STRATEGIES FOR TOLERANCE INDUCTION AND CORRECTION OF MOUSE MODEL OF HEMOPHILIA A USING LIVER DIRECTED AAV-MEDIATED GENE THERAPY AND B CELL DEPLETION

By

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To Mom

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Hemophilia A is an X-linked bleeding disorder occurring in approximately 1 in 5000 males. The disease is caused by a deficiency of clotting factor VIII (FVIII) resulting in bleeding episodes that can be fatal to an affected individual. The only treatment for hemophilia A is protein replacement therapy, which is both expensive and is often complicated by formation of antibodies against FVIII (termed “inhibitors”). Thus, therapies that address the burden of costly infusions as well as the immune response to FVIII are highly desired. The use of liver-directed adeno-associated viral (AAV) gene therapy was investigated as a means of both phenotypic correction and tolerance induction to the FVIII transgene in a hemophilia A mouse model. Treatment with a liver-specific AAV8-hFVIII vector either sensitized or tolerized BL/6-129/sv or BALB/c hemophilia A mice, respectively, to an intravenous hFVIII challenge protocol. B cell depletion concurrent with gene therapy reduced the antibody response in AAV-treated BL/6-129/sv-HA mice. All of the mice had minimal correction of phenotype correlating to ~1% of normal FVIII activity which did not persist past the hFVIII challenge. Codon-optimization of the transgene (COhFVIII) not only increased expression of hFVIII to a

peak of ~10% and persistent level of 3-5%, but also induced tolerance in both strains. Consistent with previous studies, this tolerance was found to be mediated by T regulatory cells. B cell depletion was also used to develop a protocol to prevent the development of anti-AAV capsid antibodies and as an adjuvant to reverse established inhibitors. Reduction of capsid-specific antibodies was achieved but not to a level that allowed vector re-administration. Furthermore, delivery of AAV-COhFVIII to mice with pre-existing inhibitors suggests that gene transfer with or without B cell depletion may reduce inhibitors.

These results provide a significant step forward in gene therapy for hemophilia A by demonstrating that the immune responses to FVIII may be mitigated by liver-specific expression of the transgene. Furthermore, B cell depletion (such as with the human drug rituximab) may prove to be a essential adjuvant in gene therapy for the prevention of an immune response to both the transgene and the viral vector.

CHAPTER 1
INTRODUCTION

Hemophilia

Hemophilia A is a rare X-linked recessive bleeding disorder occurring in approximately 1 in 5000 live male births. The earliest documentation of the disease dates back to the second century when a rabbi observed that sons of carriers were susceptible to bleeding episodes following circumcision [1]. The reduced ability to form a blood clot is the most salient manifestation of the disease and is due to a deficiency in clotting factor VIII (FVIII). The FVIII protein is a large (~300kD), multi-subunit protein consisting of 2332 amino acids and is produced primarily in the liver [2,3]. It exists in circulation in two parts: a heavy chain consisting of domains A1, A2 and B domains and a light chain with the A3, C1 and C2 domains (Fig. 1-1). Factor VIII is a part of the intrinsic coagulation cascade and is activated by thrombin whereupon it then works with activated factor IX (FIX) to cleave and activate factor X (FX) (Fig. 1-2**)** [4]. Activated FX cleaves pro-thrombin to thrombin, which drives the production of a cross-linked fibrin clot that ceases the flow of blood and limits blood loss. Hemophilia B is caused by a reduction in activity or absence of FIX due to mutations in the *f9* gene. Since FIX and FVIII act as cofactors at the same step in coagulation, the two diseases are clinically indistinguishable although hemophilia B is considerably more rare occurring in about 1 in 20000 males.

The congenital form of hemophilia A is caused by mutations in the FVIII gene which limit or eliminate circulating FVIII levels or activity. The FVIII gene is found on the long arm of the X chromosome at the locus Xq28 [1]. The gene itself is 186kb, of which 9kb is comprised of its 26 exons with the remainder being intervening or intronic

sequences[5]. Given that hemophilia A is an X-linked disorder, males are much more commonly affected than females and can either inherit the disease by receiving a defective allele from the mother or by a *de novo* mutation in the *f8* gene. The mutations that cause disease vary greatly but, surprisingly, the vast majority (40-50%) are of a specific type consisting of an inversion in intron 22 resulting in less than 1% of normal FVIII levels [6,7]. This is the result of homology between a region inside intron 22 of the *f8* gene and an upstream gene, "FVIII-associated gene A", which can lead to crossing over and inversion of the *f8* gene (Fig. 1-3) [6]. The remaining patients with hemophilia A harbor mutations that span the spectrum from point mutations to large deletions. Severity of disease is directly correlated with the amount of residual FVIII activity. Patients with "severe" hemophilia A have less than 1% of normal FVIII activity either from a complete absence of circulating protein or minimal functional protein. This form of the disease usually results from very disruptive mutations which either prevent FVIII from being produced or severely limit its function. Given the essential role FVIII plays in coagulation, patients with severe disease suffer from spontaneous bleeding episodes. These bleeds are the central complication in severe hemophiliacs who can suffer from frequent bleeding into soft tissues and joints. Bleeding into joints can cause significant swelling leading to arthropy, damage, and can eventually limit joint function to the point of necessitating arthoplasty [8]. These bleeding episodes can be fatal if the bleeding occurs in the brain in the form of an intracranial hemorrhage and in fact is the leading cause of death among hemophiliacs not infected with HIV [9]. The prognosis of hemophilia A improves with increasing amounts of FVIII activity so that patients with "moderate" disease (1-5% FVIII activity) have a milder phenotype characterized by

infrequent spontaneous bleeds and generally are managed only during traumatic instances. Likewise, the “mild” form of the disease (>5% FVIII activity) only requires intervention during surgery or following injury.

Treatment of Hemophilia A

The current standard of care for hemophilia is protein replacement therapy where FVIII is administered either prophylactically or on-demand. The source of FVIII has historically been derived from human plasma which carried the risk of viral infection with HIV and hepatitis C. Infection with HIV was a very serious concern amongst hemophiliacs as HIV-positivity raised the mortality rate from <1% to >10% in severe and moderate hemophilia A patients [10]. Since the institution of better screening methods following the AIDS epidemic of the early 1980s, plasma-derived FVIII is considered much safer. Recently, FVIII production has been possible *in vitro* utilizing mammalian cell culture and introduction of recombinant FVIII DNA. Either product can be used to treat hemophiliacs with equal efficacy [11]. Frequency of FVIII administration depends on the form of the disease. Mild and moderate hemophiliacs can be administered clotting factor “on-demand” during times of trauma whether in the form of injury or during surgical/dental work. For patients with severe hemophilia A, FVIII is generally recommended prophylactically to control spontaneous bleeding events before they occur. Given that FVIII has a half-life of approximately 8-12 hours, patients usually self-administer or are given FVIII infusions every other day which prevents the majority of spontaneous bleeds and better preserves joint function [1,12]. Unfortunately, production of plasma derived and recombinant FVIII is limited in the yield of FVIII. Thus, the cost of FVIII is prohibitively high. In the case of severe hemophiliacs, prophylactic FVIII administration can cost well over $100,000 per year per patient [13]. As a result,

successful treatment of hemophilia A is limited to countries that have access to expensive pharmaceuticals and widespread health insurance coverage (developed areas such as North America and Western Europe) [14,15]. It is estimated that 70% of hemophiliacs worldwide are untreated, leaving them exposed to the almost certain fatal consequences of the disease [14,15].

However, if treated with exogenous FVIII, the prognosis for patients with hemophilia A is generally good. Aside from cost, clotting factor replacement is also limited by the development of neutralizing antibodies against FVIII protein—termed "inhibitors" for their ability to inhibit FVIII activity.

Inhibitors in Hemophilia A

Inhibitors can occur in up to 30% of severe hemophilia A patients. They render treatment with FVIII ineffective and eliminate the only long-term treatment option. Cessation of bleeding can be controlled with "FVIII bypassing agents" such as activated FVII but these agents are not suitable for long-term use as they are exceedingly expensive. Patients with severe hemophilia are at greatest risk of developing inhibitors particularly those with large deletions, nonsense mutations and inversions of either intron 1 or 22 [16].

It is not entirely clear why inhibitors develop in the case of hemophilia [17]. Normal lymphocyte development causes self-reactive T and B cells to be removed by a process known as negative selection. T cells mature in the thymus and are presented a variety of self antigens by antigen presenting cells (APCs) in the thymus which express numerous self proteins driven by the pluripotent transcription factor AIRE. If a developing T cell is able to recognize and bind a self-antigen presented by these thymic APCs, the result will be cell death and elimination of the self-reactive T cell. B cells are

also similarly negatively selected in the bone marrow during development where engagement of the B cell receptor (BCR) leads to cell death. Self-reactive B cells that escape negative selection can further be selected against in the periphery where they will be rendered anergic if they encounter antigen in the absence of immunological "danger" signals and/or in the absence of help from CD4 T helper cells—which should have been selected against in the thymus if specific for self-antigens. Thus, non-affected individuals do not possess FVIII-specific T and B cells as a result of this process. Individuals with severe hemophilia A, however, may lack any FVIII antigen expression at all (due to large mutations, see above)—leading to the persistence of FVIII specific effector T and B cells and the most likely explanation for the higher percentage of severe hemophiliacs that develop inhibitors [18]. Still, the presence of FVIII in the circulation in the absence of any immunological "danger" signals should not lead to such a robust humoral immune response. It has been hypothesized that the role of FVIII in damaged surfaces may provide the immune stimulus to mount an immune response to the infused protein, but this has yet to be proven [19].

Once established, inhibitors are very difficult to eliminate. The only established method for reversing inhibitors is a process called immune tolerance induction (ITI). This involves delivering frequent high doses of FVIII until the inhibitors are cleared—which usually takes more than a year. The exact mechanism of ITI is unknown but both T cell exhaustion (as seen in chronic viral infections) and inhibition of B cell differentiation by supraphysiologic levels of FVIII have been proposed [17,20]. Approximately 60-80% of patients will experience complete remission of inhibitors but at a high cost [17,21,22]. One round of ITI can cost well over $1,000,000 in FVIII alone and

over $2,000,000 in total when considering the expense of bypassing agents before and during treatment [22]. For those that fail, a second attempt is usually made at which point supplementing ITI with immune suppressants such as the B cell-depleting biologic drug rituximab may show some benefit [23,24]. Aside from a less than perfect success rate, ITI has some considerable limitations. For one, delivering daily intravenous injections presents complications with compliance and venous access. A venous access port can be used but at the risk of infection which tends to exacerbate the existing inhibitor response and lessens the chance of success of ITI [25]. Given the high cost, number of patients that fail ITI and other complications, alternative methods to induce immunological tolerance to FVIII are desired.

AAV Gene Therapy

Adeno-associated virus (AAV) is a replication-deficient, non-pathogenic parvovirus that naturally infects humans as well as other species including non-human primates [26]. It contains a 4.7kb single-stranded genome with two open reading frames: one encoding *rep* genes required for viral replication and the other for *cap* genes which code for the three capsid genes VP1, VP2 and VP3 (Fig. 1-4). In order to generate a productive, replicative infection AAV must be in the presence of a helper virus co-infection such as adenovirus or herpes virus. In the absence of such viruses, AAV will establish a latent infection by integrating its genome into a locus on the human chromosome 19. Historically, AAV received little recognition and study due to their lack of pathogenicity. For this same reason however, interest in using these viruses as gene therapy vectors has increased drastically in the past 20 years. AAV vectors can be made by removing all native viral sequences except for the 145bp inverted terminal repeats (ITRs) which are required for packaging DNA into the viral capsid and for

initiating second-strand synthesis—the process of converting the single-stranded viral DNA into transcriptionally active double-stranded DNA. Approximately 5kb of an expression cassette coding for a therapeutic gene can be placed between these ITRs and packaged into an assembled AAV capsid. AAV vectors can be produced *in vitro* by transfecting HEK-293 cells with three plasmids: one encoding the vector genome, one coding for the missing viral genes *rep* and *cap* which are necessary to assemble the virus and a third providing adenoviral helper genes (E2a, VA, and E4). When these AAV vectors are delivered *in vivo* they efficiently target non-dividing cells where the genome can exist episomally for extended periods of time (up to >8 years in dogs) [27]. AAV vectors are notoriously inefficient at infecting APCs such as macrophages and dendritic cells which decreases the likelihood that they will be targeted by adaptive immunity [28]. AAV has also been shown to be a poor stimulant of the innate immune system compared to other vectors such as adenovirus, further limiting the likelihood of an immune response [29,30]. Initially, AAV failed to make a significant clinical breakthrough. However, recent successes in clinical trials using AAV for Leber congenital amaurosis—an inherited degenerative blindness disorder—and hemophilia B strongly support the application of AAV as a gene therapy vector [31-36].

Liver Directed AAV gene therapy for hemophilia

The liver produces many proteins destined for vascular circulation including FVIII, although other extra-hepatic cells may contribute to circulating FVIII protein [37]. In addition to being the natural site of FVIII production, the liver also has access to central circulation and possesses the cellular machinery to translate, process and secrete large proteins such as FVIII in abundance. The liver is also an attractive gene therapy target as it is recognized as an immune privileged site. This was first discovered in

experiments where allogeneic liver grafts were readily accepted in pigs as opposed to the rejection observed in other tissues [38]. This property is thought to be due to the fact that the liver is directly downstream of the gut blood flow and thus receives a vast array of innocuous food and commensal bacteria antigens that do not warrant an immune response [39,40]. The Herzog lab has previously demonstrated that AAV gene transfer directed at hepatocytes can not only provide sustained transgene expression but also renders immunological tolerance to the transgene. This tolerance is an active phenomenon mediated by $CD4^+CD25^+FoxP3^+$ T regulatory cells (Treg) which are antigen specific and mediate tolerance through a variety of mechanisms including secretion of suppressive cytokines such as IL-10, TGF-β and by expressing inhibitory surface molecules such as CTLA-4 [41-44]. Once tolerance is established, the immune system is non-responsive to antigenic challenge even in the presence of strong adjuvants [45]. This has been successful in preclinical models of hemophilia B with FIX and has also been extended to induce tolerance in autoimmune disorders such as EAE, a mouse model of multiple sclerosis [46]. Early clinical trials using AAV gene therapy in the human liver failed to show any immune response to the transgene but instead resulted in a $CD8^+$ T cell response to the AAV capsid [47,48]. However, FVIII is a much more immunogenic protein and thus there is concern of invoking an immune response to the FVIII transgene. Furthermore, if levels of FVIII are achieved in clinical trials that are ≤10%, FVIII protein therapy would still be occasionally necessitated and tolerance to FVIII would be essential in avoiding the development of inhibitors. Therefore, liver-directed AAV gene therapy for hemophilia A is an ideal protocol given the characteristics of the FVIII protein and the immune privilege of the liver.

Gene therapy with AAV has seen a recent encouraging success in clinical trials where 6 individuals with severe hemophilia B were treated with an AAV8-serotype vector carrying a genome encoding a self-complementary (double-stranded) codon-optimized FIX cDNA [36]. All 6 patients had sustained levels of FIX ranging from 2-12% which reduced or eliminated their need for supplementary FIX treatment and allowed for a dramatic improvement in quality of life—even to the point where one patient now is a marathon runner. This is a major step forward for safety and efficacy of AAV gene therapy with all treatments groups showing an improvement in quality of life. The only adverse events were the development of a capsid-specific $CD8^+$ T cell response against transduced hepatocytes in two patients, which was easily controlled with prednisone and did not negatively affect treatment efficacy.

While encouraging, extending these successes to hemophilia A presents major challenges. Preclinical studies have been limited by the large 7.3kb FVIII cDNA which exceeds the packaging capacity of AAV, the immune response to FVIII, poor activity of human FVIII in murine models and inefficient expression and secretion of FVIII protein. Despite these challenges, preclinical progress has been made to circumvent these barriers. Deletion of the non-essential B domain of FVIII reduced its cDNA size to 4.5kb which is within the limitation of AAV [49]. Alternatively, the FVIII cDNA has been divided into the heavy and light chain allowing co-delivery of two AAV vectors expressing each chain [50]. The two chains then recombine intracellularly and comprise a functional FVIII protein capable of correcting disease—although concerns about imbalances in chain expression and secretion still remain [50]. Since a minimum level of expression from the liver is required to induce tolerance, poor expression of FVIII from AAV vectors often

feed an immune response rather than prevent it. To circumvent this, broad immune suppression or gene transfer to neonatal mice (when the immune system is still developing and more permissive) are often required [51-54].

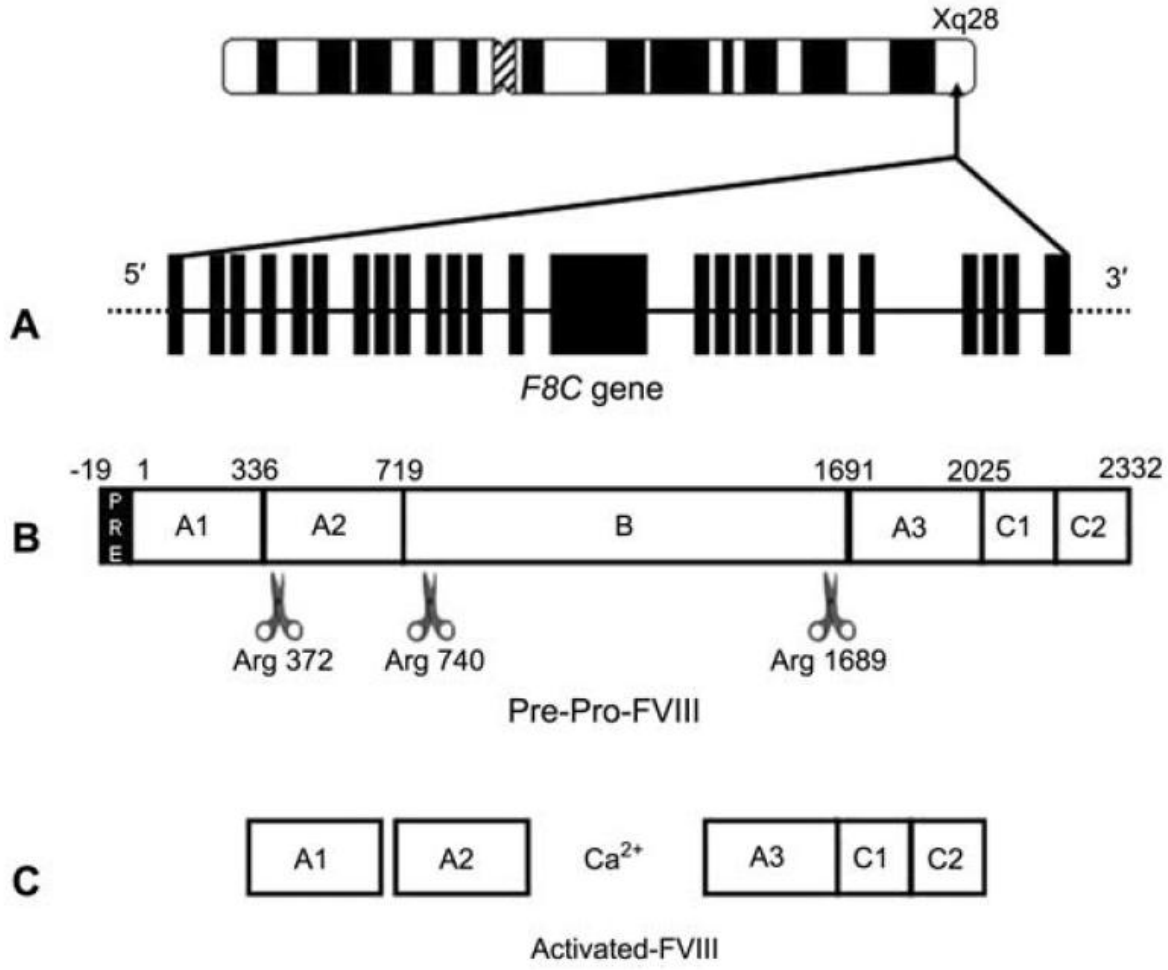

Figure 1-1. The human factor VIII protein. Diagram of the FVIII gene location on chromosome Xq26 along with its 26 exons in (A)**.** The FVIII protein before (B) and after C) proteolytic cleavage at indicated sites to the active form of FVIII as the heavy (A1 and A2) and light chains (A3, C1 and C2). From Castaldo, et al. [2].

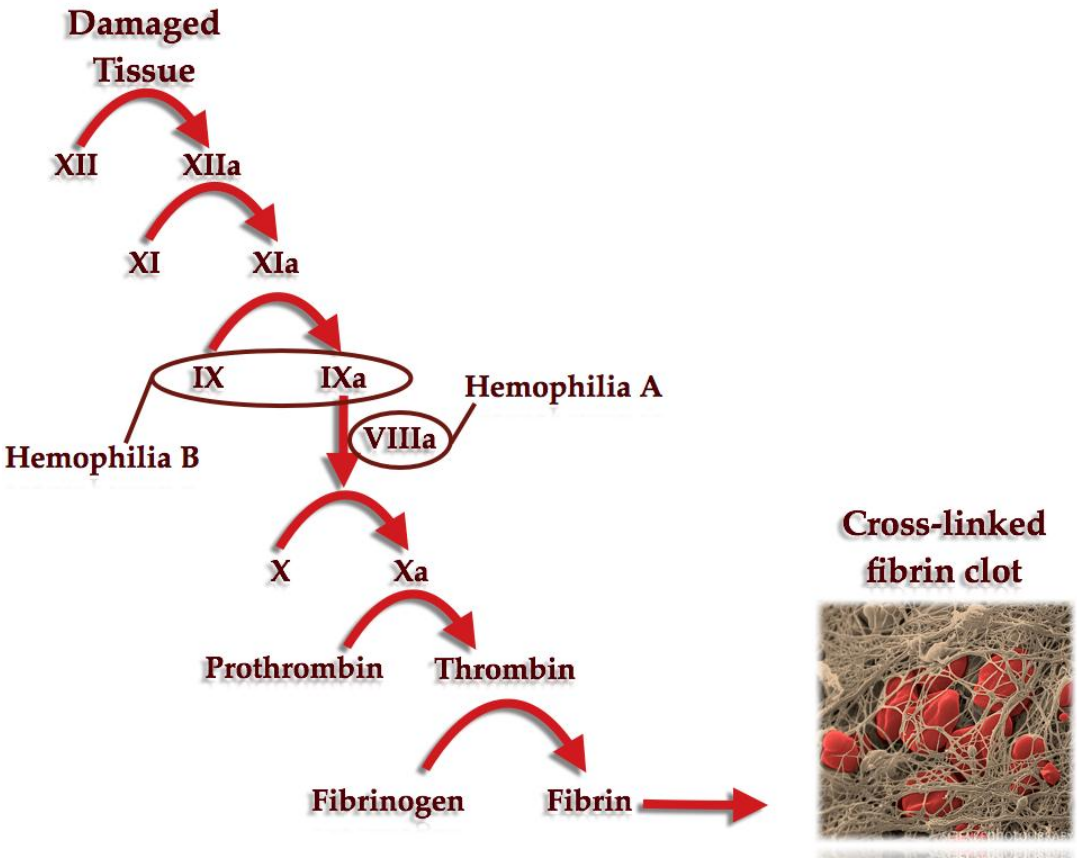

Figure 1-2. Simplified overview of the intrinsic clotting pathway.

