INDUCTION OF IMMUNE TOLERANCE TO FACTOR IX IN GENE AND PROTEIN REPLACEMENT THERAPY FOR HEMOPHILIA B

By

SUSHRUSHA NAYAK

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To my parents for their unwavering support and encouragement

ACKNOWLEDGMENTS

I take this opportunity to thank my mentor, Dr. Roland W. Herzog for his guidance and extensive support. He has always been available to answer my questions, without judgment, since the day I joined his lab. I appreciate the opportunities he has provided me to work on multiple projects and collaborate with the best researchers in the fields of gene therapy and immunology. What I have gained over my time in this lab and university transcends academic learning and cannot be encompassed in this thesis alone. I would like to thank Dr. Ou Cao, who provided me, with the essential training I required to be successful at the bench. I would also like to extend my thanks to my committee members Dr. Mark Atkinson, Dr. Barry Byrne, Dr. Bradley Fletcher and Dr. Laurence Morel who have been instrumental in guiding and directed the theme of my work and bringing this thesis to its successful completion. I thank my lab colleagues for their collaboration and for making arduous tasks easy.

I thank my parents, Dr. Rabindranath Nayak and Sukanti Nayak for always believing in me and for their incomprehensible love and support in the things I chose to do, even if they broke from tradition. I thank my brother for his camaraderie; Dr. Arun Srivastava and Dr. Jackie Hobbs for easing my transition into a new country and making me feel at home. I thank my friends in the IDP program for all the fun and intellectual conversations and Dr. Aravind Natarajan for all his support.

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Sushrusha Nayak

May 2010

Chair: Roland W. Herzog
Major: Medical Sciences— Immunology and Microbiology

Hemophilia B is an X-linked bleeding disorder affecting 1 in 30, 000 male births with varying severity, differing due to the underlying mutations in the Factor IX (F.IX) gene. Treatment of patients with severe hemophilia is complicated by the formation of neutralizing antibodies to the therapeutic Factor IX protein in gene and protein replacement therapies. Prophylactic protocols for the prevention of immune responses are key to successful therapy. Preventive protocols utilizing the immunosuppressant drug rapamycin, cytokines IL-10, IL-2, Fc-GITR-L and antigen specific CD4 epitopes were developed to induce antigen specific tolerance in a test model (DO.11.10tg Rag2-/-) BALB/c mice. The prophylactic protocol utilizing rapamycin, IL-10 cytokine, antigen specific peptide was investigated in detail. The T cell receptor (TCR) transgenic test model showed the induction of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (T_{reg}), a cell type crucial for immune tolerance. Concomitant with T_{reg} induction, depletion of antigen specific effector T cell (T_{eff}) was observed. The T_{eff} cell depletion was caused by activation induced cell death (AICD). Flow cytometric analysis further indicated differential effects of the drug cocktail on T_{reg} vs. T_{eff}. T_{reg} were more resistant to the drug-induced AICD than T_{eff}, hence allowing T_{reg} cells a proliferative advantage. Optimization studies

with differing routes of drug cocktail administration showed higher levels of T_{reg} induction and T_{eff} cell depletion in intra-peritoneal, subcutaneous and intravenous drug administration as opposed to oral gavage. Promising results from the (DO.11.10tg Rag2-/-) Balb/c mice warranted an extension to F9 knockout mice (C3H/HeJ-F9-/-), which is a Hemophilia B disease model. Optimal protocols initially identified in the TCR transgenic model were tested for and resulted in prophylactic tolerance induction to F.IX prior to adeno-associated virus serotype1 (AAV1) gene transfer to skeletal muscle in hemophilia B mice. Tolerance was maintained for > 5 months, accompanied by sustained F.IX expression. In addition to prophylactic use, the Rapa/IL-10/specific peptide protocol suppressed a pre-existing immune response initiated by AAV1-hFIX gene transfer in hemophilia B mice and maintained long-term tolerance.

The results demonstrate that the prophylactic protocol tested can successfully prevent immune responses to F.IX and may also be applicable to treatment of other inherited systemic protein disorders such as hemophilia A (Factor VIII deficiency) and Pompe disease (a lysosomal storage disorder). The success of the tolerance protocol in eliminating a pre-existing immune response raises the prospect of eventual translation to human therapy for the treatment of patients who have developed an immune response to protein replacement therapy, or correcting unintended immune responses in gene therapy.

CHAPTER 1
INTRODUCTION

Hemophilia: An Introduction to the Disease

Hemophilia is an X linked recessive hemorrhagic disorder resulting from mutations to genes encoding coagulation Factor IX (Hemophilia B) or Factor VIII (F.VIII; Hemophilia A) thereby causing a systemic protein deficiency.[1] Males are usually affected and females are typically carriers.[2] According to the world federation of hemophilia, an estimated 400,000 people around the world live with hemophilia with only 25% receiving adequate treatment around the globe. It came to the forefront as the "royal disease" manifested among many royal families in Europe. The size of the Factor IX gene is about 34kb, 92% of the gene consists of introns, while there are 8 exons. Factor IX participates in the intrinsic plasma-clotting cascade.[3] It occurs as a zymogen and is activated to Factor IXa that further activates Factor X in the presence of Factor VIIIa to further the clotting cascade, which finally results in fibrin clot formation (Fig. 1-1). "Royal disease" was recently confirmed via genotype analysis to be hemophilia B caused by mutations that led to altered RNA splicing.[4] Mutations may vary from single base substitutions, early/late deletions, missense mutations etc, which affect the severity of the disease and influence the risk of antibody formation to the missing factor upon treatment with enzyme replacement or gene therapy. Patients with hemophilia exhibit prolonged clotting times (such as activated partial thromboplastin time- aPTT) and require factor infusion following trauma or surgery. They may also exhibit spontaneous bleeds into joints and internal spaces. Less than 1% of F.VIII or F.IX activity represents severe disease, 2-5% is moderate and greater than 5% represents mild disease. An increase in the level of circulating clotting factors to above 1% reduces the incidence of bleeding and improves quality of life. There are suggestions that hemophilia A may be the more severe phenotypic form of the two hemophilia's. [4] The frequency

of Hemophilia B is about 1 in 30, 000 male births, while the frequency of hemophilia A is about 1 in 5000 male births.

Treatment of Hemophilia

Protein Replacement Therapy

Protein replacement is the choice of treatment for many systemic protein deficiencies such as the hemophilias and glycogen storage diseases (such as Pompe disease and Gaucher disease). Bleeding in hemophilia patients is currently managed by intravenous (IV) infusion of plasma-derived or recombinant protein (F.VIII or F.IX) concentrate. Children with severe hemophilia A or hemophilia B can be treated prophylactically with doses of the respective missing factor to prevent haemarthrosis and reduce the regular progression of the disease, which targets joints. Joint damage is a major cause of morbidity of the disease but hemorrhagic stroke may also occur. The treatment usually involves the early start of factor infusion after diagnosis, with an eventual dose escalation. Prophylactic treatment for hemophilia is not commonly practiced; typically, patients are treated with the missing factor in response to a bleeding episode, which does not treat joint damage that has already occurred. In the past, clotting factor from porcine, bovine or human sources raised concerns about viral infection and allergic reactions. The availability of recombinant clotting factors however has reduced this risk. Prophylactic treatment is largely not performed in the United States due to high costs associated with high doses of clotting factor required, and the risk of infection of the catheter needed for intravenous (IV) access. Only 20-25% of hemophilia patients are being treated worldwide because of the expense of purified coagulation factors. Maintenance of >1% of factor activity ameliorates disease phenotype by reducing incidences of spontaneous bleeds.[5]

In protein-based therapy for hemophilia, 20-30% of hemophilia A and 1.5-4% of hemophilia B patients form inhibitors (primarily subjects with gene deletions or premature stop codons).[6] These immune responses are dependent on $CD4^+$ T cells. Targeting F.IX-specific $CD4^+$ T cells for tolerance induction is therefore an ideal strategy. It has become clear in recent years that immune regulation plays an important role in many tolerance protocols.[7,8]

In many ways, hemophilia is an ideal model for the development of gene therapy for genetic disorders. Precise regulation of transgene expression is not required. Although normally expressed by the liver, several cell types are capable of synthesizing biologically active coagulation factor following gene transfer. In addition, there are small and large animal models of the disease (F.VIII and F.IX knockout mice and naturally occurring hemophilia A and B dogs).

Gene Therapy for Hemophilia

The prevalence of the disease (~400,000 individuals) with the low access to treatment in developing countries, combined with the very high cost of treatment begs the need for gene therapy as a treatment modality. Approaches that may be used to ensure consistent production of F.IX and F.VIII include gene modified cell based therapies and integrating or non-integrating viral or non-viral vectors. Gene therapy has the potential to provide cost effective, consistent, long-term factor replacement in these individuals.[9]

AAV-mediated gene transfer

Adeno-associated virus (AAV) is a non-pathogenic, replication-deficient virus with a 4.7-kb single-stranded DNA genome.[10] The viral genome is packaged into a viral capsid that is composed of three capsid proteins (VP-1, -2, and -3). In the absence of a helper virus, wild-type AAV undergoes latent infection, characterized by preferential integration into a specific site in human chromosome 19, *in vitro* however most vector genomes (>95%) persist in episomal forms

in vivo.[11,12] AAV replication is dependent on the presence of a helper virus such as adenovirus or herpes virus. In the AAV vector, all viral coding sequences are removed and replaced by an expression cassette for the therapeutic gene. The only remaining viral sequences are the 145-bp inverted terminal repeats (ITRs), which flank the expression cassette. AAV vectors have a packaging limit of ~5kb. The vector can be produced by triple transfection of HEK-293 cells in a helper virus-free system. One plasmid encodes the vector genome, the second provides AAV wild-type *rep* and *cap* genes required for replication and packaging of genome, and a third plasmid encodes adenoviral helper functions (E2a, VA, and E4). AAV-2 vectors efficiently transfer genes to non-dividing target cells *in vivo*. Sustained high levels of transgene expression have been observed for the life span of a mouse and for >8 years in canine models.[13-15] Low-titer Neutalizing Antibody (NAB) to AAV-2 efficiently block hepatic gene transfer.[16,17] AAV-2 has been shown to be highly inefficient in transduction of dendritic cells (DC) and other professional antigen presenting cells (APCs) *in vivo* and *in vitro*.[18,19] This lack of transgene expression in APCs (combined with reduced innate immunity)[20] reduces the likelihood of activation of cytotoxic T lymphocytes (CTL) compared to other vector systems. A completed clinical study on treatment of hemophilia B by *in vivo* viral gene transfer was based on intramuscular (IM) injection of adeno-associated virus serotype 2 (AAV-2) vector to subjects with severe hemophilia B however pre-clinical studies showed an increased risk of immune responses to F.IX in animals with F.IX gene deletions or nonsense mutations. These results make skeletal muscle less attractive as a target of gene transfer, despite the fact long-term (albeit sub-therapeutic) transgene expression has been demonstrated in the human subjects.[21]

Hepatic AAV gene transfer for treatment of hemophilia B

Hepatocytes are the normal site of F.IX synthesis. Following hepatic gene transfer with AAV-2 vector, F.IX levels of 4 to 10% were observed in hemophilia B dogs for > 8 years. This

included animals with a F.IX null mutation, lacking F.IX transcript. Muscle gene transfer however resulted in the development of high titer inhibitors and undetectable F.IX levels.[15,22] Expression of a human F.IX transgene at systemic levels of 4-8% of normal was demonstrated in non-human primates following infusion of AAV-2 vector into the portal vein.[23] Consequently, a Phase I/II clinical trial based on hepatic artery infusion of AAV vector in subjects with severe hemophilia B was performed.[16] In contrast to the muscle trial, enrollment was not restricted to subjects with F.IX missense mutation. One patient expressed F.IX at levels >10% of normal for ~1 month after gene transfer, no bleeds occurred during this period of time. However expression of F.IX was gradually lost during weeks 5-8 following gene transfer. While subjects did not have an immune response to F.IX, activation of AAV capsid-specific cytolytic $CD8^+$ T cells was observed. These $CD8^+$ T cells presumably targeted cells displaying the specific MHC class I-restricted capsid epitope.[24]

The liver has emerged as a preferable target for gene transfer, when evaluated for immune responses to the transgene product. Dr. Herzog's laboratory has previously demonstrated that hepatic AAV gene transfer can induce immune tolerance to F.IX and other proteins. Immune tolerance results in lack of $CD4^+$ and $CD8^+$ T cell responses and antibody formation even after challenge by administration of F.IX in adjuvant or by secondary viral gene transfer. The advantage of this approach is that the protocol provides therapy and immune tolerance at the same time. There was evidence for T cell anergy and deletion.[25] In addition regulatory $CD4^+CD25^+$ T cells are required for tolerance induction and actively suppress antibody formation to F.IX. These regulatory cells are induced by hepatocyte-derived transgene expression. Other data demonstrated that the protocol does not provide tolerance in all genetic backgrounds of experimental animals and varies depending upon the type of mutation.[26,27]

Additionally, the immune response to the AAV capsid in humans is a cause of concern and may limit the success of hepatic gene therapy in human subjects. Therefore, a superior protocol is needed for tolerance induction that can be applicable regardless of gene or protein based therapy and not bound by the background of the mouse or by the underlying factor mutation. The novel protocol developed in this thesis was tested for efficient immune tolerance induction to F.IX in a strain of hemophilia B mice (C3H/HeJ-F9$^{-/-}$) that had previously been refractory to hepatic induced tolerance.

Overview of Immune Responses in AAV Mediated Gene Transfer

AAV vectors are derived from a non-pathogenic replication deficient parvovirus. Numerous serotypes, mostly isolated from humans or non-human primates, have now been characterized to improve transduction of specific organs and circumvent immune responses. The popularity of AAV as a vector stems from a broad host range, non-pathogenic nature, ability to transduce dividing and non-dividing cells, low innate immunity, and low efficiency of transduction of professional APCs such as DCs or macrophages, possibly due to a post entry block.[28] Preclinical trials in animal models of human disease have shown long-term correction of genetic disease using AAV vectors, and clinical trials have been carried out in a number of diseases.[10,29] Nonetheless, immune responses to AAV vectors have been observed in animals as well as in clinical trials and may negate therapy.

Innate Immune Responses to Adeno-Associated Virus

AAV is a weak innate immunogen; microarray studies have shown that AAV does not elicit the robust type I IFN response as is seen for adenoviral vectors.[30] Similarly, cytokine and chemokine responses in the transduced tissue are limited and highly transient. AAV triggers Toll-like receptor signaling (e.g. TLR-9, which senses DNA).[31] AAV has also been shown to interact with complement proteins.[32] The complement cascade, an important component of the

innate immune system, leads to opsonization of foreign bodies and lysis of target cells. The three complement pathways include the classical, alternative, and lectin binding pathways, all of which involve C3 convertases. Recent data show that the AAV2 capsid binds to the C3 complement proteins C3, C3b, iC3b and complement regulatory factor H, hence increasing the uptake of AAV into macrophages and enhancing their activation.[32] C3-AAV capsid interactions are direct and can occur independently of anti-AAV antibodies. However, complement activation by AAV is primarily antibody dependant (classical pathway). Complement-dependent activation of macrophages is not restricted to the AAV2 serotype. For example, AAV1 and AAV8 have been found to induce inflammatory gene expression in macrophages. Deficiency of C3 or complement receptor 1/2 results in the impairment of the humoral response to AAV.[32] C3 and CR 1/2 are essential for humoral but not innate immune responses to AAV *in vivo*.

Adaptive Immune Responses to AAV Vectors and their Transgene Products

Humoral immune responses against AAV capsid or the transgene product can occur following exposure to AAV vectors. Such responses differ depending on the target organ, location within the target organ, route of administration, serotype, transgene and expression cassette, and dosing schedule of injection (Fig. 1-2).[28] Humans are natural hosts to AAV. A recent study concerning the prevalence of neutralizing antibody titers (NAB) to the various AAV serotypes spanning humans in 4 continents has shown that the most prevalent NABs are to AAV2 followed by AAV1, while AAV8 and AAV7 have the least prevalent responses.[33] Interestingly, the structurally modified AAVrh32.33 serotype was rarely neutralized by human sera.[33] However different studies in mice and rhesus monkeys showed robust T cell responses to AAVrh32.33 capsid and transgene.[34,35]

IgG1 is the predominant antibody subclass response against AAV capsid antigen in humans.[36] Pre-existing NAB may not necessarily block *in vivo* gene transfer to some organs such

as skeletal muscle following intramuscular injection. However injections into blood vessels e.g. portal or peripheral vein injections and direct injection into liver parenchyma resulted in reduced transduction due to the presence of pre-existing NAB. Local delivery of the vector outside blood vessels may reduce exposure to NAB.[16,17,21] In addition to isolation of novel serotypes, shuffling of capsid sequences between serotypes and molecular evolution techniques are being employed to create AAV particles that are more resistant to neutralization by human sera.[37]

While long-term expression in skeletal muscle and a lack of inflammatory responses were observed in a clinical trial in hemophilia B patients using an AAV-2 vector, in a subsequent trial, initial therapeutic expression of the factor IX (F.IX) transgene declined starting 6 weeks after hepatic gene transfer. This decline of F.IX expression in a patient enrolled in the highest dose cohort was accompanied by transient elevations of liver enzyme levels, suggesting destruction of hepatocytes.[38] Another subject, who had a similar low titer of pre-existing NAB to AAV-2, was subsequently treated with a somewhat lower vector dose and showed a lower, but measurable increase in liver enzyme levels, which correlated with emergence of AAV2 capsid-specific $CD8^+$ T cells in peripheral blood, indicating T cell-mediated immunity.[38] Capsid specific $CD8^+$ T cells may have been activated by the infused vector and responded to the vector-transduced hepatocytes.[39] About 2.5 years after initial vector infusion, capsid-specific functional $CD8^+$ T cells were still present and cross-reacted with a common epitope of AAV serotypes 1, 6, 7 and 8, suggesting that secondary infusions with different naturally occurring serotypes may not circumvent the T cell response.[38] AAV capsid-specific $CD8^+$ memory T cells are present in humans at very low frequency but may become reactivated upon AAV gene transfer. Hepatic AAV2 infusion over a range of doses in mice transgenic for human HLA-B*0702 MHC locus failed to elicit capsid-specific $CD8^+$ T cell responses.[38] It is likely that natural infection with

AAV in the presence of a helper virus causes T cell responses in humans, which would not be the case in animals that are not natural hosts for AAV. Although mice immunized with AAV capsid or adenoviral vectors expressing AAV capsid developed $CD8^+$ T cells against capsid epitopes, these failed to eliminate AAV transduced hepatocytes in several studies.[8,40-42] This lack of an animal model that reproduces the observations in humans has hampered preclinical studies on immune responses to AAV transduced liver. Considering that AAV vectors do not express capsid, input capsid derived from vector particles would have to be efficiently cross-presented by the transduced cell to $CD8^+$ T cells via MHC I molecules. AAV capsid is ubiquitinated and degraded by proteosomes, which may occur over a period of time.[43] These observations support a model of CTL-mediated destruction of transduced cells, but do not explain why capsid-specific $CD8^+$ T cells failed to attack AAV-transduced liver in experimental animals.

Recently, several strategies have been suggested to avoid CTL responses to AAV capsid antigen. For example, alternate serotypes that do not contain a heparin binding site are processed differently by dendritic cells and activate $CD8^+$ T cells less efficiently.[44] Elimination of surface exposed tyrosine residues on the capsid, enhanced gene transfer to the nucleus and substantially reduced accumulation of ubiquitinated capsids in the cytoplasm.[45] AAV capsids with more rapid uncoating/degradation kinetics may also prove advantageous. Mycophenolate mofetil (MMF) and Cyclosporine A blocked T cell responses at least at lower vector doses.[46,47] AAV1 mediated muscle gene transfer in lipoprotein lipase (LPL) deficiency patients resulted in capsid specific and dose dependant activation of $CD4^+$ and $CD8^+$ T cells. [48]

Disorders of the central nervous system (such as Parkinson's disease) are promising targets for AAV-based gene therapies, and despite the fact that the brain is an immune privileged site, immune responses to gene transfer vectors have been observed. AAV vectors may cause

transient innate immune responses at high vector doses in naïve mouse brain parenchyma, and additional injections to the opposite hemisphere induced a significantly greater response and reduced transgene expression.[49] It has been speculated that the antigen that sparks a brain immune response to rAAV is the capsid protein when injected into the brain striata. Consistent with this hypothesis, delayed re-administration of vector, or switching serotype for a second gene transfer to the striata neither resulted in loss of transgene expression or striatal inflammation, hence overcoming the danger of pre-existing immunity. The authors of these studies also concluded that intracellular processing of AAV capsid generates the immunogenic antigen, and that capsid serotypes that are processed more quickly than rAAV2/2 are less immunogenic.[50]

Inhibitors in Hemophilia

Inhibitors in hemophilia B occur in about 9 -23% of those with severe disease; up to 50% of patients with F.IX inhibitors have anaphylactic reactions. In protein therapy, 1.5-4% of hemophilia B and 20-30% of hemophilia A patients develop inhibitors to the therapeutic protein. These antibodies generally present during the first two years of treatment after <20 days of exposure to product. High-titer inhibitors (>10 Bethesda units, BU) prevent treatment with clotting factor products. In this case, treatment options include bypass reagents (prothrombin complex concentrates or recombinant activated factor VII, F.VIIa). Because of the expense and thrombotic risks associated with prolonged administration, these reagents are only used to provide short-term hemostasis. For example, treatment of a single bleeding episode with recombinant F.VIIa may cost >$50,000. Generally, inhibitor patients are at higher risk for bleeding-related morbidity and mortality. Short-term treatment with porcine factor products is no longer performed due to potential pathogen contamination. Immune Tolerance Induction (ITI) protocols are based on frequent high dose infusion of F.VIII or F.IX, and often require amounts of products exceeding $1,000,000. These protocols are often less effective for treatment of

inhibitors to F.IX. In addition, some hemophilia B inhibitor patients experience anaphylactic responses to F.IX. Pre-clinical studies in animals have shown that inhibitor formation in gene therapy is dependent on the gene transfer vector, route of administration, vector doses, and other factors.

Preventing Immune Responses in AAV Mediated Gene Transfer

Immune responses against AAV-encoded transgene products vary substantially and are influenced by the target organ, route of delivery and dosing schedule. For example, in Hemophilia B mice with a F.IX gene deletion, intramuscular (IM) vector administration caused a local immune response characterized by activation of $CD4^+$ T and B cells to F.IX, which eliminated systemic expression. B cell activation has also been a complication upon over-expression of erythropoietin (epo) in skeletal muscle, which induced an autoantibody against epo, resulting in autoimmune anemia. Although $CD8^+$ T cell responses occur and are of particular concern in inflamed muscle (which is typical for some forms of muscular dystrophy), these T cells are often not fully functional in healthy muscle.[47,51,52] Recent studies found that IM injection of AAV vectors often induces transgene product-specific $CD8^+$ T cell that express markers of functional exhaustion and T cell suppression, and ultimately undergo programmed cell death in skeletal muscle.[53,54] Several immune suppression protocols have been successfully used in different animal models to block humoral and cellular immune responses to transgene products expressed in skeletal muscle.[47] This thesis demonstrates the prophylactic use of immunosuppressant drug rapamycin in combination with IL-10 in presence of F.IX dominant $CD4^+$ T cell epitopes induces T_{reg} and long term tolerance to the F.IX transgene product expressed in muscles of hemophilia B mice.[55]

As opposed to muscle gene transfer, hepatic gene transfer with AAV vectors has been shown to induce immune tolerance to a number of transgene products via induction of T_{reg} and

other mechanisms.[8,56-58] In addition to direct tolerization of transgene product-specific $CD4^+$ T cells by induction of non-responsiveness (anergy) or deletion, B and T cell responses (including CTL responses) are actively suppressed by induction of $CD4^+CD25^+$ regulatory T cells (T_{reg}) with an immune suppressive phenotype.[8] Antibody-mediated depletion studies suggest that T_{reg} are critically required for tolerance to the transgene product following hepatic gene transfer.[8,59] IL-10 expression by T_{reg} and Kupffer cells may directly suppress immune responses in the liver. Once tolerance is established, the transgene product can safely be expressed in sites that would otherwise predispose to immune responses [60,61]

In some cases, one may simply avoid immunity by taking advantage of immune privileged sites. The ocular disease Leber's Congenital Amaurosis (LCA), caused by an autosomal recessive mutation of RPE65, is being successfully treated in several ongoing clinical trials. Single eye injections of rAAV2-CBSB-hRPE65 resulted in an increase of visual sensitivity in several patients.[62-65] Due to the immune privileged nature of the eye, one should be able to improve treatment by re-injection in the previously injected eye or the partner eye, as has been suggested by animal studies in sub-retinal vector administration. This route causes a deviant immune response, resulting in a lack of NAB formation. However, intravitreal administration of AAV2 vector in mice resulted in a humoral response against the capsid that blocked transgene expression on re-administration of vector in a partner eye.[66]

Immune Tolerance and Regulation of Transgene Product Directed Responses

Blocking immune responses to the coagulation factor protein is key to successful therapy for hemophilia. Induction of $CD4^+$ T cell tolerance is desirable because inhibitor formation is dependant on CD4 help. To this end, the ability to activate T_{reg} that aid in inducing/maintaining tolerance may be crucial.[67] It is known that certain types of T_{reg} can be induced, such as Th3 cells that secrete TGF-β or Tr1 (regulatory T cells type 1) that secrete large amounts of IL-10.[68-70]

Besides these induced T_{reg} that suppress by cytokine secretion, $CD4^+CD25^+$ T_{reg} cells occur naturally (5-10% of $CD4^+$ T cell population).[71] These cells are generated during thymic development to maintain tolerance to self-antigens. $CD4^+CD25^+$ T_{reg} constitutively express the IL-2R α-chain, glucocorticoid-induced tumor necrosis factor receptor (GITR)[72,73], and CTLA-4 and are potent suppressors of $CD4^+CD25^-$ T cells. Suppression occurs via a cell contact-dependent mechanism *in vitro*, albeit cytokine-mediated suppression has been postulated *in vivo*. Expression of transcription factor Forkhead Box P3 (FoxP3) defines this lineage of T_{reg} and represents a master control gene for development and function of $CD4^+CD25^+$ T_{reg}.[74,75] There is now growing evidence in the literature that this type of T_{reg} is not only naturally present but can also be induced upon presentation of exogenous antigen.[76,77] Furthermore drugs like rituximab, MMF, dexamethasone and others show some promise in reducing inhibitor responses.[78] This thesis project explores a novel strategy toward tolerance to F.IX in gene and protein replacement therapy and seeks to take advantage of immune regulation to facilitate antigen specific tolerance induction using rapamycin/cytokine/specific peptide combinations.

