA
backbone retaining a basal level of immunogenicity (Haas, Metzger et al. 2008). Some groups have also reported the importance of methylation and its inhibition of TLR9 binding; this potentially could be explored to rationally design vectors to be less immunogenic. In larger animal models in which transgene antibodies seem to be more common, the effect of CpG depletion may become more pronounced. Therefore, future studies may help to further our knowledge and ability to design less immunogenic vectors.
Figure 19 TLR9 Signaling is required for an anti-transgene antibody response.

Male C57BL/6 or TLR9 KO mice (n=5/group) age 6-8 weeks were given IV 1x10^{11} GC of (a) AAV9.CMV.hIDUA vector (b) AAV9.CB7.hIDUA vector promoter, or (c) AAV9.TBG.hIDUA vector. Mice were bled weekly, and IDUA activity and anti-transgene antibodies were determined from serum.
Figure 20 TLR9 Signaling is Required for an Anti-Transgene CTL Response.

Male C57BL/6 or TLR9 KO mice (n=5/group) age 6-8 weeks were given 1x10^{11} IV GC (a) or IM (b) AAV9 hIDUA vector with CMV or CB7 promoter. Mice were sacrificed ten days later and INF-gamma ELISPOT was performed on isolated T cells. Analysis was performed by one-way ANOVA with the Bonferroni test comparing columns in C57BL/6 mice to TLR9 KO mice for each stimulation., *P<0.05, **P<0.001, ***P<0.0001.
Figure 21 TLR9 KO B cells are intrinsically capable of mounting an anti-transgene response.

(a) 1.5E7 B cells from C57BL/6 mice or TLR9 KO mice (n=5/group) were adoptively transferred to µMT mice. One week later, mice were administered 1x10^{11} AAV9.CMV.hIDUA IV. (b) Serum IDUA activity and anti-transgene antibodies were determined every seven days.
Figure 22 CpG+ and CpG- sequences have equivalent activity *in vitro*.

HEK-293 cells were transfected with plasmid containing EF1-Alpha.coCpG+.hIDUA, EF1-Alpha.coCpG-.hIDUA, or CB7.coCpG+.hIDUA constructs in triplicate. Media was collected before transfection and each following day for two days. IDUA activity was assayed after collection of media.
Figure 23 CpG Depletion Does Not Inhibit Anti-IDUA Formation.

Male C57BL/6 mice (n=5) ages 6-8 weeks were given IV or IM 1x10^{11} AAV9 EF1α hIDUA vector with normal or CpG-depleted transgene sequence. (a, b) IDUA activity and antibodies were determined from serum. (c) T cell IFN-gamma ELISPOT was performed on splenocytes isolated from mice ten days after vector administration.
Male C57BL/6 mice (n=5) ages 6-8 weeks were given IV $1 \times 10^{11}$ of AAV9.hIDUA vector with CMV promoter, TBG promoter, or a dose of both. Serum IDUA activity and anti-transgene antibodies were determined every seven days. (a) Results show that expressing IDUA with a universal promoter antibody titer rises and serum IDUA activity lowers even when there is concurrent high levels of expression from the liver. (b) Male C57BL/6 mice (n=5) ages 6-8 weeks were given IV $1 \times 10^{11}$ of AAV9.CB7.IDUA with or without a 4X miRNA 206 sequence. Serum IDUA activity and anti-transgene antibodies were determined every seven days.
Chapter 5: Discussion

General Summary
AAV gene therapy holds promise for safe efficacious treatment of genetic disorders with limited available therapeutics. Preclinical development in many disease models, including MPS1, hemophilia, muscular dystrophy, pompe disease, have both demonstrated efficacy and the potential for an immune response to AAV mediated gene therapy (Manno, Pierce et al. 2006, Mendell, Campbell et al. 2010, Hinderer, Bell et al. 2015, Doerfler, Todd et al. 2016). Following these observations and previous challenges with adenoviral vector immunogenicity studies we have investigated the immune response and its effect on gene therapy efficacy. The goal of my dissertation work was to investigate the underlying host factors that modulate the activation of the adaptive immune response to the transgene product. With deeper understanding of these agents we could better assess the risks of each new AAV clinical product and tailor AAV treatment based on an individual’s likelihood for immune activation. Through these efforts, we may discover critical points in adaptive immune activation and lead to a safer more thought-out methodology for reducing the risk of adverse immunity to gene therapy.