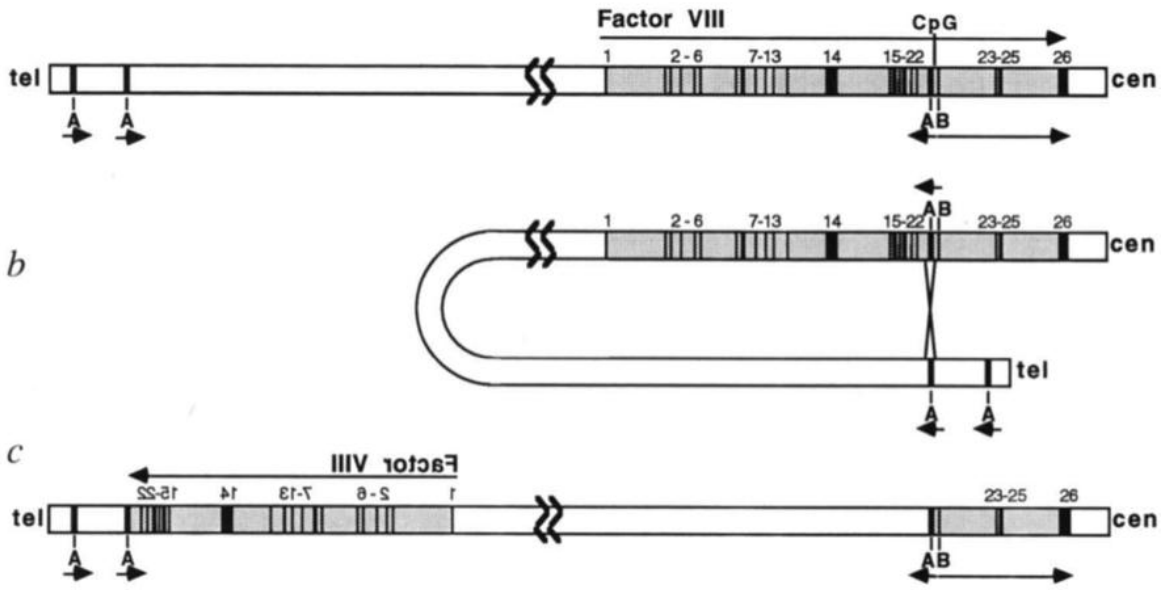

Figure 1-3. Overview of FVIII common inversion in intron 22 of the FVIII gene. Copies of the "A" gene are shown and direction of transcription is indicated by arrows. Crossover in *b* between upstream A gene and A gene within intron 22 results in inversion indicated in *c.* From Lakich, et al. [6]

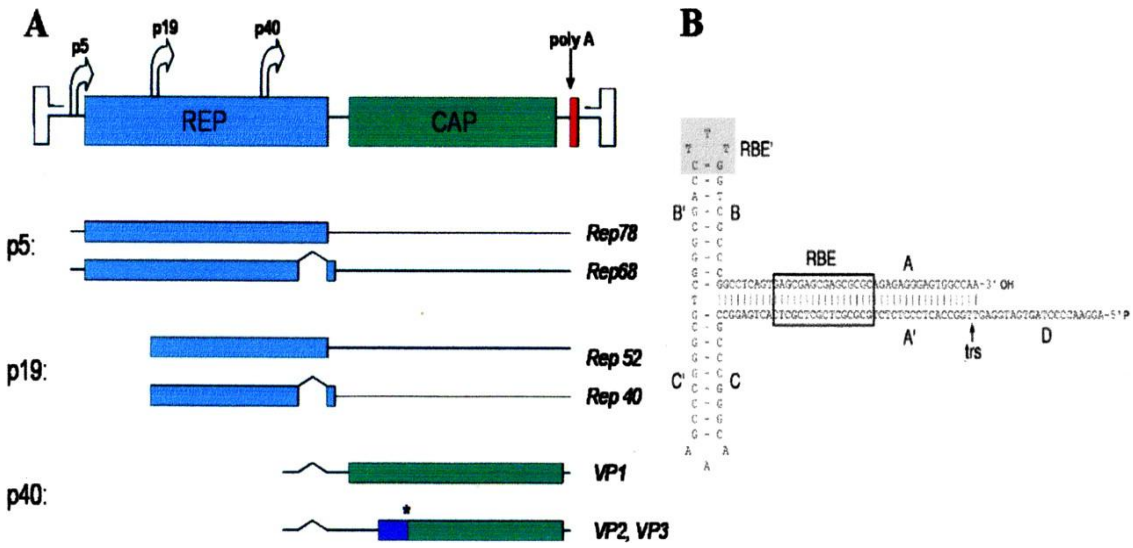

Figure 1-4. AAV genome structure. (A) Layout of the AAV genome with different transcripts (from promoters p5, p19 and p40) along with their proteins to the right of each transcript. In (B) the ITR is shown which is required for DNA packaging and second strand synthesis. From Daya and Berns [26]

CHAPTER 2
MATERIALS AND METHODS

Mouse strains

All animals used were 6-10-week old male hemophilia A mice with a deletion in Exon 16 of the *f8* gene on either BALB/c or a mixed C57BL/6-129/sv background, kindly provided by Drs. Lillicrap and Kazazian [55]. These mice were generated by inserting a neomycin cassette into exon 16 of the murine FVIII gene resulting in undetectable FVIII activity and thus providing a model of severe hemophilia A.

Ten- week-old C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at the University of Florida College of Medicine. All animal experiments were performed according to the guidelines for animal care specified by the Animal Care Services at the University of Florida. The Institutional Animal Care and Use Committee approved all protocols for the care and use of these mice.

B cell depletion and flow cytometry

Mice were depleted of B cells using 10 mg/kg of anti-murine CD20 IgG2a antibody (clone 18B12, kindly provided by Biogen Idec, Weston, MA) [56]. Mice were given doses by intravenous (*i.v.*) injection. To assess B cell depletion, lymphoid tissues were collected either 1 day or 7 weeks after the second dose. Peripheral blood was collected in heparinized microcapillary tubes via retro-orbital plexus, centrifuged at 9600 RPM for 10 minutes at 4°C to separate and remove plasma. The remaining cells were re-suspended in 150µL PBS with 1.5µL Fc block (BD Biosciences, San Jose, CA) and incubated at room temperature for 15 minutes before further staining for flow cytometry. Single cell suspensions of splenocytes and peripheral lymph node cells were made by

forcing organs through a 70μm filter, washed in PBS and centrifuged at 300x*g* for 10 minutes. This pellet was resuspended in PBS for counting and 10^6 cells in 100μL were added to 1.5μL Fc block (BD Biosciences) and incubated at room temperature for 15 minutes. Staining of all single cell suspensions was performed with fluorescently labeled antibodies for the B cell marker CD19 (clone 1D3 V450-conjugated, BD Biosciences) as well as CD3 (clone 145-2C11 FITC-conjugated, BD Biosciences) or CD4 (clone L3T4 FITC-conjugated, BD biosciences) to detect T cells. Lymphocyte gating was determined by forward-scatter and side-scatter following back gating for the area containing $CD19^+$ cells. A BD LSRII flow cytometer (BD Biosciences, Frederick, MD, USA) and FCS Express 4 software (Denovo Software, Los Angeles, CA, USA) were used for analysis.

AAV vector construction

Mice were given a tail vein injection of 10^{11}vg/mouse of AAV8 containing either wild-type or codon-optimized B domain-deleted human hFVIII (BDD-hFVIII) under a liver-specific promoter. This construct was assembled by replacing the transthyretin promoter of the vector published by Lu et al. [52] with the ApoE/hAAT enhancer/promoter (outlined in Fig. 2-1) [44]. This was accomplished by digesting the pAAV-TTR-hFVIII (from Lu, et al. [52]) plasmid with SacI and XhoI and isolating the fragment containing the AAV inverted terminal repeats (ITRs) and minimal ("mini") synthetic poly-A signal. The pAAV-hAAT-hFVIII plasmid containing the liver-specific human alpha-1 antitrypsin (hAAT) promoter and human growth hormone (hGH) poly-A was also digested with MluI/SacI and the fragment containing the hAAT promoter and hFVIII cDNA was ligated to the "mini" poly-A and backbone of the previously mentioned fragment to generate pAAV-hAAT-hFVIII-mini with an approximately 5.3kb of DNA from ITR to ITR. The codon-optimized hFVIII cDNA was synthesized by GeneArt (Regensburg, Germany) and

cloned into the wild-type construct by replacing the wild type hFVIII using ClaI/XhoI digestion and ligation into the previous plasmid (sequence in Appendix).

Viral vectors using these plasmids were produced by triple transfection of HEK-293 cells and purified by iodixonal gradient centrifugation as published previously [57]. Viral titers were assessed by quantitative slot-blot hybridization and confirmed via western blot using antibodies against viral capsid.

Blood Collection

Plasma samples were collected via either the retro-orbital plexus for ELISA-based assays and flow cytometry or via the tail vein for aPTT and Bethesda assays. For retro-orbital plexus, mice were anesthetized with isofluorane and immediately bled using heparinized microcapillary tubes. Tail vein bleeding was performed by heating the mice under a 125w lamp for 5 minutes and then anesthetizing with isofluorane. The tail was then cut using a scalpel and 250µL of blood collected in 1.5mL centrifuge tubes containing 25µL of 3.8% sodium-citrate. Tails wounds were ligated with non-absorbable sutures and cauterized with sliver-nitrate. Following collection via either method, plasma was collected by centrifuging samples at 9600 RPM in a table-top centrifuge for 10 minutes at 4°C and plasma removed by pipetting the supernatant.

Intravenous hFVIII challenge protocol

For challenges with hFVIII in mice, 1 IU BBD-hFVIII (Xyntha, Pfizer, New York, NY) was diluted in 200µL PBS and injected *i.v.* once a week for 4 weeks unless otherwise indicated.

ELISA protocols

Enzyme-linked immunosorbant assay (ELISA) for hFVIII-specific IgG1 was performed by coating 96-well plate (Corning, Tweksbury, MA) with 1ug/mL of hFVIII

(Xyntha, Pfizer, New York, NY) in coating buffer (0.68g Na_2CO_3; 3.675g $NaHCO_3$ in 500mL H_2O at pH 9.2) at 4°C overnight. Standards for IgG1 were coated onto the plate in duplicate by serially diluting mouse IgG1 (Sigma Aldrich, St. Louis, MO) in coating buffer beginning at 2000ng/mL. Plates were then washed and blocked with dilution buffer (0.05% tween 20 and 6% BSA in PBS) for 1h at room temperature. Following plate washing, plasma samples were applied in duplicate to plates at a 1:20 dilution and incubated at 37°C for 2 hours. After washing, IgG1 antibodies were detected using a rat anti-mouse IgG1 horseradish peroxidase-conjugated antibody (R&D Systems, Minneapolis, MN) diluted 1:2000 in dilution buffer for 2h at 37°C. Quantitation of antibody levels was performed by measuring optical density (OD) at 450nm of each well following room temperature incubation with SigmaFast OPD (Sigma Aldrich, St. Louis, MO) using a Bio-Rad 680 microplate reader (Hercules, CA). Calculation of antibody concentrations was performed using MPM III v1.6 software.

Anti-AAV8 IgG2a antibodies were measured in the same manner with some exceptions. First, an IgG2a standard was used (Sigma Aldrich, St. Louis, MO) at a starting concentration of 250ng/mL. Plates were coated with AAV8 virus at a concentration of $5x10^{10}$vg/mL in coating buffer. Samples were diluted at 1:10 in dilution buffer, incubated overnight at 4°C and antibodies detected with a horseradish peroxidase-conjugated rat anti-mouse IgG2a antibody (R&D Systems, Minneapolis, MN).

Plasma hFIX levels were also measured by ELISA. The same 96-well plates were coated with a monoclonal antibody recognizing human (and not murine) FIX (FIX-1 monoclonal anti-hFIX Clone HIX-1; F2645 Sigma Aldrich, St. Louis, MO) diluted 1:850

in coating buffer and incubated overnight at 4°C. After washing, plates were blocked with blocking buffer (5% non-fat dry milk in PBS with 0.05% tween 20) for 2h at room temperature. Plasma samples were diluted 1:10 in blocking buffer. A standard was generated by diluting normal human plasma (TriniCHECK Level 1; Trinity Biotech, Wicklow, Ireland) to 200ng/mL of FIX serially to 3.125ng/mL. Both samples and standards were applied to the plate in duplicate after washing and incubated overnight at 4°C. Goat anti-human FIX horseradish peroxidase-conjugated antibody was diluted 4.5µL in 10.5mL of dilution and used to detect hFIX following washing. Quantitation was performed as described above.

Clotting assays

Collected blood was used to determine activated partial thromboplastin time (aPTT) as a measurement of clotting time. Assay was performed by mixing 50µL of sample plasma with 50µL of FVIII-deficient plasma (Hematologic Technologies Inc., Vermont) and 50µL of automated aPTT reagent (TriniCLOT, Trinity Biotech, Wickow, Ireland) and incubated at 37°C for 3 minutes. Next, 50µL of 25mM calcium-chloride was added and recording of time to clot was immediately started using a fibrometer (Fibrosystem^{TM}, Block Scientific Inc.).

Bethesda assay was performed as previously described [58,59]. In detail, plasma samples were serially diluted in Imidazole buffer (MDA, Baltimore, MD) from N to N/1028 in a total volume of 30µL. Each diluted sample was mixed with 30µL of normal human plasma (TriniCHECK Level 1; Trinity Biotech, Wicklow, Ireland) and incubated at 37°C for 2 hours. Control samples of normal human were diluted in Imidazole at 1:2 and 1:4 and were also incubated at 37°C for 2 hours. After incubation, all samples were transferred to ice. Standards were also made from 20% to 0% of normal human plasma

in Imidazole. Normal aPTT was then performed on all samples, controls and standards and Bethesda units were calculated using the controls and standard so that 1 Bethesda unit equals a reduction of FVIII activity of 50% as described in Verbruggen, et al. [59].

For hFVIII % activity, two methods were used. First, a standard curve of 0-10% was made using the same BDD-hFVIII used for challenge diluted in naïve hemophilia A mouse plasma and aPTT times measured. The formula for this resulting regression was used to input the sample aPTT time and hFVIII activity determined. Alternatively, a chromogenic assay was used (Chromogenix Coatest SP4 Factor VIII, Diapharma, West Chester) and samples analyzed according to the manufacturer's protocol for 1-20% FVIII activity in a 96-well plate.

Adenovirus challenge

BALB/c-HA mice were treated with 10mg/kg αCD20 3 weeks apart as before. Seven weeks after the second injection of αCD20 mice were given 10^{11}p/ms of an E1/E3-deleted human serotype 5 adenovirus expressing the *lacZ* gene (Ad-LacZ). Two weeks after Ad-LacZ challenge, mice were bled and plasma was collected for analysis of anti-βgal antibodies and neutralizing antibodies against adenovirus.

To ascertain the level of anti-adenoviral neutralizing antibodies, plasma samples were diluted serially from 1/16-1/512 in DMEM (Life Technologies, Carlsbad, CA) and incubated with Ad-GFP in 53.5μL total volume for 1.25h at 37°C and 5%CO_2. Immediately following incubation, 14μL of the plasma/Ad-GFP was added to one well of a 96-well plate containing 10^5cells/well of HEK-293 cells in 300μL total for an MOI of 250. Each sample dilution was plated in duplicate along with uninfected wells and control wells mixed with naïve mouse plasma. After 20h of incubation at 37°C, 5%CO_2 cells were collected and analyzed via flow cytometry for GFP expression. Percent

positive cells were counted at dilutions of 1/16 and Ad-LacZ-infected plasma compared with naïve plasma.

For anti-transgene antibody levels, 96-well ELISA plates (Easy Wash, Corning, Tweksbury, MA) were coated with β-gal protein at 1ng/μL in coating buffer along with serially diluted standards of mouse IgG1 (Sigma Aldrich, St. Louis, MO) from 2000ng/mL to 15.625ng/mL and incubated overnight at 4°C overnight. After washing, plates were blocked with dilution buffer for 1h at room temperature and then washed again. Plasma samples were diluted 1:20 and added to β-gal-coated wells and incubated for 2h at 37°C. After washing, IgG1 antibodies were detected using a rat anti-mouse IgG1 horseradish peroxidase-conjugated antibody (R&D Systems, Minneapolis, MN) diluted 1:2000 in dilution buffer for 2h at 37°C. Quantitation of antibody levels was performed by measuring optical density (OD) at 450nm of each well following room temperature incubation with SigmaFast OPD (Sigma Aldrich, St. Louis, MO) using a Bio-Rad 680 microplate reader (Hercules, CA). Calculation of antibody concentrations was performed using MPM III v1.6 software.

Immunohistochemistry

For immunohistochemical staining of hFIX-producing hepatocytes, liver cryo-sections from two mice per group were obtained 10 weeks post-injection of AAV2-hFIX and analyzed for hFIX expression. Briefly, liver sections in O.C.T. medium (Sakura Finetek USA, Torrance, CA) were fixed in acetone at room temperature for 10 min, washed, and blocked with 5% donkey serum in PBS for 15 min at room temperature. Goat anti-hFIX antibody (Affinity Biologicals, Ancaster, ON, Canada) was applied in a 1:200 dilution in 2% donkey serum for 30 min after washing. Samples were washed and incubated with Alexa Fluor 568-conjugated donkey anti-goat IgG (Molecular

Probes=Invitrogen, Eugene, OR) diluted 1:200 in 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Fluorescence microscopy was performed with a Nikon E800 microscope (Nikon, Tokyo, Japan). Images were captured with a CoolSNAP-Pro camera and analyzed with Image Pro- Plus software (Media Cybernetics, Bethesda, MD). Percent positive cells were estimated by counting stained hepatocytes in 10 different frames per mouse and dividing by the average cell number per frame.

T cell assays and adoptive transfer

Single cell suspensions of splenocytes isolated from mice in each group were generated as described above and 10^6cells/well were cultured in 12 well plates in RPMI 1640 media containing 5μM β-mercaptoethanol, 100 mM insulin/transferrin/selenium, glutamine and penicillin/streptomycin either with or without 5 μg/mL of hFVIII for 48h at 37°C and 5% CO_2. Cells were then removed and stained with APC-efluor780-conjugated anti-mouse CD3 (clone 17A2, eBiosciences, San Diego, CA), Alexa700-conjugated anti-mouse CD4 (clone RM4-5, eBiosciences, San Diego, CA) and V500-conjugated anti-mouse CD8 (clone 53-6.7, BD Biosciences, San Jose, CA). These cells were sorted for $CD3^+CD4^+$ cells on an ARIA cell sorter (BD Biosciences, San Jose, CA, USA). RNA was immediately extracted from these cells using the Qiagen RNeasy isolation kit (Valencia, CA, USA) and used for quantitative RT-PCR with an SA Biosciences custom RT-PCR array (Qiagen, Valencia, CA).

For adoptive transfer, splenic $CD4^+CD25^+$ cells were purified with a magnetic isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), which achieves ~85% purity. These cells were then pooled and adoptively transferred to naïve BALB/c-HA mice at 10^6 cells/mouse via tail vein injection. Recipient mice were challenged 24 hours

later via subcutaneous injection of 1 IU hFVIII in Sigma Adjuvant System (St. Louis, MO, USA).

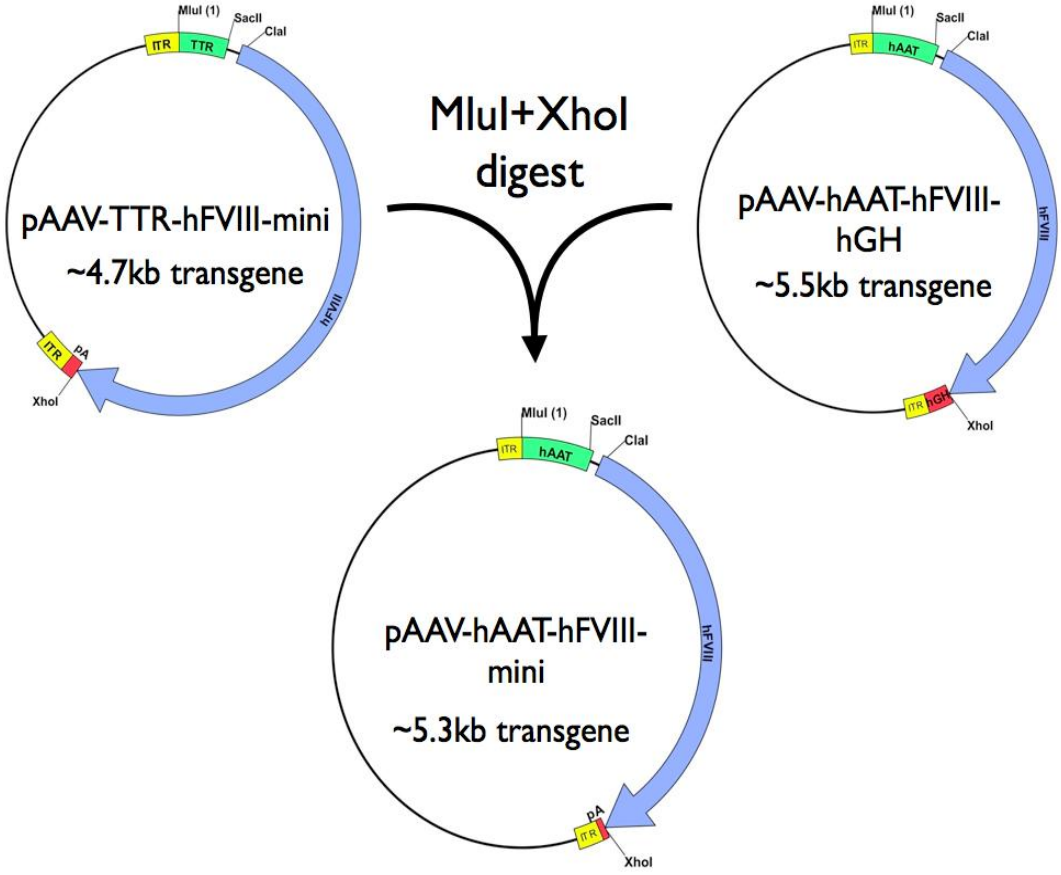

Figure 2-1. Cloning strategy for pAAV-hAAT-hFVIII-mini vector plasmid. Both pAAV-TTR-hFVIII-mini (as in Lu, et al. [52]) and pAAV-hAAT-hFVIII-hGH vector containing the ApoE/hAAT promoter with MluI and XhoI restriction enzymes. The fragment from the pAAV-TTR-hFVIII-mini containing the minimal poly-A sequence and ITRs was ligated to the pAAV-hAAT-hFVIII-hGH fragment containing the hAAT promoter and hFVIII transgene to yield the pAAV-hAAT-hFVIII-mini transgene with approximately 5.3kb of DNA between the ITRs.