Potential Roles of Rapamycin, IL-10, IL-2 and Fc-GITR-L and Flt3L

Rapamycin (sirolimus) is an immunosuppressant used to prevent transplant rejection. It is a macrolide antibiotic produced by *Streptomyces hygroscopicus*. Rapamycin suppresses T cell signaling in response to cytokines and growth factors through binding to intracellular protein FKBP12 (which in turn inhibits the protein kinase mTOR), but does not interfere with TCR signaling or calcineurin activity. Rapamycin inhibits IL-2 stimulated signal transduction by causing dephosphorylation of p70 S6 kinase and of eIF-4E BP1, which are both regulated by mTOR. These effects lead to a block in cell cycle progression and consequently to inhibition of T cell proliferation.[25,79,80] Interestingly, rapamycin does not prevent induction of T_{reg} such as

$CD4^+CD25^+$ T cells, nor does it prevent activation induced cell death (AICD).[25] Published data from Dr. Roncarolo's lab demonstrated that *in vitro* antigen presentation in the presence of rapamycin leads to selective expansion of $CD4^+CD25^+$ T_{reg}.[81] Rapamycin was recently found to ameliorate induced autoimmune encephalomyelitis (EAE) in mice with the elimination of $CD4^+$CD45RB(high) effector T cells and selective expansion of T_{reg} cells bearing the $CD4^+$CD45RB(low)$FoxP3^+CD25^+CD103^+$ phenotype.[82]

The cytokine IL-10 has immunomodulatory effects and can be produced by various cell types including dendritic cells, macrophages, various subsets of T cells and B cells produce it. IL-10 is known to inhibit MHC class II and co-stimulatory molecules B7-1/B7-2 expression on APCs. It also limits the production of pro-inflammatory cytokines like IL-1, IL-6, IL-12 and TNF-α. IL-10 can act directly on $CD4^+$ T cells, inhibiting IL-2 production and hence inhibiting proliferation. It can also act via autocrine signaling in DC to prevent chemokine production and prevent its trafficking to lymphnodes.[83] Rapamycin combined with interleukin IL-10 applied in a non-specific fashion prevented Type 1 Diabetes via increasing $CD4^+CD25^+$ T_{reg} and T Regulatory Type 1 (Tr1) cell numbers in the absence of chronic immunosuppression in non-obese diabetic (NOD) mice.[7]

IL-2 is a secreted cytokine produced by several cell types including $CD4^+$ T cells, some other cell types like $CD8^+$ T cells, NK and DCs. IL-2 is known to have autocrine as well as paracrine function. It is important for the regulation of immune responses and proliferation of T and B lymphocytes. The alpha chain of the IL-2 receptor (IL-2R; CD25) is a known marker for $FoxP3^+$ T_{reg}. T_{reg} do not express IL-2 and are thus dependant on exogenous IL-2 produced by other cells. IL-2 plays an important role in controlling the proliferation and survival of T_{reg}.[83] IL-2 and IL-2R knockout mice exhibit severe autoimmunity due to the absence of

$CD4^+CD25^+FoxP3^+$ T_{regs}. IL-2 is also known to potentiate activation induced cell death in T cells.[84]

Glucocorticoid-Induced TNF Receptor (GITR) is expressed on normal T lymphocytes and in high levels on T_{reg} cells. The corresponding ligand for GITR (GITR-L) is expressed on DCs and other APCs. Initially it was hypothesized that that the interaction of GITR-L on DCs with GITR of T_{reg} resulted in the abrogation of T_{reg} suppression. However, GITR-specific antibodies have been found to stimulate proliferation of T_{reg} cells in the presence of IL-2 but in the absence of a TCR signal. Soluble GITR-L has been observed to cause proliferation of T_{reg} cells in the presence of IL-2 and rIL-2 can break the anergic state of T_{reg}.[85] Others have shown that GITR-L is important in rendering $CD25^-$ effector T cells resistant to suppression by T_{reg}. [86] The mechanism of GITR/GITR-L control of immune mechanisms is not well understood. Fc-GITR-L is a chimeric molecule generated by Dr. Gongxian Liao from the laboratory of Dr. Cox Terhorst, Beth Israel Deaconess Medical Center, Harvard Medical School. It consists of the Fc region of an antibody, fused with GITR-L. The Fc-GITR-L molecule was observed to induce $CD4^+FoxP3^+$ T_{reg} proliferation *in vitro and in vivo*.[87]

Fms-like tyrosine kinase 3 ligand (Flt3L) is a cytokine required for DC homeostasis in lymphoid tissues. Genetic deletion of Flt3L or treatment with Flt3 tyrosine kinase inhibitors has been observed to cause a reduction of plasmacytoid dendritic cells (pDCs) and DCs in lymphoid organs. Increased levels of Flt3L cause a massive proliferation of pDCs and conventional DCs (cDCs) in lymphoid and non-lymphoid organs. Repeated injections of Flt3L indirectly lead to an expansion of peripheral naturally occurring T_{reg}.[88] Flt3L exists in a soluble or membrane-bound form. It is expressed by multiple tissue stromal cells and by activated T cells.[89]

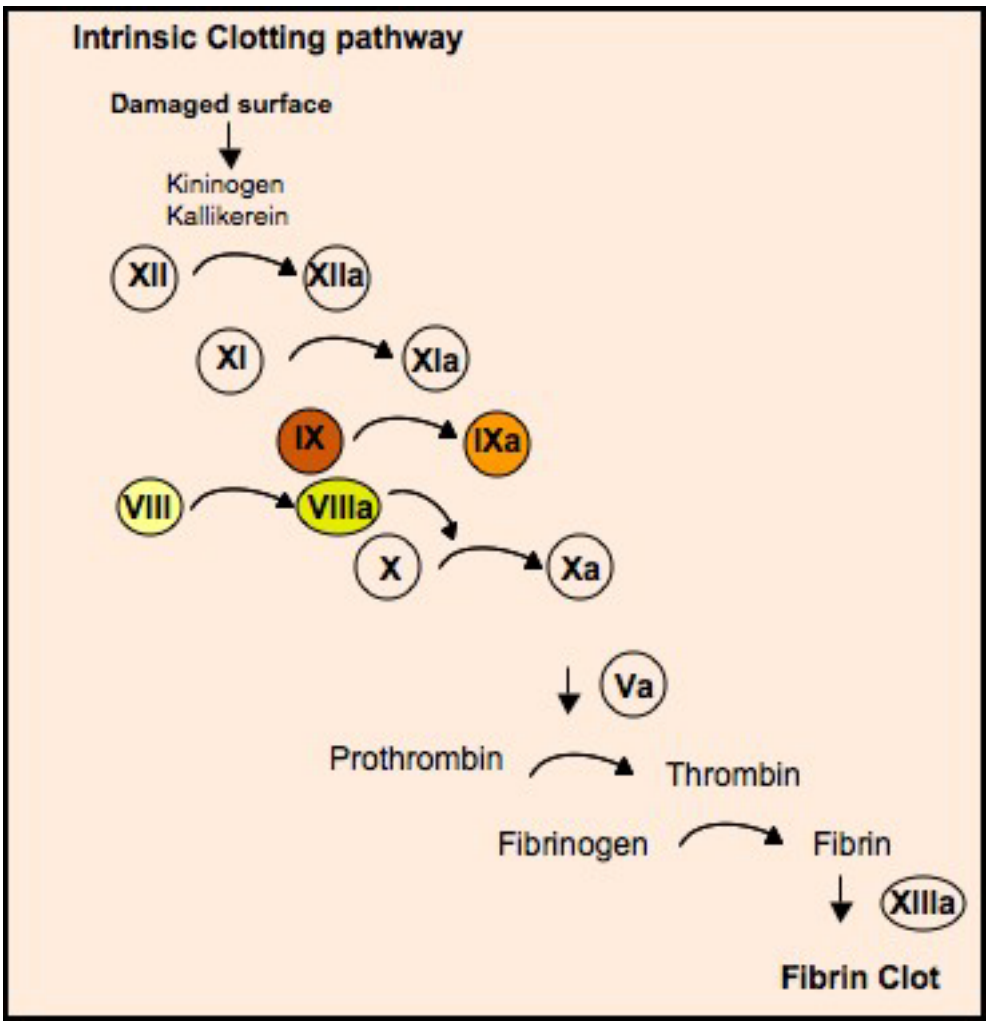


Figure 1-1. Simplified diagram of the intrinsic pathway in the blood-clotting cascade.

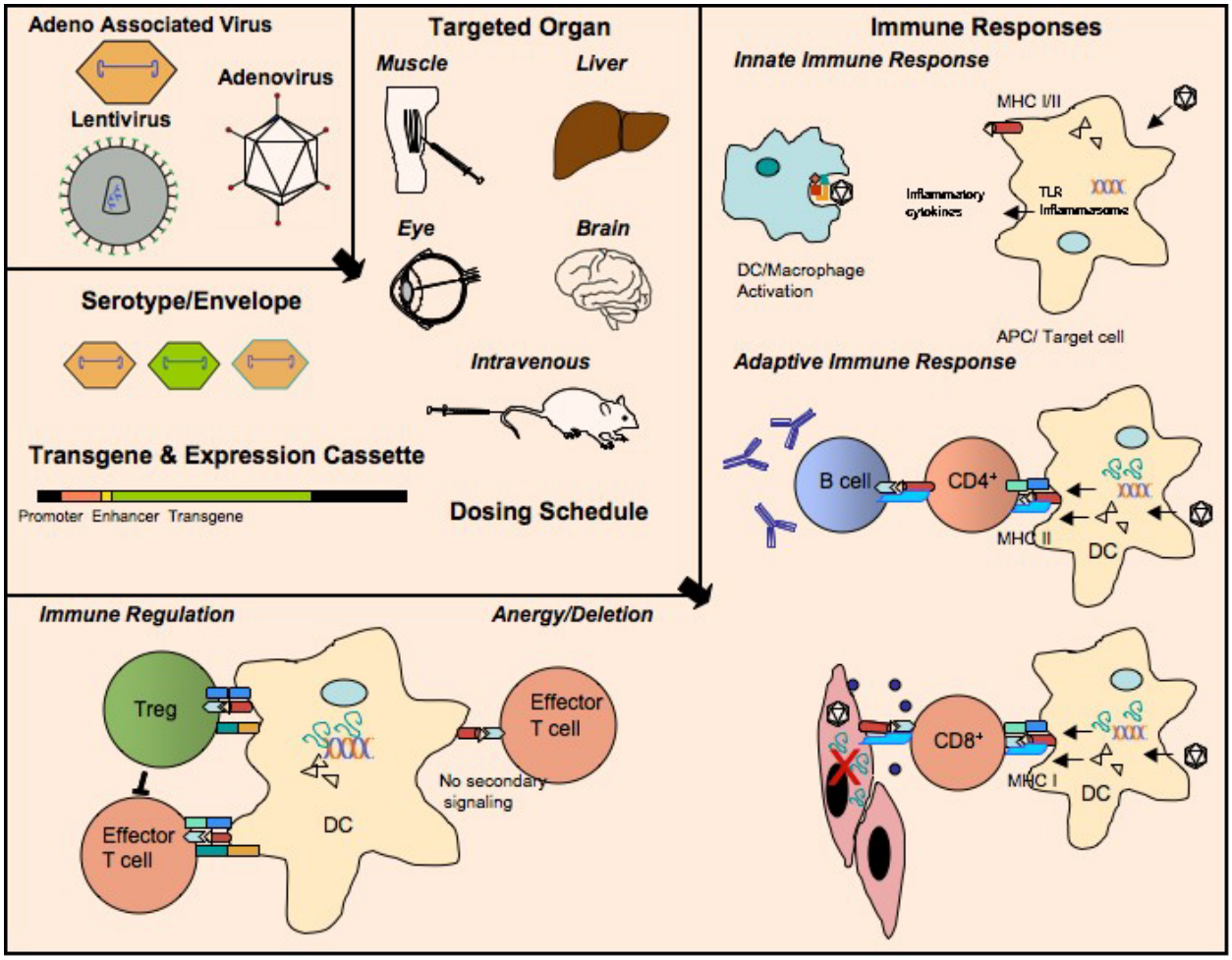


Figure 1-2. Overview of immune responses to viral vectors. Targeting specific organs, engineering viral envelopes, switching serotypes, modifying the transgene cassette, utilizing organ specific promoters or tolerizing dosing schedules increase possibility of immune avoidance or cause immune suppression to the viral vector and transgene product.

CHAPTER 2
MATERIALS AND METHODS

Reagents

Peptide A2-54 (TEQKRNVIRIIPHHNYNAAI), the dominant $CD4^+$ T cell epitope for human F.IX (hF.IX) in C3H/HeJ mice, peptide 2A-58 (NKYNNHDIALLELDEPLVLNS), an epitope for C57BL/6 mice and chicken ova peptide- amino acids 323 to 339 (ISQAVHAAHAEINEAGR) were synthesized by Anaspec (San Jose, CA) or the ICBR protein core facility at University of Florida (Fig.2-1). [90] Murine IL-10 (Sigma Aldrich, St. Louis, MO), recombinant mouse IL-2 (Roche Diagnostics, Mannheim, Germany), human FLT3L carrier free (Cell signaling technology, MA) rapamycin (LC Laboratories, Woburn, MA) stock solution were made in 0.02% Carboxy-methyl cellulose (CMC) and 0.25% Tween80 (Sigma, St. Louis, MO). Rapamycin solution was sonicated before being aliquoted and stored at -20^{o}C. Recombinant coagulation Factor IX (Benefix; Wyeth, Philadelphia, PA) was sterile diluted Phosphate buffered saline (PBS). AAV-CMV-hF.IX vector was as previously published.[91] It contains the hF.IX cDNA (plus a 1.4-kb portion of the F9 gene) expressed from the cytomegalovirus immediate-early enhancer/promoter. AAV serotype 1 vector was produced by triple transfection of HEK-293 cells, purified by gradient centrifugation, and titered by quantitative slot-blot hybridization. [92] Peptide, interleukin and virus dilutions were made in Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen, NY) unless otherwise stated.

Mouse Strains

Male BALB/c mice transgenic for chicken ovalbumin-specific DO11.10 $CD4^+$ TCR (OVA peptide, amino acids 323-339) and knock out for Rag-2 gene (recombinase activating gene 2) were used. These DO11.10-tg Rag-$2^{-/-}$ animals do not develop endogenous mature T or B cells.

BALB/c DO11.10-tg Rag-2$^{-/-}$ males were obtained from Taconic (Germantown, NY). These animals were used to characterize the mechanism of drug action.

In F.IX knockout mice on a C3H/HeJ background (C3H/HeJ-F9$^{-/-}$) the promoter and the first three exons of the F9 gene were replaced by neoΔHPRT, which is a functional neo gene plus a partially deleted hypoxanthine phosphoribosyl transferase minigene as published. [93] These mice show no transcription of the F9 gene and are completely F.IX antigen deficient. These have <1% clotting activity and are susceptible to internal hemorrhage.

Male mice homozygous for Rag1^{tm1Mom} on a C57BL/6 background (B6.129S7-*Rag1*^{tm1Mom}/J) were from Jackson Laboratories (Bar Harbor, ME). These animals do not produce mature T cells or B cells and have no CD3^{+} or T cell receptor (TCR) alpha-beta positive cells. The thymus of the mutant mice contains 15 to 130 times fewer cells than heterozygous or wild type siblings. Their thymocytes are CD8^{-}CD4^{-}, and most are IL-2 receptor-positive.

Male C3H/HeJ and Male C57BL/6 were from Jackson Laboratories (Bar Harbor, ME). Animals were housed in specific pathogen free conditions and in accordance to local IACUC protocols.

Administration of Drugs

Drug cocktails were administered intraperitoneal (IP) in 200 μl of sterile PBS 3-times per week at the following doses. Ovalbumin (ova) peptide (amino acid residues 323-339) or hF.IX-specific peptide (2A-54) were given at 100 μg/dose, rapamycin at 4 mg/kg/dose, and IL-10 at 50 ng/kg/dose, IL-2 at 50ng/kg/dose, FLT3L at 2μg/dose. Experiments involving different routes of injection included IP, Intravenous (IV), and subcutaneous (SQ) on the back of the neck region and oral gavage (oral). Oral gavage of rapamycin was given in 100 μl volumes everyday for 1 month or thrice a week, using a special feeding needle with an olive, round tip (Fine Science

Tools). Viral vector diluted in PBS was administered at $1x10^{11}$ vector genomes (vg)/mouse in 100μl volume, by intramuscular (IM) injection into quadriceps and tibialis anterior muscles using a Hamilton syringe or by IV administration in the case of AAV8 serotypes. [91] Splenic capsule injections of viral vector in hemophilic mice were done 1 hour after infusion of normal mouse plasma to preclude excessive bleeding and increase survival. Platelet activating factor antagonist (Anti-PAF; 50 μg/mouse; CV3988, Sigma, USA) was prepared in absolute ethyl alcohol and PBS. Anti-histamine Triprolidiene hydrochloride (Sigma) was prepared in PBS; a final dose of 100 μg/mouse was injected in conjunction with anti-PAF via tail vein injection. The anti-histamine combination was used 5 minutes before hemophilic mice were treated with hF.IX whole protein at the 4^{th} tail vein injection in order to avoid hypersensitivity reactions.

Blood Sample Collection

Blood samples were obtained using heparinized microhematocrit capillary tubes (Fisherbrand, Fisher Scientific, PA) via the retro-orbital plexus for ELISA or via tail-bleed (after heating the mice for a few minutes under a 125W heating lamp and anesthetizing with isofluorane) into 3.8% sodium citrate buffer for clotting assays or into EDTA tubes; 1.6 mg EDTA/ml blood (Sarstedt, Germany) for whole blood analysis. The tail wound was then sutured and sealed with silver nitrate.

Analysis of Plasma Samples

Immunocapture assay for determination of anti-hF.IX titers was performed as follows. Microtiter plates were coated with recombinant human FIX protein (1 μg/ml, Benefix: Wyeth, Philadelphia, PA), and mouse plasma samples were applied at a 1:20 dilution. In parallel, wells were coated with 2-fold serial dilutions of purified mouse immunoglobulin IgG (Sigma-Aldrich, St Louis, MO) starting at 2000 ng/ml for generation of a standard curve. Anti-hF.IX was detected

with rat anti mouse horseradish peroxidase–conjugated antibody at 1:2000 dilution (R&D, USA). Antibody levels were measured by optical density (OD) reading at 450 nm following incubation with o-phenylene diamine substrate (Sigmafast OPD: Sigma-Aldrich) using a microplate reader (Bio-Rad, Hercules CA).

Levels of hF.IX were determined by an Enzyme linked Immunosorbent Assay (ELISA) as follows. Microtiter plates were coated with 1:850 dilution of mouse monoclonal anti hF.IX (FIX-1 monoclonal anti-hF.IX Clone HIX-1; F2645 Sigma). Blocking buffer (5% non-fat milk in wash buffer) was used to block the plate. The standard was diluted from 200 to 3.125 μg/ml (TriniCHECK Level 1; Trinity Biotech, Wicklow, Ireland). Samples were diluted to 1:10. Dilute goat anti-human FIX-HRP (Enzyme Research: GAFIX-APHRP 230) 4.5 μl in 10.5ml dilution buffer. F.IX levels were measured using O-phenylenediamine tablets (Sigmafast OPD from Sigma) with a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 450 nm.

Clotting Assays

Activated partial thromboplastin time (aPTT) was measured with a fibrometer (Fibrosystem; BBL, Cockeysville, MD). Briefly, 50 μl of plasma sample was incubated at 37^0C with 50 μl of F.IX deficient plasma (Hematologic Technologies Inc., Vermont) and 50 μl TriniCLOT automated aPTT reagent (Trinity Biotech, Wickow, Ireland) for 3 minutes. Fifty microliter of 25mM $CaCl_2$ is added and the time taken for clotting to occur is measured using the fibrometer (Fibrosystem^{TM}, Block Scientific Inc.).

Bethesda assay for measuring the titer of inhibitory antibodies was performed as published.[91] Briefly, Sample dilutions are made using Imidazole buffer (MDA, Baltimore, MD) ranging from N to N/8. Control samples at 1:2 and 1:4 dilutions of normal human plasma (TriniCHECK Level 1, Trinity Biotech, Ireland) and Imidazole buffer were made. Thirty

microliter of TriniCHECK was added to all experimental samples and control samples with 60 μl to the last dilution of experimental sample. All samples and controls were incubated at 37^0C for 2 hrs and then transferred to ice. Standards were prepared from 20% to 0% of normal human plasma in Imidazole buffer. Sixty microliter of F.IX deficient plasma was added to all standard tubes. Coagulation times were then determined as aPTT. Residual activity was computed on a standardized chart. One Bethesda unit (BU) represents a residual hF.IX activity of 50%.

Flow Cytometry

Cells were isolated from the spleen, inguinal, popliteal lymph nodes, thymus and peripheral blood using standard protocols. Briefly, spleen/lymph nodes were isolated and stored in cold PBS and single cell suspensions were prepared using a 70 μm cell strainer (BD Bioscience, MA). Red blood cells are lysed using an ammonium chloride based lysis buffer (Pharmlyse; BD Biosciences, MA), remaining cells were washed with cold PBS or 2-MLC medium (heat inactivated 2 % fetal calf serum, sodim pyruvate, HEPES, penicillin, streptomycin, non essential amino acids and 2-β-mercaptoethanol added to Dulbecco's modification of Eagle's medium). Lymph nodes were incubated in collagenase D (Roche) 1 mg/ml for 30 min before straining. Viable cells were enumerated using a hemocytometer in the presence of trypan blue to differentiate between the live and dead cells. Staining for surface and intracellular molecules was carried out according to manufacturers' protocols. Antibodies against mouse antigens CD4 (conjugated to APC or FITC), CD69-PE, CD62L-APC, CD44-PE, CTLA-PE, CD95-PE, CD25 (-PE or –FITC), FoxP3 (-Alexa Fluor 647 or –FITC) were from eBioscience (San Diego, CA). Anti-murine GITR-cy7 was from BD Biosciences, USA. Isotype controls included Rat IgG1-FITC, Rat IgG2b-PE-Cy7, Rat IgG2a-FITC, Rat IgG2b-PE, Rat IgG2a- APC, Rat IgG2b- PE Cy7, Rat IgG2a-Alexa Fluor 647. Other controls were unstained splenocytes and anti-Rat Ig

Compensation Particles (BD Biosciences/Pharmingen). Apoptosis was measured with the AnnexinV-PE Apoptosis detection kit (BD Biosciences/Pharmingen, USA). Data acquisition was carried out using the BD LSR II and BD FACSCalibur system. Data analysis was done with FACS DIVA 6.1 and Cell Quest software.