Gene Therapy, Immunology and TRL9

Previous work performed in the Wilson laboratory had shown that CD8+ T cells required inflammatory signaling in addition to the presence of their specific antigen to become active and attenuate transgene expression of highly immunogenic bacterial
protein (Somanathan, Breous et al. 2010, Breous, Somanathan et al. 2011). As described in chapter 2, I opted to develop a model to explore transgene-specific immunity against the mitochondrial protein OTC and to further investigate the influence of specific host factors that are critical to immune activation. Initially I sought to develop a scheme similar to ones used previously to activate CD8+ T cells against a specific transgene by immunizing C57Bl/6 mice with Ad5 vector expressing hOTCco, then harvest the T cells for adoptive transfer (Somanathan, Breous et al. 2010). Our first experiments did not yield the anticipated result, as T cell activation, measured by IFN-γ ELISPOT, did not induce a robust OTC-specific response immune response. Inducing and immune response required a more robust methodology, first we administered the immunogenic AAVrh32.33, then 12 weeks later administered both Ad5 and TLR9 agonist concurrent with vector and for three days following (Figure 2 a,b) (Mays, Vandenberghe et al. 2009). These results indicated that we were not activating naive hOTC-specific T cells, but rather breaking tolerance of anergized T cells which could have occurred due to the highly conserved nature of OTC between species (P11725, P00480) (Lang, Georgiev et al. 2006). Under such stringent conditions necessary to generate a population of OTC-specific CD8+ T cells I did not consider it feasible to continue with that methodology. Further, I opted against using cells that had previously been exposed to the transgene as this is not what I anticipate will occur in AAV gene therapy for the patients most likely to have an adverse T cell activation, namely, those that express no protein from the mutated gene. To develop our model I took advantage of the transgenic OT-1 mouse which contains genes for a T cell receptor that specifically binds the immune-dominant epitope
of chicken ovalbumin (SIINFKEL) by generating a construct with a SIIFEKEL tag attached to the c-terminus of hOTCco (Figure 11 a) (Hogquist, Jameson et al. 1994). Though the epitope tag did diminish OTC activity (Figure 3), biogenesis of the protein and its translocation to the mitochondria remained intact, which was important for our design study. Maintenance of the biogenesis pathway was important since I expected protein degradation and processing into antigen to be similar to the untagged hOTCco (Yewdell, Reits et al. 2003). To investigate the influence of TLR9 on the activation of a cell mediated immune response to the transgene product we packaged hOTCco-SIINFEKL into an AAV8 vector (Figure 5). C57Bl/6 mice were given IV either TLR9, OT-1 CD8+ T cells or both and analyzed for toxicity by evaluation of transaminase levels and the presence of genome copies four weeks after adoptive transfer. Elevations of AST and ALT were only observed in the group of mice that received both transgene specific T cells and TLR9 agonist indicating a requirement for a naïve CD8+ T cell population, and TLR9 can provide the necessary inflammatory signals to activate a cellular response. Further, we observed both a transgene mRNA and genome copy loss indicating clearance of the episomes potentially driven by cell death.

To evaluate the number of naïve T cells required to initiate such an immune response we titrated the number of CD8+ T cells that were adoptively transferred to recipient mice. Surprisingly even at the lowest dose of 3,000 transferred cells we observed a rise in transaminase levels (AST and ALT) (Figure 6 a). This low dose also resulted in a decrease of GC, although not as dramatic as that observed in the higher dose.
groups. These findings were corroborated by our experiment designed to examine the activation of the adoptively transferred cells infiltrating the liver, in which the majority of cells were activated (Figure 9 d). We investigated the importance of naïve T cells in developing a cell mediated response by withholding TLR9 agonist until three weeks after the adoptive transfer to allow for CD8⁺ OT-1 T cells to come in contact with the SIINFEKL antigen presented by liver cells. While toxicity was reduced with transaminase elevation being substantially decreased in the late inflammation group; protein expression was not consistent between mice in the group despite genome copies and RNA expression being similar. These findings suggest that antigen naïve T cells are able to generate a more robust immune response compared to T cells that have previously been exposed to a transgene product prior to an inflammatory event.