CHAPTER 3
TRANSIENT B CELL DEPLETION IN COMBINATION WITH LIVER-DIRECTED GENE THERAPY

Background

Given the high cost, invasiveness and long time to remission of inhibitors, strategies to improve ITI or alternative means to induce tolerance to FVIII are highly desirable. One approach is through the use of the biologic drug rituximab - a monoclonal chimeric antibody directed against human CD20 originally developed to treat B cell lymphoma. Rituximab efficiently depletes CD20-expressing B cells via several mechanisms that include complement fixation, antibody-mediated cellular cytotoxicity and direct induction of apoptosis [60]. CD20 is expressed from the early pre-B cell stage to mature B cells and short-lived plasma cells but not by long-lived plasma cells. Rituximab has been investigated for use in antibody-mediated autoimmune diseases such as acquired hemophilia, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, myasthenia gravis and others [61]. Several case reports and one national survey have revealed that rituximab can improve ITI in hemophilia A patients, especially in cases where patients have previously failed traditional ITI [24,62,63]. Successful reversal of an inhibitor against factor IX (FIX) that had formed in a non-human primate after gene therapy was also reported using rituximab combined with cyclosporine A [64]. However, pre-clinical studies using αCD20 in hemophilic animals or in gene therapy for hemophilia are very limited. B cell depletion as a potential means of preventing (rather than reversing) inhibitor formation has also not been studied.

Importantly, liver-directed gene therapy with adeno-associated virus (AAV) can provide both long-term phenotypic correction and immune tolerance to FIX in hemophilia B animal models [44]. Success in animal models has led to two clinical trials

for hemophilia B using liver-directed AAV gene therapy [65]. Hemophilia A has been more difficult to treat with AAV gene therapy due to the increased immunogenicity of FVIII, limitations in the packaging capacity of AAV and poor expression of FVIII. Typically, transient immune suppression, high vector doses, the use of canine FVIII (which has a higher specific activity in mice than hFVIII) and mice of C57BL/6 strain background (which is more promiscuous to hepatic AAV transduction), or a combination of these methods was needed to achieve long-term correction in hemophilia A mice [50-52]. Here, we investigate liver-directed AAV gene therapy in different strains of hemophilia A mice to induce tolerance to hFVIII either alone or in combination with transient B cell depletion.

Study Design for Transient B Cell Depletion and Gene therapy

We sought to identify pathways toward immune tolerance to hFVIII in gene therapy and to determine the effect of transient B cell depletion on hFVIII-specific immune responses using an anti-murine CD20 monoclonal antibody (αCD20) comparable to rituximab. Hemophilia A mice on either a mixed BL/6-129/sv (BL/6-129/sv-HA) or a BALB/c (BALB/c-HA) background were divided into two treatment groups (Fig 3-1). "AAV8-F8+αCD20" mice were given 10 mg/kg of αCD20 one week prior to receiving 10^{11}vg/mouse of an AAV8 vector expressing B domain-deleted hFVIII under the liver-specific hAAT promoter (AAV8-hF8). Two weeks following AAV8-hF8 injection (3 weeks following initial αCD20 injection), mice received another dose of αCD20. Mice in the group "AAV8-F8" received only the AAV8-hF8 vector (but not αCD20). Ten weeks following vector administration, all mice were challenged with weekly i.v. infusions of 1 IU hFVIII per mouse for 4 weeks (which reliably results in inhibitor formation in both strains). In case of BALB/c-HA mice, another 1-month

challenge with weekly hFVIII injections was performed starting 22 weeks after gene transfer, and blood was collected within 15 min after the fourth hFVIII injection.

B Cell Depletion with αCD20

B cell depletion in lymphoid organs was assessed 1 day and 7 weeks following the second αCD20 injection. CD19+ lymphocytes were largely absent from all sites investigated one day after the first or second αCD20 dose (Fig. 3-2 and data not shown for 1d post first injection). At 7 weeks after the second αCD20 dose (concurrent with initial hFVIII challenge), this B cell population had returned to normal levels in the spleen and lymph nodes and near normal levels in peripheral blood (Fig. 3-2)**.** Absolute T cell numbers, as measured by total CD3+ cells per 10,000 total cells in each tissue, were not reduced by treatment and were only significantly different from control mice in the lymph node where αCD20-treated mice had higher T cell numbers (Fig. 3-3). To further investigate immune competence at 7 weeks post last αCD20 injection (the time of hFVIII challenge) a separate set of mice were administered 10^{11}vp/mouse of adenovirus expressing the β-gal transgene. Following this adenoviral challenge, neither neutralizing antibodies against the adenovirus capsid nor antibodies against the transgene were reduced in αCD20-treated mice (Fig. 3-4). Considering the data in figures 3-2 to 3-4, mice were deemed to be immune competent at time of hFVIII challenge (7 weeks post-αCD20) in all subsequent experiments.

B Cell Depletion Concurrent with Gene Transfer Renders BL/6-129/sv-HA Mice Hyporesponsive to hFVIII

BL6-129/sv-HA showed modest correction of clotting times below that of untreated mice but this correction was minimal (≤1% of normal FVIII activity) regardless of αCD20 treatment (Fig. 3-4 A). Without further manipulation, this level of correction was

sustained for at least 4 months. However, those mice that were challenged with supplementary hFVIII therapy lost correction concurrent with the development of inhibitors (Fig. 3-4 C). “Control” age-matched mice (no gene transfer) receiving identical hFVIII challenge had an average Bethesda titer of 154±44 BU, whereas mice receiving AAV8-hF8 had a higher average titer of 336±88 ng/mL, suggesting that AAV-hF8 treatment primed the mice against hFVIII. However, transient B cell depletion with αCD20 at the time of AAV8-hF8 gene transfer resulted in subsequent hypo-responsiveness to hFVIII with a significantly lower Bethesda titer (22±11 BU). These titers were 15 times lower than in mice receiving AAV8-hF8 without αCD20 and 7-fold lower than mice receiving only protein challenge. These results were also reflected in total circulating anti-hFVIII IgG1 as determined by ELISA (Fig. 3-4 B). Mice in the “αCD20 + AAV8-F8” group had a mean IgG1 titer of 1609±868 ng/mL, which was significantly lower than both control and AAV8-hF8 mice (which had titers of 5988±1520 ng/mL and 7001±867 ng/mL, respectively).

Gene Transfer Renders BALB/c-HA Mice Hyporesponsive to hFVIII regardless of B Cell Depletion

The above experiments were repeated in hemophilic mice carrying the same mutation on a BALB/c background. All BALB/c-HA mice receiving AAV8-hF8 had ~1% FVIII activity, which was again sustained in the absence of further manipulation (Fig 3-5A). Coagulation times of mice challenged with hFVIII increased to or near the uncorrected range. However, 8/9 mice in the “AAV8-F8 + αCD20” group and 5/6 mice in the “AAV8-F8” group had at most low-titer inhibitors (<5 BU), and undetectable anti-hFVIII IgG1 (Fig. 3-5 B and C). This was in contrast to control mice (no gene transfer or αCD20), which developed significantly higher anti-hFVIII IgG1 and significantly higher

inhibitor titers (11-fold and 4-fold higher BU than “AAV-F8” and “AAV8-F8 + αCD20” mice, respectively) (Fig. 3-5B and C). Another control group, “αCD20” mice, had received the same doses of αCD20 (but no gene transfer) followed by hFVIII challenge (following identical time lines). These mice developed similar Bethesda titers as the other control mice (Fig 3-5B), albeit anti-FVIII IgG1 titers were somewhat lower (1067±297 ng/mL for “αCD20” vs. 2786±693 ng/mL for control; Fig. 3-5B).

We next wanted to determine if hypo-responsiveness would be maintained after another round of challenge, and if the supplementary exogenous hFVIII could correct the aPTT times of vector-treated mice. The lack of humoral immune response was maintained in all but one mouse in the “AAV8-F8” group as measured by hFVIII-specific IgG (data not shown). In naïve BALB/c-HA mice, injection of 1 IU hFVIII resulted in correction of the aPTT to 49±2 sec at a 15-min time point (Fig. 3-5D). Mice in “AAV8-F8” and “AAV8-F8 + αCD20” groups also showed correction with average clotting times of 58±3 sec and 60±4 sec, respectively. While this level of correction was not quite as good as for naïve mice, the difference did not reach statistical significance. No or marginal correction was achieved in control mice that had formed high-titer inhibitors (Fig. 3-5D). In summary, we conclude that treatment of BALB/c-HA mice with AAV8-hFVIII with or without B cell depletion confers a level of long-term tolerance to hFVIII that protects from high-titer inhibitors in subsequent protein therapy, thereby allowing for correction of coagulation with exogenous clotting factor.

T Cell Tolerance to FVIII in BALB/c-HA Mice

Inhibitor formation is dependent on T help and may be controlled by Treg. The BALB/c-HA strain was chosen for analyses of T cell responses to FVIII because this strain responded better to tolerance induction and, representing a pure strain

background, could be used for adoptive transfer studies. Quantitative RT-PCR on *in vitro* hFVIII-stimulated CD4+ splenocytes was performed to test for induction of T cell tolerance. Responses in mice previously treated with AAV8-hFVIII + αCD20, AAV8-hFVIII only, or control mice (that had received protein challenge only) were measured after the last round of challenge with hFVIII protein had been completed. Bulk splenocytes were stimulated *in vitro* with hFVIII and subsequently sorted based on CD4 expression. RNA from CD4+ T cells was used in qRT-PCR to analyze the transcription of 9 different genes related to T cell immunity. CD4+ cells from control mice, which had developed high-titer inhibitors, showed increased transcription of a mix of Th1, Th2 and suppressive cytokine/marker genes including IL-2, IL-4, IL-13, IFN-γ, FoxP3, CTLA-4, and IL-10, and more modestly of TGF-β (Fig. 3-6A). Mice receiving AAV8-hFVIII alone showed a substantial reduction in their IL-2 and IL-10 responses and a partial reduction in IL-4 and IL-13 gene expression, while IFN-γ and also Treg markers remained similarly up-regulated as in controls. The “AAV8-F8 + CD20” group showed a broad T cell unresponsiveness. Only IL-10 and CTLA-4 were marginally induced in response to FVIII.

The potential role of Treg in tolerance was further investigated by adoptive transfer of CD4+CD25+ cells harvested from “AAV8-F8 + αCD20” or “AAV8-F8” mice after the first challenge. Adoptive transfer of cells from either group only modestly suppressed anti-hFVIII formation in recipient mice (which did not reach statistical significance, Fig. 3-6B).

hFVIII protein administration during recovery of B cells fails to achieve lasting tolerance

Next we investigated whether B cell depletion could be combined with hFVIII protein administration to induce tolerance to hFVIII. Mice from each strain were given αCD20 as before at week 0 and 3. Starting at week 7 (4 weeks past the last αCD20 dose), when B cells were still recovering, mice were given 4 weekly IV doses of 1 IU of hFVIII. Mice were bled 2 weeks later to test for antibody formation. Initially, mice treated with αCD20 were either non-responsive or hyporesponsive to challenge. Only 1 of 8 BL/6-129/sv-HA mice had detectable anti-FVIII antibodies (513 ng/mL; 3 BU), whereas 3/7 BALB/c-HA mice had detectable (albeit low-titer) inhibitors (Fig. 3-7 A and B). As expected, control mice (no αCD20 treatment) developed high-titer inhibitors. The hFVIII treatment protocol was repeated at weeks 14-17 and 21-24. After the second challenge, only 2 of the BL/6-129/sv-HA mice remained unresponsive, whereas all other mice developed an antibody response similar to controls after the first challenge. As expected, control mice showed further increases in both IgG and Bethesda titers. By the third challenge, all mice treated with αCD20 had developed a humoral immune response to hFVIII similar in magnitude to the controls after two challenges (Fig. 3-7 A and B). Therefore, transient B cell depletion merely delayed the antibody response to hFVIII protein.

Interestingly, when comparing the magnitude of both IgG1 titers and Bethesda titers for mice in these experiments, the two strains show several differences. First, BL/6-129/sv-HA mice have higher anti-hFVIII IgG1 and Bethesda titers (Figs. 3-4 B and C; 3-5 B and C). Furthermore, IgG1 and Bethesda titers after one 4-week challenge only positively correlate in BL/6-129/sv-HA mice (Fig. 5-7A). BALB/c-HA mice eventually

do form a positive correlation but only when data from multiple rounds of challenge are included—again highlighting the lack of a robust Th2/antibody response of this strain to hFVIII challenge and perhaps explaining the greater permissiveness to tolerization (Table 3-2 and Discussion).

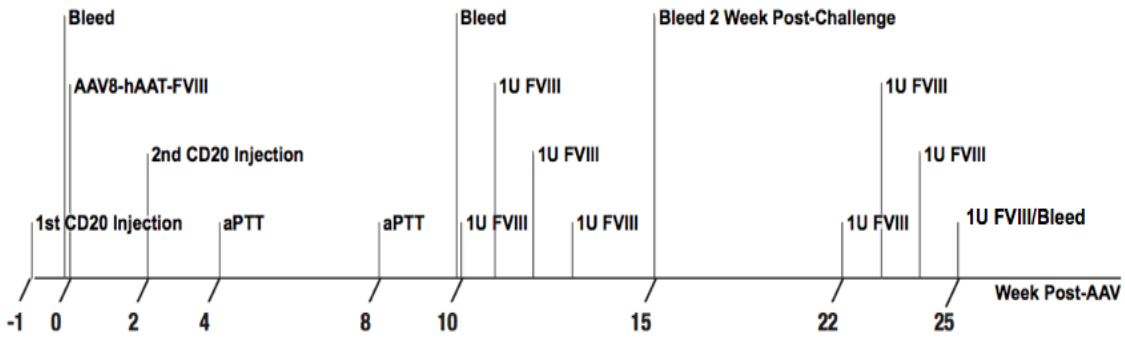

Figure 3-1. B cell depletion and gene therapy protocol. Hemophilia A mice on either a BL/6-129/sv or BALB/c background were administered 10mg/kg anti-mCD20 IgG2a 1 day prior to receiving 10^{11}vg/ms AAV8 expressing hFVIII under the liver-specific hAAT promoter (AAV8-hAAT-FVIII). Mice were bled at indicated time points to assess B cell recovery and to monitor clotting times (via aPTT). At week 10, mice were “challenged” with 1U hFVIII i.v. once per week for 4 weeks. Two weeks following challenge, mice were bled again to monitor phenotypic correction as well as immune responses to hFVIII. A subset of BALB/c mice were again challenged as before starting at week 22 with the exception that mice were bled 10-15 minutes immediately after the last challenge to monitor clotting correction with exogenous hFVIII.

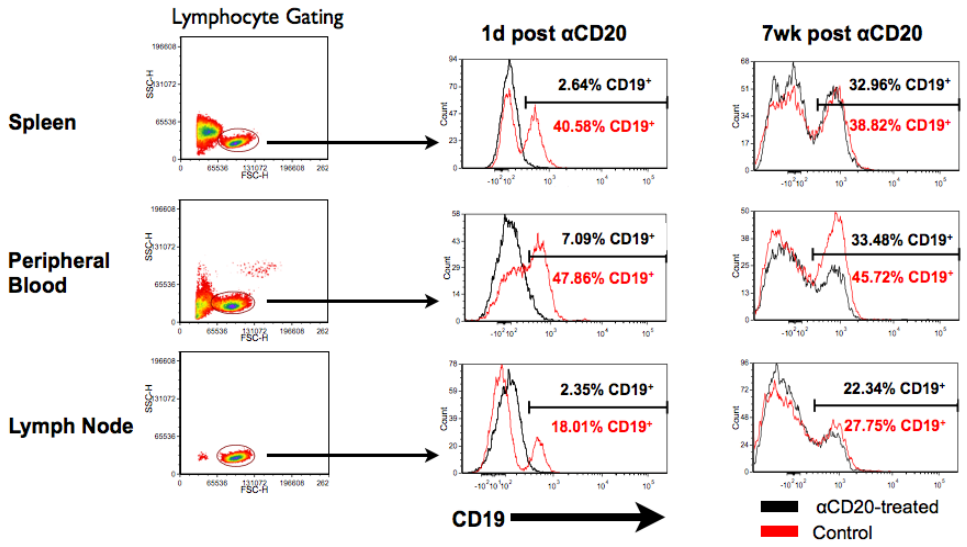

Figure 3-2. B cell depletion following αcd20 administration. Mice were i.v. injected with 10mg/kg αCD20 IgG2a 3 weeks apart and sacrificed at indicated time points for flow cytometry analysis of B cell populations in the spleen, lymph node and peripheral blood. Left panel shows the gating strategy for live lymphocytes based on forward and side scatter while the middle and right panels show percent $CD19^+$ lymphocytes for each organ at indicated time points following αCD20 administration. Each plot is a representative image of three mice in each group.

Table 3-1. $CD3^+$ T cells following B cell depletion.

Tissue	$CD3^+$/10,000 cells Control	$CD3^+$/10,000 cells CD20
Spleen	2616± 119	3556± 878
Lymph Node	6703± 39	8751± 270*
Peripheral Blood	2089± 844	1784± 312

T cells populations were determined in indicated organs 1 day following a second injection of αCD20 in BALB/c-HA mice. T cells were determined by $CD3^+$ lymphocytes (lymphocytes determined by FSC/SSC gate as in Fig. 4-2) per 10,000 total cells. Means with standard deviation are shown for each subset. * indicates $p<0.05$ vs. control as determined by t-test.

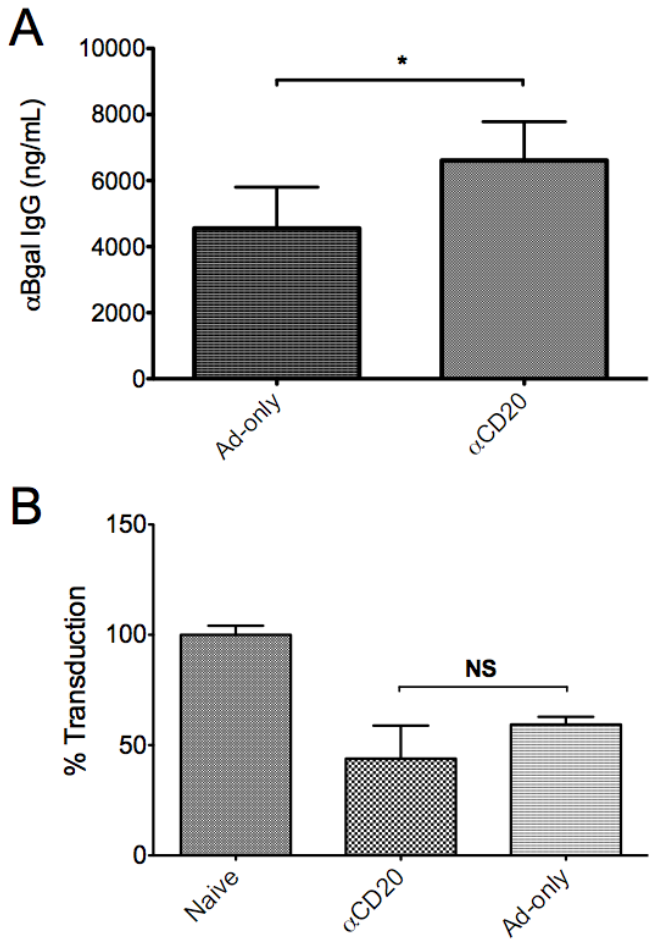

Figure 3-3. Immune response to Ad-LacZ in B cell depleted mice. Seven weeks following a second αCD20 injection according to the schedule in Fig. 4-1 mice were given 10^{11}vp/ms of Ad-LacZ. Four weeks after challenge, mice were bled to assess antibodies against the β-gal transgene via ELISA in (A) and development of neutralizing antibodies in (B**).** For (B), mouse plasma from naïve untreated mice ("Naïve"), αCD20-treated mice also receiving Ad-LacZ ("αCD20") or naïve-challenged mice that did not receive αCD20 but were challenged with Ad-LacZ ("Ad-LacZ") was diluted 1:16 and incubated with Ad-GFP at 37°C for 1 hour prior to infection of HEK-293 cells. After 24 hours incubation, cells were analyzed for GFP expression by flow cytometry and percent transduction was determined by comparison to incubation with naïve mouse plasma. Data shown is mean ±SD. Comparison of means of "Control" to "αCD20" groups was conducted by t-test where * indicates p-value <0.05. NS = not significant.