Cytokine Secretion Assay

Mouse Cytokine Secretion Assay-Detection Kit (PE) (Miltenyi Biotech, Auburn, CA) was used as per the manufacturer's recommendations. Briefly, spleens from DO11.10-tg $Rag^{-/-}$ mice were processed for cell culture using standard protocols. The cells were re-suspended in 5-MLC medium at 1 x 10^7 cells/ml and added to a round-bottom 96-well culture plate (1 x 10^6/well in triplicate) for *in vitro* stimulation with ova peptide (final concentration of 10 μg/ml). Three to six hours after peptide stimulation, IL-4 and IL-10 expressing $CD4^+$ T cell were identified by flow cytometry using anti-murine CD4-FITC, anti-murine IL4-PE, and anti-murine IL-10-APC.

Adoptive Transfer Assay

Male C3H/HeJ mice were treated with the Rapa/IL-10/2A54 drug protocol for 3 weeks, after which splenocytes were isolated. $CD4^+CD25^+$ cells were isolated using magnetic cell sorting regulatory T cell isolation kits (Mylteni Biotech, Auburn, MA). Isolated $CD4^+CD25^+$ T cells were enumerated using a hemocytometer and Trypan blue staining. Live $CD4^+CD25^+$ T cells (1x10^6 cells) were injected into recipient mice. Control animals were injected with $CD4^+CD25^-$ or $CD4^-$ cells. Animals were challenged with F.IX in complete Freunds Adjuvant (cFA; Sigma, St. Louis, MO), which was prepared by mixing 1ml of cFA with 1ml of diluted F.IX antigen in PBS (5 μg/100 μl). The mixture is sonicated and loaded in syringes. Fifty microliter of the mixture is injected subcutaneously into each of 4 spots on each mouse giving a

total dose of 200 μl for each mouse Fig. 2-3. Plasma is collected 3 weeks after challenge for further analysis.

In vitro Suppression Assay

Splenic $CD4^+$ cells were isolated using negative selection; $CD25^+$ T cells were isolated from the enriched $CD4^+$ population by positive selection using magnetic cell sorting (Miltenyi Biotech, Auburn, MA). Cells were resuspended in 2-MLC media and plated in triplicates in flat-bottom 96-well plates with a gradient of ratios of regulatory T cells, effector T cells and accessory cells. The Celltitre96 Aqueous Non-Radioactive Cell Proliferation assay (Promega, Madison, WI) was used to assess suppression of effector T cell proliferation in the presence of T_{reg} cells Fig. 2-2. The plate was read at 490 nm using an ELISA reader (Biorad, Hercules CA).

Statistical Analysis

Results are represented in means ±SD. Assessment of significant differences between means was done by unpaired student's t-test. *P* values of <0.05 were considered significant.

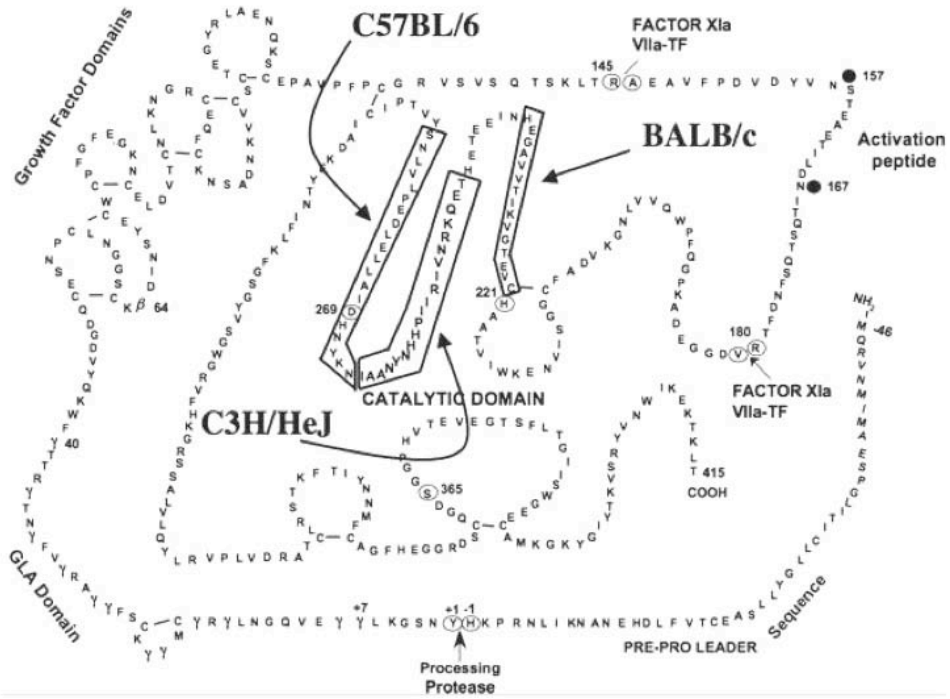


Figure 2-1. Diagram of human factor IX primary amino acid sequence with $CD4^+$ T cell epitopes. Boxes indicate peptides that contain dominant $CD4^+$ T cell epitopes in hemophilia B mice on C57BL/6, C3H/HeJ and BALB/c genetic background. Also indicated are domains and protease cleavage sites and numbering of some reference amino acids.[90]

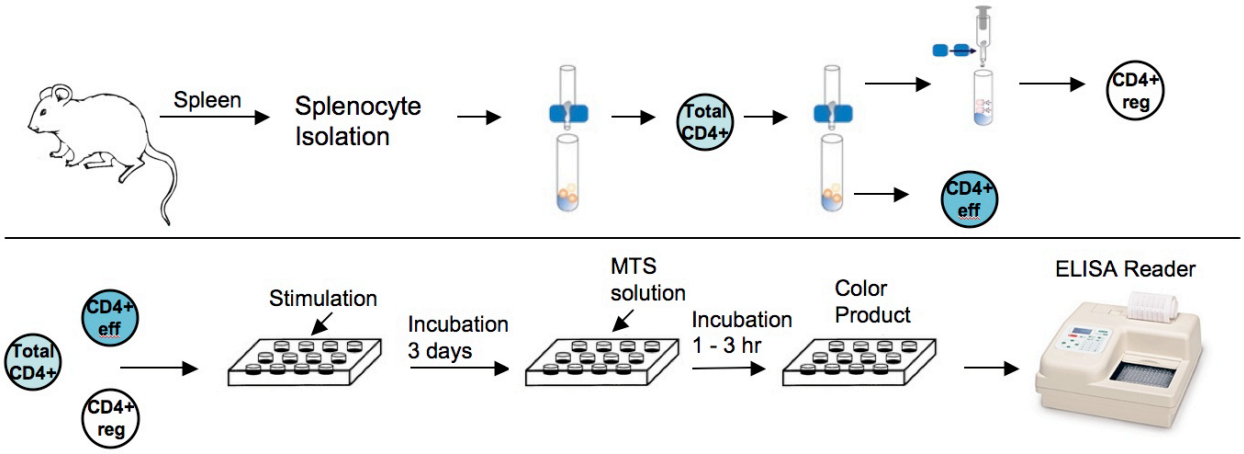


Figure 2-2. Diagrammatic representation of the effector T cell suppression assay

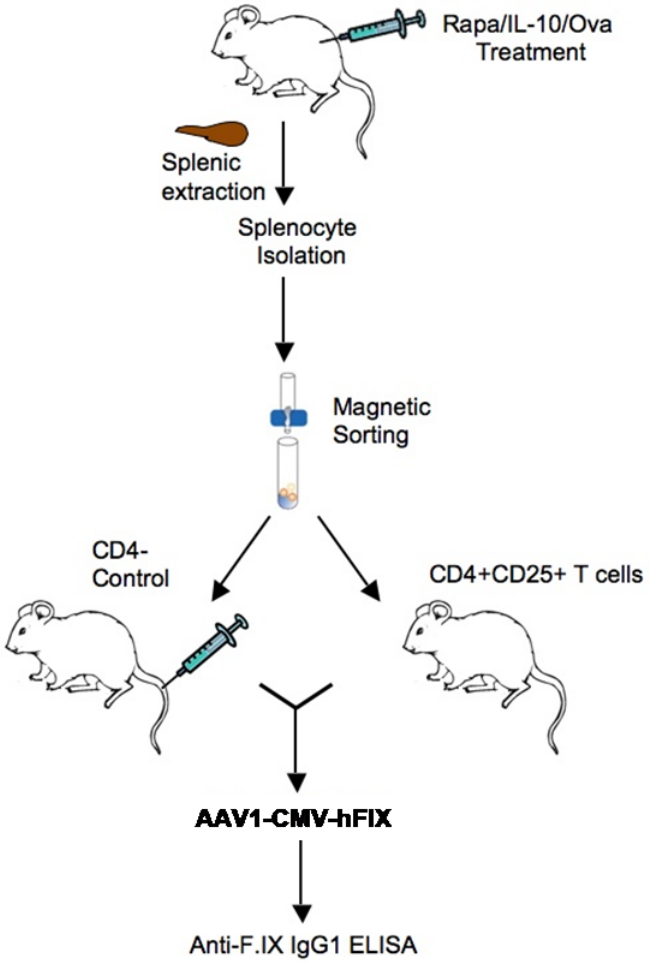


Figure 2-3. Diagrammatic representation of the adoptive transfer assay

CHAPTER 3
ESTABLISHMENT AND MECHANISTIC INVESTIGATION OF NOVEL TOLERANCE PROTOCOLS

Introduction

Therapeutic proteins introduced via protein or gene replacement therapy may be recognized as non-self in patients whose underlying mutations are null mutations. T cells are often critical components of these adaptive immune responses. Antigen presenting cells such as DCs pick up the offending protein that is not recognized as "self" by the patients' immune system. The protein is then processed and presented via MHC Class II on their surface. T lymphocytes confer cell-mediated immunity. Antigen specific $CD4^+$ T cells recognize the MHC Class II-antigen complex and are signaled to proliferate. $CD4^+$ T cells induce the proliferation of B cells that produce copious amounts of antigen specific antibody. The antibody produced is secreted and functions by neutralizing the therapeutic protein. The process of neutralization renders the injected protein or transgene product non-functional and hence ineffective. In order to combat this neutralization, higher factor doses at more frequent treatment intervals are required to provide therapy in patients with low titer antibodies. This increase in dosing for patients with anti-F.IX or anti-F.VIII inhibitors results in increased costs and reduced quality of life. Bypassing reagents such as activated prothrombin complex concentrates and recombinant factor VIIa (rF.VIIa) are used in patients with high titer antibodies. These bypassing reagents however have to be limited in dosing and frequency as they are associated with unwanted thrombosis. rF.VIIa has to be administered every 2-3 hours, which leads to problems with venous access. Immune tolerance induction therapy (ITI) involves the administration very high concentrations of factor possibly multiple times a day for many months to years. This therapy works for a percentage of F.VIII deficient patients but largely does not work for F.IX deficient

patients. A more effective protocol is therefore desirable to tolerize patients to the therapeutic proteins.

The Herzog lab has previously demonstrated the importance of $CD4^+CD25^+$ T_{reg} in tolerance induction to F.IX. Based on the hypothesis is that a clinically feasible protocol can be designed to reliably induce immune tolerance to coagulation factors; a novel and promising approach to efficiently induce antigen-specific $CD4^+$ T cell tolerance by transient administration of a cocktail of a specific peptide antigen, an immune suppressive drug, and a cytokine was developed. The function of the drug cocktail was to delete effector $CD4^+$ T cells that mediate the immune response while simultaneously increasing the number of functional T_{reg} identified as $CD4^+DC25^+FoxP3^+$. An approach based on TCR transgenic mice to study $CD4^+$ T cell responses to a systemically delivered peptide was adapted. In order to identify such a drug or drug/cytokine combination best suited to achieve these results, DO11.10 TCR transgenic BALB/c mice deficient in Rag-2 were used. These animals do not have any endogenous rearranged T cell receptors (TCR) and also lack T_{reg}.[94]

The DO11.10 TCR (encoded by rearranged Vα13 and Vβ8.2 genes) is specific for a chicken ovalbumin (ova) peptide ISQAVHAAHAEINEAGR (ova amino acids 323-339) presented by the MHC class II molecule $I\text{-}A^d$ to $CD4^+$ T cells, and is expressed in >90% of T cells in the thymus of DO11.10 TCR-transgenic mice. Ova-specific $CD4^+$ cells can be tracked and counted by FACS analysis using dual antibody stain (anti-murine CD4 and DO11.10 clonotype-specific KJ1-26 monoclonal antibody). For example, conclusive evidence for generation of $CD4^+CD25^+$ T_{reg} cells can be obtained in DO11.10 TCR transgenic BALB/c mice deficient in Rag-2 (and thus devoid of re-arranged endogenous TCR to other antigens). These mice, as shown by others, are deficient in $CD4^+CD25^+$ T_{reg} (<1% of $CD4^+$ T cells instead of the

normal 5-10%) due to lack of TCRs to self-antigen, and therefore contain almost exclusively $CD4^+CD25^-$ ova-specific T cells. The investigation detailed below tested whether this strain could be useful to screen for antigen/drug combinations that may be optimal for induction of $CD4^+$ T cell tolerance.

Depletion T_{eff} Cells and Induction of $CD4^+CD25^+FoxP3^+$ T_{reg} via Rapamycin/IL-10/Specific-peptide Protocol.

Using DO11.10-tg Rag-$2^{-/-}$ BALB/c mice, ova peptide (100 μg/mouse/dose) was co-administered with rapamycin (4 mg/kg/dose) by intraperitoneal (IP) injection. Administration was carried out 3 times per week for a total of 4 weeks (n=4 mice). Spleens were harvested and flow cytometry was utilized to quantify the proportion of $CD4^+CD25^+FoxP3^+$ cells. In an identical set of experiments, IL-10 (50 ng/kg/dose) in combination with ova peptide was administered; rapamycin was left out in this group. Finally, a combined protocol was tested, in which mice received ova peptide, rapamycin and IL-10 along with the specific peptide at the above-mentioned doses. Control mice received a mixture of rapamycin, IL-10 and an irrelevant peptide.

The two groups of mice that received the specific peptide and rapamycin showed a marked reduction (up to 4-fold) in ova-specific $CD4^+$ T cells when compared to the rapa/IL-10/irr treated controls (Figs. 3-1 and 3-2)**.** The effect was greater when IL-10 was included (rapa/IL-10/ova specific peptide group) . $CD4^+CD25^+FoxP3^+$ regulatory T cells were induced in all groups of mice except for the Rapa/IL-10/Irr peptide treated controls. The T_{reg} induction observed was 18-fold of $CD4^+$ T cells and up to 148-fold of total splenocytes in the Rapa/IL-10/ova specific peptide treated mice and exceeded the Rapa/ova and IL-10/ova mice by a fair margin (Figs. 3-1, 3-2). Effects of rapamycin/IL-10 were dependent on TCR signaling and therefore antigen-specific, as can be observed in the differing effects as a result of the presence or absence of the

specific ova peptide. IL-10/ova peptide-treated animals showed a Th2 response as was determined by the induction of $CD4^+IL\text{-}10^+IL\text{-}4^+$ cells (Fig. 3-3). Upon *in vitro* stimulation with ova, we were unable to detect $IL\text{-}10^+$ or $IL\text{-}4^+$ T cells in any experimental group, except for IL-10/ova-treated mice, which showed an ova-specific $CD4^+IL\text{-}10^+IL\text{-}4^+$ response, indicating Th2 activation (Fig.3-3). A substantial decrease in $CD4^+$ T cells was again seen in this assay for rapamycin/IL-10/ova-treated mice. No evidence for a Tr1 response was seen in rapamycin/IL-10/ova peptide treated mice, which had the strongest decrease in $CD4^+$ T cells and the most robust induction of $CD4^+CD25^+FoxP3^+$ cells. These increases in T_{reg} observed on administering the novel rapamycin/IL-10/specific peptide protocol are ~3-fold greater than had achieved previously by the Herzog lab with hepatic ova gene transfer in this strain of mice. Virtually all $CD4^+CD25^+$ expressed FoxP3. This protocol can serve as a powerful tool to shift the balance from transgene product-specific effector to regulatory T cells prior to therapy.

Effect of Rapamycin/IL-10/Specific-peptide in Lymphoid Organs

Next, the effect of the most optimal protocol on different lymphoid organs was investigated. Rapamycin/IL-10/ova was administered IP 3-times per week for 3 weeks to DO11.10-tg Rag-$2^{-/-}$ mice. Control animals received an irrelevant hF.IX peptide instead of ova. Subsequent flow cytometric analysis showed that $CD4^+$ cell frequencies were markedly reduced in peripheral blood and secondary lymphoid organs (spleen, inguinal and popliteal lymph nodes) and slightly reduced in the thymus (Fig. 3-4). Mice receiving an irrelevant peptide showed minor reduction (spleen) or no change (lymph nodes and thymus) in $CD4^+$ T cell numbers compared to naïve mice (Fig. 3-4). Mice treated with rapamycin/IL-10/irrelevant peptide, similar to naïve mice, had again no detectable $CD4^+CD25^+FoxP3^+$ cells (<0.4%) in any of the organs analyzed. In contrast, a substantial induction of T_{reg} to ~20% of $CD4^+$ T cells was observed in peripheral blood and all secondary lymphoid organs in rapamycin/IL-10/ova-treated mice (Fig. 3-5). T_{reg}

were also detectable in the thymus, albeit at lower frequency (~0.9%, with one animal achieving 7% of $CD4^+$ T cells compared to 0.1-0.3% in control mice, (Fig. 3-6 C).

Activation and Apoptosis of Ova-specific $CD4^+$ T Cells.

DO.11.10-tg RAG-$2^{-/-}$ mice were injected IP 3-times per week for 1 week with rapamycin/IL-10/ova. When analyzed 1 day after the last drug administration, splenic $CD4^+$ T cells showed an up-regulation of activation markers like CD25, CD44, and CD69, and the down-regulation of marker CD62L (which mediates homing of lymphocytes to sites of infection and is lost after activation occurs) compared to control mice receiving an irrelevant hF.IX peptide instead of ova, indicating T cell activation due to the drug cocktail administration (Fig. 3-7).

Rapamycin/IL-10/ova-treated mice also showed a 2- to 3-fold increase in Fas $(CD95)^+$ and in apoptotic (Annexin V^+) splenic $CD4^+$ T cells compared to controls (Fig. 3-8 A-B). These cells stained double positive for CD90 and Annexin V (Fig. 3-8 C). Taken together, the data demonstrate induction of activation induced cell death. As a result of Activation induced cell death, the portion of apoptotic $CD4^+$ T cells increased with increasing doses of drug cocktail administration hence resulting in a decline of $CD4^+$ T cell numbers over time (Fig. 3-9).

Induced T_{reg} Suppress T_{eff} and Show Lower Rate of Apoptosis.

While antigen presentation in the presence of rapamycin and IL-10 induced apoptosis of $CD4^+$ T cells and consequently a decline in $CD4^+$ T cell numbers, T_{reg} were induced to high frequency; therefore, there had to be a differential effect of the drug cocktail on T_{eff} vs. T_{reg} cells. To test this hypothesis, we measured the frequency of apoptotic cells among T_{reg} and T_{eff} cells 3 weeks after treatment with rapamycin/IL-10/ova (specific peptide) cocktail. In 3 of 4 mice, splenic $CD4^+CD25^-FoxP3^-$ T_{eff} cells distinctly contained a higher proportion of Annexin V^+ (an apoptosis marker) cells compared to $CD4^+CD25^+FoxP3^+$ T_{reg} (Fig. 3-10). These data indicate a higher resistance of T_{reg} to antigen/rapamycin-induced apoptosis. As expected, rapamycin/IL-

10/irrelevant peptide treated control animals showed only a low level of apoptotic $CD4^+$ cells (which are $CD25^-FoxP3^-$).

To demonstrate that the T_{reg} cells induced by this novel antigen specific protocol are functional and suppressive, an *in vitro* suppression assay was performed on splenocytes isolated from DO.11.10-tg RAG-$2^{-/-}$ mice after 3 weeks of treatment with rapamycin/IL-10/ova. While the tolerized splenocytes failed to proliferate upon stimulation with ova, T cell proliferation was observed in splenocyte cultures depleted for $CD4^+CD25^+$ cells (Fig. 3-11). When purified T_{reg} were added back to the depleted cells, proliferation was again suppressed (Fig. 3-11).

Route Optimization of Rapamycin/IL-10/Specific-peptide Protocol

In order to optimize the rapamycin/IL-10/specific peptide tolerance protocol, various routes of drug cocktail administration were tested. The routes tested included intraperitoneal, tail vein, subcutaneous and oral gavage. In the case of oral gavage, only rapamycin was administered orally while IL-10 cytokine and the specific peptide were administered by IP injections due to concerns about their stability in acidic and basic environments of the stomach and intestines. The different routes of administration of the drug combination (3 inj/week for 3 weeks) in T_{reg}-deficient DO11.10-tg Rag$2^{-/-}$ mice showed that T_{reg} induction was more efficient in IP injections (4.7x) compared to naïve controls, followed by subcutaneous (3.3x) and tail vein injections (2.8x). Deletion of T_{eff} was more pronounced via IP (2.7x) followed by subcutaneous (2.3x) and tail vein (1.7x) injections. Oral gavage of rapamycin (3inj/week for 3 weeks) along with IP injections of IL-10/ova specific peptide showed the least deletion (Fig. 3-12 A) accompanied by the lowest induction of $CD4^+CD25^+FoxP3^+$ cells (Fig. 3-12 B), possibly due to lower bioavailability. In summary, the route of administration of drug combination influences the outcome of the treatment modality.

Alternative Cytokines for Induction of T_{reg} in the Tolerance Protocol

DO11.10-tg Rag-2 $^{-/-}$ BALB/c mice were administered IP with Flt3L (2 μg/mouse/dose) co-administered with rapamycin (4 mg/kg/dose) and specific ova peptide (100 μg/mouse/dose) by intraperitoneal (IP) injection. Administration was carried out 3 times per week for a total of 3 weeks. In parallel, Fc-GITR-L fusion protein (200 μg/mouse/dose), which has been previously shown to increase levels of T_{reg}, was administered with rapamycin and ova peptide. Alternatively IL-2 cytokine, which is known to be necessary for expansion of T_{reg} was injected (50 ng/kg/dose) along with rapamycin and specific ova peptide. An additional group of mice was given rapamycin/IL-10/ova peptide for comparison of T_{reg} induction, and a naïve control group was also included. Each treatment group included n=4 mice. Spleens were harvested, and flow cytometry was carried out to quantify $CD4^+$ T effector cells and $CD4^+CD25^+FoxP3^+$ T_{reg}.

Rapa/Flt3L/ova administration resulted in a 4.5 fold deletion of CD4+ T cells, and a concomitant 19-fold increase in $CD4^+CD25^+FoxP3^+$ regulatory T cells. Rapa/Fc-GITR-L/ova treatment resulted in a 5.5fold reduction in effector T cells with a 18-fold increase in $CD4^+CD25^+FoxP3^+$ T reg. Rapa/IL-2/ova peptide administration resulted in 2-fold deletion of $CD4^+$ effector T cells and an 8-fold increase in T_{reg} cells (Fig. 3-13). The combination of specific peptide with rapamycin and Flt3L was statistically most significant and therefore shows considerable promise due to synergistic effects of rapamycin and Flt3L on the induction of T_{reg} cells.

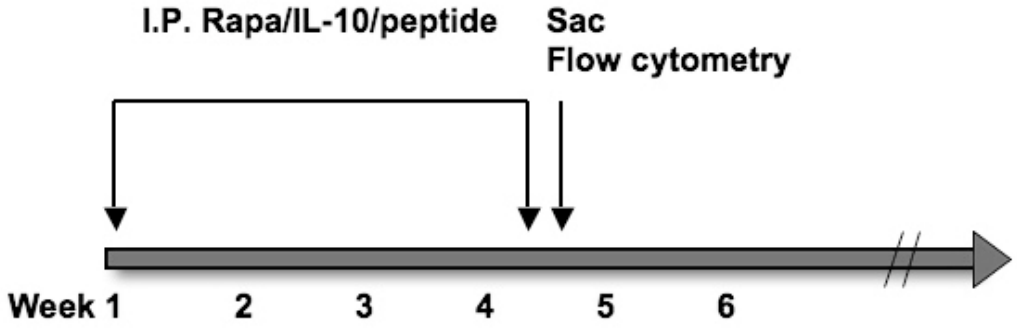
Transient Non-specific Changes in Lymphocyte and Neutrophil Counts in Immune Competent Mice

Some general immune suppression is expected by the rapamycin/IL-10 protocol in immune competent mice since $Rag^{+/+}$ mice have TCRs for multiple antigens and mTOR is also expressed in T and B cells. Wild-type C3H/HeJ mice were injected IP 3-times per week with ramapycin/IL-

10 and hF.IX-specific A2-54 instead of ova peptide for 4 weeks. Immediately after treatment, a 3- to 4-fold decrease in $CD4^+$, $CD8^+$, and $B220^+$ splenocytes compared to untreated controls was observed, which was independent of the co-administered peptide (Fig. 3-14 A-C). However, by 5 months after treatment, B and T cell numbers were similar to controls (Fig. 3-14 D-F).