Clearance of transduced cells by an adaptive immune response has been a concern for the development of AAV-based gene therapy; in our experiments we address this likelihood. C57Bl/6 mice were given IV AAV8.TBG.hOTCco-SIINFEKL and fourteen days later administered CD8⁺ OT-1 T cells and TLR9 agonist. Mice from this group and control groups were then sacrificed on days 3, 7 and 17 following adoptive transfer. Liver sections were analyzed for CD8⁺ T cell infiltration (Figure 8 a) noting extreme infiltration in the TLR9 treated group into regions beyond the periportal or pericentral regions. We also observed some infiltration in the periportal liver region in mice administered only AAV8.TBG.hOTCco-SIINFEKL and T cells indicating some homing to the liver but potentially no activation. Death markers capsase9 and TUNEL staining
were performed on liver sections, however, no differences were observed between groups including a control AAV8.TBG.hOTCco-SIINFEKL only treated cohort. The possibility that we missed the time-point of maximal cell death led us to using a marker for cell growth KI-67 to evaluate the regeneration of the liver (Figure 8 b). Upon examination we observed a large percentage of hepatocytes marked as having left G₀ of the cell cycle in mice that received TLR9 agonist indicating that active division was occurring to replace cells lost as a consequence of the immune response. Further, we observed significant GC loss in TLR9 treated mice corroborating our KI67 data suggesting that transduced cells were lysed by the infiltrating CD8⁺ T cells (Figure 8c). Whereas previous loss of transgene expression was not highly correlated with loss of GC and was potentially caused by shut-off of transcription, here we have evidence that massive cell death can occur prior to regeneration of hepatocytes (Somanathan, Breous et al. 2010, Breous, Somanathan et al. 2011).

To confirm that the adoptively transferred T cells were migrating to the liver, mice were given IV AAV8.hOTC.SIINFEKL. Fourteen days later, mice were administered TLR9 and CFSE labeled CD8⁺ OT-1 T cells. Seven days following adoptive transfer, at peak liver infiltration, mice were sacrificed and liver and spleen were harvested for lymphocyte isolation. In mice receiving TRL9 agonist, the CD8⁺ T cell population, as a percentage of total lymphocytes, was increased as was the amount of SIINFEKL-tetramer positive cells (Figure 9a, b). In the liver, the majority of infiltrating T cells were transgene-specific when TLR9 was given, however since the number CFSE
positive cells was reduced in this cohort, we hypothesize that by this point of the immune activation the transferred T cells may have undergone many rounds of division resulting in dilution of the CFSE to below our threshold for positive detection (Figure 9 b,c). This indicates that most of the transferred cell population remaining had expanded as opposed to only a subset of that population suggesting that most of the T cells that survive migrate to the liver.

Finally it was important to determine if TLR9 signaling in CD8+ T cells was responsible for immune activation. C57Bl/6 or TLR9 KO mice were administered AAV8 hOTCco-SIINFEKL. Fourteen days later, mice were given OT-1 T cells and TLR9 agonist. In this experiment TLR9 KO mice that received the CD8+ T cells will only have signaling in the adoptively transferred cells. We found that transaminase levels did not elevate in TLR9 -/- mice given both agonist and T cells (Figure 10 a). GC and mRNA levels were similar to vector only control in treated TLR9 KO (Figure 10 b). Taken together, these results demonstrate the need for TLR9 signaling extrinsic to the T cells that will become activated. This is important as during gene delivery TLR9 signaling should occur only in cells transduced by the vector or potentially by the antigen presenting cells that take up vector particles.