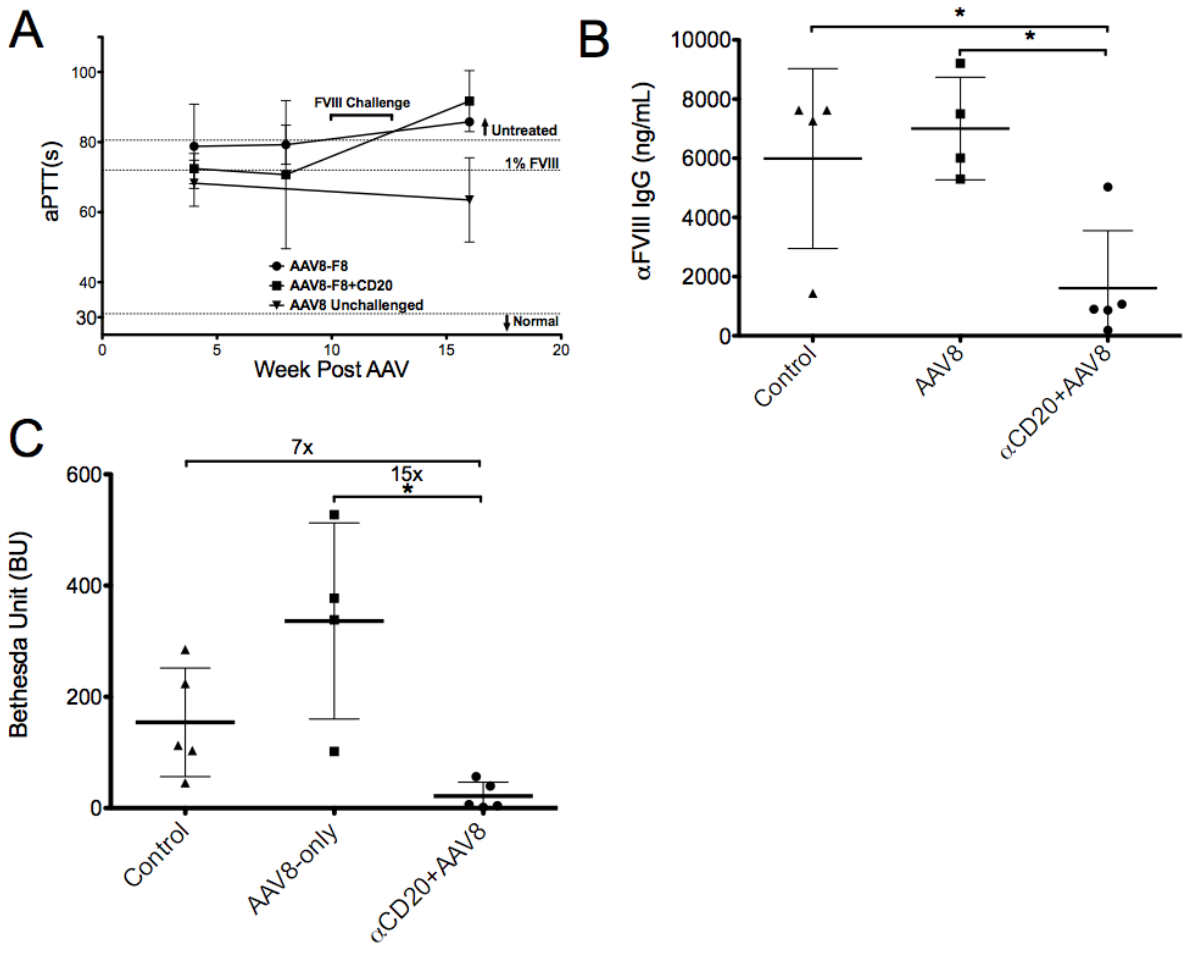

Figure 3-4. B cell depletion and gene transfer in BL/6-129/sv-HA mice. BL/6-129/sv-HA mice were treated as described and illustrated in Fig. 4-1. (A) Correction of clotting times as measured by aPTT. Dotted lines represent expected clotting times of an untreated HA mouse, a mouse with 1% hFVIII activity and of a normal mouse. (B) FVIII-specific IgG1 2 weeks following challenge with hFVIII as measured by ELISA. (C) Inhibitors of hFVIII as measured by Bethesda assay 2 weeks after challenge. * indicates p-value of <0.05 following ANOVA analysis with Tukey post-hoc analysis. Data points in A are mean ±SD. Horizontal bars in B-C indicate mean ±SD.

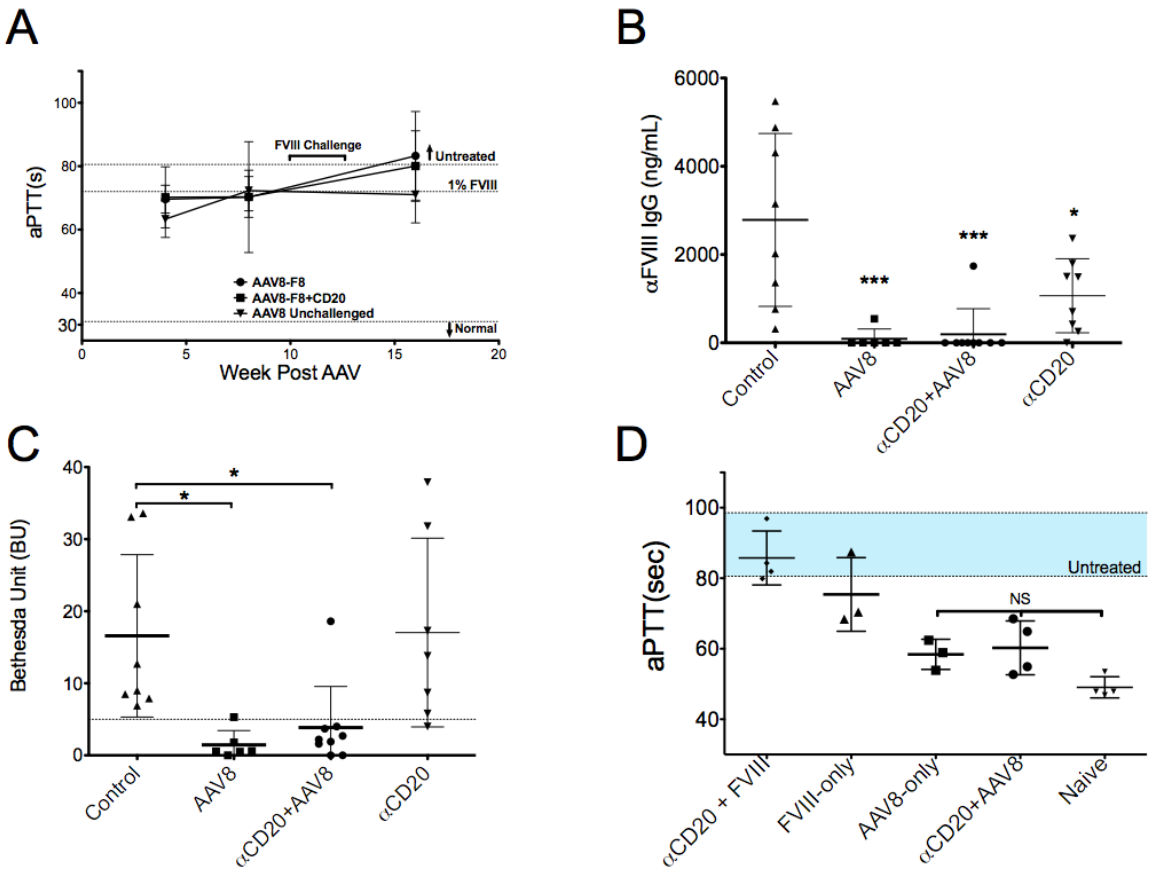

Figure 3-5. B cell depletion and gene transfer in BALB/c-HA mice. BALB/c-HA mice were treated as described and illustrated in Fig. 4-1. (A) Correction of clotting times as measured by aPTT. Dotted lines represent expected clotting times of an untreated HA mouse, a mouse with 1% hFVIII activity and of a normal mouse. (B) FVIII-specific IgG1 2 weeks following challenge with hFVIII as measured by ELISA. (C) Inhibitors of hFVIII as measured by Bethesda assay 2 weeks after challenge. (D) Mice were again challenged on the same 4 week course. However, on the fourth and final injection, mice were bled 10-15 minutes after hFVIII infusion and this plasma was used in aPTT to determine the level of correction induced by the infused hFVIII. * indicates p-value of <0.05 following ANOVA analysis with Tukey post-hoc analysis. Data points in A are mean ±SD. Horizontal bars in B-D indicate mean ±SD.

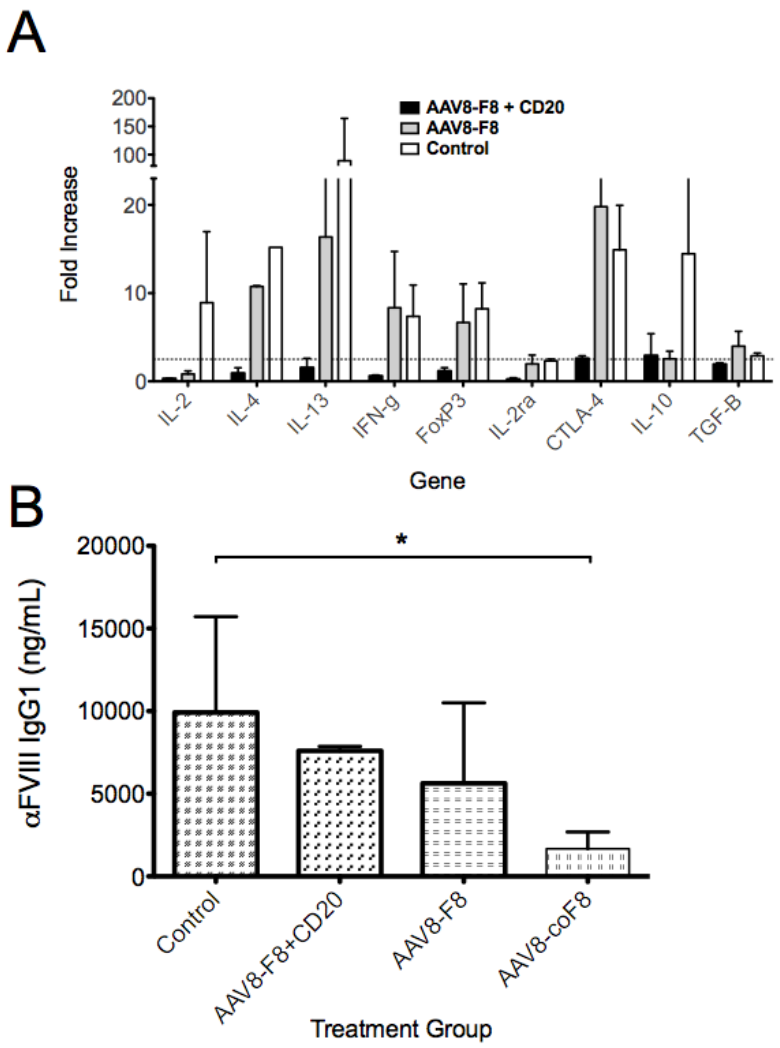

Figure 3-6. T Cell Responses in BALB/c-HA mice. (A) Following the second round of challenge, BALB/c-HA mice were sacrificed and splenocytes stimulated *in vitro* with 5μg/mL of hFVIII for 48h. Following stimulation, cells were purified for $CD3^{+}CD4^{+}$ T cells by FACS and RNA was immediately extracted for use in qRT-PCR to determine fold increase in transcription of several genes. (B) Following one round of challenge according to the schedule in Fig. 4-1, mice from indicated treatment groups were sacrificed and $CD4^{+}CD25^{+}$ splenocytes were purified, pooled by treatment group and adoptively transferred to naïve BALB/c-HA mice. One day after transfer, mice were challenged with 1U hFVIII in adjuvant subcutaneously. 5 weeks following s.c. challenge plasma antibody titers were measured by ELISA. * indicates $p<0.05$ as measured by Student's T-test. Bars indicate mean ±SD.

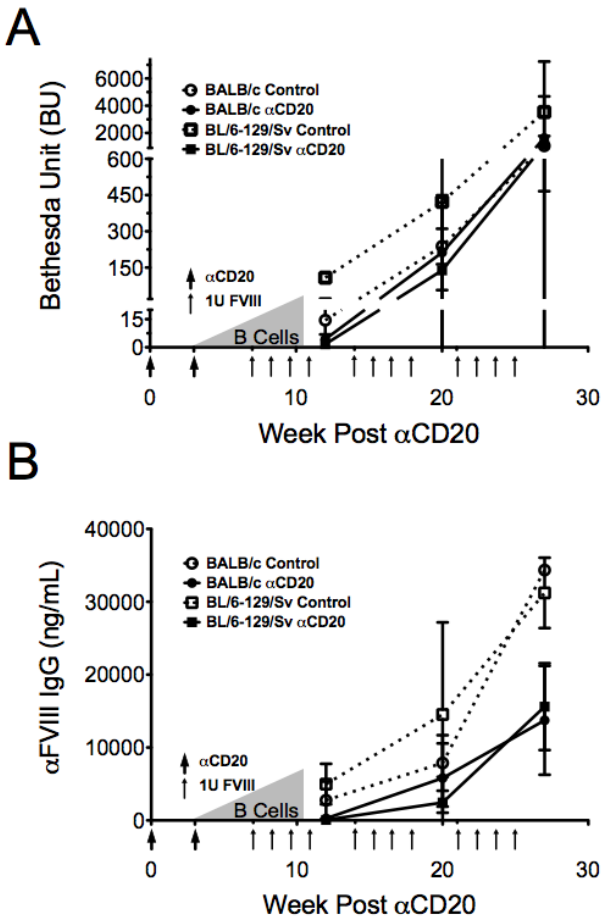

Figure 3-7. Anti-CD20 treatment to prevent antibody formation in hFVIII protein replacement therapy. BL/6-129/sv-HA and BALB/c-HA mice were treated with αCD20 antibody as outlined in Fig. 4-1 (indicated with large arrows) followed by 4 weeks of hFVIII challenge (indicated by small arrows) beginning at 4 weeks after the second αCD20 administration. Mice were treated with hFVIII twice more following the same schedule. Antibody formation against hFVIIII was measured by Bethesda assay (A) and anti-hFVIII IgG1 ELISA (B) two weeks after each 4-week challenge. Control mice did not receive αCD20. Gray triangles represent B cell recovery. Data are mean ± SD for n= 3-5/group.

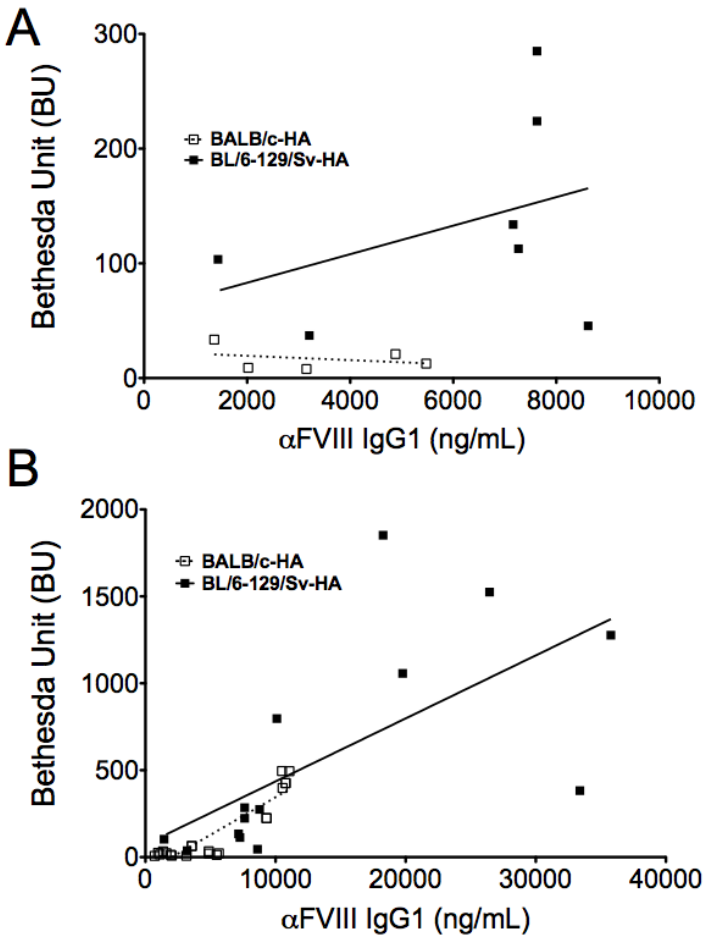

Figure 3-8. Correlation between anti-FVIII IgG1 and Bethesda in BALB/c-HA and BL/6-129sv-HA mice. Mice were challenged with standard 1U hFVIII/ms/week *i.v.* for 4 weeks and bled 2 weeks following the fourth challenge. (A) Correlation between hFVIII IgG1 titers as measured by ELISA and Bethesda titers after only 1 round of hFVIII challenge with regression line shown for each strain. (B) Correlation between hFVIII IgG1 and Bethesda titer including data from one to three challenges.

Table 3-2. Comparison of immune response to hFVIII in BALB/c and BL/6-129/sv hemophilia A mice.

Cytokine/Marker	BL/6-129/sv	BALB/c
IL-2	++	+
IFN-γ	-	+
IL-4	++	++
IL-6	++	-
IL-10	+/-	++
IL-13	-	++
IL-17	-	-
TGF-β1	-	+/-
FoxP3	-	+
Il2ra (CD25)	-	+/-
CTLA-4	-	++

Data from figure 3-6A for BALB/c-HA mice and previously published data for BL/6-129/sv mice from Moghimi, et al. [66] comparing cytokine and surface marker upregulation as measured by quantitative RT-PCR following *in vivo* hFVIII challenge and *in vitro* restimulation of splenocytes.

CHAPTER 4
CODON OPTIMIZATION OF HUMAN FVIII TRANSGENE IMPROVES EXPRESSION AND INDUCTION OF TOLERANCE IN HEMOPHILIA A MICE REGARDLESS OF STRAIN BACKGROUND

Background

Our previous experiments with AAV8 expressing a wild-type FVIII cDNA demonstrated that immunological tolerance or hyporesponsiveness can be achieved depending on strain background and concurrent administration of the B cell-depleting αCD20 antibody. However, this was always in the context of minimal (1% or less of normal FVIII activity), transient correction that did not withstand intravenous FVIII challenge. Any FVIII activity below 10% would necessitate on-demand infusion with FVIII in the case of trauma or surgery. Thus, even if the levels of correction seen in our previous studies were translated to human trials, the lack of tolerance seen in BL/6-129/sv-HA mice would be unacceptable—as the development of inhibitors would not only render the gene therapy benefits moot but would also prevent treatment with exogenous FVIII. Prior work in our lab has shown a correlation with increased levels of antigen expression and increased tolerance induction following liver-directed gene therapy with AAV vectors [67]. Wild-type hFVIII has notoriously low expression which has hampered clinical trials for hemophilia A regardless of gene delivery platform [68,69]. The barriers to expression seem to be at translation and secretion steps of the protein as mRNA levels do not correlate well with FVIII antigen levels *in vivo* [37]. Several attempts have been made to improve expression for gene therapy purposes including directed engineering of the hFVIII cDNA to more closely resemble porcine FVIII which secretes at a much higher rate [70,71]. While these modifications have yielded drastic improvements in the expression of FVIII, the use of porcine FVIII which differs in amino acids from wild

type human FVIII may be less desirable due to the uncertain immunological consequences of delivering a partially novel antigen in the case of mild or moderate hemophiliacs.

Thus a strategy to improve transgene expression which does not alter the amino acid sequence of the protein would be ideal. One such strategy is codon optimization of the transgene which may increase expression of the transgene at the translational level. The process of codon optimization is based on the premise that different species will have preferences for certain codons for the same amino acid, known as “codon-usage bias”. Thus, codon optimization alters the cDNA to incorporate those codons most likely to be used in a given species thereby improving the rate at which a protein is translated [72]. Furthermore, the cDNA can be further optimized to remove cryptic splice sites, reduce secondary structure of the mRNA and optimize GC content all in hopes of increasing the efficiency of gene expression while preserving the amino acid sequence of the original cDNA [72,73]. This strategy has been successful for multiple proteins utilized across various gene delivery platforms including a recent report where codon-optimization of hFVIII improved expression from a lentiviral vector [54,73-78].

An alternative approach is to increase the number of transcriptionally active vector genomes following gene transfer. Our AAV8-hFVIII constructs carry a single-stranded genome, which must be converted to double-stranded DNA before transcription in a process called “second strand synthesis”. The Srivastava lab has previously shown that this is a rate limiting step and can be overcome by removing a DNA-binding protein that binds to the single-stranded genome and prevents second strand synthesis [79]. Removal of this protein can be facilitated by dephosphorylation of FKBP52 via protein

phosphatase 5 (PP5). This PP5 protein can be provided by co-administering a “helper” AAV virus carrying a double-stranded (or “self-complementary”, sc) transgene for PP5 which will be transcribed and translated immediately (Fig. 4-1). This strategy has been shown to increase expression of GFP but it’s utility for secreted, therapeutic transgene is unknown. Thus, we investigated whether use of a helper virus containing sc-PP5 transgene or a codon-optimized hFVIII transgene could improve expression from our AAV8 vector and if this would impact tolerance induction to the transgene.

Codon optimization improves hFVIII expression from AAV8 and yields long term correction of phenotype at therapeutic levels

We tested an AAV8 vector containing codon-optimized hFVIII (AAV8-COhFVIII) under the same liver-specific promoter as our vector containing the wild-type hFVIII transgene (AAV8-hFVIII). Doses of this vector identical to those used with AAV8-hFVIII resulted in drastic improvements of clotting times in both strains, with average aPTTs declining from 78±8 sec to 37±2 sec in the BL/6-129/sv-HA mice and from 90±8 sec to 43±2 sec in BALB/c-HA mice after 4 weeks (Fig. 4-2A). Clotting times generally rose over the following 4 weeks but then stabilized. Means of 53±4 sec in BL/6-129/sv-HA and 61±6 sec for BALB/c-HA mice were measured at 18 weeks. FVIII activity was then measured with one of two functional assays. First by chromogenic assay, which indicated that FVIII activity peaked at 12.7±5.1% stabilizing to 0.8±1.7% for BL/6-129/sv-HA mice and 16.6±2% stabilizing to 0.7±1.4% in BALB/c-HA mice (Fig. 4-2B). Alternatively, a standard curve was generated by diluting hFVIII in naïve hemophilia A mouse plasma and measuring the aPTT, a method which we found to be more sensitive and precise. With this method peak hFVIII activities were 10.4±6% and 8±1.6% in BL/6-

129/sv-HA and BALB/c-HA mice, respectively, with a decline to 3.8±2.8% and 2.1±2.5% (Fig. 4-2C).

Immunological tolerance to hFVIII following AAV8-COhFVIII gene transfer

Factor VIII activity in this range would result in a vast improvement in quality of life for an individual with severe hemophilia A but again would necessitate occasional infusion of hFVIII. Thus it was essential to investigate tolerance to *i.v.* hFVIII following AAV8-COhFVIII gene therapy. Following challenge with hFVIII protein, 3 of 4 mice in the BL/6-129/sv showed no antibody response to hFVIII with the other having a muted IgG1 titer of 603 ng/mL compared with an average of 5988±3040 ng/mL in control animals challenged in the same manner (Fig. 4-3A and 3-4B). This animal also had a low Bethesda titer of 1.4 BU (Fig. 4-3B), resulting in a longer aPTT (64.9 sec) compared to the other mice at 18 weeks post vector injection. In BALB/c-HA mice, no anti-hFVIII antibodies were detected in 2/4 mice, while the other 2 mice had very low but detectable antibody titers after challenge (830 and 443 ng IgG1 anti-hFVIII/mL, corresponding to 1.4 and 0.6 BU, Fig. 4-3A and B). Average anti-FVIII titers of the control group were 2785±693 ng/mL or 17±5 BU (Fig 3-5 B and C). In summary, a highly significant 9 to10-fold reduction in antibody response to hFVIII protein was achieved with the AAV8-COhFVIII construct including a complete lack of response to hFVIII protein in 5/8 mice.