Complete blood counts showed that all animals treated with rapamycin/IL-10 (n=6) developed neutrophilia, which was observed up to 4 months after treatment but dissipated 7 months after treatment (this is expected since rapamycin is used for treatment of neutropenia) [95]. Peripheral blood analysis included White Blood Cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, RBC and platelet counts, hemoglobin, mean cell hemoglobin concentration (MCHC), Reticulocyte Count Hematocrit, packed cell volume, nucleated erythrocytes, mean cell or corpuscular volume, mean platelet volume (MPV), and mean cell or corpuscular hemoglobin. Low MCHC and polycythemia was found at random among both treated and untreated, age-matched control animals at both time points. Although platelet counts were normal, MPV was low in 40% of C3H/HeJ mice regardless of treatment. All other parameters were within the normal range for mice.

A



B

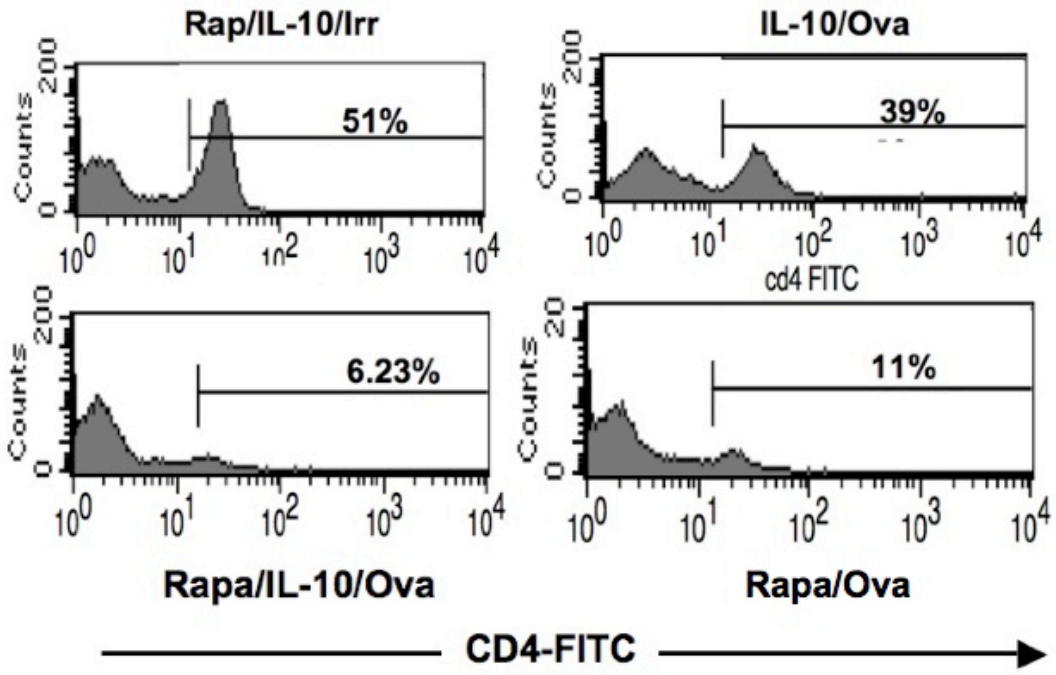


Figure 3-1. Flow cytometric analysis of splenocytes from DO.11.10-tg $Rag^{-/-}$ BALB/c mice injected IP 3-times/week for 4 weeks with rapamycin/IL-10/specific peptide (ova), rapamycin/ova, IL-10/ova or control rapamycin/IL-10/irrelavant (2A-54; "irr") peptide. **A**) Experimental design, **B**) Representative examples of $CD4^+$ T cells percentage of total gated lymphocytes as a function of treatment**.**

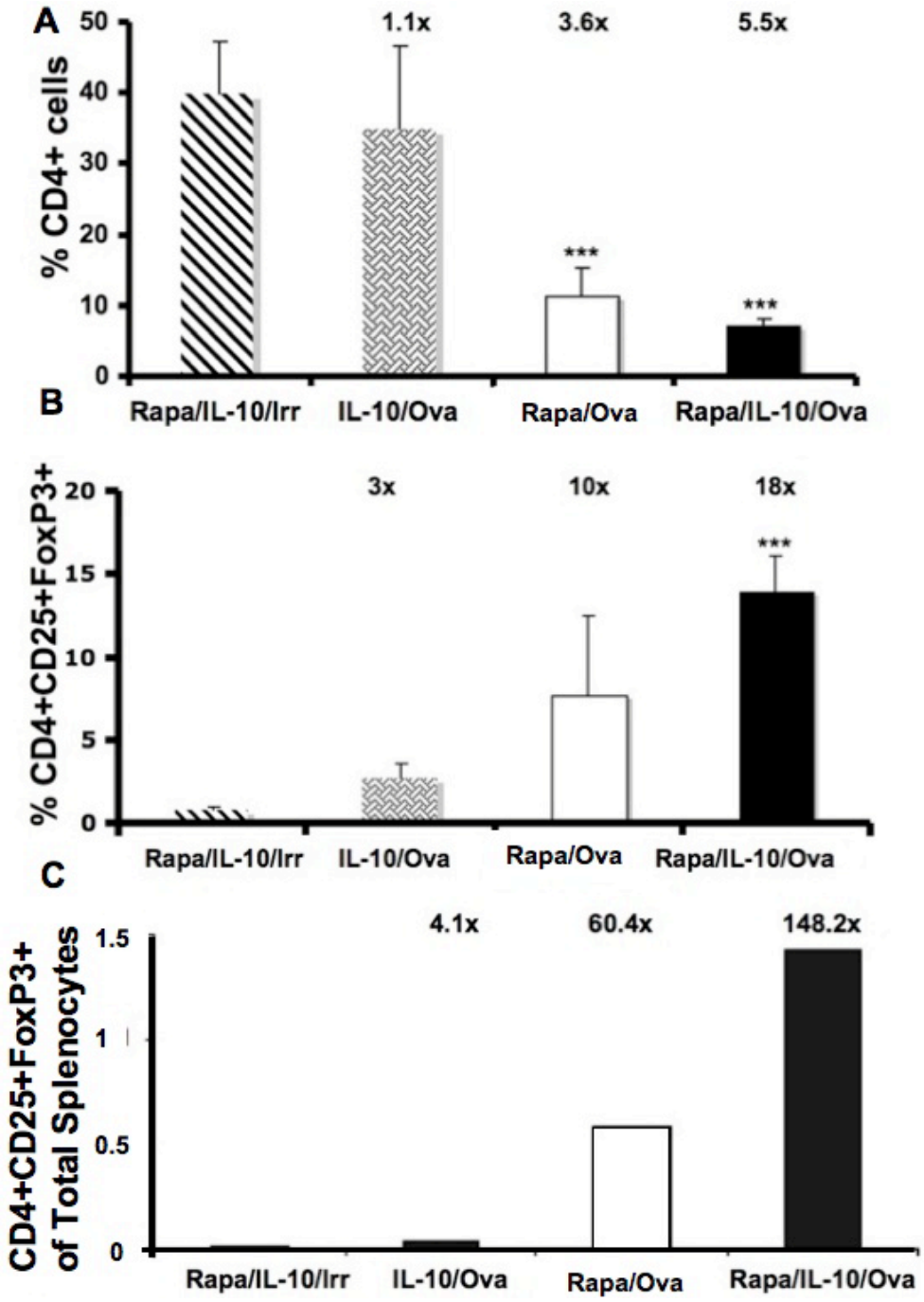


Figure 3-2. Average cell percentages from flow cytometric analysis of splenocytes in DO.11.10-tg $Rag^{-/-}$ BALB/c mice. IP injections 3-times/week for 4 weeks with rapamycin/IL-10/specific peptide (ova), rapamycin/ova, IL-10/ova or control rapamycin/IL-10/irrelavant (2A-54; "irr") peptide. **A**) Percentage $CD4^+$ T cell deletion; fold decrease compared to control **B**) $\%CD25^+FoxP3^+$ T reg cells gated on $CD4^+$ T cells; fold increase compared to controls **C**) $CD25^+FoxP3^+/CD4^+$ T cells gated on total splenocytes. $P<0.0005$ ***.

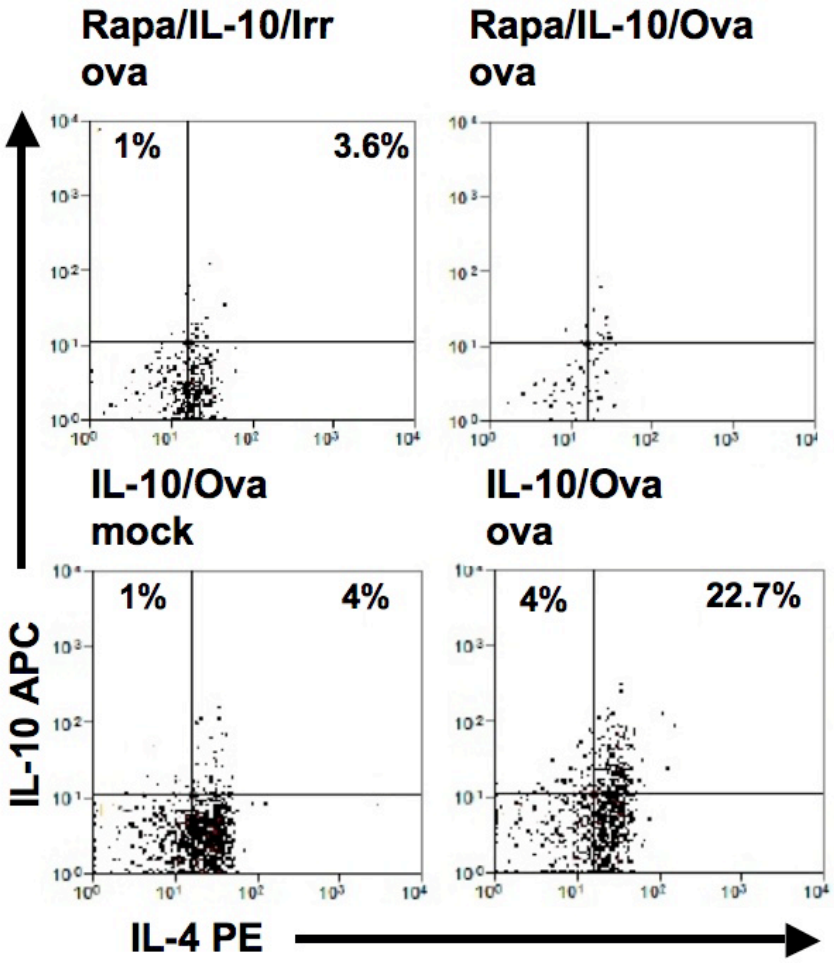


Figure 3-3. Cytokine capture in treatment groups. Flow cytometric analysis of splenocytes from DO.11.10-tg Rag$^{-/-}$ BALB/c mice injected IP 3-times/week for 4 weeks with A) rapamycin/IL-10/irrelavant (2A-54; "irr") peptide *in vitro* stimulated with ova **B**) rapamycin/IL-10/specific peptide (ova) *in vitro* stimulated with ova, **C**) IL-10/ova stimulated with mock media or **D**) IL-10/ova stimulated with ova.

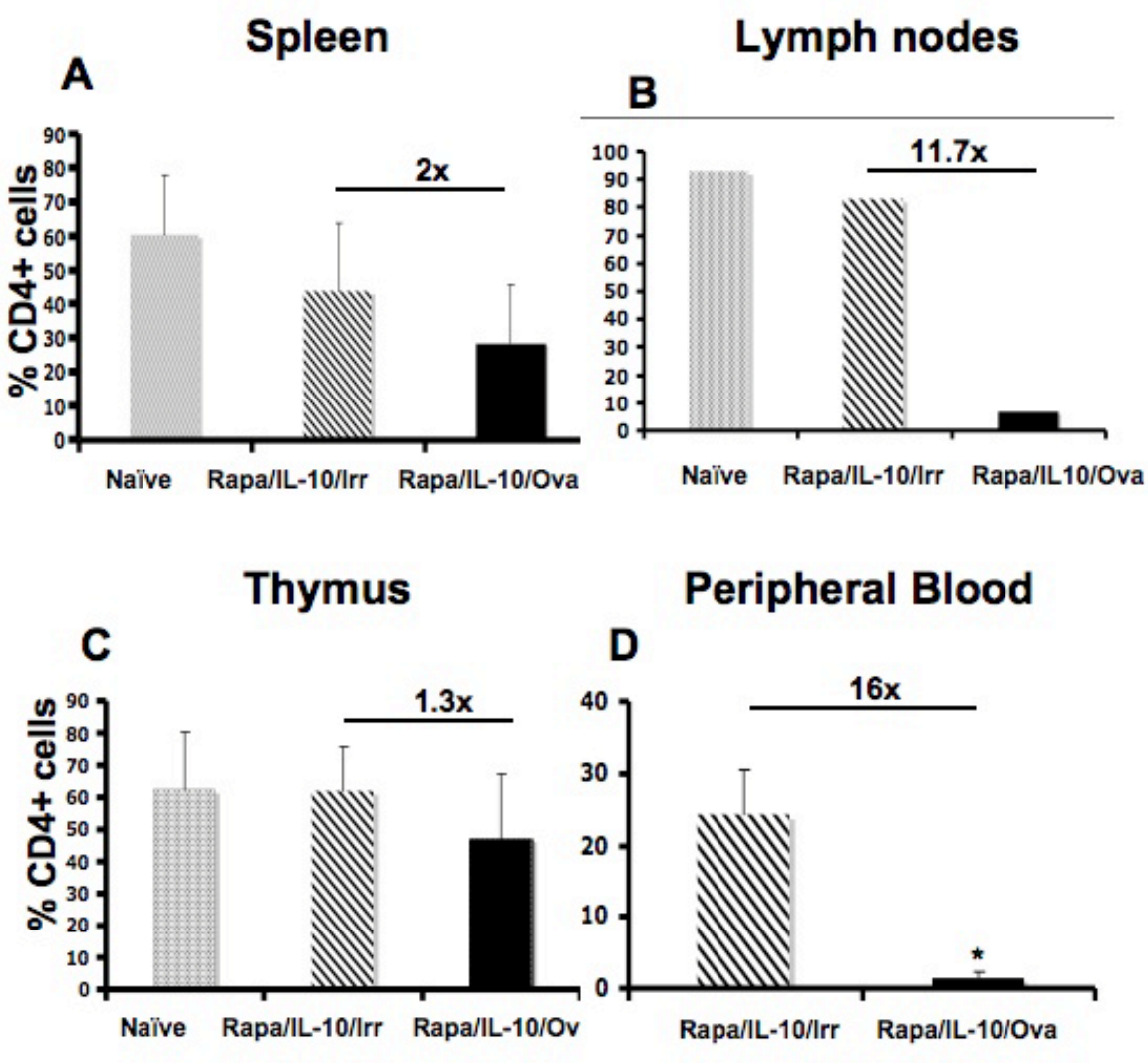


Figure 3-4. Flow cytometric analysis of $CD4^+$ T effector T cell deletion. in DO.11.10-tg $Rag^{-/-}$ mice were treated for 3 weeks with rapamycin/IL-10/ova or rapmycin/IL-10/A2-54 "Irr" peptide or in naïve mice. **A**) Splenocytes **B**) popliteal and inguinal lymph nodes, **C**) Thymus and **D**) peripheral blood were analyzed for percentage of $CD4^+$ T cell depletion. The figure demonstrates fold decrease of $CD4^+$ T cells compared to irrelevant peptide treated controls.

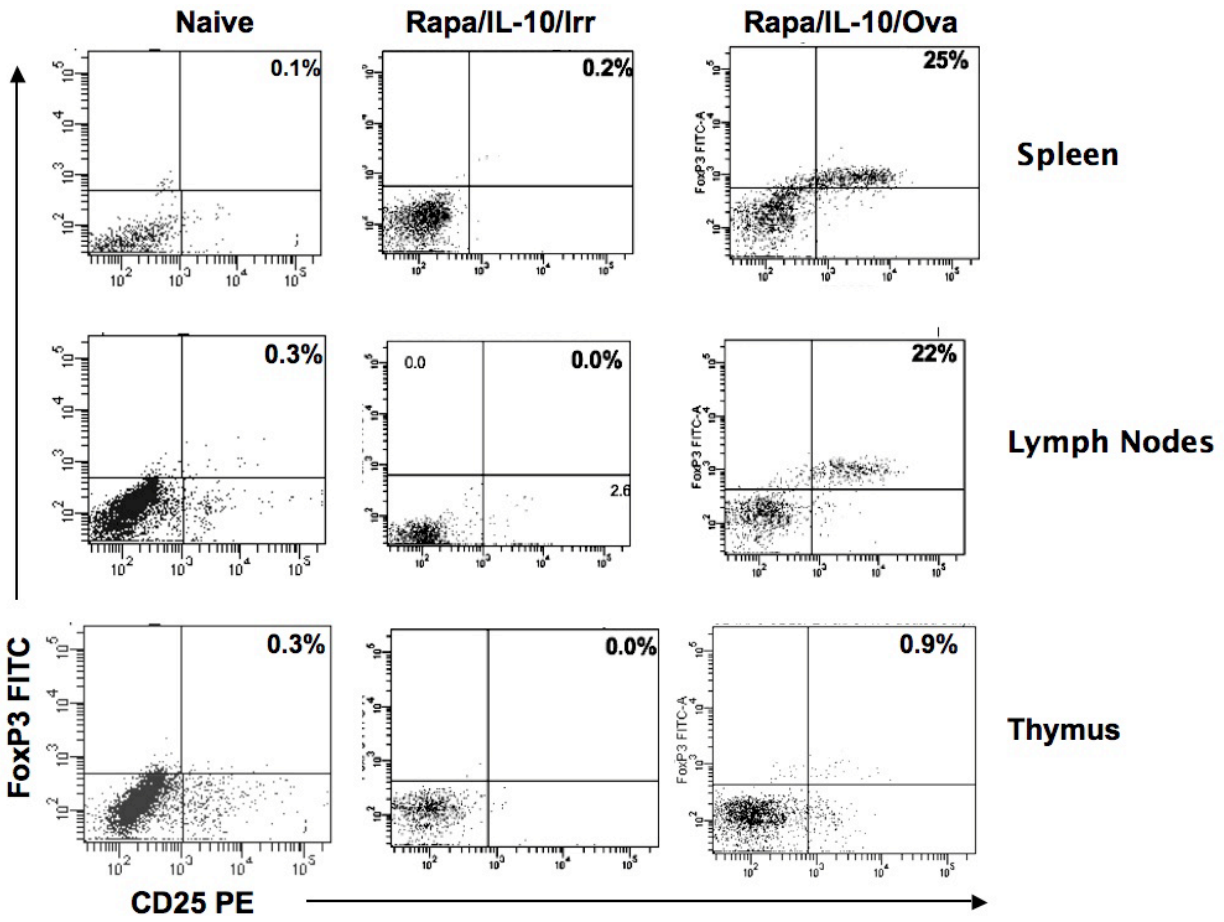


Figure 3-5. Flow cytometric analysis of $CD4^+$ T cell deletion in lymphoid organs. DO.11.10-tg $Rag^{-/-}$ mice were treated for 3 weeks with rapamycin/IL-10/ova or rapmycin/IL-10/A2-54 peptide or naïve (no treatment). Representative examples of $CD4^+$ cells of total gated lymphocytes depletion in spleen, pooled inguinal and popliteal lymph node cells and thymus.

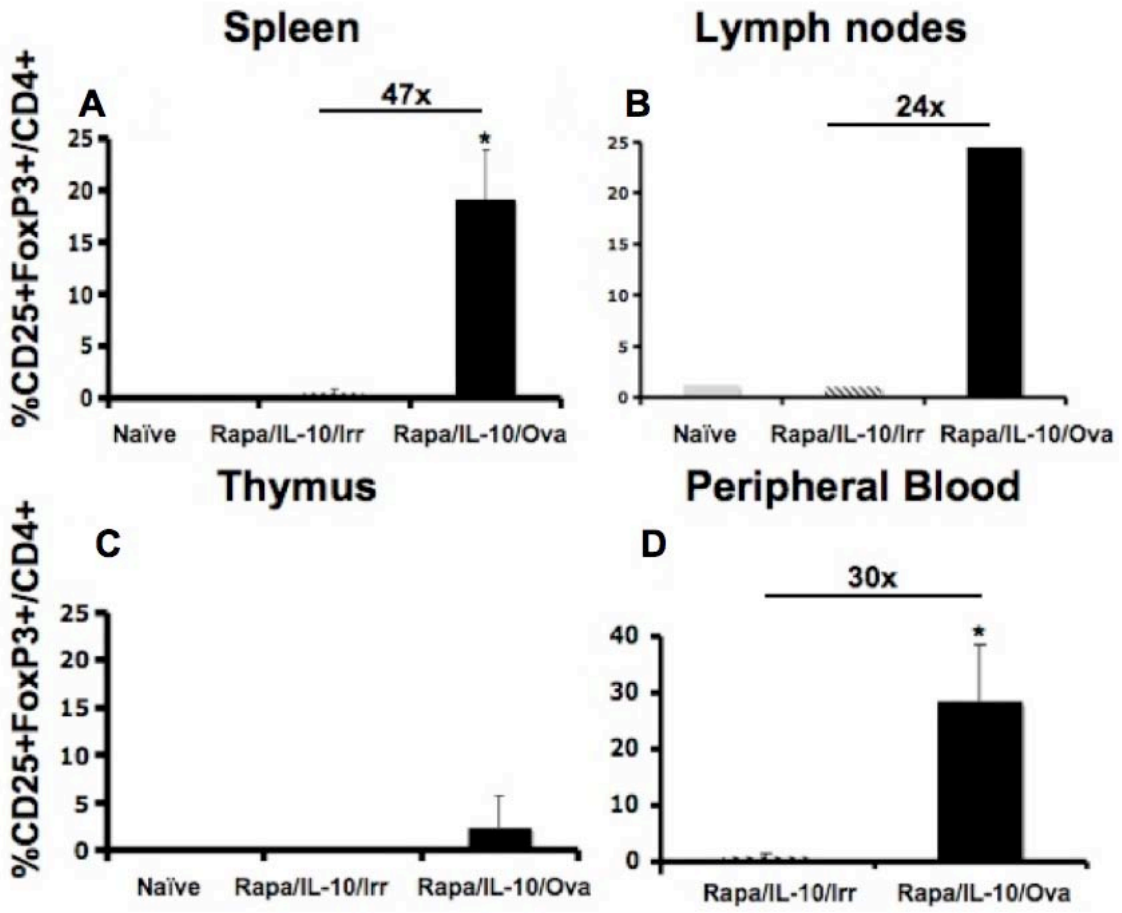


Figure 3-6. Flow cytometric analysis of regulatory T cell induction. DO.11.10-tg $Rag^{-/-}$ mice were treated for 3 weeks with rapamycin/IL-10/ova or rapmycin/IL-10/A2-54 "Irr" peptide or in naïve mice. **A**) Splenocytes **B**) popliteal and inguinal lymph nodes**, C**) Thymus and **D**) peripheral blood were analyzed for percentage of $CD25^{+}FoxP3^{+}$ T cells gated on $CD4^{+}$ cells. The figure demonstrates fold increase of regulatory T cells compared to irrelevant peptide treated controls.