These results establish a method for investigating a cytotoxic T cell response to a mitochondrial targeted gene therapy of the liver. We observed that it was necessary to have both a strong inflammatory event and transgene specific T cells. Our evidence suggests that TLR9 could provide that inflammatory signaling, and as both other groups
and our own investigation into the humoral response has shown AAV provides a sufficient amount of DNA to initiate such a response (Zhu, Huang et al. 2009). If we are able to either avoid or suppress this signaling we would anticipate a much reduced immune response to the transgene product. An important question for the field would be how long does the vector DNA remain in endosomes after transduction and the likely hood that it could lead to inflammation in the months following gene therapy. The requirement for having a pool of naïve antigen specific T cells also provides optimism as in the third chapter we describe a mechanism that could potentially reduce the number of these T cells from individuals with a PTC mutation.

**Tolerance to Epitopes C-Terminal of a PTC**

One guiding principal for the dangers of transgene-specific T cell activation is how the genotype of the afflicted individual can alter the outcome. Specifically, that people with missense mutations that are expected to allow reduced or non-functional protein expression will retain tolerance to the transgene product as both negative selection and anergy should still be occurring; compared to those that lack expression of protein altogether whom we would assume lack those mechanisms for preventing an auto-immune response to gene therapy. In our work on OTC we thought it was important to explore the potential effects of a patient’s genotype on the outcome of gene therapy as most individuals in OTC tend to have private mutations as opposed to some disorders that have a single mutation predominate, such as the delta 508 CFTR mutation in cystic fibrosis. A potential area of concern was related to missense mutations resulting in
proteins that could aggregate or in some other way cause cellular stress in ways similar to the Z allele of ATAT (Carrell and Lomas 2002, Flotte, Trapnell et al. 2011). In less extreme forms, misfolding can lead to endoplasmic reticulum stress releasing oxygen species, which could lead to a pre-inflammatory environment which could be prone to initiating an immune response upon AAV mediated gene therapy (Nakagawa and Yuan 2000, Malhotra, Miao et al. 2008). This would deviate from our understanding that any specific missense mutation would not carry an increased risk of immune activation. To investigate these potential effects we chose to create a panel of hOTCco-SIINFEKL constructs that contained missense mutations or nonsense mutations originally discovered in individuals with OTC deficiency (Table 1). In our initial screen of these mutants we found that only one of our selected missense mutants had increased antigen presentation as measured by IFN-γ ELISPOT, which was anticipated since this category of mutation should not interfere with translation and the mutation, R26Q, occurs in the leader sequence of OTC potentially interfering with biogenesis. (Grompe, Muzny et al. 1989) (Figure 11 a, b). Of noted interest were the results obtained from our nonsense mutant constructs, where each was capable of expressing antigen that is C-terminal to the PTC.

This result contrasted our assumed model where antigen presentation should not occur, and indicated that expression of epitopes in spite of a PTC could occur and that potential exists for tolerance either peripheral or central to transpire in individuals with nonsense mutations (Figure 25 a). The source of antigens presented on MHC has been contested in the past with two competing theories; one holds that antigens presented are
primarily from fully formed proteins degraded by the proteasome as part of the natural turnover cycle (Colbert, Farfan-Arribas et al. 2013) the second states that the primary reservoir of epitopes are generated from DRiPS, defective ribosomal products that include early terminated peptides chains (Yewdell, Anton et al. 1996, Dolan, Li et al. 2010, Dolan, Li et al. 2011). In our model, it is possible that one or both are occurring, read-through of the PTC by incorporation of a cognate tRNA would allow for continuous synthesis of our constructs with the finished protein being degraded by turnover processes (Goodenough, Robinson et al. 2014), another option is that translation is being initiated downstream of the PTC creating peptide chains that are immediately sent to the proteasome for degradations (Bazykin and Kochetov 2011, Kochetov, Prayaga et al. 2013) (Figure 25 b, c). Our investigation required the production of several more constructs to interrogate these hypotheses and we took advantage of inducing PTC at different location or in volume. Our in vitro work demonstrated that read-through was unlikely to be occurring, our construct with three mutations, CLP109-111X, did not have any decrease in T cell activation compared to a single stop codon at position 109 and we believe it unlikely that cognate tRNA incorporation would occur at the same rate in both instances (Figure 12 a). Conversely, mutation K353X is one removed from the start of the SIINFEKL epitope and effectively shut down expression of the peptides measured by our T cell assay. We believe this demonstrates that by having more gene sequence between the PTC and epitope one increases the chance of an alternative start site initiating translation, whereas if read-through we the dominant cause of expression position would have less of an effect. Finally using a +1 frame shift mutation at P110 we show that C-
terminal expression occurs, this is important as the frame shift generates 17 PTC, in addition even if all stop codons were read-through the downstream product would be out of frame and SIINFEKL would never be translated, therefore we conclude that enough translation events to generate a pool of antigens must begin C-terminal to the PTC (Figure 12 a).