Adoptive transfer of CD4+CD25+ Treg was also performed as above with tolerant AAV8-COhFVIII-treated BALB/c-HA mice serving as donors. Recipient mice showed a significantly lower antibody response to FVIII compared to mice receiving CD4+CD25+ T cells from naïve mice (Fig. 3-6B). Therefore, the use of COhFVIII induced a more potent Treg response than wild-type cDNA consistent with our previous studies that suggest a positive correlation between expression levels and tolerance induction.

Co-administration of an AAV helper virus encoding a sc-PP5 transgene under a liver-specific promoter improves hFIX expression but does not effect expression of COhFVIII

Given the relative insensitivities of our FVIII activity assays, especially near or below 1%, we first wanted to test the helper virus strategy with a hFIX vector since hFIX can be detected in wild-type C57BL/6 mice with a quantitative ELISA. We used an AAV2 vector carrying a single-stranded hFIX transgene under the same liver-specific promoter as our hFVIII vectors and delivered this *i.v.* at a dose of 10^{10}vg/ms or $5x10^{10}$vg/ms with or without 10^{10}vg/ms of a scAAV8-PP5 vector expressing PP5 under the liver-specific transthyretin (TTR) promoter. At a dose of 10^{10}vg/ms of scAAV2-hFIX, administration of a helper virus was able to increase expression of hFIX from sub-therapeutic to therapeutic levels (216 ± 22 vs. 30 ± 11 ng/mL, 4–5% vs. <1% of normal human FIX levels) (Fig. 4-4A). This enhancement was similar even when helper virus at 10^{9}vg/ms was used (Fig. 4-4A), resulting in systemic hFIX expression of 300 ± 90 ng/mL (~6% of normal) by week 8. A 5-fold higher dose of ssAAV2-F.IX vector alone was required to achieve similar therapeutic levels. However, at this higher dose the helper virus only marginally increased FIX levels (Fig. 4-4A).

To investigate whether this enhancement was caused by an increase in hFIX-producing hepatocytes or by increased expression from a similar number of cells, liver tissue was collected at 10 weeks post injection from mice receiving a high or low dose of ssAAV2-hAAT-F.IX with or without 10^{10} vg of scAAV8-TTR-PP5, and immunofluorescence staining for hFIX was performed. The percentage of hFIX-positive hepatocytes in each group is shown in Fig. 4-4B along with representative images from each group. The results of fluorescence staining correlate with systemic hFIX expression in that the helper virus had the greatest effect in mice receiving the lower

dose of ssAAV2-FIX, bringing the percent positive hepatocytes from <1% without helper virus to 3–5% with the helper virus (Fig. 4-4B). The PP5-mediated increases in hFIX-expressing hepatocytes were less substantial for high-dose ssAAV2-hAAT-F.IX-treated mice (Fig. 4-4B).

Co-administration of scAAV8-TTR-PP5 helper virus does not augment AAV8-COhFVIII expression but does improve tolerance induction

We next wanted to determine if PP5 could be used to increase a sub-therapeutic dose of AAV8-COhFVIII to therapeutic levels. To do this, we delivered 10^{10}vg/mouse of AAV8-COhFVIII with or without 10^{10} scAAV8-TTR-PP5 to BALB/c-HA mice. Clotting times were approximately 1% FVIII activity for all mice and co-administration of helper virus failed to improve hFVIII expression at any time point as determined by aPTT (Fig. 4-5A). All mice returned to baseline at 39 weeks similar to the low-expressing AAV8-hFVIII vector-treated mice which failed to show long-term correction following hFVIII challenge. However, all mice given both AAV8-COhFVIII and scAAV8-TTR-PP5 had hFVIII-specific IgG1 titers that were below the detection limit of our ELISA two weeks following hFVIII challenge (Fig. 4-5B). On the other hand, 3/4 mice given AAV8-COhFVIII alone developed IgG1 antibodies against hFVIII following *i.v.* challenge ranging from 533-2850ng/mL (Fig. 4-5B). This is on the low end of what was observed in mice given 10-times more AAV8-hFVIII (which also had correction in the 1% range). All mice had Bethesda titers below 5 units but mice in the group treated with only AAV8-COhFVIII had the highest peak titer of 4.5BU. Thus, mice supplemented with the helper PP5 vector had a slightly better immune profile similar to mice treated with a log higher dose of AAV8-hFVIII but failed to show any improvement in phenotypic correction.

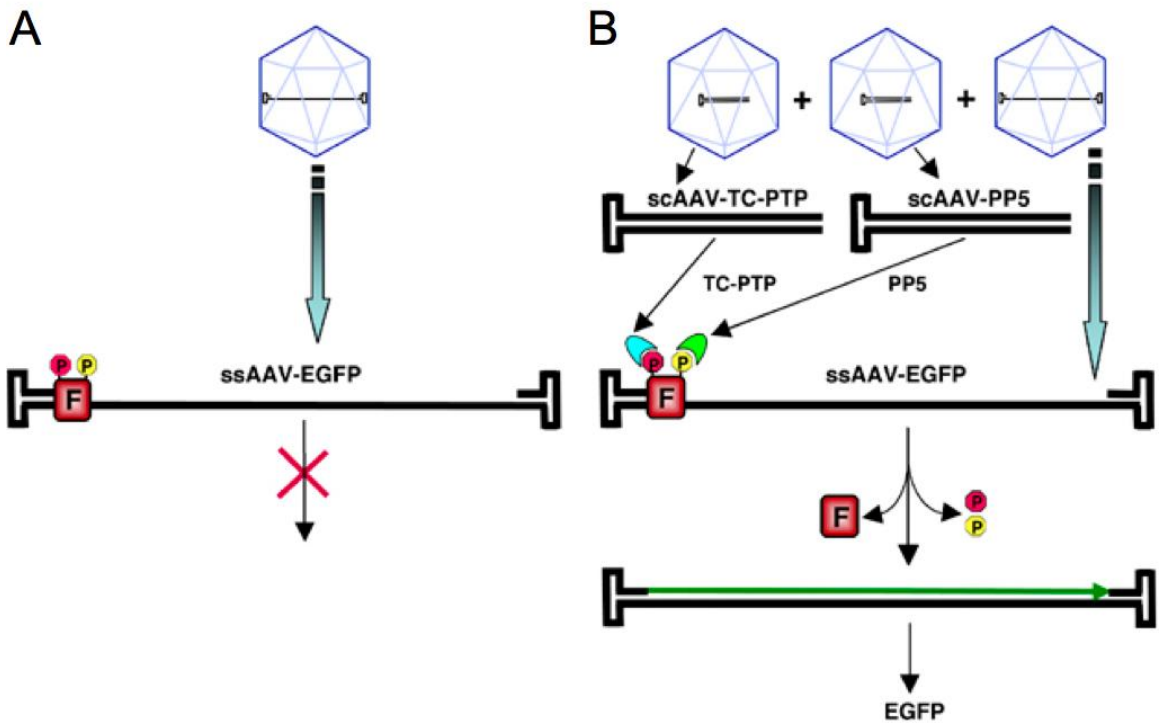

Figure 4-1. Improved expression with delivery of “helper” AAV virus. Second-strand synthesis of a single-stranded viral genome can be inhibited by binding of FKBP52 in (A). Co-administration of a double-stranded, self-complementary, vector can provide expression of one of two phosphatases capable of de-phosphorylating FKBP52 and causing its subsequent release from the single-stranded DNA in (B). Thus, second-strand synthesis may occur and facilitate expression of the double-stranded DNA. From Jayandharan, et al. [79].

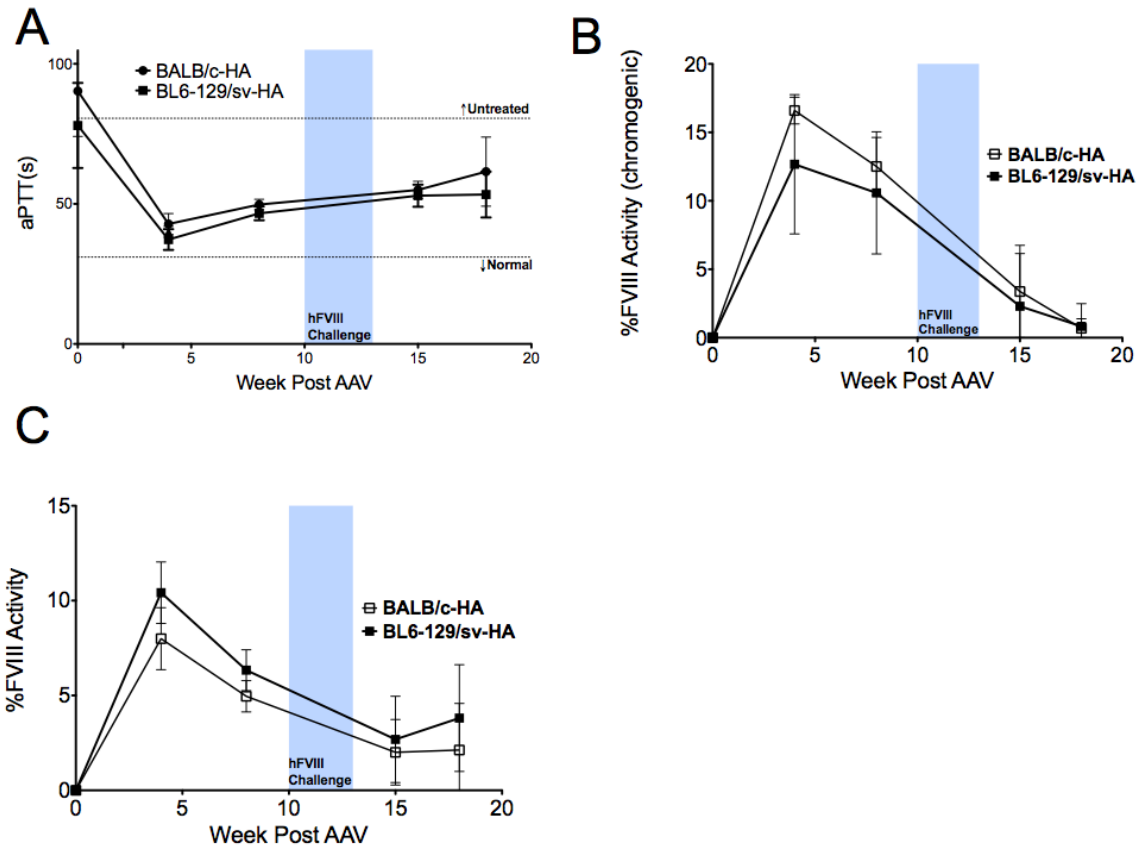

Figure 4-2. Expression of codon-optimized hFVIII. A dose of 10^{11}vg/ms of AAV8-COhFVIII was delivered *i.v.* to both BL/6-129/sv and BALB/c hemophilia A mice and plasma collected to determine phenotypic correction and FVIII activity. (A) Phenotypic correction of clotting deficiency as measured by aPTT. Dotted lines represent untreated hemophilia A mice and normal mouse clotting times while blue shaded area indicates period of hFVIII challenge with 1U hFVIII/mouse/week *i.v.* (B) FVIII activity as measured by chromogenic assay following delivery of AAV8-COhFVIII. (C) FVIII activity as measured by aPTT-based standard curve generated by diluting hFVIII in naïve hemophilia a mouse plasma. Data points are plotted as mean ±SD.

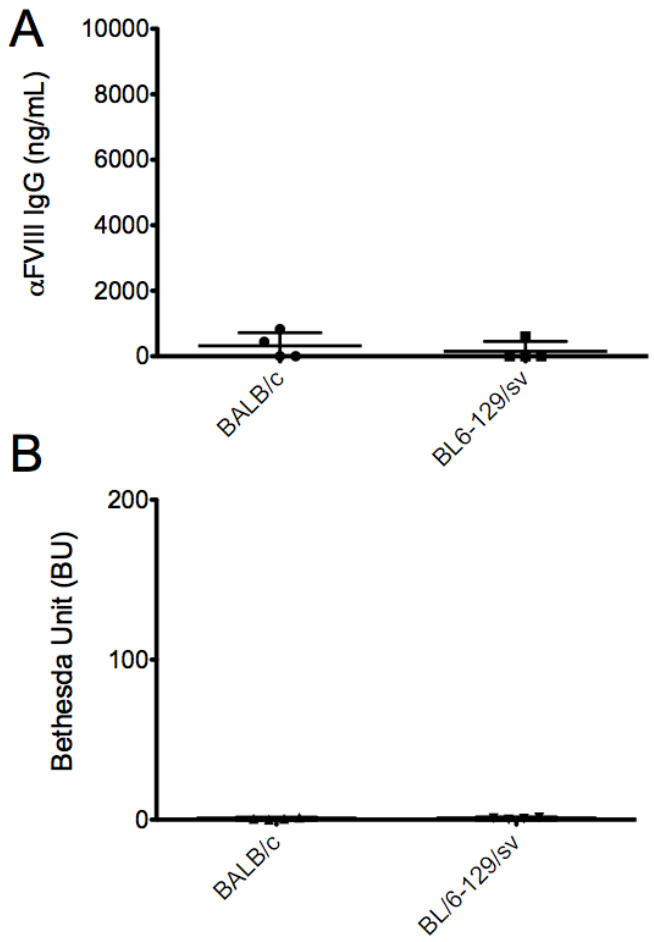

Figure 4-3. Immune responses following AAV8-COhFVIII gene transfer. Ten weeks following *i.v.* delivery of 10^{11}vg/mouse of AAV8-COhFVIII, mice were challenged with 1U hFVIII/mouse/week *i.v.* and immune responses to hFVIII measured by hFVIII-specific IgG1 ELISA in (A) and by Bethesda assay in (B). Both graphs are plotted with the same y-axis range as in Fig. 4-4 for comparison of scale. Bars indicate mean ±SD.

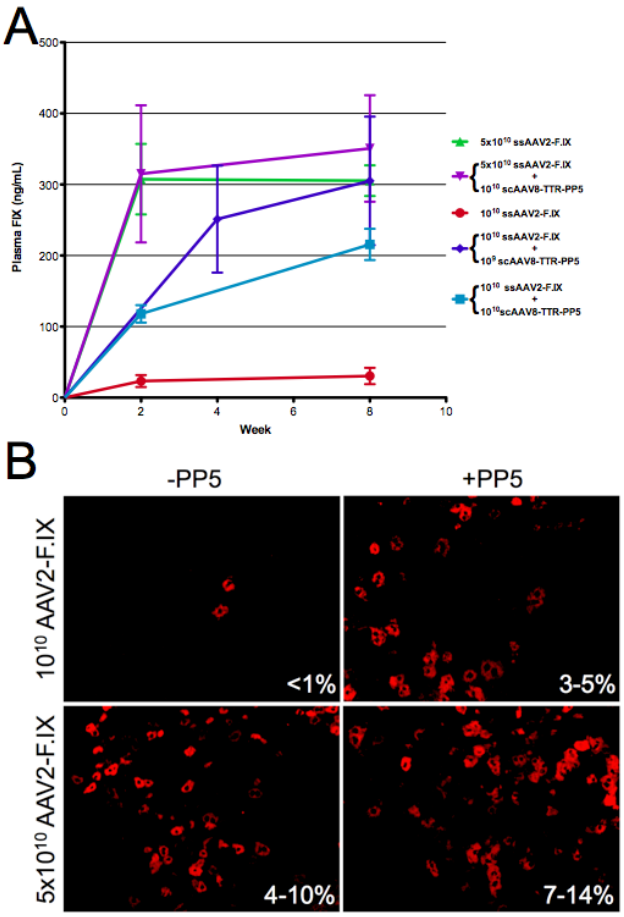

Figure 4-4. scAAV8-TTR-PP5 improves FIX expression at low doses. (A) Mice on C57/BL6 background were given indicated doses (vg/ms) of AAV2-hFIX with or without indicated doses scAAV8-TTR-PP5 intravenously. Plasma was monitored for hFIX concentration by ELISA and data points are plotted as mean ±SD. (B) Representative images from mouse livers at 10 weeks post injection. Livers were stained for hFIX-expressing cells and percentage of hFIX-positive cells was determined for multiple mice. Ranges of percent positive cells are indicated in lower right corner of each panel.

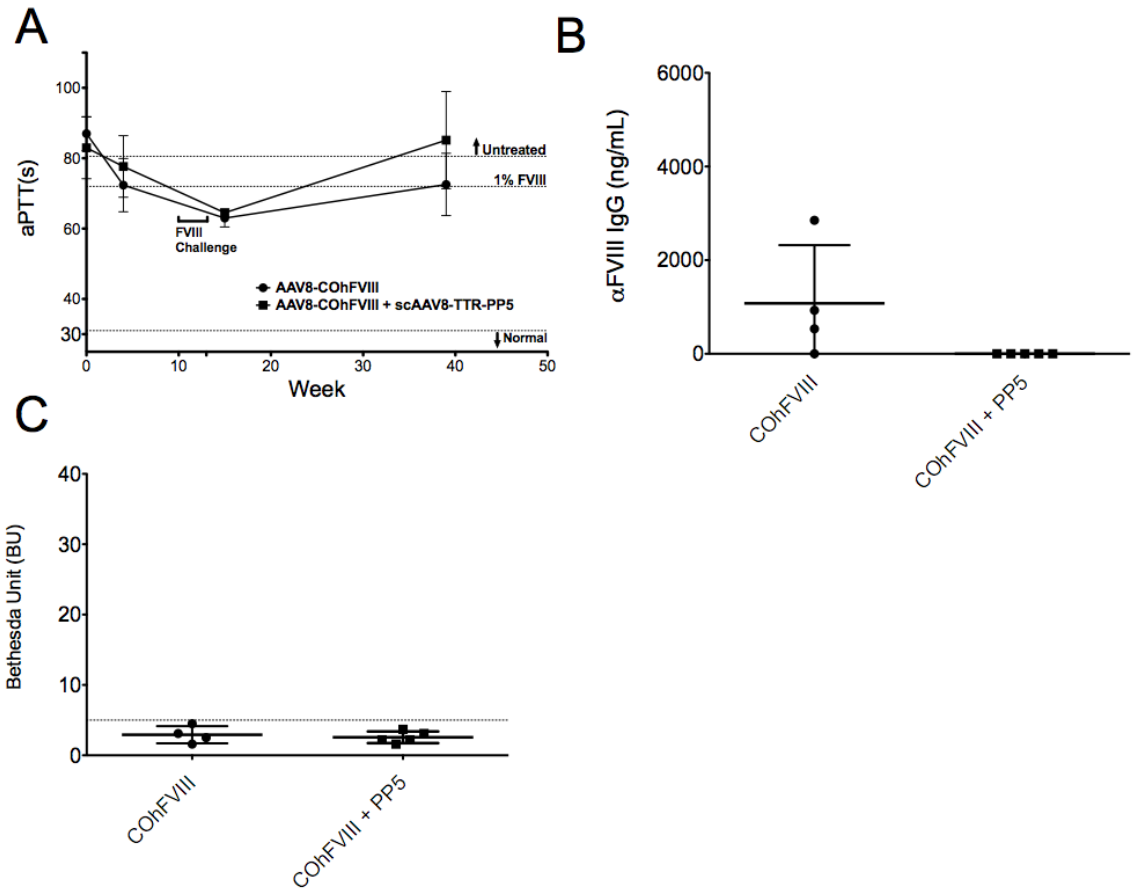

Figure 4-5. scAAV8-TTR-PP5 fails to improve AAV8-COhFVIII expression. BALB/c-HA mice were injected *i.v.* with 10^{10}vg/ms of AAV8-COhFVIII alone or in conjunction with 10^{10}vg/ms of scAAV8-TTR-PP5. (A) Mice were monitored for hFVIII expression by correction of clotting time as measured by aPTT. Two weeks following 4 week challenge of 1U hFVIII/ms/week mice were bled and checked for immune responses to hFVIII by measuring IgG1 antibodies to hFVIII by ELISA in (B) and inhibitors by Bethesda assay in (C).

CHAPTER 5
ALTERNATIVE STRATEGIES FOR B CELL DEPLETION COMBINED WITH GENE THERAPY

Background

While our data suggests a role for B cell depletion in gene therapy to correct hemophilia A and induce tolerance to hFVIII, there may be additional areas in the field of gene therapy where this specific immune suppression is helpful. For example, since AAV naturally infects humans there is a large majority of the population which possesses pre-existing immunity to the virus in the form of neutralizing antibodies against the capsid. In fact, worldwide the presence of neutralizing antibodies directed against AAV2—the serotype that naturally infects humans—ranges from 30-60% [80]. Neutralizing antibodies to the AAV capsid can limit or preclude treatment with AAV gene therapy vectors thus substantially reducing the pool of potential patients if measures to circumvent this problem are not found. Neutralizing antibodies against serotypes that do not normally infect humans, such as AAV5 or AAV8, are less prevalent although there is some cross-reactivity and antibodies against each are still found in a significant portion of the population [80]. However, AAV8 is able to bypass pre-existing immunity to AAV2 in non-human primate models and has been used in the most recent clinical trial for hemophilia B with success [36,81]. Still, administration of any AAV virus will inevitably lead to the development of neutralizing antibodies which will prevent future administration of the same vector should the patient require multiple treatments with the same vector or a vector with a different transgene but with the same AAV capsid [82,83]. Reduction of these neutralizing antibodies has been demonstrated in one study using non-human primates that had anti-AAV8 antibodies due to prior exposure to i.v. administered vector [84]. In this study, Mingozzi *et al.* used the B cell-depleting drug rituximab combined with

cyclosporine A to reduce low-titer anti-AAV antibodies enough to allow for successful re-administration of an AAV vector. However, this strategy was unsuccessful in another animal which had high-titer anti-AAV antibodies demonstrating the limited utility of this type of post-hoc immune suppression. Preventing the development of anti-vector antibodies by immune suppression at the time of AAV delivery may be more feasible. One such study found immune suppression with cyclosporine A and a non-depleting CD4 antibody spanning one day prior and one week after delivery of AAV8 was able to prevent the development of neutralizing antibodies to the viral capsid and allowed for re-administration with an AAV8 vector 100 days later [85]. Importantly, the second injection of AAV8 allowed for transgene expression indistinguishable from mice receiving the vector for the first time.

Transient immune suppression, in particular B cell depletion with rituximab, has also received attention from the hemophilia A community as an adjunct to traditional ITI. While rituximab alone fails to eradicate inhibitors in hemophilia A patients, the literature suggests a slight advantage for using rituximab during ITI especially in patients who have previously failed ITI alone, but failure rates remain fairly high [23,24,62,63]. Given that up to 30% of severe hemophilia A patients, the most likely candidates for gene therapy, have established inhibitors, it is prudent to determine if liver-directed gene therapy can reverse these inhibitors and if this can be augmented with immune suppression such as antibody-mediated B cell depletion.