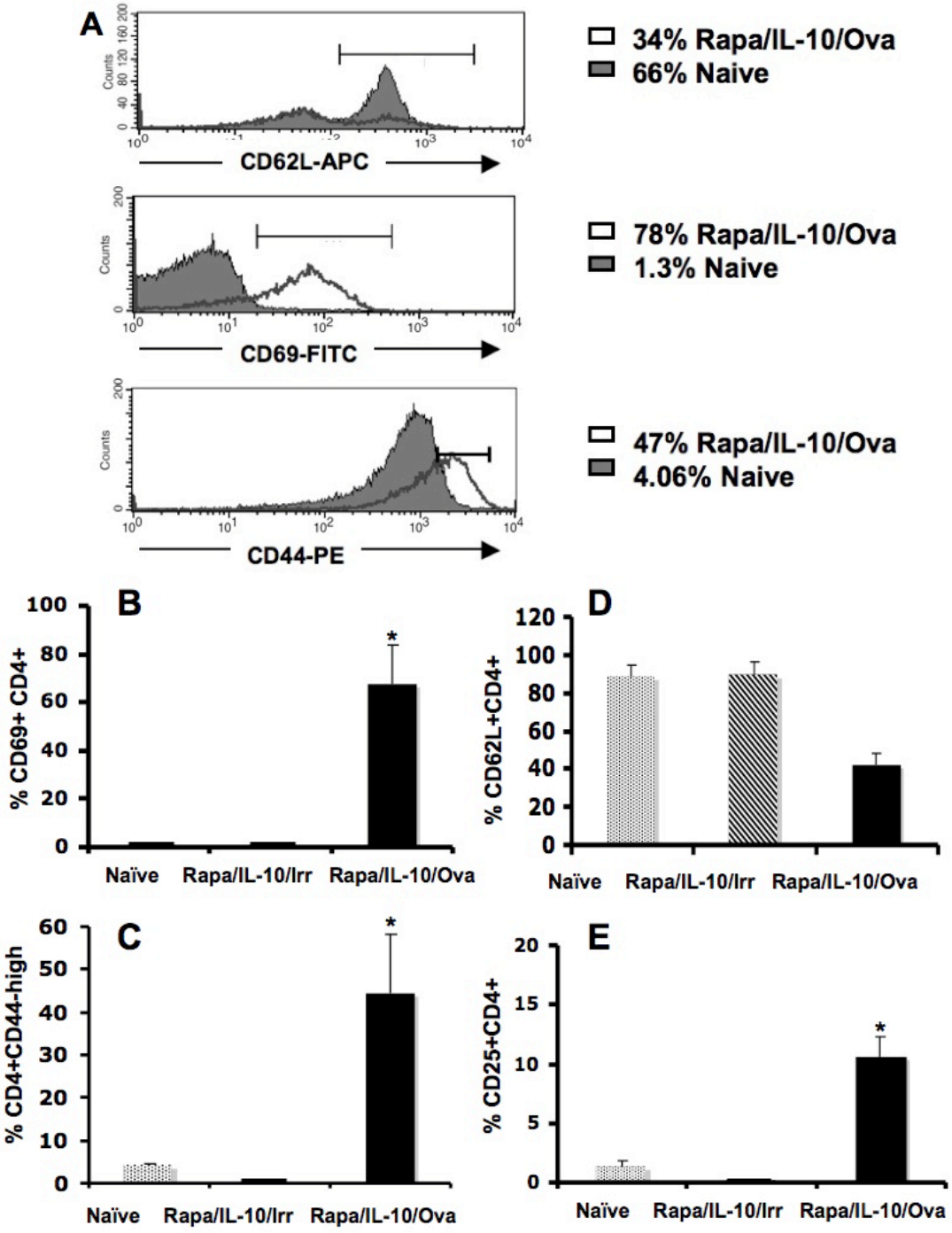


Figure 3-7. Measurement of T cell activation in splenocytes. DO.11.10-tg $Rag^{-/-}$ mice were treated for 1 week with rapamycin/IL-10/ova specific peptide or irrelevant peptide (control). **A**) Overlay of representative examples of CD62L, CD69, and CD44 expression in CD4+ T cells from a single treated and or naïve mouse. Graphs show summaries of results (averages ±SD, n=4 per group) for percent $CD69^+$ **B**), CD44high **C**), $CD4^+$ **D**) $CD62L^+$, and $CD25^+$ **E**) of $CD4^+$ cells. * indicates a significant difference with $P<0.05$.

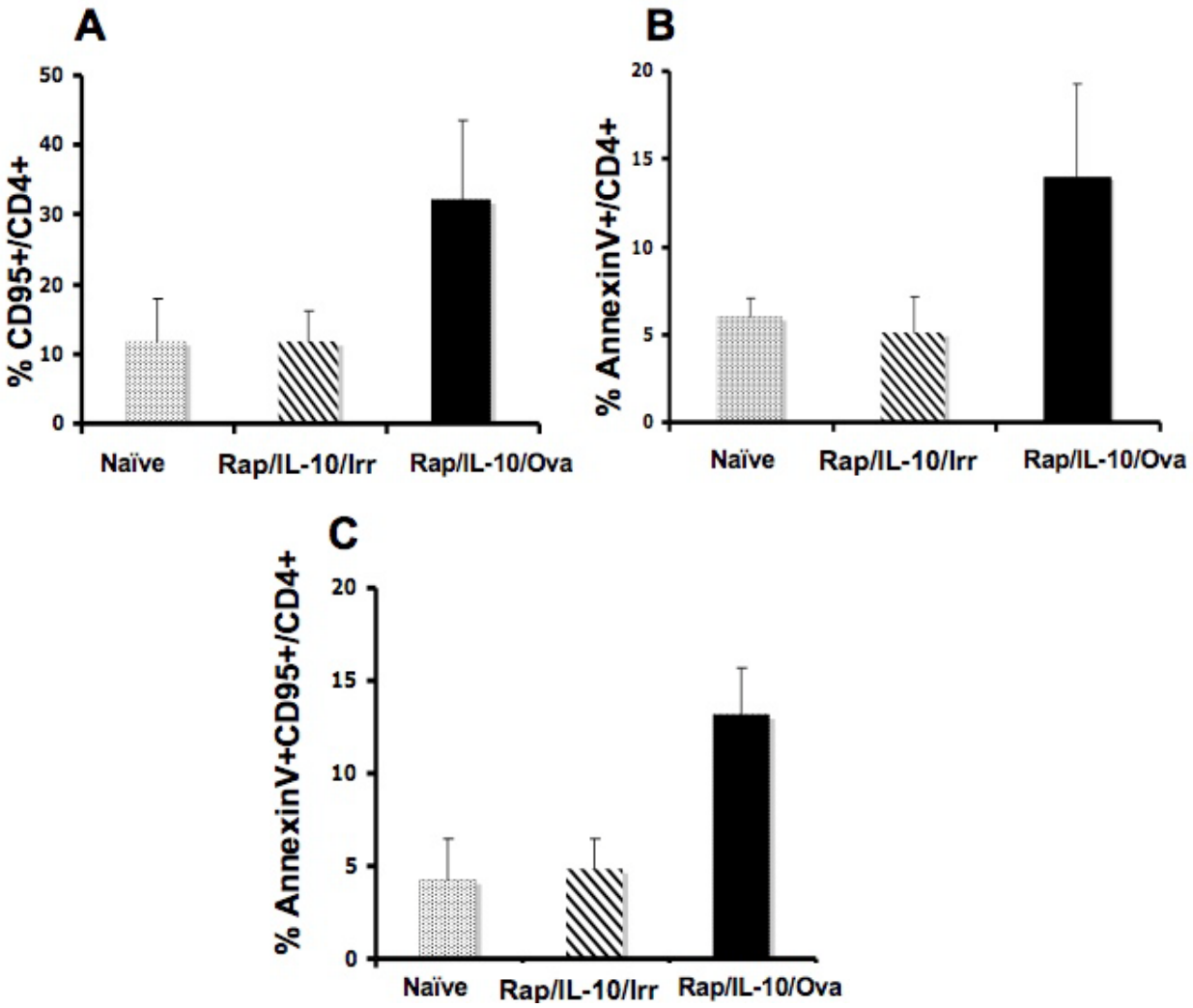


Figure 3-8. Measurement of apoptosis and Fas (CD95) expression in splenocytes. DO.11.10-tg $Rag^{-/-}$ mice were treated for 1 week with rapamycin/IL-10/ova, rapapamycin/IL-10/A2-54, or untreated (naïve) mice. **A**) Percent CD95+ of CD4+ T cells. **B**) Percent $AnnexinV^{+}PI^{-}$ of $CD4^{+}$ T cells. **C**) Percent $AnnexinV^{+}PI^{-}CD95^{+}$ of $CD4^{+}$ T cells.

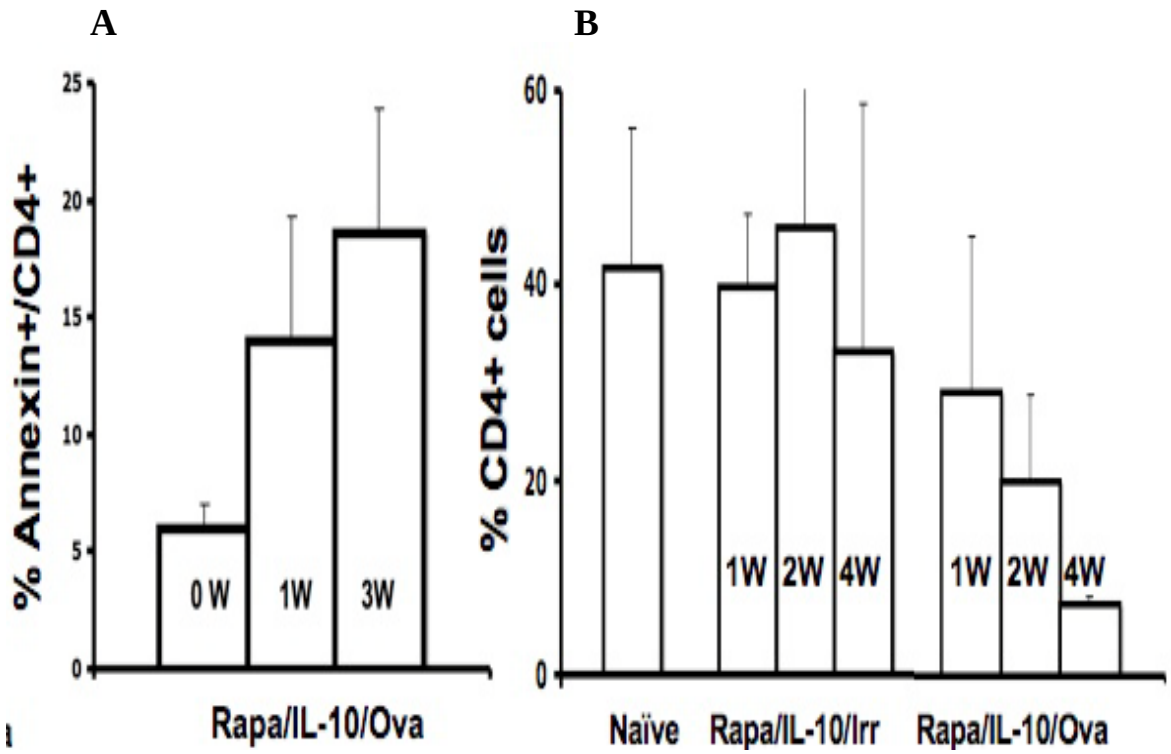


Figure 3-9. Activation induced cell death (AICD) timeline. **A**) Increase in apoptotic cell numbers as a function of time (duration of treatment) with rapamycin/IL-10/ova. **B**) Change in ova-specific $CD4^+$ T cell frequency as a function of time during treatment with specific or non-specific protocol, and comparison to untreated naïve mice.

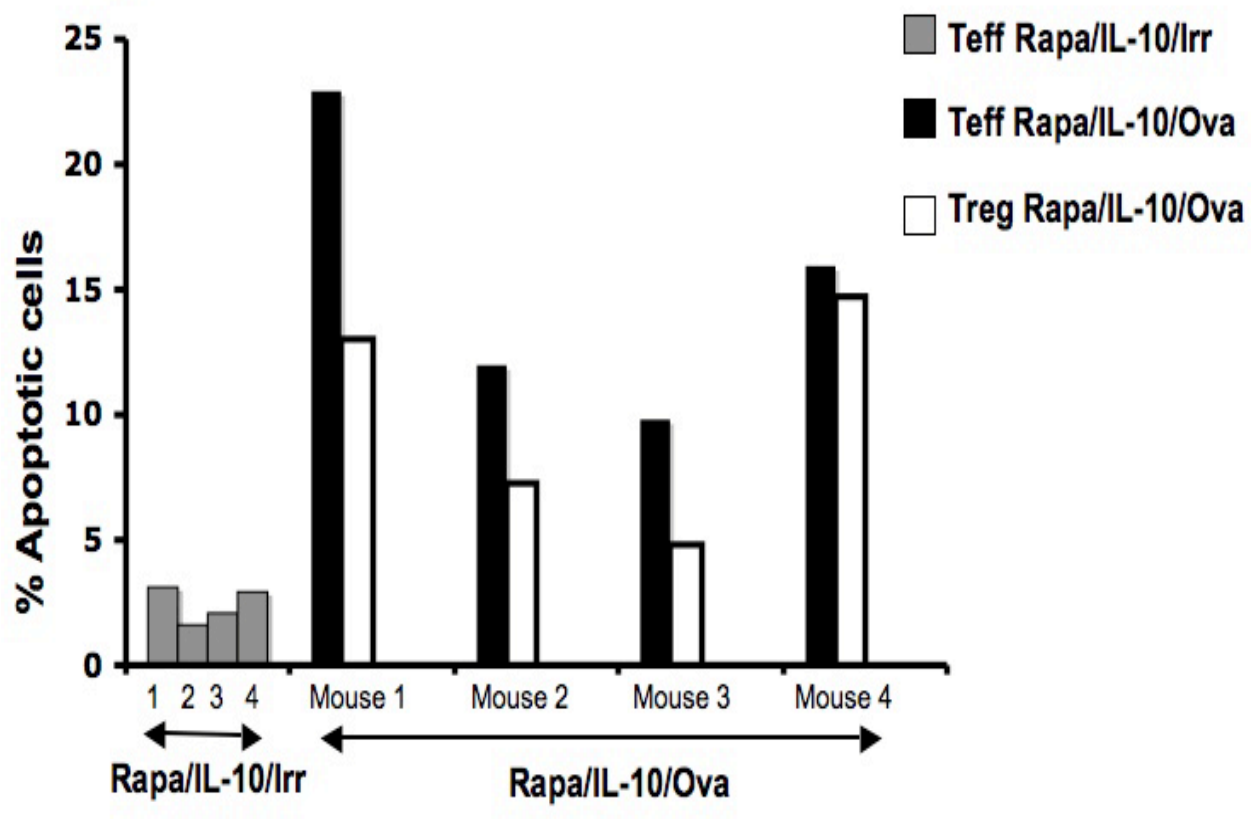


Figure 3-10. Differential effects of treatment on T_{eff} and T_{reg}. Percent apoptotic ($AnnexinV^{+}$) cells among splenic T_{eff} ($CD4^{+}CD25^{-}FoxP3^{-}$) and T_{reg} ($CD25^{+}CD25^{+}FoxP3^{+}$) from DO.11.10-tg $Rag^{-/-}$ mice after 3 weeks of treatment with rapamycin/IL-10/ova compared to $CD4^{+}$ T cells from or control rapamycin/IL-10/A2-54 treated mice. Shown are results for individual mice.

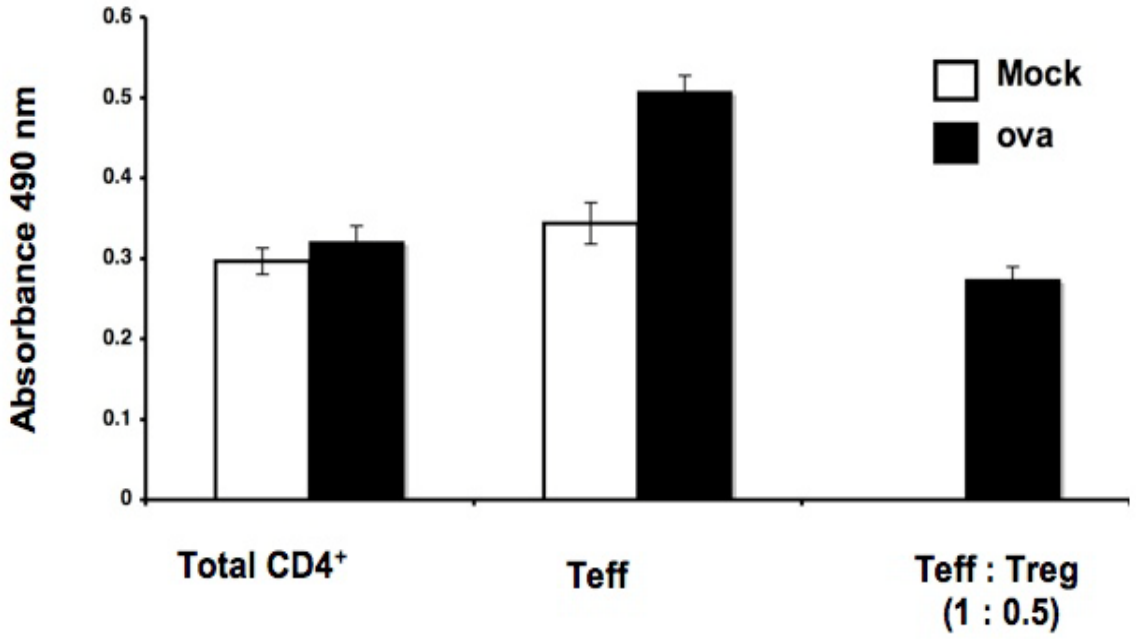


Figure 3-11. Ova-specific functional T cell proliferation assay. Complete splenocyte cultures ("total $CD4^+$"), $CD25^+$ cell-depleted cultures ("T_{eff}"), and depleted cultures reconstituted with $CD4^+CD25^+$ T cells at a ratio of 0.5:1 when compared to CD4+CD25- cell number ("T_{eff} : T_{reg}; 1 : 0.5"). Splenocytes were from DO11.10-tg $Rag^{-/-}$ mice treated for 3 weeks with rapamycin/IL-10/ova. Results are average ±SD for quadruplicate cultures.

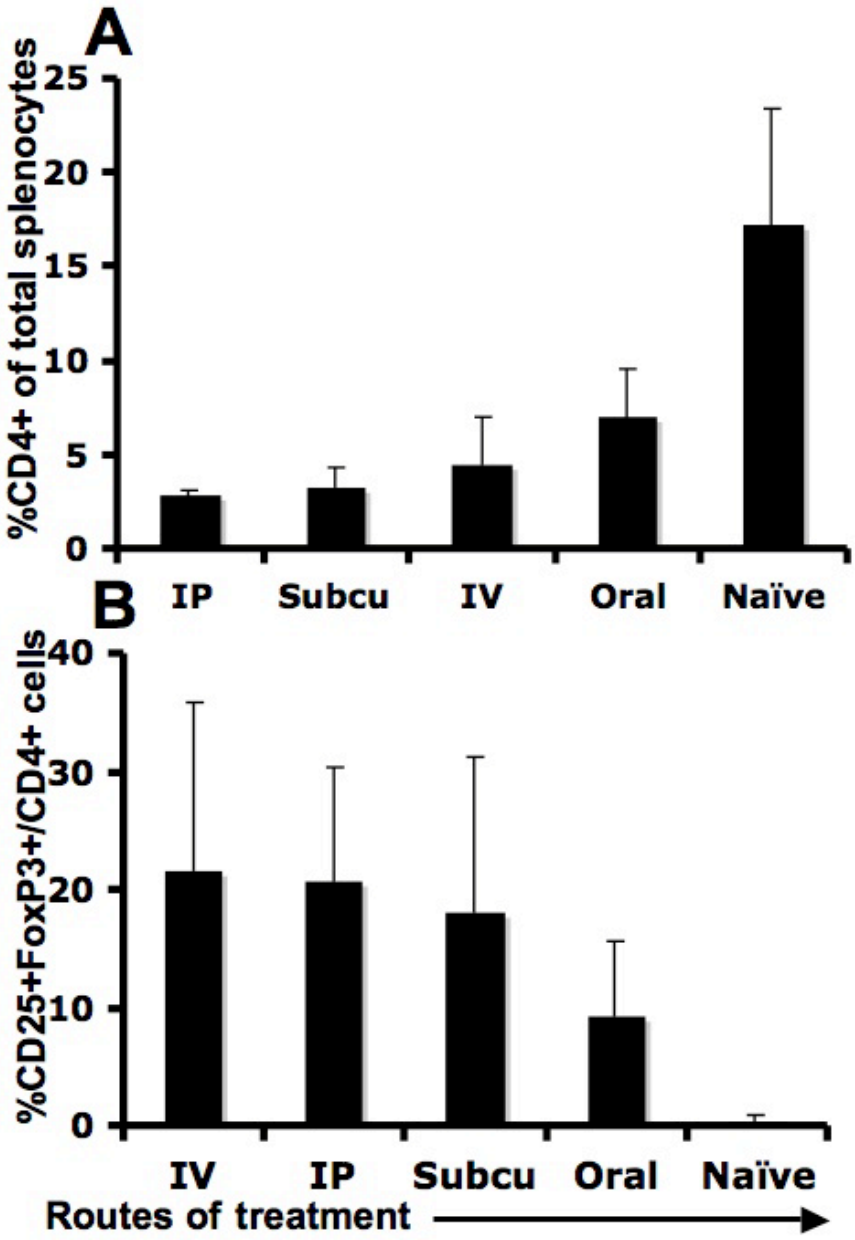


Figure 3-12. Optimization of Rapa/IL-10/specific peptide tolerization protocol utilizing different routes of injection. DO.11.10-tg Rag$^{-/-}$ mice (n=4) were treated for 3 weeks with Rapa/IL-10/specific peptide after which the spleens were harvested and flow cytometry was used to determine **A**) percentage deletion of CD4^{+} T effector T cells and **B**) CD4^{+}CD25^{+}FoxP3^{+} T regulatory cells.

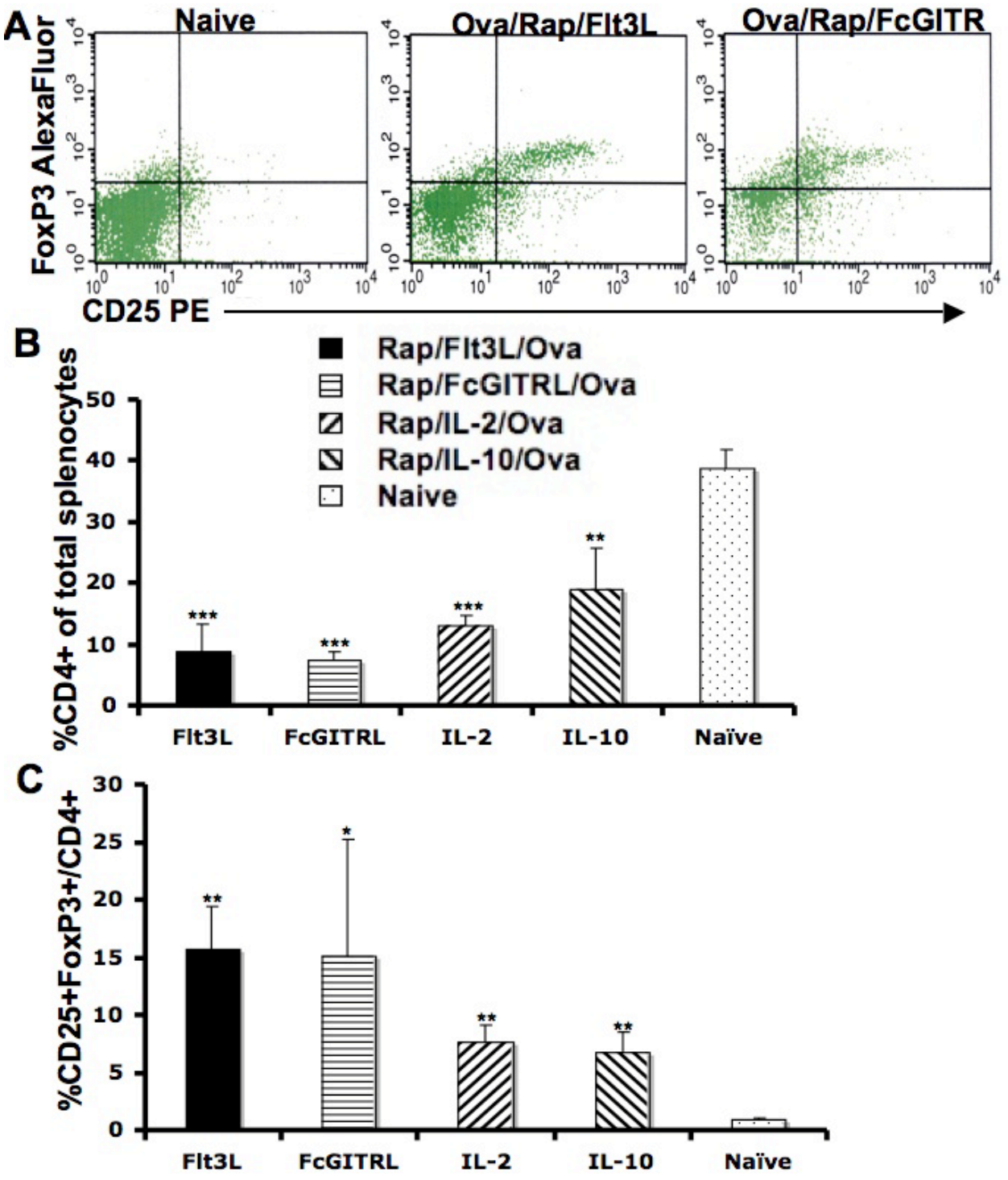


Figure 3-13. Optimization of alternate drug combinations. Flow cytometric analysis of splenocytes from DO.11.10-tg $Rag^{-/-}$ mice injected IP for 3 weeks with Ova/Rapa in combination with one of the following drugs: Flt3L, Fc-GITR-L, IL-2 or IL-10. **A**)Representation of $CD25^{+}FoxP3^{+}/CD4^{+}$ T cells **B**) Percentage $CD4^{+}$ T cells of total splenocytes **C**) Percentage $CD25^{+}FoxP3^{+}$ cells gated on $CD4^{+}$ T cells. P<0.0005 ***; P<0.005 **; P<0.05 *.