We attempted to identify the site of alternative translation initiation and to accomplish this we created two panels of constructs, one where each methionine downstream of the C109X mutation was substituted with a stop codon, and a second where each methionine is changed to an arginine. Our results showed that alternative initiation did not require an AUG site as all our Met-Arg substitution constructs did not prevent C-terminal expression (Figure 12 c). From our Met-Stop mutants we saw that only the final mutation M338X inhibited translation, indicating that either it or the M335X mutation were involved in some capacity to start translation as the intervening codons are not common alternative start codons such as CUG or GUG (Figure 12 b) (Starck, Jiang et al. 2012). Taken together we hypothesize that multiple codons contribute to translation initiation including both canonical and non-canonical start codons.

While these observations are interesting with respect to antigen presentation and offer more evidence for the importance of the DRiP theory to explain the source of antigen presentation, we think that in the context of gene therapy it could be very important to considering how we think individual mutations can influence the outcome of gene therapy. To determine if these phenomena occur in an animal model, we packaged
the C109X construct into an Ad5 vector and injected into mice. We observed an immune activation against SIINFEKL as measured by the INF-γ ELISPOT assay against both the WT and mutant gene containing vectors indicating that epitopes C-terminal to a PTC were expressed and presented on MHC class I to CD8+ T cells (Figure 14).

Induction of tolerance to the transgene product is an important potential outcome of our work. If antigen presentation is occurring even in individuals with a PTC or other null mutation, tolerance could still be maintained if alternative translation occurs at a significant enough rate to either create central tolerance and thereby deplete the body of transgene specific T cells or by peripheral tolerance inducing anergy in transgene specific T cells. To explore these possibilities we used a model of peripheral tolerance and anergy where expression of an antigen from the liver will induce tolerance to antigens in the transgene product by exposure of naïve T cells to their epitopes without major inflammation signals (Lin, Hensley et al. 2007). To determine if tolerance to epitopes C-terminal to a PTC could induce tolerance similar to normal protein we created AAV8 vectors containing hOTCco-SIINFEKL constructs with stop codon mutations. Mice were given the AAV vectors and then challenged with Ad5 hOTCco-SIINFEKL, followed by analysis by INF-γ ELISPOT assay. The results showed that a successful reduction of SIINFEKL specific cells occurred, providing evidence that an individual with a PTC could still induce tolerance to their defective protein (Figure 15 a). Next, we challenged a second cohort of mice with Ad5 OVA to prevent activation of hOTC specific CD4+ CD25+ T-regulatory cells that can be induced by liver directed AAV gene transfer. This
was important as OTC-specific T regulatory cells could have become active against N-terminal OTC epitopes; by using Ad5 OVA to provide the SIINFEKL we avoided that possibility. Again we showed that the PTC containing constructs reduce T cell activation and lower the amount of SIINFEKL specific T cells (Figure 15 a, b).

Taken as a whole these studies provide insight into the source of antigen presented on MHC class 1 and the potential for tolerance to intracellular proteins that are expressed in the liver. Our work provides strong evidence that a large contributor to the antigen pool comes from defective ribosomal products, specifically from peptides derived from start sites within the gene. Peptide synthesis starting from internal start sites was sufficient to create the requisite amount of peptides to load onto MHC class 1 and present antigen, even with the possibility of mRNA degradation of mutant transcripts by nonsense mediated decay. While other groups have observed presentation from peptides formed N-terminal to a PTC this is the first evidence that epitopes found downstream of a PTC could lead to T cell activation (Apcher, Daskalogianni et al. 2011). A clinically relevant application is that antigen presentation of epitopes C-terminal to a PTC could allow for T cell tolerance to be generated to the defective gene’s product. Based on these results we should reevaluate how individuals are chosen for clinical trials. Patients that contain a PTC mutation in the defective gene should not be denied the opportunity to contribute to the advancement of gene therapy development and the potential of receiving a positive health outcome.
Anti-Transgene Antibodies