Optimization of a CD20 protocol to prevent neutralizing antibodies against AAV8 capsid

First we wanted to determine if αCD20-mediated B cell depletion could be used as a sole agent in a protocol to prevent the development of AAV8 antibodies in our

hemophilia A mice with the ultimate goal of successful readministration. We have previously demonstrated that a single 10mg/kg dose of αCD20 can deplete B cells in spleen, lymph node and peripheral blood 24h post-injection. Therefore, we attempted to use a single dose 1 day before administering 10^{11}vg/mouse of AAV8-COhFVIII *i.v.* into hemophilia A mice and measured plasma concentrations of anti-AAV8 IgG2a antibodies 4 and 8 weeks post vector injection by ELISA (Fig. 5-1A). This single dose protocol failed to prevent or reduce antibody concentrations as mice given αCD20 and AAV8-COhFVIII had antibody titers (453 ±113ng/mL) not significantly different than control mice given only AAV8-COhFVIII vector at 316 ±267ng/mL (Fig. 5-1A and B). Antibodies at this level completely blocked transduction of an AAV8-hFIX vector given at the same dose *i.v.* whereas this dose gives high (mean of 2506ng/mL) expression in hemophilia A mice (Fig. 5-1D).

This may have failed to prevent antibody formation since B cell recovery begins at approximately 3 weeks post αCD20 injection, which may not be enough time for capsid antigen to be cleared. Thus we tried a similar protocol where αCD20 was again administered one day before and 2 weeks after administration of 10^{11}vg/ms of AAV8-COhFVIII (Fig. 5-2A). Again, this protocol failed to prevent antibody formation as titers were similar in both vector-only and αCD20-supplemented mice (Fig. 5-2B and C).

Administration of αCD20 24 hours prior to vector administration may not provide sufficient time for complete B cell depletion in all immune compartments. Therefore, we modified the protocol so that the first dose of αCD20 was given 1 week prior to vector as well as two weeks following AAV8-COhFVIII (Fig. 5-3-A). Using this protocol, antibody titers were significantly lower at 4 weeks post-vector in mice given αCD20 (223

±95ng/mL) compared to vector-only mice (410 ±47ng/mL, Fig. 5-3-B and C). However, at 8 weeks both groups had decreased titers and were not significantly different (means of 80 ±21ng/mL and 89 ±9ng/mL for αCD20 and vector-only groups, respectively).

Given the indication that this protocol could reduce anti-capsid antibodies, we next wanted to determine if a lower vector dose could allow for complete elimination of the antibody response and if this would allow for re-administration and "boosting" of phenotypic correction. Dosing and timing of αCD20 was the same as in Fig. 5-3 except with only $2.5x10^{10}$vg/ms of AAV8-COhFVIII delivered intravenously (Fig. 5-4A). With this lower dose, anti-AAV8 Ig2a antibodies were undetectable by ELISA at 4 and 8 weeks for αCD20-treated mice whereas vector-only mice had titers of 178 ±21ng/mL and 330 ±101ng/mL at 4 and 8 weeks, respectively (Fig. 5-4B and C). Clotting correction as measured by aPTT was modest, as expected at this lower dose, with clotting times reduced to 61 ±8s and 54 ±8s for αCD20 and vector only mice, respectively. We then delivered another dose of $2.5x10^{11}$vg/ms of AAV8-COhFVIII at week 10. This did not result in further reduction of clotting times (Fig. 5-4D) as αCD20 mice maintained clotting times of 64 ±5s and vector-only mice had an average clotting time of 60 ±9s at 4 weeks "post-boost". Several issues could have contributed to the lack of improvement in clotting times following secondary gene transfer. First, although antibody titers were below our limit of detection, there were still some OD values above zero in αCD20-treated mice even though these were comparable to pre-injection levels. Therefore, perhaps even the presence of minimal antibody levels are enough to neutralize the small dose of AAV used in this experiment. Alternatively, our aPTT assay may not be sensitive enough to detect the additional FVIII of the "boost" given that there is little

distinction between the aPTT clotting times between 1-5% FVIII activity (the range of correction seen at this dose). Thus, either a larger second dose or use of a transgene with a more sensitive assay (such as FIX) may be warranted.

Use of αCD20 and AAV8-gene therapy to reverse inhibitors in hemophilia A mice

Given that up to 30% of severe hemophiliacs—the patient population most likely to be candidates for gene therapy—will develop inhibitors, it is vital to determine the effects of gene therapy on a pre-existing antibody titer and if liver-directed gene transfer will be able to mitigate these inhibitors. In the presence of inhibitors, any FVIII produced by gene transfer will be neutralized and the therapy rendered null. To test this, we developed inhibitors in BALB/c-HA mice with weekly infusions of 1U hFVIII/ms/week as before. One week before gene transfer, mice were boosted with a single 2U dose of hFVIII (Fig. 5-5A). Mice were then either given 10^{11}vg/ms of AAV8-COhFVIII *i.v.* alone or 1 day after 10mg/kg of αCD20. Mice in the αCD20-treated group were given another dose of antibody 2 weeks after the first. Mice were monitored weekly for anti-hFVIII antibody titers via IgG1 ELISA and every 4 weeks by ELISA and Bethesda titer. In both groups, IgG1 titers decreased steadily over 4 weeks from an average of 5976± 4865ng/mL to 2060± 1644ng/mL in mice treated with only AAV8-COhFVIII and from 7305± 3657ng/mL to 1595± 794ng/mL in mice treated with both AAV8-COhFVIII and αCD20. Thus, after 4 weeks liver-directed gene therapy was able to reduce but no completely eliminate hFVIII antibodies in BALB/c-HA mice regardless of the use of αCD20. Given this initial data though, long-term follow-up is warranted.

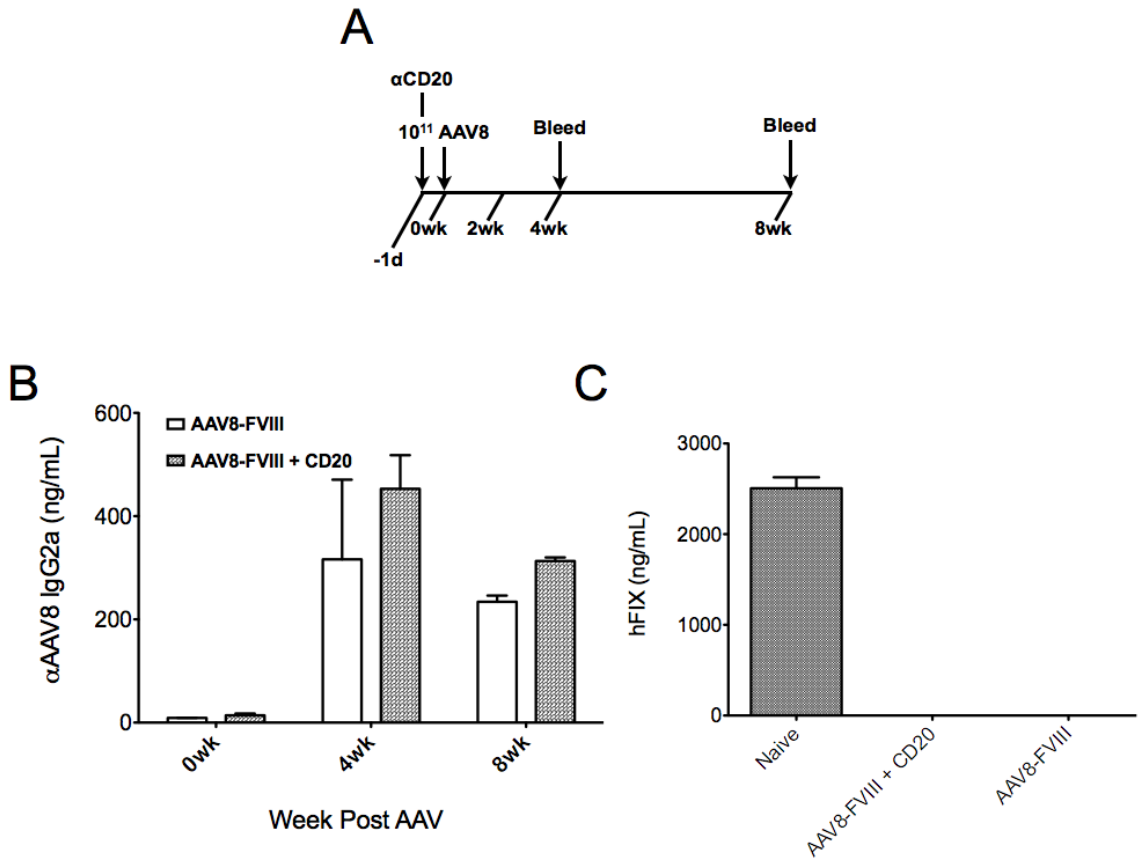

Figure 5-1. Single dose αCD20 B cell depletion does not prevent AAV8 antibodies. (A) Timeline of protocol using single 10mg/kg dose of αCD20 IgG2a 1 day before administration of 10^{11}vg/ms AAV8-COhFVIII. Mice were bled at 4 weeks and 8 weeks post vector injection and plasma analyzed for anti-AAV8 IgG2a antibodies by ELISA in (B). (C) Plasma hFIX levels as measured by ELISA 3 weeks following re-administration of an AAV8-hFIX vector (10^{11}vg/ms) at 10 weeks post AAV8-COhFVIII.

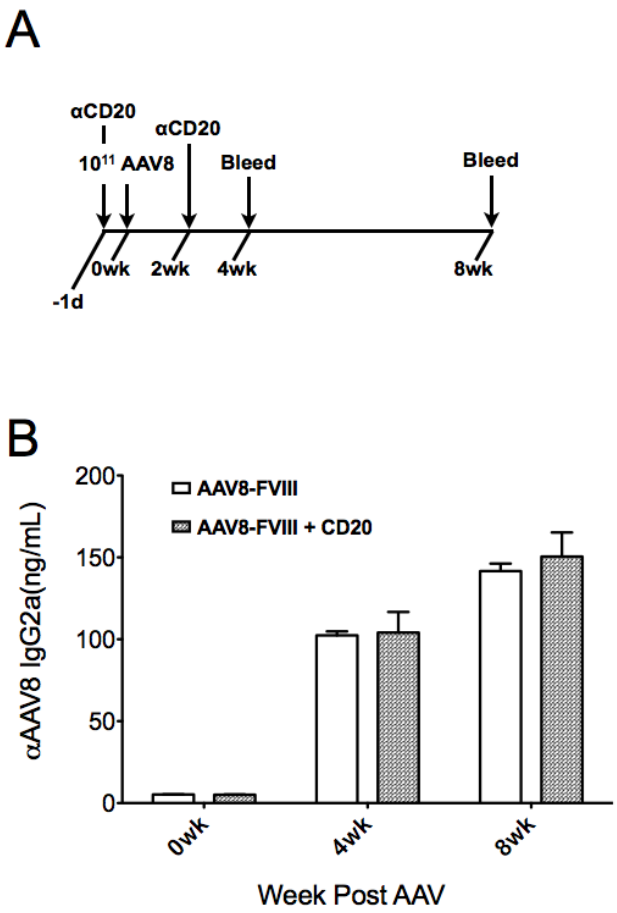

Figure 5-2. Two doses of αCD20 also fails to prevent antibodies to AAV8. (A) BALB/c-HA mice were given 10mg/kg αCD20 1 day before and 2 weeks after *i.v.* injection of 10^{11}vg/ms of AAV8-COhFVIII. At 4 weeks and 8 weeks mice were bled and plasma analyzed for antibodies to AAV8 capsid by ELISA. Antibody titers are shown by optical density (B).

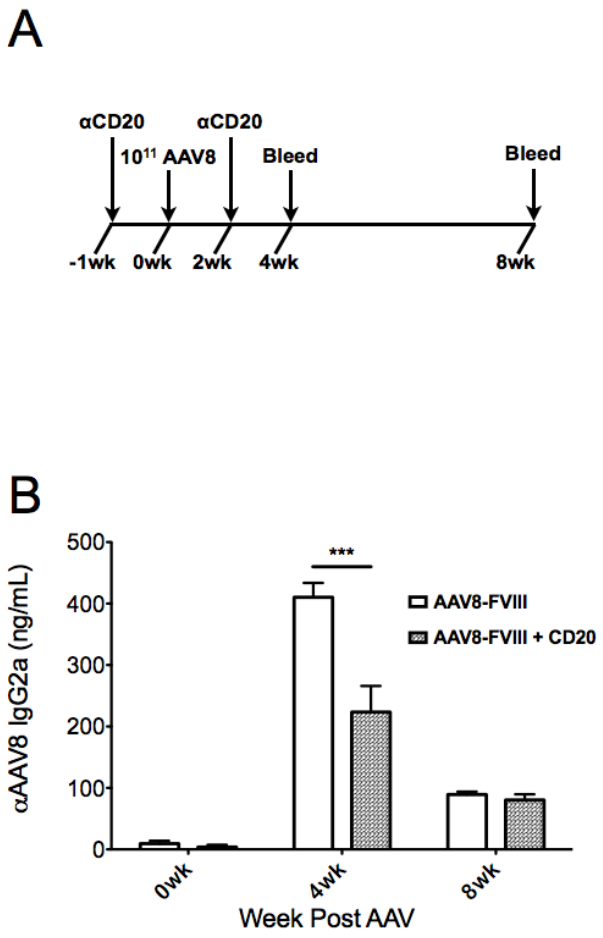

Figure 5-3. Alternate two-dose αCD20 protocol reduces anti-AAV8 antibodies. (A) BALB/c-HA mice were given 10mg/kg αCD20 1 week before and 2 weeks after *i.v.* injection of 10^{11}vg/ms of AAV8-COhFVIII. At 4 weeks and 8 weeks post vector, mice were bled and plasma analyzed for antibodies to AAV8 capsid by ELISA. Antibody titers are shown in (B). "***" is $p<0.001$ by t-test.

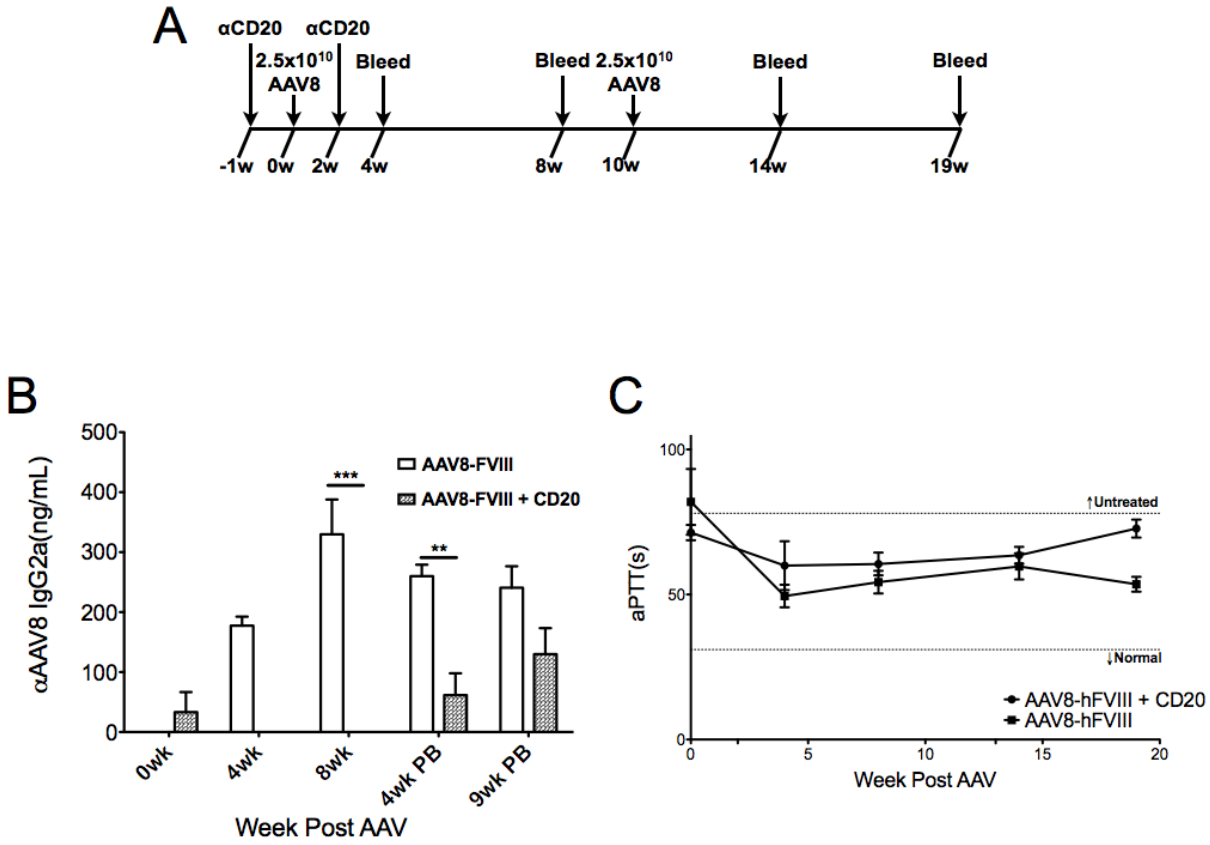

Figure 5-4. Lower doses of AAV8-COhFVIII allow for prevention of antibodies without improvement after re-administration. (A) BALB/c-HA mice were given 10mg/kg αCD20 1 week before and 2 weeks after i.v. injection of $2.5x10^{10}$vg/ms of AAV8-COhFVIII. At 4 weeks and 8 weeks post vector, mice were bled and plasma analyzed for antibodies to AAV8 capsid by ELISA. At 10 weeks, mice received another i.v. injection of $2.5x10^{10}$vg/ms AAV8-COhFVIII. Antibody titers are shown in (B) where "PB" indicates "post-boost" (second AAV8-COhFVIII injection). (C) Clotting times as measured by aPTT for mice treated with $2.5x10^{10}$vg/ms of AAV8-COhFVIII at week 0 and week 10.

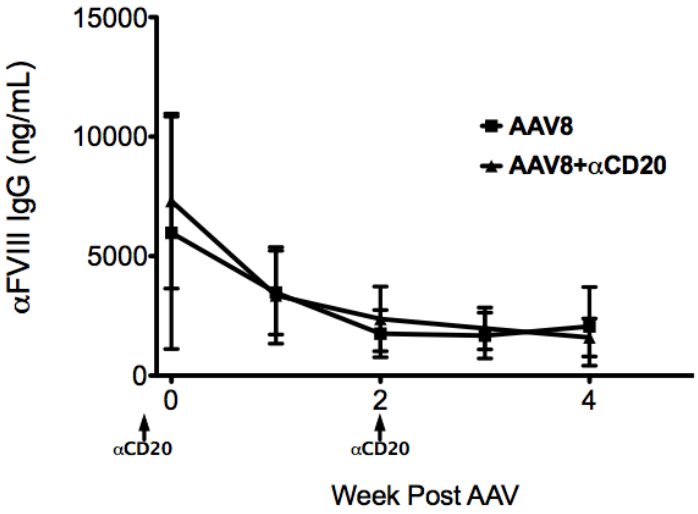

Figure 5-5. Liver-directed gene therapy is able to reduce antibody titer in hFVIII-challenged BALB/c-HA mice. BALB/c-HA mice were challenged with 1U hFVIII/week/ms for 4 weeks and antibody titers measure 2 weeks later (time 0 on graph). Mice were then administered either 10mg/kg αCD20 followed by 10^{11}vg/mL AAV8-COhFVIII i.v. one day later or just the same dose of vector alone. αCD20-treated mice also received an addition dose of αCD20 at 2 weeks post-AAV. Plasma concentrations of hFVIII-specific IgG1 were measured weekly up to 4 weeks post-AAV by ELISA. Data is plotted as mean ±SD.

CHAPTER 6
DISCUSSION

For many monogenetic diseases, gene therapy represents the only long-term hope for a safe, non-invasive cure. However, the main barrier standing between success and failure of these treatments remains the immune system. Whether it is pre-existing immunity to the viral capsid as is the case for AAV, the response to the viral vector in adenovirus or lentivirus or the response to the transgene in a number of disorders, most prominently in hemophilia A. However, there is a large percentage of gene therapy research dedicated to overcoming these hurdles. Hemophilia A is complicated by the chance of a pre-existing immune response to the viral vector or to the transgene (hFVIII) prior to gene therapy as well as the possibility of an immune response to the transgene after vector delivery. Thus, specific attention must be paid to learning how to manipulate the immune system to avoid these complications and careful choice of vector and target tissue will be critical if success is to be realized in this disease.

Gene transfer may tolerize or sensitize mice depending on genetic background

If gene transfer only accomplished expression at the lower end of the therapeutic range, e.g. <5% of normal, this would occasionally necessitate supplementary clotting factor administration. Hence, it is imperative that tolerance is maintained under these circumstances. Previously, sustained correction of murine hemophilia A with AAV vectors has been shown using either cFVIII or murine FVIII, but neither study tested the effect of FVIII challenge after gene therapy [86,87]. Others have combined gene transfer with immune suppression to avoid inhibitor formation [50-52,86]. Our study shows that lack of inhibitor formation against FVIII after hepatic AAV gene transfer does not necessarily

constitute humoral immune tolerance as mice in both strains treated with a low-expressing vector maintained correction in absence of but not after challenge with hFVIII (Figs. 3-4A and 3-5A). In fact, in the BL/6-129/sv-HA strain, mice were predisposed to heightened inhibitor formation in subsequent treatment with hFVIII protein, while gene transfer had the desired tolerogenic effect in BALB/c-HA mice. Clearly, high levels of hepatic expression promote tolerance regardless of the strain background (Fig. 4-2 and 4-2), which is consistent with our earlier findings on factor IX [44]. However, within the range of sub-optimal expression, two possibilities may explain the differing responses between the two strains. Somewhat higher expression in BALB/c-HA mice may have been partially effective in tolerance induction, while expression in the BL/6-129/sv-HA strain may have been just below this threshold and thus sensitized the mice. Alternatively, similarly low levels of expression may sensitize animals with higher immune responsiveness to FVIII, while having a more tolerogenic effect in a strain with lower responsiveness.