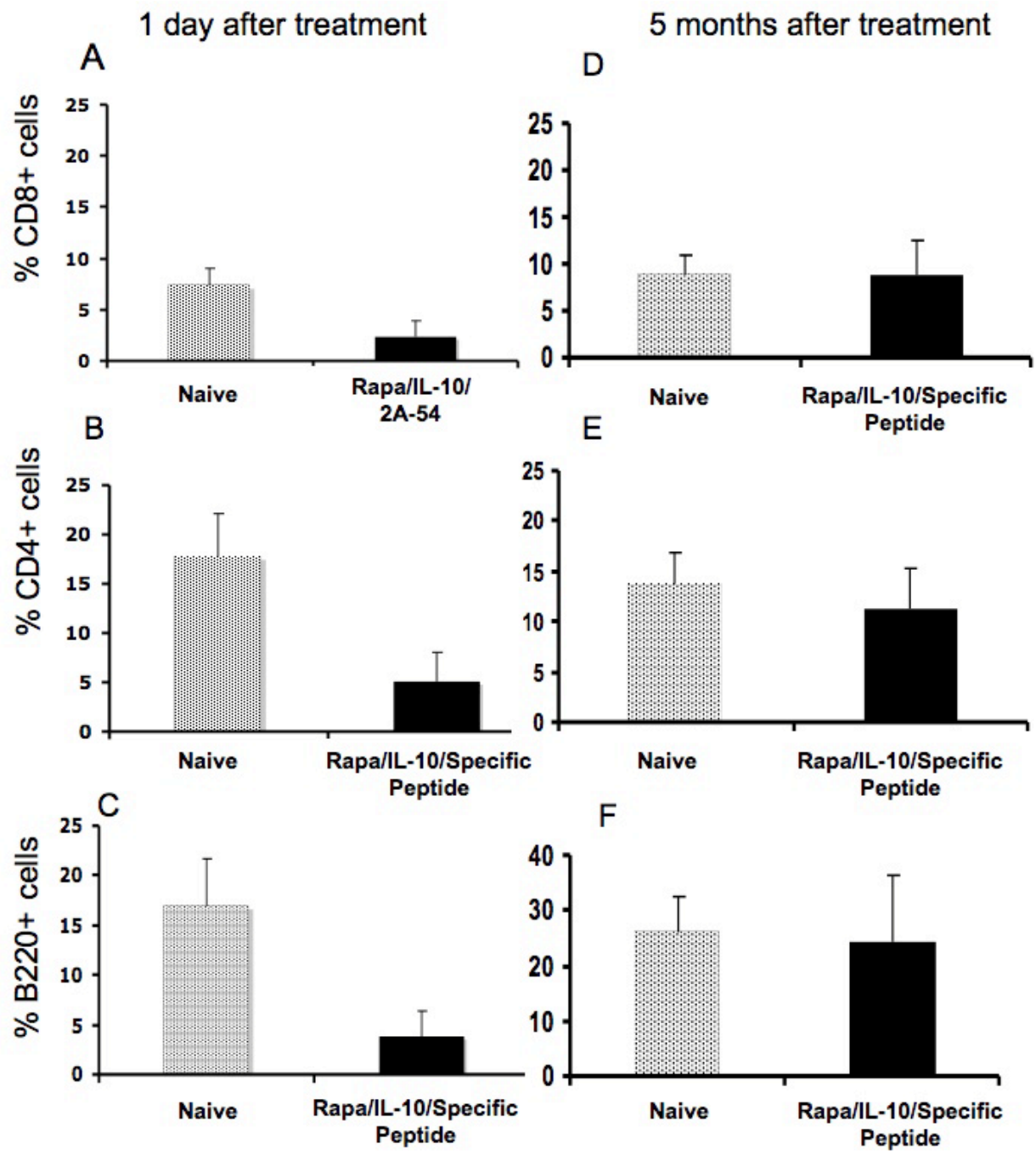


Figure 3-14. Effect of tolerance protocol on general immune suppression in immune competent mice over time. Flow cytometric analysis of splenocytes from C3H/HeJ mice injected IP for 3-weeks with Rapa/IL-10/specific peptide protocol. Splenocytes were isolated 1 day after the end of treatment or 5 months after the end of treatment. **A,B**) $CD8^+$ cells **C,D**) $CD4^+$ cells **E,F**) $B220^+$ cells

CHAPTER 4
INDUCTION OF TOLERANCE IN HEMOPHILIA B DISEASE MICE RECEIVING GENE THERAPY

Introduction

The novel tolerance protocol formulated using Rapamycin and IL-10 (or IL-2 or Fc-GITR-L) in the presence of specific peptide induced $CD4^+CD25^+FoxP3^+$ T_{reg} and deleted antigen specific $CD4^+$ effector T cells as observed in the test DO.11.10-tg $Rag^{-/-}$ mice (Chapter 3) . In order to extend these findings to a relevant disease model, hemophilia B mice were used to test and optimize the protocol. F.IX protein is a vitamin K dependant protein that participates in the coagulation pathway in plasma. It is a serine protease zymogen that converts to activated Factor IXa after vascular injury. The F9 gene DNA sequence is about 1.4 kilo bases in length and can be packaged in an AAV vector.[96] Successful gene therapy in the absence of antibody formation in severe hemophilia patients will reduce costs associated with protein replacement therapy and improve the standard of living. Patients with early stop codons and gene deletions develop a neutralizing antibody response against F.IX upon expensive therapeutic protein replacement therapy.

The hemophilia B mice used in my experiments are on a C3HeJ background and are knockouts for F.IX (C3H/HeJ F9-/-). They fail to produce hepatic F.IX mRNA and have no circulating F.IX protein. These mice exhibit phenotypic characteristics for severe disease such as prolonged bleeds from tail clips, spontaneous internal bleeds and increased mortality (possibly from bleeding due to fight wounds). The Herzog lab was previously unable to completely inhibit the neutralizing antibody response to F.IX by AAV2 gene therapy in C3H/HeJ F9-/- (hemophilia B) mice although the protocol was successful using an AAV8 vector.[97] Hepatic injection of AAV2-hF.IX containing human α_1-antitrypsin promoter (ApoE/hAAT) after nasal administration of specific peptide suppressed the immune response and long-term F.IX expression was

observed. [90] Muscle is an alternative target organ for gene therapy as it can produce biologically active F.IX upon gene transfer. Muscle gene transfer was used in a phase I clinical trial although it resulted in <1% detectable F.IX which was sub-therapeutic. F.IX muscle gene transfer in dogs has resulted in long-term therapeutic F.IX levels >4 yrs after gene delivery. AAV1-CMV-hF.IX when injected IM has consistently led to high levels of neutralizing antibody production against hF.IX in C3H/HeJ F9-/- mice as detected by the Bethesda assay. This muscle gene transfer model was used to test the novel drug cocktail/specific peptide combinations for prevention of the anti-hF.IX immune response in the hemophilia B mice.

Testing a Short Tolerance Protocol in C57BL/6 Mice

Before moving into a therapeutic setting in hemophilia B mice, the Rapa/IL-10/specific peptide tolerance protocol was tested in immune competent C57BL/6 mice to observe the reduction or elimination of an anti-F.IX IgG1 response to AAV2-CMV-F.IX administration in muscle. Control animals that received only the gene transfer formed high titer anti-hF.IX IgG1 levels (4-36 μg/ml plasma in 5/6 mice Fig. 4-1A) as measured by a sandwich ELISA. An additional group containing experimental mice (n=5) received rapamycin (4mg/kg), IL-10 (50ng/kg) and hF.IX-specific peptide ($CD4^+$ T cell epitope in C57BL/6 mice; 2A58). The dosing of the mixture was 3-times a week for 6 weeks. The AAV2-CMV-h.F.IX vector was administered IM during the third week of treatment. Only 1/5 mice developed an anti-F.IX antibody in the tolerized group Fig. 4-1B. Thus, the prophylactic protocol efficiently prevented an antibody response in immune-competent mice; these results encouraged the extension of the protocol to a therapeutic hemophilia B model.

Prevention of Inhibitor Formation in Intramuscular Gene Therapy for Hemophilia B

In order to test this prophylactic protocol in a therapeutic setting, hF.IX-specific peptide (2A-54, containing the dominant $CD4^+$ T cell epitope in C3H/HeJ mice) was co-administered

with rapamycin and IL-10 cytokine 3-times per week for 4 weeks to C3H/HeJ hemophilia B mice (n=4 per experimental group). These mice have a targeted F9 gene deletion and show stronger adaptive immune responses to F.IX than other inbred strains [98,99]. After the third week of treatment, AAV1-CMV-hFIX vector ($1x10^{11}$ vg/mouse) was injected to intramuscularly (IM) as illustrated in Fig. 4-2A. Control mice received vector only, or rapamycin, IL-10 treatment, except that an irrelevant (ova) peptide was used instead of 2A-54 (n=4 per experimental group). An additional group was given rapamycin with 2A54 specific peptide only. Without immune suppression, mice developed inhibitors of 4-19 BU within 1 month after gene transfer (Fig. 4-2B). Mice treated with the hF.IX tolerance protocol (Rapa/IL-10/2A54) had at best very low titer inhibitors (<2 BU, the background of the assay) for the duration of the experiment (5.5 months after gene transfer, Fig.4-2B). Importantly, non-specifically immune suppressed mice (Rapa/IL-10/Irrelavent ova peptide) formed low-titer inhibitors of 4-5 BU, and therefore showed only partial suppression of the inhibitor response. Systemic hF.IX levels in hF.IX-tolerized mice (Rapa/IL-10/2A54) were stable and on average 200-250 ng/ml (4-5% of normal human levels), while both control groups showed substantially lower expression of on average 0-50 ng/ml (Fig. 4-2C). Clotting times (aPTTs) were partially corrected (~50 sec) in the hF.IX-tolerized group compared to an average of 65-80 sec in the control groups, which is identical to untreated mice (Fig. 4-2D). While hF.IX levels and aPTTs did not improve, inhibitory antibody titers in animals without immune suppression were unchanged for 3.5 months and subsequently declined to lower titers, similar to previously published data (Fig. 4-2B). [91] In the non-specifically suppressed group (Rapa/IL10/ova), 3 of 4 animals also showed a decline in Bethesda titers by 6 months, resulting in increased hF.IX expression and improvement of the aPTTs, while one animal still showed 5 BU and lacked systemic expression (Fig. 4-2D and data not shown). One group treated

with rapamycin and specific peptide without the cytokine IL-10 (Rapa/2A54) showed some reduction of neutralizing antibody levels compared to the untreated controls and lowered clotting times and improved hF.IX expression, although it was not as effective as the treatment group that included IL-10. Non-hF.IX specific effects in rapamycin/IL-10 treated immune competent mice included transient reduction in B and T cell frequencies and transient neutropenia.

Induction of Functional T_{reg} via the Prophylactic Tolerance Protocol

C3H/HeJ $F9^{-/-}$ mice were treated with rapamycin/IL-10/2A-54 or rapamycin/IL-10/ova for 4 weeks. After tolerization, $CD4^{+}CD25^{+}$ T_{reg} cells were isolated via MACS. T_{reg} cells ($1x10^{6}$) were transferred to C3H/HeJ mice. The recipient mice were given an IM injection of AAV1-CMV-hF.IX ($1x10^{11}$ vg/mouse) and later tested for anti-F.IX IgG1. The graph (Fig. 4-3) indicates a reduction in levels of anti-hF.IX antibodies in the recipients of the $CD4^{+}CD25^{+}$ T_{reg} cells from tolerized animals when compared to controls. Regulatory T cells induced by rapamycin/cytokine/specific peptide treatment suppress antibody formation against hF.IX as evidenced by this adoptive transfer experiment.

Modified Tolerance Protocol Using an Alternate Route of Tolerance Induction

Rapamycin is approved by the FDA for oral administration in patients during transplantation procedures. Therefore, modified version of the novel rapamycin, IL-10, specific peptide protocol was tested for oral tolerance. Rapamycin (4 mg/kg) was orally administered using a gavage needle 3 times/week for 4 weeks while a mixture of IL-10 (50 ng/kg) and specific peptide (hF.IX $CD4^{+}$ epitope for C3H/HeJ background; 2A54; 100 μg/dose/mouse) was injected IP concomitant with the oral gavage of rapamycin. An IM injection of AAV1-CMV-hF.IX vector ($1x10^{11}$vg) was given at the third week of tolerization. Control animals received IM gene transfer only. The tolerized group showed a lower anti-F.IX IgG1 response averaging <5000 ng/ml over 4 months compared to the gene transfer controls (~6000-20,000 ng/ml; Fig. 4-4). A

reduction in neutralizing antibody titer was also observed (~4-5 BU) in treated animals vs. 7-8 BU in controls. There was an increased expression of hF.IX expression in tolerized animals (~90-140 ng/ml) and controls (~10-100 ng/ml) over a 4-month period. The marginal reduction of the immune responses may be attributed to the lower bioavailability of the rapamycin via the oral route. Alternatively, the separation of the drug combination in the oral and IP routes may affect their synergistic activity.

Modified Tolerance Protocol Utilizing an Alternate Cytokine

One group of C3H/HeJ F9-/- mice was co-administered with rapamycin (4 mg/kg), IL-2 (50 ng/kg) and hF.IX specific peptide for C3H/HeJ background (100 μg/dose/mouse). The treatment was given thrice a week for 4 weeks. At the third week of tolerization, AAV1-CMV-hF.IX vector ($1x10^{11}$vg) was administered IM. A control group received gene transfer only. The tolerized group had a lower neutralizing antibody response (<= 2.5 BU) compared to gene therapy only controls (3.2-13.5 BU). An increase in average hF.IX levels was observed in the tolerized animals (~160-200 ng/ml) over a 4 month period; control animals showed F.IX expression from 50 ng at 1 month and a gradual increase to ~150ng/ml was observed 4 months after gene transfer. This increase in hF.IX levels was observed in other experiments as well and may be attributed to the constant long-term presence of the hF.IX protein expressed by vector transduced cells, eventually leading to some state of hypo-responsiveness. Minimal correction of clotting time was observed in the tolerized animals (60.8- 64.5 sec aPTT) over the 4-month period. Controls showed clotting times (aPTTs) of 68.5-72 sec (Fig. 4-5). This reduction in clotting times however, was not as effective as the rapamycin/IL-10/specific peptide protocol. In addition to promoting proliferation of T_{reg}, IL-2 also promotes proliferation of T_{eff}. Deletion of

T_{eff} by the dose of rapamycin administered may have been insufficient to completely control the anti-F.IX immune response.

Prevention of Inhibitor Formation in Hepatic Gene Therapy for Murine Hemophilia B

Rapamycin/IL-10/Specific Peptide Treatment

Hepatic route of vector administration has previously been shown to be less immunogenic than muscle, possibly resulting from the unique environment of the liver, which promotes an induction of T_{reg} following liver gene transfer. Although AAV2-mediated hepatic gene transfer induces robust immune tolerance to hF.IX in several strains of F9-/- mice, an immune response is observed against exogenous hF.IX in F9-/- mice of C3H/HeJ background.

Expecting to improve tolerance to the hF.IX transgene via AAV2-F.IX liver gene transfer, The rapamycin, IL-10, specific peptide IP protocol was combined with liver gene transfer. Rapamycin, IL-10 and the aforementioned specific peptide 2A54 was administered to C3H/HeJ F9-/- mice 3 times/week for 4 weeks. At the third week, AAV2-hAAT-F.IX was administered to the liver via splenic capsule injection. Control animals received the liver gene transfer only (included in Fig. 4-8). Contrary to expectations, the treatment group showed a decrease in hF.IX levels (~10-40ng/ml) compared to controls (~50-110 ng/ml) over a 3-month period although these results lack significance (average data not shown) Only 1 mouse from the tolerized group had the expected increased F.IX expression accompanied by lowered clotting times (Fig. 4-6). The anti-F.IX IgG1 values and neutralizing antibody response (BU) did not show a significant difference between the control and drug cocktail treated groups, which begged the question whether rapamycin was in some way impeding gene transfer to the liver or had a deleterious effect on F.IX expression in a non-immune dependant manner?

To test the possibility of a non-immune mediated suppression of liver cell transduction by the rapamycin/IL-10/specific peptide protocol, C57BL/6 $Rag^{-/-}$ mice (n=5) were treated with

rapamycin, IL-10 and a specific peptide (2A58; hF.IX specific CD4+ T cell epitope for the C57BL/6 mice). The treatment was done 3 times/week for 4 weeks. At the third week AAV2-hAAT-hF.IX ($1x10^{11}$vg) was administered to the liver via splenic capsule injection. Control animals received the liver gene transfer only. hF.IX levels of 1200 ng/ml were observed in the treatment group as well as the control group (Fig 4-7). The result indicates that transduction of hepatocytes is not hampered by the drugs. It can be inferred that the reduction in the hF.IX production in the treated mice was immune function related and may differ depending on the genetic background of the animal albeit the mechanism remains unclear.

Fc-GITR-L treatment

In order to test the fusion protein Fc-GITR-L for its ability to suppress an immune response to AAV2-hAAT-hF.IX in hemophilia B mice; Fc-GITR-L (200 μg/mouse/dose) was injected IP into hemophilia B (C3H/HeJ F9-/-) mice. Seven doses of the fusion protein were administered (200 μg/mouse/dose) 3-4 days apart over a period of 1 month. After 4 injections, AAV2-hF.IX ($1x10^{11}$ vg) was administered to the liver via splenic capsule injection.
Here, we tested whether the induction of $CD4^{+}FoxP3^{+}$ T_{reg} cells by *in vivo* Fc-GITR-L administration could suppress the response, hence augmenting therapy in this strain of hemophilia B mice. Consistent with the Herzog lab's previous findings, C3H/HeJ F9-/- mice expressed only low levels of hF.IX following gene transfer and developed inhibitors of 4-11 BU by 3 months (Fig. 4-8). In contrast, administration of Fc-GITR-L (every 3-4 days for 4 weeks) combined with gene transfer (which was performed after the 4^{th} Fc-GITR-L injection) directed substantially higher levels of hF.IX expression at 1- and 2-month time points (Fig. 4-8**)**. However, expression decreased to the low levels seen in control mice by 3 months (Fig.4-8). In agreement with these data, coagulation times were significantly shortened by 2 month, i.e. the peak of hF.IX expression, but then gradually increased (Fig. 4-8). Antibodies against hF.IX

formed by 5-6 months in 4/5 mice (1-1.6 μg/ml serum), resulting in low-titer inhibitors of 2-3 BU in 2/5 mice by 6 months. Therefore, the effect of Fc-GITR-L treatment in suppressing anti-F.IX responses was transient.

Inhibition of an Established Anti-F.IX Immune Response by the Rapamycin/IL-10/Specific Peptide Protocol

It is of interest for treatment of hemophilia and other systemic protein deficiencies that this novel tolerance protocol can, not only be used prophylactically but also to reverse established immune responses. To address this point, the short-term rapamycin/IL-10/specific peptide protocol was tested in the case of an ongoing response in hemophilia B mice induced by IM gene transfer of AAV1-CMV-hF.IX $1x10^{11}$ vg/mouse. Briefly, C3H/HeJ $F9^{-/-}$ mice were injected with AAV1-CMV-hF.IX $1x10^{11}$ vg/mouse IM and tested for the presence of high titer anti-hF.IX IgG1 that was found to be between 3000 ng/ml to 8000 ng/ml (n=12; Fig. 4-9). The corresponding neutralizing antibody level was ~7-11 BU (Fig 4-9), and there was no hF.IX detectable in circulation at this time point. After confirming the presence of high-titer anti-F.IX antibody, half of the animals (n=6) animals were treated 3 times per week for 4 weeks with rapamycin/IL-10/2A54 specific peptide, at doses as mentioned before. Tolerized mice showed a robust drop in neutralizing antibody levels, which remained low for 6 months. By contrast, controls that received gene transfer only showed persistent high levels of neutralizing antibody levels (~7 BU) through the 6-month period (Fig. 4-9). hF.IX levels in the tolerized mice were between 200 and 600 ng/ml during the 6 months as compared to the controls that did not have detectable levels (Fig. 4-9). In agreement with the above observations, clotting times were observed from an average of 80 seconds before tolerization to ~62 seconds after tolerization; clotting times remained low at the 6 month time point. The short-term protocol is important since it may be helpful in reversing an existing anti-F.IX immune responses in protein replacement

therapy patients who have already developed an immune response (experiments are ongoing). Clearly, future gene therapy trials can benefit from these observations if an immune response is inadvertently induced against the therapeutic protein (or vector capsid?). The long-term beneficial effects of this short protocol are encouraging.

An additional experiment in hemophilia B mice wherein animals with an immune response to AAV1-CMV-hF.IX ($1x10^{11}$ vg/mouse) IM injection were administered with a splenic injection of AAV8-haat-hF.IX ($1x10^{11}$ vg/mouse) did not result in a significant decrease of the IgG1 response against hF.IX.

Prevention of an Anti-F.IX Immune Response in F.IX Protein Therapy

The rapamycin/IL-10/specific peptide protocol was tested in the context of protein therapy in C3H/HeJ $F9^{-/-}$ mice. Mice were treated with the drug-cytokine-specific peptide combination 3 times a week for 4 weeks. After the third week of tolerization the animals were challenged with 1 IU hF.IX protein (Benefix) once a week for 4 weeks (1 IP to prime the immune system, followed by 3 IV injections). Control animals received IV protein challenge only. Three out of five control animals died shortly after the 4^{th} injection, possibly due to an allergic reaction. The remaining mice had neutralizing antibody responses of 5-7 BU (Fig 4-10). There was no mortality among the tolerized mice. Tolerized mice also showed a markedly reduced neutralizing antibody response <2 BU (Fig. 4-10). An initial experiment in normal C3H/HeJ mice was unsuccessful since the animals did not develop a detectable immune response to IV injections of 1 IU hF.IX via tailvein injections, which was further reflected in C3H/HeJ F9-/-. Hence the mice from this background were primed with 1 IP injection of 1 IU F.IX followed by three IV injections of hF.IX to observe a strong immune response. Interestingly this protocol resulted in an acute hypersensitivity response accompanied by lower body temperature, piloerection and death in a large percentage of animals.

Anaphylactic responses to therapeutic proteins in patients with inhibitors seriously complicates therapy. IgE mediated hypersensitivity, IgG1 and IgG4 all have been implicated as a cause for the anaphylactic response in hemophilia B patients and the role of complement activation has been postulated although the precise mechanism remains unclear. In hemophilia B anaphylaxis is often associated with large/complete gene deletions or early stop codon mutations.[100] Combinations of antihistamines and corticosteroids have been utilized during desensitization treatments in patients before ITI.[100] Similarly, in order to reduce the mortality rate in the hF.IX injected C3H/HeJ $F9^{-/-}$ animals, a combination of anti-PAF (50 μg/mouse) and anti-histamine Triprolidiene hydrochloride (100μg/mouse) was administered via tail vein 5 minutes prior to hF.IX protein challenge at the 4^{th} injection. This treatment prolonged the life of the animals and showed an anti-hF.IX IgG1 response (17600 ng/ml SD 5400; n=16). Mice that did not receive the anti-histamine, anti-PAF combination resulted in higher immune responses although 2/6 mice died before blood samples could be collected. The single dose of the drug combination resulted in 100% survival after hF.IX challenge as opposed to 40% mortality in control animals. In a related experiment 6 out of the 16 C3H/HeJ $F9^{-/-}$ mice previously injected with hF.IX and showing a high IgG1 response against hF.IX were orally administered with rapamycin 100 μg/kg thrice a week combined with IV administration of 0.1 IU hF.IX twice a week. All animals died by the second week of tolerization, due to severe allergic reactions to hF.IX indicating that the tolerizing protocol was ineffective in adequately suppressing immune responses against hF.IX at the given doses. Other experiments are in progress to test the role of IP administration of rapamycin/IL-10/specific $CD4^{+}$T cell epitope in diminishing an existing immune response to protein therapy.