The humoral response is the other major branch in the adaptive immune system and while it shares many systems with the cytotoxic response including assistance in activation via CD4+ T cell help it seems to have a different threshold for activation based results from pre-clinical models and enzyme replacement therapy (Nayak, Doerfler et al. 2014, Hinderer, Bell et al. 2015, Doerfler, Todd et al. 2016). We believed that immune activation was strongly tied to signaling through TLR9 an endosomal protein that becomes active upon binding unmethylated CpG motifs. Signaling through TLR9 results in translocation of NF-Kb to the nuclease and up-regulation of pro-inflammatory genes, which could cause activation of antigen presenting cells and lead to an adaptive immune response. AAV vector genome can provide unmethylated CpG to TLR9 if the vector genome is released during its transition from the cell surface to the nucleus. Previously we had investigated the importance of TLR9 signaling on the transduction of β-glycosidase in mouse muscle and demonstrated that removing CpG motifs prevented loss of transduction due to T cell activation (Faust, Bell et al. 2013). In a furtherance of that study we chose to investigate the impact of TLR9 signaling on gene therapy of MPS1 by transduction of the IDUA gene. While not part of its normal biogenesis pathway, IDUA, can be secreted through Golgi transport and then taken back up by other cells due to its binding of mannose-6-phosphate, this allows for cross correction of the disease but also leaves it vulnerability to a humoral response (Huang, Wong et al. 1997).
To investigate the effect of TLR9 on the humoral response to IDUA we administered either C57Bl/6 mice or TLR9 KO mice with AAV9 vector containing the IDUA transgene. Using three different cohorts we varied the promoters between groups to have two universal expressers (CB7, CMV) and one liver restricted expresser (TBG) (Figure 19). These studies contribute two important findings, first we demonstrate that location of transgene expression has a great deal of impact on antibody formation with expression from liver peaking high and maintaining stable with no anti-transgene antibody generation compared to either universal expresser that peak and fall off presumable due to the corresponding rise in antibody titer. The second finding was that without TLR9, mice could not mount a humoral response against the transgene product. To control for any B cell developmental differences in TLR9 KO mice we adoptively transferred both KO mice B cells and C57Bl/6 B cells to µMT mice which lack B cells due to loss of the IgM gene. In both groups mice were capable of generating an antibody response that attenuated transgene expression indicating that the TLR9 KO B cells are comparable functional to WT mice (Figure 21). We also confirmed that TLR9 signaling is vital to T cell activation by measuring IFN-γ ELISPOT of splenocytes following AAV9.IDUA transduction (Figure 20). Together these results demonstrate a very important finding that the unmethylated AAV vector genome is not only the primary immunogenic material, but that TLR9 signal is necessary to generate a humoral response. It would be invaluable to the field if the of AAV if vector immunogenicity could be decreased, and our lab has had experience in CpG depletion of AAV vectors a method that is possible due to multiple codons available for the same tRNA.
We systematically removed all CpG motifs from the IDUA gene while still retaining the same protein coding sequence. For the poly A and promoter we used ones previously generated to be CpG less as both CMV and CB7 contain large CpG islands and their removal would most likely impair functionality of the promoter (Faust, Bell et al. 2013). C57Bl/6 mice were administered AAV9.Ef1α.IDUA with either WT or CpG depleted IDUA gene sequence and our results showed that there was no difference between the two groups in either antibody titer or transgene activity (Figure 22 a, b). In addition T cell activation against the transgene as measured by the INF-γ ELISPOT assay did not shown any meaningful differences. Interestingly, unlike other universal promoters the EF1α vector resulted in a lower antibody titer and maintained stable levels of transgene activity. One possible explanation for this and the results as a whole is that the CpG islands in CMV and CB7 were more important for TLR9 activation than the CpG motifs found in IDUA. This may mean that using promoters with lower CpG content and avoidance of CpG islands could be as effective as CpG depletion.