Consistent with data by others, BALB/c-HA mice had lower inhibitor responses against hFVIII, and T helper responses differed between strains [88]. BL/6-129/sv-HA mice, after 4 weeks of hFVIII protein therapy, had 10-fold higher inhibitor titers compared to BALB/c-HA. However, IgG formation against hFVIII was only 2-fold higher (compare Figs. 3-4A and 3-5A). In contrast to BL/6-129/sv-HA, BALB/c-HA mice only showed a good correlation between BU and IgG titers after a longer course of hFVIII administration (Fig. 3-7). Previously, we documented that $CD4^+$ T cell responses against hFVIII in BL/6-129/sv-HA mice were strongly biased to Th2, characterized by IL-4 and IL-6 production while lacking expression of suppressive/regulatory cytokines [66].

BALB/c-HA mice, however, have a more mixed response, which includes expression of Th1, Th2 and suppressive molecules (Fig. 3-6A and Table 3-2). This difference in the T helper response likely explains differences in inhibitor responses and in ability to induce tolerance in the 2 strains. Despite evidence for Th1 activation as part of the $CD4^+$ T cell response in BALB/c-HA, we found that mice of both strains formed IgG2a against hFVIII only rarely and inconsistently (data not shown)**,** suggesting that antibody formation is primarily driven by the Th2 component, thus consistently resulting in high-titer IgG1.

Utility of transient B cell depletion for tolerance to hFVIII

Rituximab has been used to treat patients with inhibitors with varying degrees of success [24,62,63]. In acquired hemophilia, a rare form of the disease where inhibitors appear in hemostatically normal individuals, treatment with rituximab alone can eradicate inhibitors [89]. This response is long-lasting and has been observed to last up to 5.5 years [89]. In contrast, rituximab use in X-linked hemophilia A is usually concurrent with traditional ITI. In this setting, data is limited largely to case reports and well-controlled studies are lacking. However, a consecutive national cohort analyzed in the UK investigating the use of rituximab as an adjunct to traditional ITI suggests a modest benefit of B cell depletion for eradicating inhibitors in patients who have previously failed ITI [63]. Still, a number of these individuals (6/10) relapsed and required additional immune therapy [63]. The data in this thesis also reveal limitations for the use of αCD20. While B cell depletion effectively prevented anti-hFVIII formation in protein therapy, such a protocol failed to induce lasting hyporesponsiveness, suggesting that protein administration fails to tolerize the newly emerging B cells. This conclusion is supported by a recent clinical study where immune responses to neoantigens following Rituximab treatment were muted during B cell depletion but returned to near normal once the

effects of the drug waned [90]. Furthermore, our experiments using B cell depleting αCD20 in conjunction with gene therapy suggest its usefulness when there is concern of low-efficacy gene therapy priming the immune system to hFVIII. This indicates a role of B cells in the generation of a primary immune response to hFVIII. This was not due to long-term effects of αCD20 as transient B cell depletion did not reduce the ability of control animals to form antibodies once B cell frequencies had recovered. To further confirm this point, we administered an adenoviral vector expressing β-galactosidase 7 weeks after the second dose of αCD20. The mice formed neutralizing antibodies against the virus and antibodies against β-galactosidase at titers indistinguishable from control mice (Fig. 3-3A and B).

Low levels of hepatic expression resulted only in a partial reduction of the T cell response to FVIII in BALB/c-HA mice, despite the protective effect against inhibitor formation. The reduction of the Th2 response, in conjunction with preserving Treg responses, was likely instrumental in achieving this effect. Nonetheless, gene transfer combined with αCD20 treatment induced a broad T cell unresponsiveness. Interestingly, up-regulation of Treg markers was also abrogated. This was somewhat reflected in adoptive transfer experiments, where CD4+CD25+ cells from AAV8-F8-only treated mice showed a trend for greater suppression compared to cells from “AAV8-F8 + αCD20” mice, although this did not reach statistical significance. In contrast, high expression from the codon-optimized construct increased the suppressive response in the presence of B cells, which raises the question of how the combination of these two approaches may shape the response. B cells play a dual role in the immune response as antigen presenting cells and as producers of antibodies. Zhang *et al.* demonstrated

in mice that treatment with αCD20 similarly prevented a secondary immune response to protein challenge in hFVIII-primed hemophilia A mice [91]. Interestingly, these authors found that IgG1 instead of IgG2a αCD20 spared marginal zone B cells and caused accumulation of Treg after further exposure to hFVIII. Furthermore, a recent study by Xiang et. al demonstrated an effect of B cell depletion on T cell function and phenotype [92]. In their model, Treg were initially reduced following B cell depletion but actually increased when B cells had recovered, and there was also evidence for emergence of suppressive B cells. Thus, a more differential method for B cell targeting may further improve tolerance induction by this approach.

Tolerance induction with codon-optimized hFVIII

It is encouraging that a more effective codon-optimized hFVIII expression cassette induced humoral tolerance, resulting in sustained correction of coagulation after challenge with hFVIII protein, independent of the strain background. This is significant in light of the fact that hFVIII has been notoriously difficult to express in mice at levels high enough for phenotypic correction. Often, alternative species FVIII have been used such as canine FVIII or porcine FVIII because of either higher expression or superior secretion [50,87,93,94]. Here, we were able to achieve tolerance to hFVIII and long-term phenotypic correction with a single AAV vector expressing codon-optimized hFVIII in the absence of immune suppression. Codon optimization is a process whereby the cDNA to be expressed is analyzed and altered to improve expression based on a variety of factors including but not limited to: usage of species-specific codons, increased GC content, removal of cryptic slice sites, and stabilizing RNA structure [73]. This process has been used in the context of gene therapy to improve expression of several transgenes from different vectors [54,74,75,77,78,95]. Only one study used this approach for hFVIII

expression. Investigators observed up to a 44-fold increase in FVIII expression following delivery of a codon-optimized hFVIII via lentivirus to neonatal hemophilia A mice [54]. However, the immunological consequences of increased expression in the context of a mature immune system were not investigated. Here, we demonstrate that a COhFVIII construct improves not only efficacy but also tolerance induction in adult hemophilia A mice of two different strains. In adult animals, Treg induction is a critical component of tolerance induction by hepatic gene transfer. Our adoptive transfer experiments showed that use of COhFVIII facilitated Treg induction, thereby conferring better suppression of antibody formation against hFVIII.

Although the 4-5% long-term expression we observed in mice is therapeutic, the likelihood that these levels would translate directly to human application of this strategy is unlikely. Thus, we attempted to further increase our hFVIII transgene expression by co-administration of a "helper" AAV8-PP5 vector. This vector has been able to increase second strand synthesis and therefore expression of single-stranded vectors including GFP and in this thesis, hFIX [79,96]. While this helper virus increased the expression of low dose ssAAV2-hFIX vector by approximately 5-fold (Fig. 4-3A), this strategy did not have similar effects on a higher dose of the same vector or on our ssAAV8-COhFVIII vector (Figs. 4-3A and 4-4A). This could be for several reasons. First, the murine liver is much more permissive to AAV8 where a dose at this level should transduce >90% of hepatocytes [67]. In addition, AAV8 genomes are delivered more efficiently to the nucleus [97]. Therefore, the number of transcriptionally active vector genomes may not be a limiting factor in our AAV8-COhFVIII transgene. Also, given that our transgene was codon-optimized, it is likely that there is efficiency of translating the available transcripts

is high thus overcoming the known translational barrier for hFVIII production [37]. What this may reveal is that for hFVIII, a significant barrier may be post-translational. Indeed, studies with porcine FVIII suggest that targeted modifications in the amino acid sequence of hFVIII can greatly increase its expression and that this increase is due to enhanced secretion of the protein as opposed to greater transcription or translation [71,93]. Thus further studies that aim to increase hFVIII expression should incorporate considerations for enhanced protein secretion.

Utility of B cell depletion for the prevention of anti-AAV capsid antibodies and to reverse inhibitors in hemophilia A mice

Even in the most successful clinical trials with hemophilia B, transgene expression levels were high enough to remove the need for supplemental clotting factor in 2 of 6 patients [36]. While gene therapy did reduce the need for clotting factor in the other patients, the main advantage of AAV gene transfer would be to have a one-time or extremely infrequent treatment that is a replacement for protein therapy. Therefore, one strategy might be to give repeated administrations of the same vector for increased protein expression and decreased dependence on protein therapy. Unfortunately, upon delivery of AAV, neutralizing antibodies will inevitably be formed against the vector capsid and will preclude addition treatment with the same vector. In this thesis, we attempted to develop a protocol that would prevent the formation of capsid antibodies using only a single drug agent analogous to the drug rituximab—a clinically safe biologic with wide use and minimal side effects. Ideally, one would use as few doses of immune suppressant as possible to prevent capsid antibodies and thus we tried a single dose of αCD20 given one day before gene transfer. This did not reduce the anti-capsid antibody and the levels seen at 8 weeks (211-325ng/mL) prevented detectable expression from

an AAV8-hFIX vector delivered at 10 weeks after the initial injection (Fig. 5-1B-D). B cells are expected to begin recovering by 3 weeks and as they recover they may allow for antibodies to form against capsid antigen that may still be present. However, even an additional αCD20 dose given 2 weeks after vector administration—removing B cells for approximately 5 weeks after AAV injection—failed to prevent or reduce antibody development (Fig. 5-2 B and C). Alternatively, giving αCD20 one week rather than one day before initial AAV delivery did reduce antibody titers at 4 weeks post-injection but they were still present and were not significantly different at 8 weeks from control mice given only AAV8-COhFVIII (Fig. 5-3B and C). This suggests that B cell depletion may not be robust at 24h post injection and suggests that a αCD20 dose should be given at least one week prior to vector. Finally, the only protocol that eliminated AAV8 capsid antibodies below our limit of detection was 2 doses of αCD20 given one week prior and two weeks following a reduced dose of AAV8-COhFVIII at $2.5x10^{11}$vg/ms (Fig. 5-4 B and C). However, administration of another dose of the same vector and dose did not further decrease the clotting times of mice given αCD20 and AAV8-COhFVIII. This may be due to a lack of sensitivity of the aPTT to detect small changes in FVIII activity, or there may be still be a small neutralizing antibody titer that prevented this small amount of vector from transducing mouse hepatocytes. In conclusion, it appears that AAV capsid antigen is present for greater than 5 weeks post injection at a moderate, 10^{11}vg/ms, dose of virus. Furthermore, complete depletion of B cells must be accomplished at the time of vector injection. Both of these observations argue for more aggressive dosing of αCD20 to prevent antibodies and perhaps earlier re-administration of secondary vector while B cells are still depleted.

Thus far, patients enrolled in clinical trials for hemophilia A and B have been inhibitor-negative given the uncertain effect that gene therapy will have on an individual with an existing immune response to the clotting factor. However, up to 30% of severe hemophilia A patients have inhibitors and it is these patients who have the poorest prognosis and are most burdened by heavy factor use. It is also these patients that would benefit the most from a protocol that could not only correct disease but reverse their inhibitors as well. Our attempts to model this in mice demonstrated that antibody titers could be reduced in the short-term (Fig. 5-5) but not completely eliminated. Similar experiment in dogs revealed that most inhibitors were gone in most dogs by 5 weeks, inhibitors in one dog were not reversed for 80 weeks after gene transfer [98]. Thus, prolonged observation of these mice is warranted and underway.

Future Directions

The work presented in this thesis highlights a number of challenges left to overcome in the field of gene therapy for hemophilia A which still include the limited expression and inherent immunogenicity of FVIII. We were able to achieve sustained expression of 3-5% hFVIII activity in a murine model of hemophilia A with a scalable vector dose. Furthermore, this expression maintained tolerance to hFVIII throughout the length of this experiment. If this result were exactly recapitulated in humans it would represent a drastic improvement in the quality of life of a person with severe hemophilia A and would greatly reduce their dependence on expensive and invasive clotting factor—as seen in the recent human trials for hemophilia B [36]. However, it is unclear if these results will translate directly and therefore further improvement of expression at the same or lower doses is highly desired. Codon-optimization seemed to provide an enhancement of expression at the transcriptional and translational level but confirmation

of which of these will be important for identifying what, if any, improvements can be made to the cDNA to improve expression at these steps. Furthermore, it is known that secretion of hFVIII is a limiting step and therefore use of FVIII cDNA carrying modifications to the amino acid sequence—and not just cDNA sequence—that enhance secretion may prove fruitful in this vector system. Replicating modifications such as those by *Doering et al.* in our codon-optimized hFVIII protein could further improve the phenotypic correction beyond what was seen in the experiments presented here [70]. Expression could also be improved by use of alternative viral capsids including those modified to decrease ubiquitin-meidated degradation by removing phosphorylation sites on the viral capsid surface [99].

Another important aspect will be determining the effect of B cell depletion on tolerance induction following liver-directed AAV gene therapy. While we found that our optimized vector induced tolerance to hFVIII in both high and low-responding strains of mice in the absence of any immune suppression, there were still a few mice with low-level antibody responses to hFVIII challenge. Given that B cell depletion has mixed effects on T cell responses—especially Treg—and that rituximab has a history of use in hemophilia A as well as AAV gene therapy, it will be prudent to evaluate the use of concomitant use of B cell depletion and delivery of a tolerizing gene therapy vector [100].

Finally, up to 30% of severe hemophilia A patients have pre-existing antibodies against hFVIII. To date, clinical trials evaluating gene therapy for hemophilia have involved inhibitor-free patients. However, patients with inhibitors represent the highest-risk patients with the fewest treatment options. It will be interesting to also investigate whether or not our optimized vector can reverse inhibitors in mice of either strain and if

B cell depletion will have any role in the efficacy of this treatment. There has been success in a canine model of hemophilia A where investigators were able to reverse inhibitors in 4 hemphilia A dogs after giving liver-directed gene therapy [98]. In this study there was indication that long-established inhibitors may be more difficult to eradicate than recently-developed inhibitors as the time to reverse inhibitors in the former was 80 weeks opposed to 4-5 weeks for the latter. However, more complete characterization of this model in mice is necessary as is an understanding of the mechanism behind eliminating hFVIII-specific B cells. Our codon-optimized vector and mouse strains should allow for better observation of this phenomenon in the future.

In conclusion, the immune response remains a major hurdle for gene therapy and particularly for hemophilia A gene therapy. Obtaining high-level, liver-specific expression of the hFVIII transgene appears to be crucial to eliciting both tolerance and long-term correction. Choice of vector, route of administration, transgene modifications and transient immune suppression all must be considered for effective and safe delivery of future vectors. Several hurdles remain such as pre-existing immunity to viral vectors and the poor expression of hFVIII transgenes and our findings here should help clear these hurdles as the possibility for treating hemophilia A with AAV gene therapy becomes a reality.

APPENDIX
SEQUENCE OF CODON-OPTIMIZED HUMAN FVIII

5’-
GGGCAGTGAGCGGAAGGCCCATGAGGCCAGTTAATTAAGAGGTACCATCGATGC
CACCATGCAGATCGAGCTGTCTACCTGCTTCTTCCTGTGCCTGCTGCGGTTCTGCT
TCAGCGCCACCCGGCGGTACTACCTGGGCGCCGTGGAACTGAGCTGGGACTACA
TGCAGAGCGACCTGGGGGAGCTGCCCGTGGACGCCAGATTCCCCCCAAGAGTGC
CCAAGAGCTTCCCCTTCAACACCTCCGTGGTGTACAAGAAAACCCTGTTCGTCGAG
TTCACCGACCACCTGTTCAATATCGCCAAGCCCAGACCCCCCTGGATGGGCCTGC
TGGGCCCTACAATCCAGGCCGAGGTGTACGACACCGTGGTCATCACCCTGAAGAA
CATGGCCAGCCACCCCGTGTCCCTGCACGCCGTGGGCGTGTCCTACTGGAAGGC
CTCTGAGGGCGCCGAGTACGACGACCAGACCAGCCAGCGCGAGAAAGAGGACGA
CAAAGTCTTTCCTGGCGGCAGCCATACCTACGTGTGGCAGGTCCTGAAAGAAAAC
GGCCCTATGGCCTCCGACCCCCTGTGCCTGACCTACAGCTACCTGAGCCACGTGG
ACCTGGTCAAGGACCTGAACAGCGGCCTGATCGGCGCCCTGCTCGTGTGTAGAG
AGGGCAGCCTCGCCAAAGAGAAAACCCAGACCCTGCACAAGTTCATCCTGCTGTT
CGCCGTGTTCGACGAGGGCAAGAGCTGGCACAGCGAGACAAAGAACAGCCTGAT
GCAGGACCGGGACGCCGCCTCTGCCAGAGCCTGGCCTAAGATGCACACCGTGAA
CGGCTACGTGAACAGAAGCCTGCCCGGACTGATCGGCTGCCACCGGAAGTCCGT
GTACTGGCACGTGATCGGCATGGGCACCACCCCCGAGGTGCACAGCATCTTTCTG
GAAGGCCACACCTTCCTCGTGCGGAACCACAGACAGGCCAGCCTGGAAATCAGC
CCTATCACCTTCCTGACCGCCCAGACACTGCTGATGGACCTGGGCCAGTTCCTGC
TGTTTTGCCACATCAGCAGCCACCAGCACGACGGCATGGAAGCCTACGTGAAGGT
GGACAGCTGCCCCGAGGAACCCCAGCTGCGGATGAAGAACAACGAGGAAGCCGA
GGACTACGACGACGACCTGACCGACAGCGAGATGGACGTCGTGCGCTTCGACGA
CGACAACAGCCCCAGCTTCATCCAGATCAGAAGCGTGGCCAAGAAGCACCCCAAG
ACCTGGGTGCACTATATCGCCGCCGAGGAAGAGGACTGGGACTACGCCCCTCTG
GTGCTGGCCCCCGACGACAGAAGCTACAAGAGCCAGTACCTGAACAATGGCCCC
CAGCGGATCGGCCGGAAGTACAAGAAAGTGCGGTTCATGGCCTACACCGACGAG
ACATTCAAGACCAGAGAGGCCATCCAGCACGAGAGCGGCATCCTGGGCCCCCTG
CTGTATGGCGAAGTGGGCGACACCCTGCTGATCATCTTCAAGAACCAGGCCAGCC
GGCCCTACAACATCTACCCCCACGGCATCACCGACGTGCGGCCCCTGTACAGCAG
ACGGCTGCCCAAGGGCGTGAAGCACCTGAAGGACTTCCCCATCCTGCCCGGCGA
GATCTTCAAGTACAAGTGGACCGTGACCGTGGAAGATGGCCCCACCAAGAGCGAC
CCCAGATGCCTGACCCGGTACTACAGCAGCTTCGTGAACATGGAACGGGACCTGG
CCTCCGGGCTGATCGGCCCTCTGCTGATCTGCTACAAAGAAAGCGTGGACCAGCG
GGGCAACCAGATCATGAGCGACAAGCGGAACGTGATCCTGTTCAGCGTGTTCGAT
GAGAATCGGTCCTGGTATCTGACCGAGAATATCCAGCGGTTCCTGCCCAACCCTG
CCGGCGTGCAGCTGGAAGATCCCGAGTTCCAGGCCAGCAACATCATGCACTCCAT
CAATGGCTACGTGTTCGACAGCCTGCAGCTGAGCGTGTGCCTGCACGAGGTGGC
CTACTGGTACATCCTGAGCATCGGCGCCCAGACCGACTTCCTGAGCGTGTTCTTC
AGCGGCTACACCTTCAAGCACAAGATGGTGTACGAGGATACCCTGACCCTGTTCC
CCTTCTCCGGCGAAACCGTGTTCATGAGCATGGAAAACCCCGGCCTGTGGATTCT
GGGCTGCCACAACAGCGACTTCCGGAACCGGGGCATGACCGCCCTGCTGAAGGT

GTCCAGCTGCGACAAGAACACCGGCGACTACTACGAGGACAGCTATGAGGACATC
AGCGCCTACCTGCTGAGCAAGAACAACGCCATCGAGCCCAGAAGCTTCAGCCAGA
ACCCCCCCGTGCTGAAGCGGCACCAGAGAGAGATCACCCGGACCACCCTGCAGT
CCGACCAGGAAGAGATTGATTACGACGACACCATCAGCGTCGAGATGAAGAAAGA
GGATTTCGACATCTACGACGAGGACGAGAACCAGAGCCCCCGGTCCTTCCAGAAG
AAAACCCGGCACTACTTCATTGCCGCCGTGGAAAGACTGTGGGACTACGGCATGA
GCAGCAGCCCCCACGTGCTGCGGAACAGAGCCCAGAGCGGCAGCGTGCCCCAGT
TCAAGAAAGTGGTGTTCCAGGAGTTCACCGACGGCAGCTTCACCCAGCCCCTGTA
TCGGGGCGAGCTGAACGAGCACCTGGGACTGCTGGGACCTTACATTAGAGCCGA
GGTGGAAGATAACATCATGGTCACCTTCAGAAACCAGGCCTCCAGACCCTACAGC
TTCTACAGCAGCCTGATCAGCTACGAAGAGGACCAGCGGCAGGGCGCCGAACCC
CGGAAGAACTTCGTGAAGCCCAACGAGACTAAGACCTACTTCTGGAAGGTGCAGC
ACCACATGGCCCCCACAAAGGACGAGTTCGACTGCAAGGCCTGGGCCTACTTCTC
CGATGTGGACCTGGAAAAGGACGTGCACTCTGGCCTGATTGGACCTCTGCTCGTC
TGCCACACCAACACCCTGAACCCCGCCCACGGCCGGCAGGTCACAGTGCAGGAA
TTTGCCCTGTTCTTCACCATCTTCGATGAGACAAAGAGCTGGTACTTCACCGAGAA
CATGGAAAGAAACTGCAGAGCCCCCTGCAACATCCAGATGGAAGATCCTACCTTC
AAAGAGAACTATCGGTTCCACGCCATCAACGGCTACATCATGGACACCCTGCCCG
GCCTGGTCATGGCCCAGGATCAGAGAATCCGGTGGTATCTGCTGAGCATGGGCA
GCAACGAGAACATCCACAGCATCCACTTCAGCGGCCACGTGTTCACAGTGCGGAA
GAAAGAAGAGTACAAGATGGCCCTGTACAACCTGTACCCCGGCGTGTTCGAGACA
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GAGCATCTGCACGCCGGAATGAGCACCCTGTTTCTGGTGTACAGCAACAAGTGCC
AGACCCCTCTGGGCATGGCCAGCGGCCACATCCGGGACTTCCAGATCACCGCCT
CCGGCCAGTACGGCCAGTGGGCCCCTAAGCTGGCCCGGCTCCACTACAGCGGCA
GCATCAACGCCTGGTCCACCAAAGAGCCCTTCAGCTGGATCAAGGTGGACCTGCT
GGCCCCTATGATCATCCACGGAATCAAGACCCAGGGCGCCAGACAGAAGTTCAGC
AGCCTGTACATCAGCCAGTTCATCATCATGTACAGCCTGGACGGCAAGAAGTGGC
AGACCTACCGGGGCAACAGCACCGGCACCCTGATGGTGTTCTTCGGCAACGTGG
ACAGCAGCGGCATCAAGCACAACATCTTCAACCCCCCCATCATTGCCCGGTACAT
CCGGCTGCACCCCACCCACTACAGCATCCGGTCCACCCTGCGGATGGAACTGATG
GGCTGCGACCTGAACTCCTGCAGCATGCCCCTGGGGATGGAAAGCAAGGCCATC
AGCGACGCCCAGATCACAGCCAGCAGCTACTTCACCAACATGTTCGCCACCTGGT
CCCCAAGCAAAGCCCGCCTGCATCTGCAGGGCAGAAGCAATGCCTGGCGGCCTC
AGGTCAACAACCCCAAAGAATGGCTCCAGGTGGACTTTCAGAAAACCATGAAGGT
CACAGGCGTGACCACCCAGGGCGTGAAAAGCCTGCTGACCTCTATGTACGTGAAA
GAGTTCCTGATCAGCAGCAGCCAGGACGGGCACCAGTGGACCCTGTTCTTTCAGA
ACGGCAAAGTGAAAGTGTTCCAGGGCAACCAGGACTCCTTCACCCCCGTGGTCAA
CTCCCTGGACCCTCCACTGCTGACCAGATACCTGAGAATCCACCCCCAGTCTTGG
GTGCACCAGATCGCCCTGAGAATGGAAGTGCTGGGATGCGAGGCCCAGGATCTG
TACTGACTCGAGCTCATGGCGCGCCTAGGCCTTGACGGCCTTCCGCCAATTCGCC
-3’

LIST OF REFERENCES

1 Marshall A. Lichtman, T. J. K., Uri Seligsohn, Kenneth Kaushansky, Josef T. Prchal. in *Williams Hematology* Vol. 8 Ch. Hemostasis and Thrombosis, (McGraw-Hill Medical, 2010).