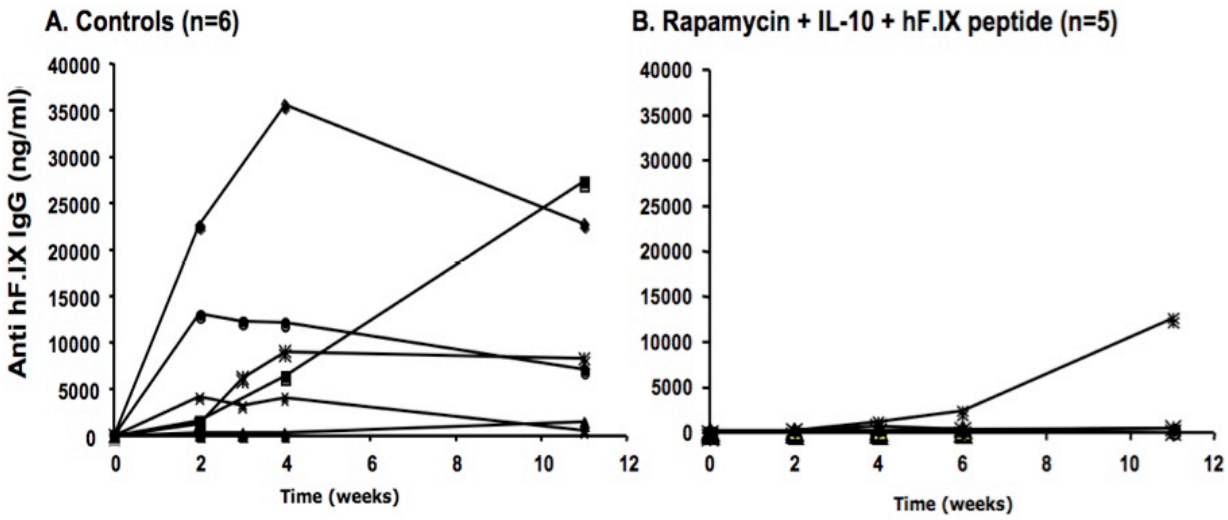


Figure 4-1. Anti-hF.IX formation after IM injection of AAV2-CMV-hF.IX in C57BL/6 mice. The mice were treated IP with Rapamycin/IL-10/B6CD4 specific peptide 3times per week for 4 weeks **B**). During the third week of tolerization, AAV2-CMV-hF.IX $1X10^{11}$ vg/mouse was injected IM. Control animals did not receive immun0modualtion **A**).

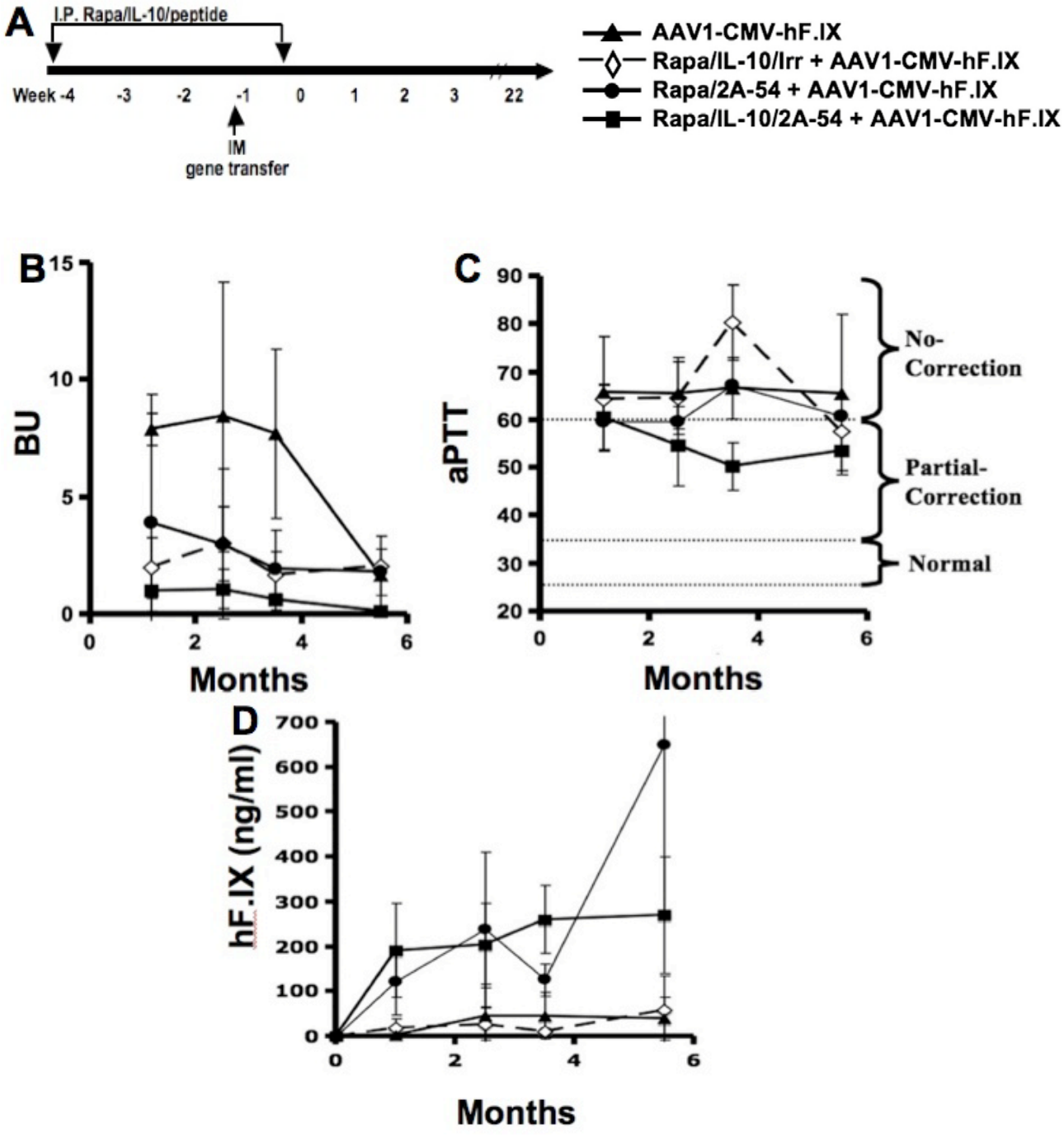


Figure 4-2. Prevention of inhibitor formation in hemophilia B mice via rapamycin/IL-10/specific peptide protocol. C3H/HeJ $F9^{-/-}$ mice were treated with rapamycin/IL-10/2A-54 or rapamycin/IL-10/ova for 4 weeks. After the third week of treatment, AAV1-CMV-hF.IX ($1x10^{11}$vg/mouse) was injected IM **A**). A third group received IM gene transfer only. Shown are the average results ±SD, (n=4 per group) for inhibitory antibody titers (in BU; **A**), hF.IX plasma levels (ng/ml; **B**), and clotting times **C)**; aPTT in sec, which is 25-35 sec for wild-type mice and >60 sec for untreated hemophilia B mice) as a function of time after vector administration.

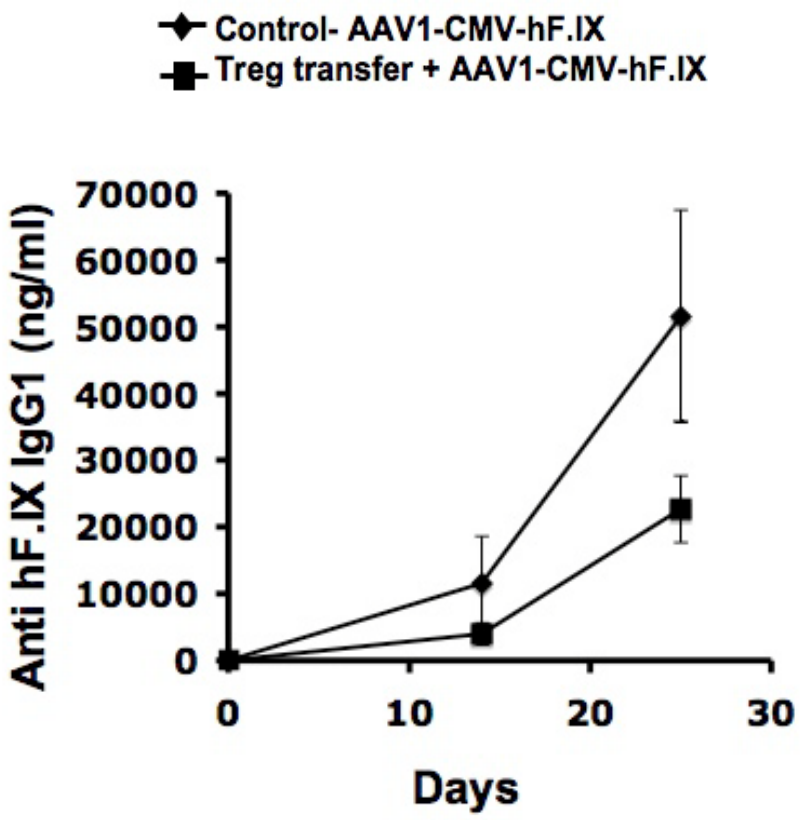


Figure 4-3. T_{reg} induced by rapamycin/cytokine/specific peptide protocol are functional and can be adoptively transferred. C3H/HeJ $F9^{-/-}$ mice were treated with rapamycin/IL-10/2A-54 or rapamycin/IL-10/ova for 4 weeks. After tolerization, CD4+CD25+ T_{reg} cells were isolated via MACS. $1x10^6$ T_{reg} cells were transferred to C3HeJ mice. The recipient mice were given IM injection of AAV1-CMV-hF.IX and later tested for anti-F.IX IgG1 responses shown as averages (ng/ml) ±SD, (n=4 per group).

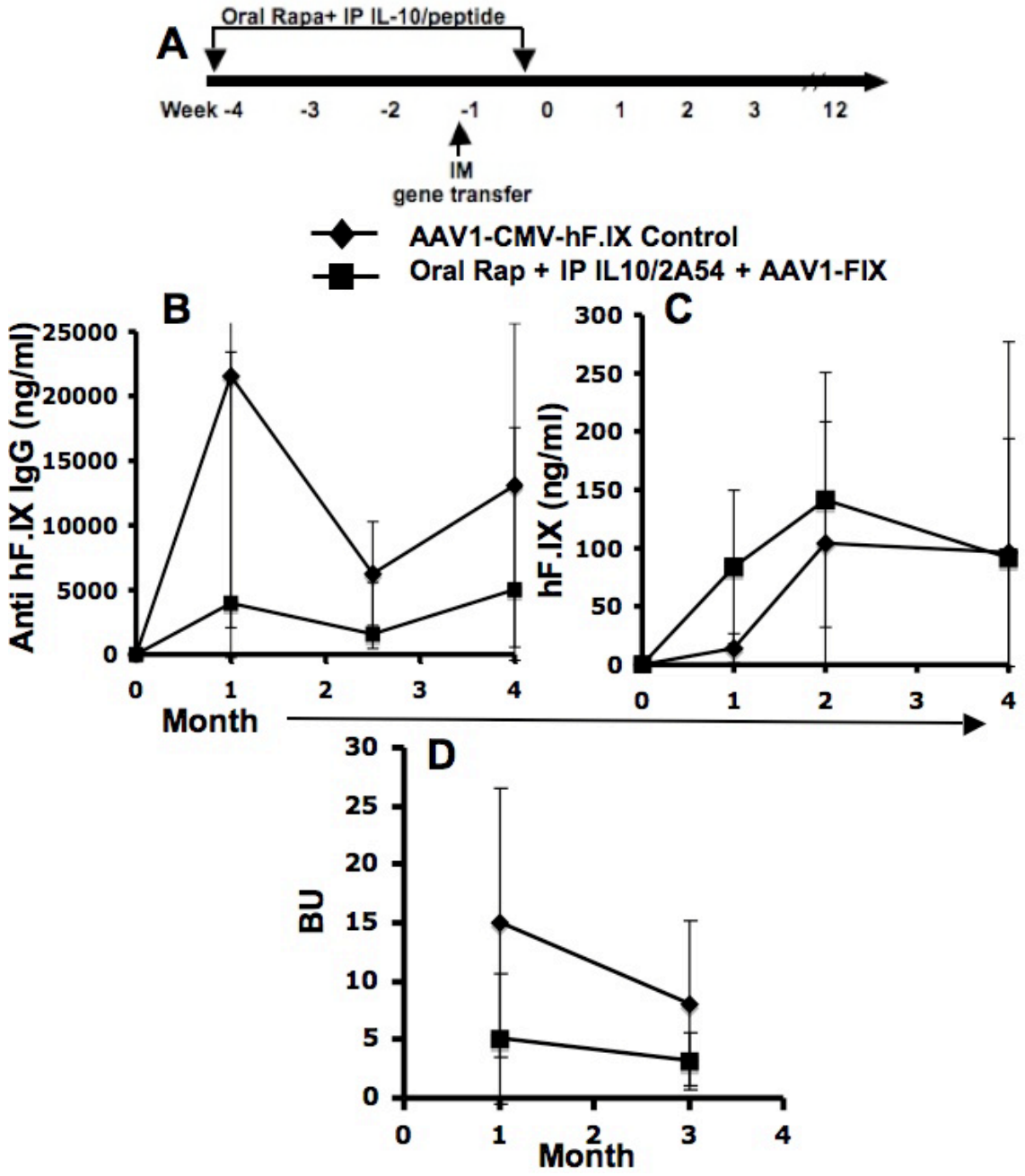


Figure 4-4. Partial suppression of inhibitor formation in hemophilia B mice via oral administration of rapamycin and concurrent IP IL-10/specific peptide treatment. C3H/HeJ F9$^{-/-}$ mice were treated with rapamycin by oral gavage while IL-10/2A-54 were injected IP 3 times/week for 4 weeks. After the third week of treatment, AAV1-CMV-hF.IX ($1x10^{11}$vg/mouse) was injected IM **A**). Control group received IM gene transfer only. The average results ±SD, (n=6 per group) for anti-h.FIX IgG1 antibody (ng/ml; **A**), hF.IX plasma levels (ng/ml; **B**), and inhibitory antibody titers (in BU; **C**).

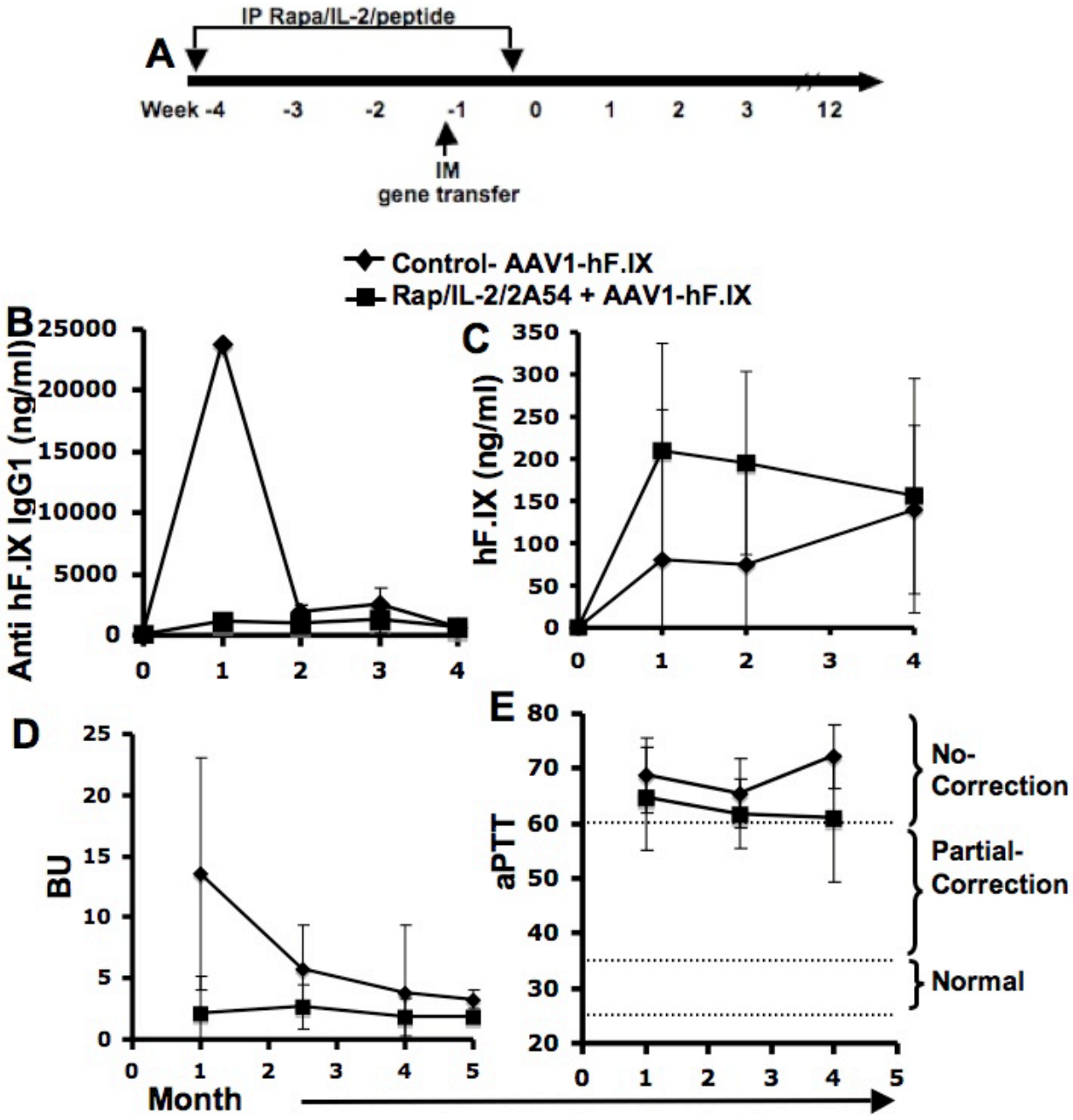


Figure 4-5. Partial suppression of inhibitor formation in hemophilia B mice via rapamycin/IL-2/specific peptide protocol. C3H/HeJ $F9^{-/-}$ mice were treated with rapamycin/IL-2/2A-54 or rapamycin/IL-2/ova for 4 weeks. After the third week of treatment, AAV1-CMV-hF.IX ($1x10^{11}$vg/mouse) was injected IM **A**). Control group received IM gene transfer only. The average results ±SD, (n=6 per group) for inhibitory antibody titers (in BU; **A**), hF.IX plasma levels (ng/ml; **B**), and clotting times (**C**; aPTT in sec).

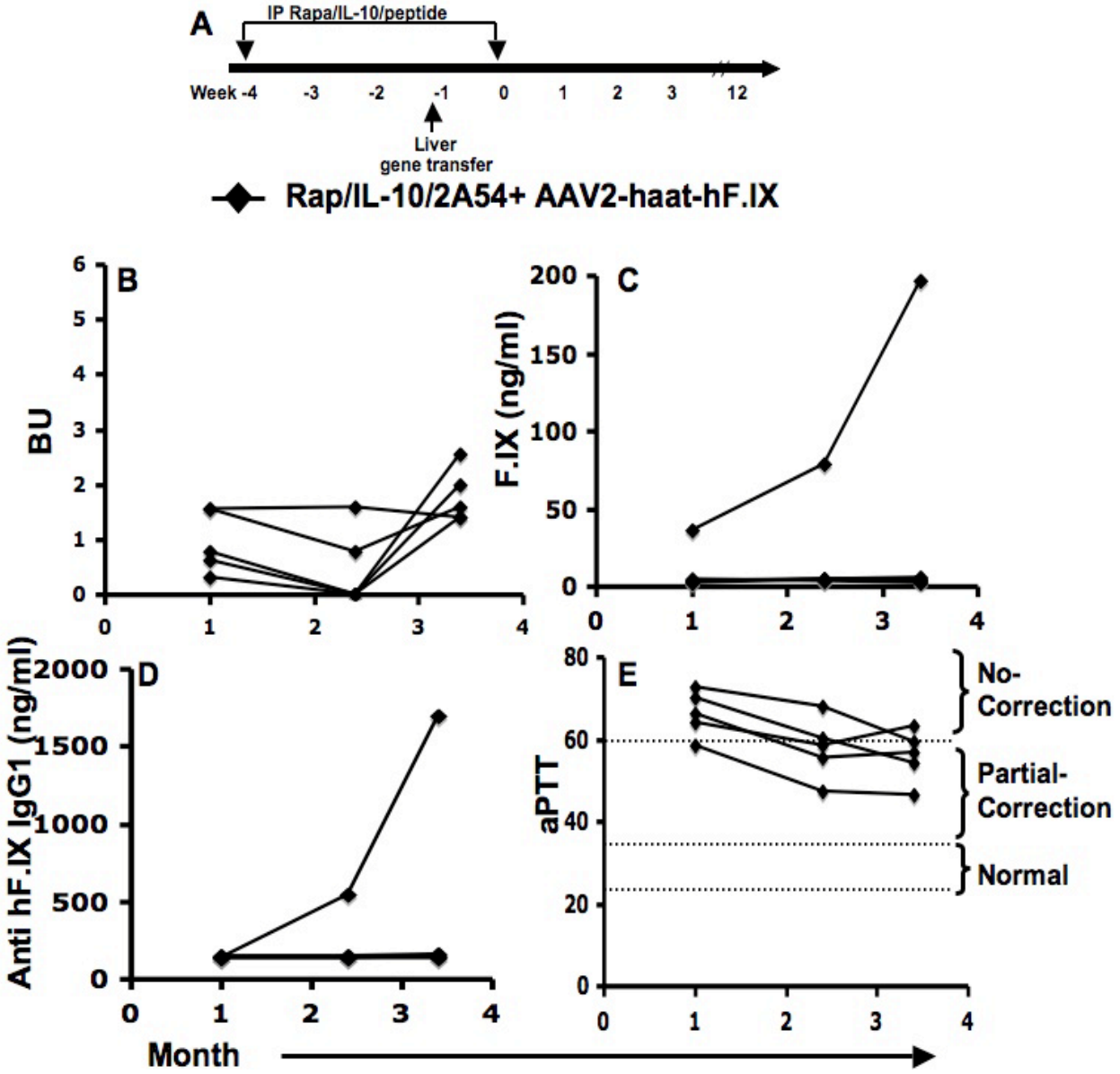


Figure 4-6. Prevention of inhibitor formation in hemophilia B mice via rapamycin/IL-10/specific peptide protocol in liver gene transfer. C3H/HeJ $F9^{-/-}$ mice were treated with rapamycin/IL-10/2A-54 or rapamycin/IL-10/ova for 4 weeks. After the third week of treatment, AAV2-haat-hF.IX ($1x10^{11}$vg/mouse) was injected via spleen capsule **A**). A third group received IM gene transfer only. Shown are the average results for individual mice for inhibitory antibody titers (in BU; **B**), hF.IX plasma levels (ng/ml; **C**), anti-hF.IX IgG1 (ng/ml; **D)** and clotting times (**E**; aPTT in sec) as a function of time after vector administration.