The location of transgene expression has also been noted to have an influence on the immune response to transgene expression. Most of the research to date has been focused on the ability of the liver to “tolerize” the body against a secreted product, less has been explored into which cell type of expression result in such antibody responses (Dobrzynski, Fitzgerald et al. 2006, Cao, Dobrzynski et al. 2007, Doerfler, Todd et al. 2016). As we observed in Chapter 4 anti-IDUA antibody responses could be modulated by the promoter used to drive expression, with more universal promoters allowing for
robust formation of anti-transgene antibodies. To determine if expression from the liver
was able to tolerize or that expression from other locations would overwhelm any T
regulatory cell activation and push the immune system toward a humoral response we
treated mice with equal amounts of both TBG and CMV driven AAV9 IDUA vectors.
From our results we observed a similar response to (Doerfler, Todd et al. 2016) where
despite having a large dose of liver specific expression it seems that the driving force of
anti-body formation is expression from non-liver tissues (Figure 24 a). One tissue group
that we hypothesized was the most important for antibody formation to a secreted
transgene delivered by AAV9 is the muscle as it is both highly transduced and capable of
expressing MHC class II (Vandendriessche, Thorrez et al. 2007, Zincarelli, Soltys et al.
2008) (Englund, Lindroos et al. 2001). We injected mice with AAV9.CB7.IDUA vectors
some of which contained a micro RNA site (miR206) that is targeted only in muscle
(Greig, Peng et al. 2014) and observed that mice injected with the miR206 containing
vectors had both lower anti-IDUA antibodies and higher long term expression. This
result is important as it demonstrates that expression from the muscle may be the most
important transduction target that results in the generation of anti-transgene antibodies. In
future work, if we can achieve cross correction with a transgene we may want to limit the
organs of transduction to exclude the muscle or diminish expression from that location by
inclusion of micro RNA sites.

These results describe the vital role TLR9 and location of transgene expression
play in the generation of a humoral immune response to a transgene product. As
demonstrated in our knockout experiments TLR9 is both necessary and sufficient to activate B cell secretion of anti-IDUA antibodies, provided that transgene expression is not restricted to the liver. One attempt to evade the TRL9 mediated B cell activation was CpG depletion of our transgene construct, our results show no improvement was made in avoiding antibody detection compared to our control. We did observe that changing from a CpG rich CB7 promoter, to a CpG depleted EF1α promoter we had a dramatic effect on serum IDUA activity and antibody-formation, however the interpretation of this result is somewhat complicated by the fact that despite both being considered “universal expressers” there may be small difference in expression from different cell types that could impact on the result. We further interrogated our observation and found that for AAV9 vectors, expression from the muscle was an important factor in formation of a robust anti-IDUA immune response. This has important implications for delivery of gene therapy where we would surmise that expression of secreted transgene would be more tolerated if it only originated from the liver. I would recommend that for a therapy where a cross correction strategy is viable one should either restrict expression to the liver, or design the vector construct as to avoid transgene expression from skeletal muscle cells.

Final Remarks

Throughout this dissertation, my extensive investigation of the adaptive immune response to transgene products following AAV transduction have given us important insight into the factors which influence the outcome. I determined influential host factors that are required for adaptive immune activation, giving us the ability to design better
AAV vector constructs in hopes of reducing the likelihood of an adverse immune response in patients. My hope is that my research will contribute to the safety and effectiveness of the AAV gene therapy delivery platform and will allow for a broader group of individuals to be candidates for treatment.
Generation of epitopes from a gene first requires transcription of the gene into messenger RNA and the translation of the mRNA into a polypeptide. (A) The classical model of antigen presentation in genes that contain a PTC is that epitopes C-terminal to the stop codon will not be generated and thus not presented as antigen. (B) One model that allows for antigen presentation of these C-terminal epitopes is by initiation of protein synthesis downstream of the PTC allowing for generation of the antigen. (C) A second model for antigen presentation of epitopes C-terminal to a PTC is during translation when a stop codon would incorporate a stop-tRNA into the peptide sequence, instead a cognate tRNA would be put in place and allow for continued synthesis of the protein.
BIBLIOGRAPHY


123