2 Castaldo, G. *et al.* Haemophilia A: molecular insights. *Clinical chemistry and laboratory medicine : CCLM / FESCC* **45**, 450-461, doi:10.1515/CCLM.2007.093 (2007).

3 Vehar, G. A. *et al.* Structure of human factor VIII. *Nature* **312**, 337-342 (1984).

4 van Dieijen, G., Tans, G., Rosing, J. & Hemker, H. C. The role of phospholipid and factor VIIIa in the activation of bovine factor X. *The Journal of biological chemistry* **256**, 3433-3442 (1981).

5 High, K. A., & Roberts, H. R. *Molecular basis of thrombosis and hemostasis*. 6th edn, (M. Dekker, 1995).

6 Lakich, D., Kazazian, H. H., Jr., Antonarakis, S. E. & Gitschier, J. Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nature genetics* **5**, 236-241, doi:10.1038/ng1193-236 (1993).

7 Higuchi, M. *et al.* Molecular characterization of severe hemophilia A suggests that about half the mutations are not within the coding regions and splice junctions of the factor VIII gene. *Proc Natl Acad Sci U S A* **88**, 7405-7409 (1991).

8 Luck, J. V., Jr. *et al.* Hemophilic arthropathy. *The Journal of the American Academy of Orthopaedic Surgeons* **12**, 234-245 (2004).

9 Darby, S. C. *et al.* Mortality rates, life expectancy, and causes of death in people with hemophilia A or B in the United Kingdom who were not infected with HIV. *Blood* **110**, 815-825, doi:10.1182/blood-2006-10-050435 (2007).

10 Darby, S. C. *et al.* The impact of HIV on mortality rates in the complete UK haemophilia population. *AIDS* **18**, 525-533 (2004).

11 Seremetis, S. *et al.* Human recombinant DNA-derived antihaemophilic factor (factor VIII) in the treatment of haemophilia A: conclusions of a 5-year study of home therapy. The KOGENATE Study Group. *Haemophilia : the official journal of the World Federation of Hemophilia* **5**, 9-16 (1999).

12 Manco-Johnson, M. J. *et al.* Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *The New England journal of medicine* **357**, 535-544, doi:10.1056/NEJMoa067659 (2007).

13 Globe, D. R., Curtis, R. G. & Koerper, M. A. Utilization of care in haemophilia: a resource-based method for cost analysis from the Haemophilia Utilization Group Study (HUGS). *Haemophilia : the official journal of the World Federation of Hemophilia* **10 Suppl 1**, 63-70 (2004).

14 O'Mahony, B. & Black, C. Expanding hemophilia care in developing countries. *Seminars in thrombosis and hemostasis* **31**, 561-568, doi:10.1055/s-2005-922228 (2005).

15 Stonebraker, J. S., Brooker, M., Amand, R. E., Farrugia, A. & Srivastava, A. A study of reported factor VIII use around the world. *Haemophilia : the official journal of the World Federation of Hemophilia* **16**, 33-46, doi:10.1111/j.1365-2516.2009.02131.x (2010).

16 Gouw, S. C. *et al.* F8 gene mutation type and inhibitor development in patients with severe hemophilia A: systematic review and meta-analysis. *Blood*, doi:10.1182/blood-2011-09-379453 (2012).

17 Waters, B. & Lillicrap, D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *Journal of thrombosis and haemostasis : JTH* **7**, 1446-1456, doi:10.1111/j.1538-7836.2009.03538.x (2009).

18 van Haren, S. D., Wroblewska, A., Fischer, K., Voorberg, J. & Herczenik, E. Requirements for immune recognition and processing of factor VIII by antigen-presenting cells. *Blood reviews* **26**, 43-49, doi:10.1016/j.blre.2011.10.001 (2012).

19 Lollar, P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *Journal of thrombosis and haemostasis : JTH* **2**, 1082-1095, doi:10.1111/j.1538-7836.2004.00802.x (2004).

20 Hausl, C. *et al.* High-dose factor VIII inhibits factor VIII-specific memory B cells in hemophilia A with factor VIII inhibitors. *Blood* **106**, 3415-3422, doi:10.1182/blood-2005-03-1182 (2005).

21 Di Michele, D. M. Immune tolerance induction in haemophilia: evidence and the way forward. *Journal of thrombosis and haemostasis : JTH* **9 Suppl 1**, 216-225, doi:10.1111/j.1538-7836.2011.04349.x (2011).

22 Odeyemi, I. A. & Dano, A. M. Optimising immune tolerance induction strategies in the management of haemophilia patients with inhibitors: a cost-minimisation analysis. *Current medical research and opinion* **25**, 239-250, doi:10.1185/03007990802633863 (2009).

23 Franchini, M. *et al.* Immune tolerance with rituximab in congenital haemophilia with inhibitors: a systematic literature review based on individual patients' analysis. *Haemophilia : the official journal of the World Federation of Hemophilia* **14**, 903-912, doi:10.1111/j.1365-2516.2008.01839.x (2008).

24 Streif, W. *et al.* Inhibitor treatment by rituximab in congenital haemophilia A - Two case reports. *Hamostaseologie* **29**, 151-154 (2009).

25 Santagostino, E. & Mancuso, M. E. Barriers to primary prophylaxis in haemophilic children: the issue of the venous access. *Blood transfusion = Trasfusione del sangue* **6 Suppl 2**, s12-16 (2008).

26 Daya, S. & Berns, K. I. Gene therapy using adeno-associated virus vectors. *Clinical microbiology reviews* **21**, 583-593, doi:10.1128/CMR.00008-08 (2008).

27 Niemeyer, G. P. *et al.* Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood* **113**, 797-806, doi:10.1182/blood-2008-10-181479 (2009).

28 Jooss, K., Yang, Y., Fisher, K. J. & Wilson, J. M. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *Journal of virology* **72**, 4212-4223 (1998).

29 McCaffrey, A. P. *et al.* The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 931-941, doi:mt200837 [pii]10.1038/mt.2008.37 (2008).

30 Zaiss, A. K. *et al.* Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. *Journal of virology* **76**, 4580-4590 (2002).

31 Ashtari, M. *et al.* The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest* **121**, 2160-2168, doi:10.1172/JCI57377 (2011).

32 Bennett, J. *et al.* AAV2 gene therapy readministration in three adults with congenital blindness. *Science translational medicine* **4**, 120ra115, doi:10.1126/scitranslmed.3002865 (2012).

33 Cideciyan, A. V. *et al.* Human RPE65 gene therapy for Leber congenital amaurosis: persistence of early visual improvements and safety at 1 year. *Human gene therapy* **20**, 999-1004, doi:10.1089/hum.2009.086 (2009).

34 Maguire, A. M. *et al.* Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet* **374**, 1597-1605, doi:10.1016/S0140-6736(09)61836-5 (2009).

35 Simonelli, F. *et al.* Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 643-650, doi:10.1038/mt.2009.277 (2010).

36 Nathwani, A. C. *et al.* Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B. *The New England journal of medicine*, doi:10.1056/NEJMoa1108046 (2011).

37 Hollestelle, M. J. *et al.* Tissue distribution of factor VIII gene expression in vivo--a closer look. *Thrombosis and haemostasis* **86**, 855-861 (2001).

38 Calne, R. Y. *et al.* Induction of immunological tolerance by porcine liver allografts. *Nature* **223**, 472-476 (1969).

39 Crispe, I. N. *et al.* Cellular and molecular mechanisms of liver tolerance. *Immunological reviews* **213**, 101-118 (2006).

40 Mackay, I. R. Hepatoimmunology: a perspective. *Immunol Cell Biol* **80**, 36-44, doi:1063 [pii] (2002).

41 Dobrzynski, E. *et al.* Induction of antigen-specific CD4+ T-cell anergy and deletion by in vivo viral gene transfer. *Blood* **104**, 969-977, doi:10.1182/blood-2004-03-08472004-03-0847 [pii] (2004).

42 Hoffman, B. E. *et al.* Nonredundant roles of IL-10 and TGF-beta in suppression of immune responses to hepatic AAV-factor IX gene transfer. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 1263-1272, doi:10.1038/mt.2011.33 (2011).

43 LoDuca, P. A., Hoffman, B. E. & Herzog, R. W. Hepatic gene transfer as a means of tolerance induction to transgene products. *Curr Gene Ther* **9**, 104-114 (2009).

44 Mingozzi, F. *et al.* Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *The Journal of clinical investigation* **111**, 1347-1356, doi:10.1172/JCI16887 (2003).

45 Cao, O. *et al.* Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer. *Blood* **110**, 1132-1140 (2007).

46 Luth, S. *et al.* Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. *J Clin Invest* **118**, 3403-3410 (2008).

47 Manno, C. S. *et al.* Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**, 342-347, doi:nm1358 [pii]10.1038/nm1358 (2006).

48 Mingozzi, F. *et al.* CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* **13**, 419-422, doi:nm1549 [pii]10.1038/nm1549 (2007).

49 Gnatenko, D. V. *et al.* Human factor VIII can be packaged and functionally expressed in an adeno-associated virus background: applicability to haemophilia A gene therapy. *British journal of haematology* **104**, 27-36 (1999).

50 Sarkar, R. *et al.* Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood* **103**, 1253-1260, doi:10.1182/blood-2003-08-2954 (2004).

51 Jiang, H. *et al.* Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood* **108**, 107-115, doi:10.1182/blood-2005-12-5115 (2006).

52 Lu, H. *et al.* Complete correction of hemophilia A with adeno-associated viral vectors containing a full-size expression cassette. *Human gene therapy* **19**, 648-654, doi:10.1089/hum.2007.0182 (2008).

53 Hu, C. & Lipshutz, G. S. AAV-based neonatal gene therapy for hemophilia A: long-term correction and avoidance of immune responses in mice. *Gene therapy*, doi:10.1038/gt.2011.200 (2012).

54 Ward, N. J. *et al.* Codon optimization of human factor VIII cDNAs leads to high-level expression. *Blood* **117**, 798-807, doi:10.1182/blood-2010-05-282707 (2011).

55 Bi, L. *et al.* Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* **10**, 119-121, doi:10.1038/ng0595-119 (1995).

56 Uchida, J. *et al.* The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* **199**, 1659-1669, doi:10.1084/jem.20040119 (2004).

57 Zolotukhin, S. *et al.* Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene therapy* **6**, 973-985, doi:10.1038/sj.gt.3300938 (1999).

58 Cao, O. *et al.* Impact of the underlying mutation and the route of vector administration on immune responses to factor IX in gene therapy for hemophilia B. *Mol Ther* **17**, 1733-1742, doi:10.1038/mt.2009.159 (2009).

59 Verbruggen, B. *et al.* The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thrombosis and haemostasis* **73**, 247-251 (1995).

60 Verweij, C. L. & Vosslamber, S. New insight in the mechanism of action of rituximab: the interferon signature towards personalized medicine. *Discov Med* **12**, 229-236 (2011).

61 Weiner, G. J. Rituximab: mechanism of action. *Semin Hematol* **47**, 115-123, doi:10.1053/j.seminhematol.2010.01.011 (2010).

62 Aleem, A. *et al.* Rituximab as a single agent in the management of adult patients with haemophilia A and inhibitors: marked reduction in inhibitor level and clinical improvement in bleeding but failure to eradicate the inhibitor. *Haemophilia* **15**, 210-216, doi:10.1111/j.1365-2516.2008.01865.x (2009).

63 Collins, P. W. *et al.* Rituximab and immune tolerance in severe hemophilia A: a consecutive national cohort. *J Thromb Haemost* **7**, 787-794, doi:10.1111/j.1538-7836.2009.03332.x (2009).

64 Nathwani, A. C. *et al.* Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* **107**, 2653-2661, doi:10.1182/blood-2005-10-4035 (2006).

65 High, K. A. Gene therapy for haemophilia: a long and winding road. *J Thromb Haemost* **9 Suppl 1**, 2-11, doi:10.1111/j.1538-7836.2011.04369.x (2011).

66 Moghimi, B. *et al.* Induction of tolerance to factor VIII by transient co-administration with rapamycin. *Journal of thrombosis and haemostasis : JTH* **9**, 1524-1533 (2011).

67 Cooper, M. *et al.* Improved induction of immune tolerance to factor IX by hepatic AAV-8 gene transfer. *Human gene therapy* **20**, 767-776, doi:10.1089/hum.2008.161 (2009).

68 Powell, J. S. *et al.* Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood* **102**, 2038-2045, doi:10.1182/blood-2003-01-0167 (2003).

69 Roth, D. A., Tawa, N. E., Jr., O'Brien, J. M., Treco, D. A. & Selden, R. F. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *The New England journal of medicine* **344**, 1735-1742, doi:10.1056/NEJM200106073442301 (2001).

70 Doering, C. B. *et al.* Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A. *Molecular therapy : the journal of the American Society of Gene Therapy* **17**, 1145-1154, doi:10.1038/mt.2009.35 (2009).

71 Doering, C. B., Healey, J. F., Parker, E. T., Barrow, R. T. & Lollar, P. Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *The Journal of biological chemistry* **279**, 6546-6552, doi:10.1074/jbc.M312451200 (2004).

72 Gustafsson, C., Govindarajan, S. & Minshull, J. Codon bias and heterologous protein expression. *Trends in biotechnology* **22**, 346-353, doi:10.1016/j.tibtech.2004.04.006 (2004).

73 Fath, S. *et al.* Multiparameter RNA and codon optimization: a standardized tool to assess and enhance autologous mammalian gene expression. *PloS one* **6**, e17596, doi:10.1371/journal.pone.0017596 (2011).

74 Foster, H. *et al.* Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 1825-1832, doi:10.1038/mt.2008.186 (2008).

75 Li, C., Xiao, P., Gray, S. J., Weinberg, M. S. & Samulski, R. J. Combination therapy utilizing shRNA knockdown and an optimized resistant transgene for rescue of diseases caused by misfolded proteins. *Proc Natl Acad Sci U S A* **108**, 14258-14263, doi:10.1073/pnas.1109522108 (2011).

76 Ng, Y. Y. *et al.* Correction of B-cell development in Btk-deficient mice using lentiviral vectors with codon-optimized human BTK. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* **24**, 1617-1630, doi:10.1038/leu.2010.140 (2010).

77 Padegimas, L. *et al.* Optimization of hCFTR Lung Expression in Mice Using DNA Nanoparticles. *Mol Ther* **20**, 63-72, doi:10.1038/mt.2011.196 (2012).

78 Wang, L. *et al.* Preclinical evaluation of a clinical candidate AAV8 vector for ornithine transcarbamylase (OTC) deficiency reveals functional enzyme from each persisting vector genome. *Mol Genet Metab* **105**, 203-211, doi:10.1016/j.ymgme.2011.10.020 (2012).

79 Jayandharan, G. R., Zhong, L., Li, B., Kachniarz, B. & Srivastava, A. Strategies for improving the transduction efficiency of single-stranded adeno-associated virus vectors in vitro and in vivo. *Gene therapy* **15**, 1287-1293, doi:10.1038/gt.2008.89 (2008).

80 Calcedo, R., Vandenberghe, L. H., Gao, G., Lin, J. & Wilson, J. M. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* **199**, 381-390, doi:10.1086/595830 (2009).

81 Davidoff, A. M. *et al.* Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. *Molecular therapy : the journal of the American Society of Gene Therapy* **11**, 875-888, doi:10.1016/j.ymthe.2004.12.022 (2005).

82 Wang, L. *et al.* AAV8-mediated hepatic gene transfer in infant rhesus monkeys (Macaca mulatta). *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 2012-2020, doi:10.1038/mt.2011.151 (2011).

83 Wang, L. *et al.* Impact of pre-existing immunity on gene transfer to nonhuman primate liver with adeno-associated virus 8 vectors. *Human gene therapy* **22**, 1389-1401, doi:10.1089/hum.2011.031 (2011).

84 Mingozzi, F. *et al.* Pharmacological Modulation of Humoral Immunity in a Nonhuman Primate Model of AAV Gene Transfer for Hemophilia B. *Molecular therapy : the journal of the American Society of Gene Therapy*, doi:10.1038/mt.2012.84 (2012).

85 McIntosh, J. H. *et al.* Successful attenuation of humoral immunity to viral capsid and transgenic protein following AAV-mediated gene transfer with a non-depleting CD4 antibody and cyclosporine. *Gene therapy* **19**, 78-85, doi:10.1038/gt.2011.64 (2012).

86 Ishiwata, A. *et al.* Liver-restricted expression of the canine factor VIII gene facilitates prevention of inhibitor formation in factor VIII-deficient mice. *J Gene Med* **11**, 1020-1029, doi:10.1002/jgm.1391 (2009).

87 Sarkar, R., Xiao, W. & Kazazian, H. H., Jr. A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype. *J Thromb Haemost* **1**, 220-226 (2003).

88 Qadura, M. *et al.* Immunoglobulin isotypes and functional anti-FVIII antibodies in response to FVIII treatment in Balb/c and C57BL/6 haemophilia A mice. *Haemophilia* **17**, 288-295, doi:10.1111/j.1365-2516.2010.02397.x (2011).

89 Singh, A. G., Hamarneh, I. S., Karwal, M. W. & Lentz, S. R. Durable responses to rituximab in acquired factor VIII deficiency. *Thromb Haemost* **106**, 172-174, doi:10.1160/TH11-02-0097 (2011).

90 Pescovitz, M. D. *et al.* Effect of rituximab on human in vivo antibody immune responses. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2011.08.008 (2011).

91 Zhang, A. H., Skupsky, J. & Scott, D. W. Effect of B-cell depletion using anti-CD20 therapy on inhibitory antibody formation to human FVIII in hemophilia A mice. *Blood* **117**, 2223-2226, doi:10.1182/blood-2010-06-293324 (2011).

92 Xiang, Y. *et al.* The Dual Effects of B Cell Depletion on Antigen-Specific T Cells in BDC2.5NOD Mice. *J Immunol*, doi:10.4049/jimmunol.1103055 (2012).

93 Doering, C. B., Healey, J. F., Parker, E. T., Barrow, R. T. & Lollar, P. High level expression of recombinant porcine coagulation factor VIII. *J Biol Chem* **277**, 38345-38349, doi:10.1074/jbc.M206959200 (2002).

94 Gangadharan, B., Parker, E. T., Ide, L. M., Spencer, H. T. & Doering, C. B. High-level expression of porcine factor VIII from genetically modified bone marrow-derived stem cells. *Blood* **107**, 3859-3864, doi:10.1182/blood-2005-12-4961 (2006).

95 Sun, B. *et al.* Immunomodulatory gene therapy prevents antibody formation and lethal hypersensitivity reactions in murine pompe disease. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 353-360, doi:10.1038/mt.2009.195 (2010).

96 Jayandharan, G. R. *et al.* Optimized adeno-associated virus (AAV)-protein phosphatase-5 helper viruses for efficient liver transduction by single-stranded AAV vectors: therapeutic expression of factor IX at reduced vector doses. *Human gene therapy* **21**, 271-283, doi:10.1089/hum.2009.100 (2010).

97 Thomas, C. E., Storm, T. A., Huang, Z. & Kay, M. A. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *Journal of virology* **78**, 3110-3122 (2004).

98 Finn, J. D. *et al.* Eradication of neutralizing antibodies to factor VIII in canine hemophilia A after liver gene therapy. *Blood* **116**, 5842-5848, doi:10.1182/blood-2010-06-288001 (2010).

99 Markusic, D. M. *et al.* High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 2048-2056, doi:10.1038/mt.2010.172 (2010).

100 Xiang, Y. *et al.* The dual effects of B cell depletion on antigen-specific T cells in BDC2.5NOD mice. *J Immunol* **188**, 4747-4758, doi:10.4049/jimmunol.1103055 (2012).

BIOGRAPHICAL SKETCH

Brandon Sack was born in Longwood, Florida to Diane and Jerome Sack. He grew up in and around central Florida always enjoying the outdoors and in particular the water. He was first introduced to the world of science by his mother who provided him with a microscope and a pack of slides on which to explore everything from pond scum to dog hair. He carried this interest into the University of Florida from which he received a Bachelor of Science degree in biology and a minor in classical studies. He joined the University of Florida IDP in August of 2007, was awarded a Grinter fellowship and a University of Florida Alumni Fellowship to support his research. He eventually joined the lab of Dr. Roland Herzog following rotations with Dr. Kenneth Warrington and Dr. Dietmar Siemann. The Herzog lab focused on gene therapy for hemophilia and the immunological consequences thereof. He received recognition for his work by being awarded an NIH Infection Disease and Microbiology Training Grant in 2009 and the Medical Guild silver medal in 2012. During his tenure he published one first-author research paper, one co-author research paper, a first-author review article and one first-author book chapter.

Brandon accepted a post-doctoral research position at the Seattle Biomedical Research institute where he will provide immunological insight into the development of a malaria vaccine. He hopes to continue working in academic research involving global health challenges while also teaching and preparing future generations of scientists through teaching and mentoring.