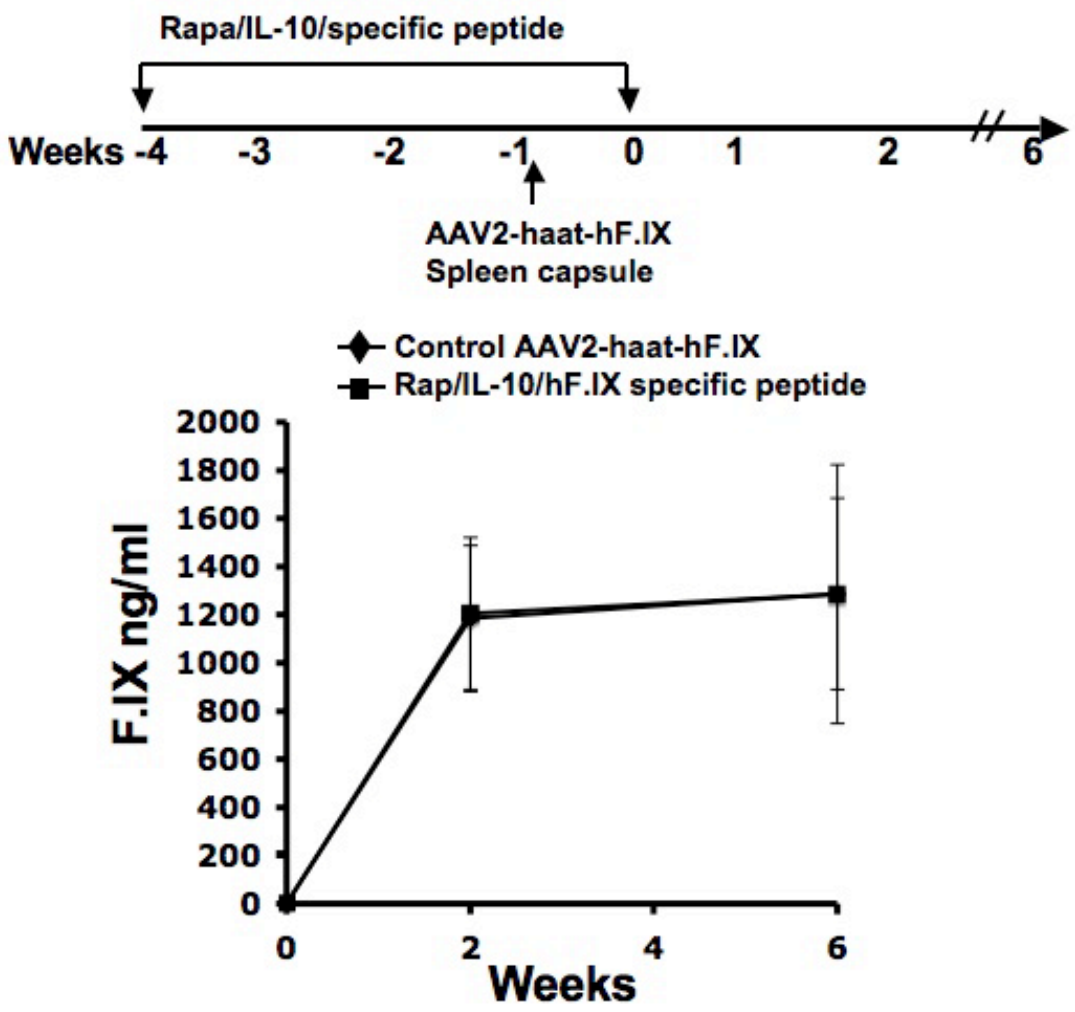


Figure 4-7. Rapamycin/IL-10/specific peptide protocol evaluation in liver gene transfer to $Rag^{-/-}$ mice. C57BL/6 $Rag^{-/-}$ mice (n=5) were treated for 4 weeks with Rap/IL-10/hF.IX specific peptide; Control and treatment groups were given liver gene transfer (AAV2-haat-hF.IX) $1x10^{11}$vg/mouse) via spleen capsule injection and tested for hF.IX expression by ELISA.

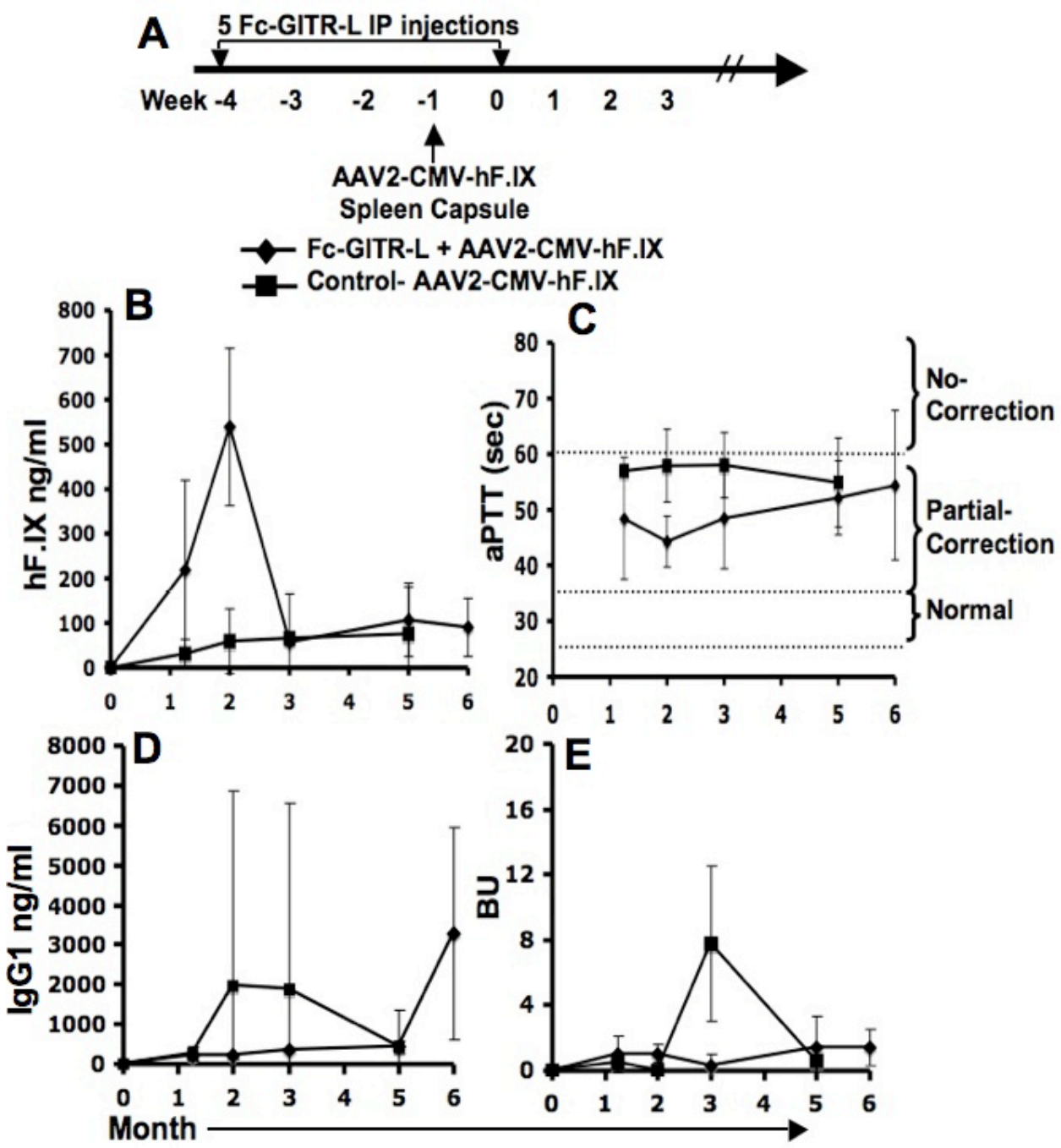


Figure 4-8. Delay of immune response to hF.IX in hemophilia B mice following administration of Fc-GITR-L. C3H/HeJ F-IX$^{-/-}$ mice (n=5) were treated with 7 doses of Fc-GITR-L IP, AAV2-hAAT-hF.IX was injected into the liver via spleen capsule injection 2 weeks into treatment. Controls (n=7) received gene transfer only.

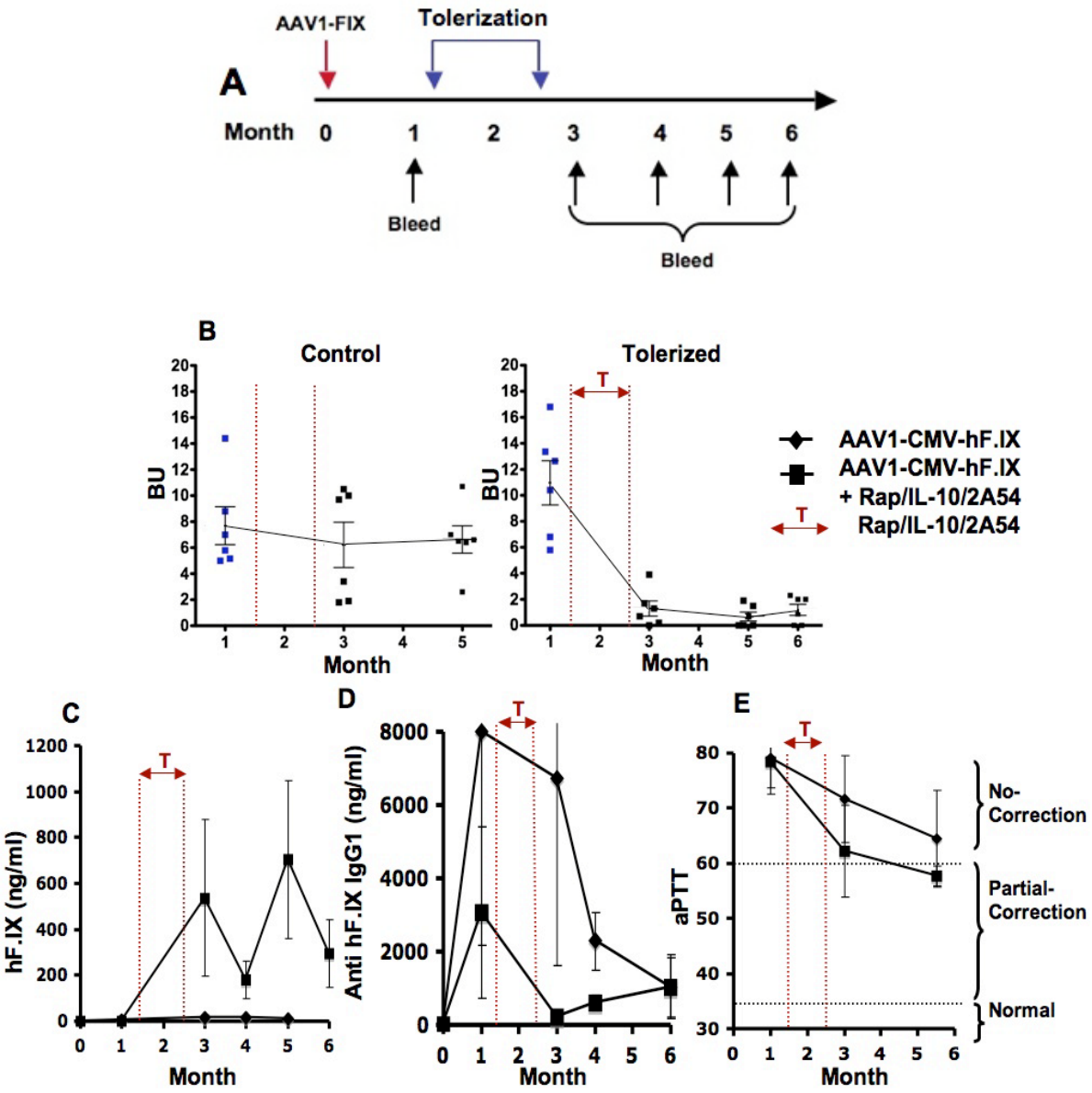


Figure 4-9. Elimination of a pre-existing/ongoing anti-hF.IX immune response. C3H/HeJ F9$^{-/-}$ mice were injected IM with AAV1-CMV-hF.IX ($1x10^{11}$vg/mouse) to generate an immune response. After the proven existence of an immune response, animals were given rapamycin/IL-10/specific peptide for 4 weeks. Controls only received gene transfer **A**). Average results ±SEM, (n=6 per group) for inhibitory antibody titers (in BU; **B**), hF.IX plasma levels (ng/ml; **C**), anti-hF.IX IgG1 antibody (ng/ml; **D**) and clotting times (aPTT; **E**) as a function of time after vector administration are shown.

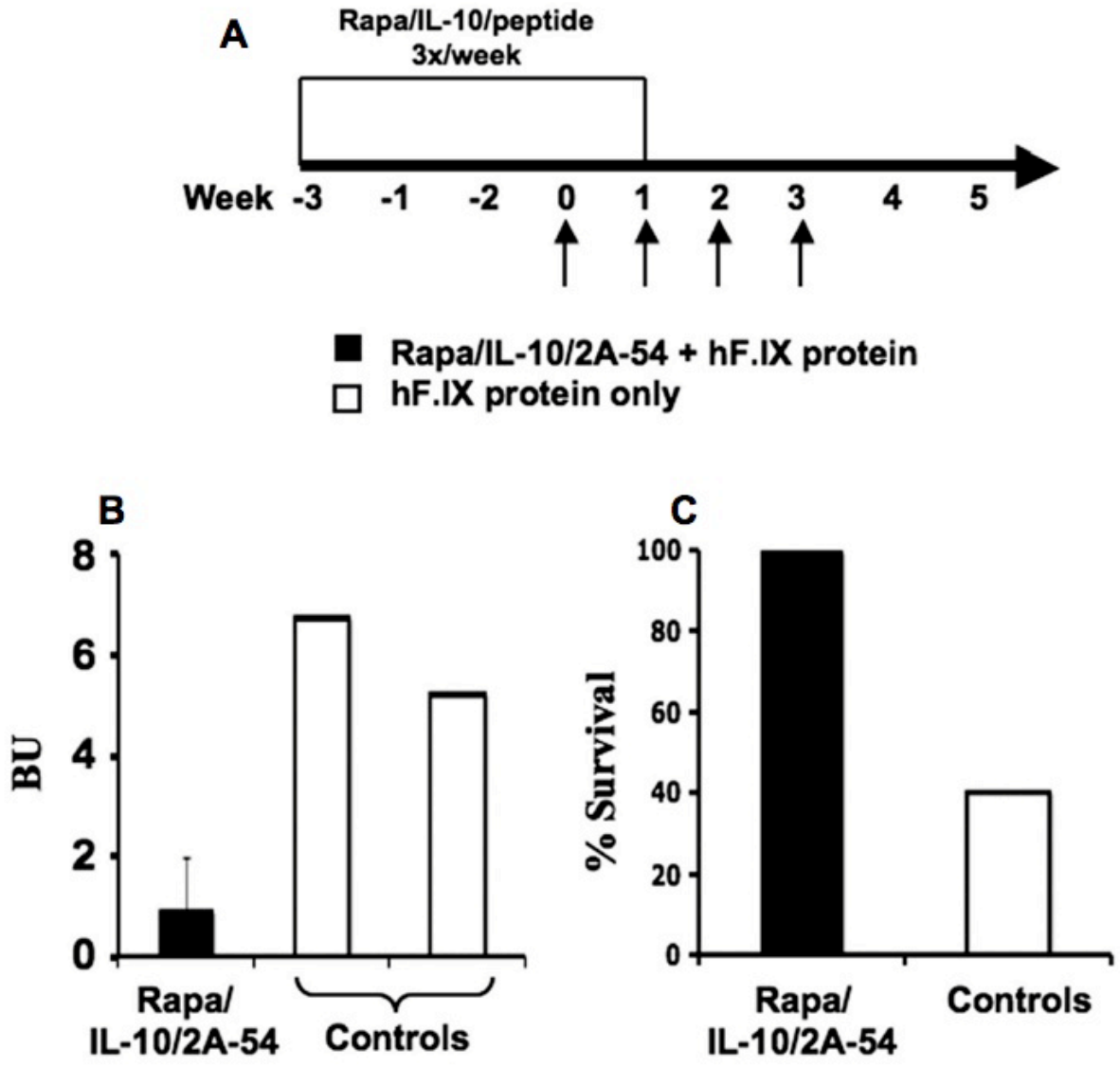


Figure 4-10. Prevention of inhibitor formation in hemophilia B mice receiving protein therapy. C3H/HeJ F9-/- mice were treated with rapamycin/IL-10/2A-54 for 4 weeks. **A**). After the third week of treatment, weekly administration of Benefix (arrows) was initiated. **B).** Inhibitor titers (average ±SD; n=5) after the 4th protein injection were compared to control mice that had received protein therapy only (results shown for 2 surviving individual mice). **C**). Survival of tolerized and control C3H/HeJ F9-/- mice after 4 injections of Benefix (n=5 per group)

CHAPTER 5
DISCUSSION

The immune system has evolved to fight off infections. In order to achieve an effective and specific response and to create memory, antigen presentation to T lymphocytes results in the generation of effector and memory T cells. $CD4^+$ T cells provide help for activation of B cells, which subsequently form antibodies. However, if the adaptive response is directed against a therapeutic protein, it can become a major complication and source of immunotoxicity in gene and protein replacement therapies for genetic disease. Hemophilic patients with inhibitor formation are at a higher risk for bleeding-related morbidity and mortality. Patients at high risk for antibody formation in protein replacement therapy for hemophilia and lysosomal storage disease (LSD) would benefit from prophylactic immune tolerance protocols. Furthermore, safety of gene therapies, which offer the chance of long lasting cures for genetic disease, would increase if such protocols were developed. The possibility of eliminating a pre-existing or ongoing immune response must be emphasized, in patients who have previously developed an immune response to protein treatment.

Creating an Optimal Shift From an Effector to a Regulatory T Cell Population

Experiments presented in this thesis illustrate the usefulness of the highly defined DO.11.10-tg $Rag2^{-/-}$ mouse model to screen for useful drugs and define their mechanism of action. The drug cocktail protocols proposed were evaluated and are found to be effective in directing a shift from a T_{eff} to a T_{reg} response upon specific antigen presentation to $CD4^+$ T cells (Fig. 5-1). Changing the balance of the immune system in favor of regulation is critical for tolerance to the therapeutic protein. Rapamycin, and even more so the combination of rapamycin and IL-10, had a substantial effect by deleting ova-specific $CD4^+$ T cells while allowing induction of $CD4^+CD25^+FoxP3^+$ T_{reg} cells. In the absence of the specific antigen (ova in this

case), the drugs did not change the ova-specific T cell population. The presence of the antigen specific $CD4^+$ T cell epitope provides direction to the immune response due to the mechanism of depletion of effector $CD4^+$ T cells. Mechanistically, the data demonstrates activation of $CD4^+$ T cells upon *in vivo* antigen presentation in the presence of rapamycin/IL-10 and the specific peptide, resulting in apoptotic cell death (AICD). At the same time, we found a greater resistance to antigen/rapamycin-induced apoptosis in T_{reg} compared to T_{eff}, which explains why T_{reg} induction and expansion is successful in regimens that utilize rapamycin. Rapamycin/FKBP12 complex inhibits IL-2 stimulated signal transduction by preventing phosphorylation of p70 S6 kinase and of eIF-4E BP1, which are both regulated by mammalian target of rapamycin (mTOR), leading to a block in cell cycle progression. A recent study has shown that IL-2 stimulation of T_{reg}, compared to T_{eff}, leads to preferential use of the STAT5 rather than the mTOR pathway, which results in resistance to inhibition of mTOR [101]. The T_{eff} cell population regained responsiveness to their specific antigen when T_{reg} were depleted while T_{reg} were able to dampen the antigen specific immune response on in vitro stimulation. This is similar to previously published findings wherein the depletion of $CD25^+$ T_{reg} using anti-CD25 antibody resulted in an increased immune response.[94] T_{reg} induction by repeated antigen/rapamycin administration was more robust than in hepatic ova gene transfer published previously, which shows the usefulness of the DO.11.10-tg $Rag2^{-/-}$ mouse experimental system for comparison between different treatment approaches. [8]

Different routes of administration of the Rapamycin/IL-10 specific peptide protocol resulted in a differential induction of T_{reg} and varying depletion of T_{eff}. Intraperitoneal, intravenous and subcutaneous methods of administration proved to have a significantly better effect than oral gavage. This difference in results depending on routes may be due to the

bioavailability of the drugs or metabolism in the digestive system or due the splitting of the rapamycin (oral gavage) and IL-10/specific peptide (IP) which would indicate the importance of the presence of the three components of the cocktail *in vivo* at the same time for adequate antigen presentation, T_{eff} cell deletion and T_{reg} induction.

IL-2 cytokine has been previously shown to induce T_{reg}. It is also known to encourage T_{eff} cell proliferation and is produced by T cells for autocrine and paracrine function. The cytokine IL-2 tested in combination with rapamycin and specific peptide showed better T_{reg} cell induction than IL-10 in DO.11.10-tg $Rag2^{-/-}$ mice although its effects were not as significant as the combination of rapamycin and IL-10 in hemophilia B mice; possibly due to the otherwise immune compromised transgenic model system. In addition, IL-2 is known to induce the expansion of T_{reg} and T_{eff} and hence there is a possibility of some "runaway" T_{eff} cell proliferation that slightly dampened the overall antigen specific immune suppression. Flt3L is known to induce T_{reg} and hence is likely to work synergistically with rapamycin. Flt3L in combination with rapamycin and specific peptide resulted in a very significant T_{eff} cell deletion accompanied by a robust T_{reg} cell increase and may be a promising candidate for further testing in hemophilia and other diseases. Fc-GITR-L also showed promising T_{reg} induction accompanied by antigen specific T_{eff} deletion in combination with rapamycin and specific peptide although its effects tended to have a more variable effect from one mouse to another.

T_{reg} are Critical for Many Tolerance Induction Protocols

Usefulness of the rapamycin/IL-10/antigen cocktail for therapeutic application was demonstrated in the hemophilia B mouse model, where inhibitor formation was effectively blocked using a short (4-week) tolerance protocol starting 3 weeks prior to muscle-directed gene transfer. Immune tolerance protocols that utilize immune suppressive drugs will be acceptable for treatment of hemophilia, only if they are limited in time. A recent study by Lillicrap and

colleagues accomplished this goal in hemophilia A mice by short-term administration of low doses of a non-Fc-receptor binding anti-CD3 monoclonal antibody. This protocol caused a shift in the Th1/Th2 ratio during the response to F.VIII, and tolerance to F.VIII was dependent on T_{reg} function. [102] Transient blockade of ICOS signaling, a co-stimulatory pathway during T cell activation, at the time of hepatic gene transfer with plasmid DNA also blocked inhibitor formation to F.VIII and resulted in T_{reg} induction.[103] In contrast to these studies, utilizing more recently developed and still experimental reagents, our investigation established the usefulness of a drug, rapamycin, which is well established in clinical practice. Therefore, translational research could take advantage of a wealth of experience in humans (including pediatric patients). Furthermore, this protocol resulted in only moderate general immune suppression. Oral administration may also result in Tr1 activation, which was not seen in these experiments or by lab colleagues in the spleen, but which may add another useful subset of regulatory T cells.[104] Finally, the importance of T_{reg} function during immune suppression with rapamycin upon F.IX gene transfer is also supported by data in non-human primates.[46]

Implications for Translational Research

Prevention of inhibitor formation upon muscle gene transfer required co-administration of the immune modulatory drugs rapamycin/IL-10 and the hF.IX-specific peptide. Without the specific antigen, the drugs were only partially effective in blocking the response to hF.IX expressed in skeletal muscle. These findings indicate that antigen-specific immune tolerance protocols that are initiated prior to the onset of therapy may have more beneficial effects than general immune suppression applied at the time of gene transfer. In this regard, peptide administration is highly effective, because the peptide can be directly loaded onto MHC molecules without a need for antigen processing and because the risk of inducing an adaptive response against the cognate protein antigen in the early phase of the protocol (before tolerance

is established) is substantially reduced.[90] However, translation to the clinic would require knowledge of $CD4^+$ epitope for each patient being treated, which although possible is not economically feasible at this point in time. Therefore, it is desirable to develop and optimize the protocol using the entire protein as the antigen for induction of T cell tolerance. However two attempts to devise a protocol with different concentrations of the entire hF.IX protein instead of the $CD4^+$ T cell epitope in hemophilia B mice were not successful. Additional trials in Hemophilia A mice with heat inactivated protein or various low doses of whole protein in IP or IV routes of injection in combination with rapamycin and IL-10 were also not successful, indicating the difficulty of using the whole protein in the tolerization protocol. Nonetheless Dr. Babak Moghimi succeeded in inducing tolerance to F.VIII in Hemophilia A mice using a combination of oral treatments with rapamycin and IV whole F.VIII protein (given at a low dose) in protein replacement therapy (manuscript in preparation). These data show that oral administration of rapamycin and use of protein antigen can be effective but requires significant optimization.

The use of rapamycin/IL-10/specific peptide protocol in protein replacement therapy for hemophilia B when delivered via IP administration caused decreased mortality and decreased neutralizing antibody responses when treatment was started before challenge with hF.IX. Control animals receiving hF.IX challenge only developed severe allergic reactions, accompanied by lower body temperature and piloerection, eventually leading to death; while tolerized animals remained healthy. An effort to reduce or eliminate an ongoing immune response in hemophilia B mice brought on by hF.IX administration, by oral administration of rapamycin in combination with IP injections of small doses of whole F.IX protein resulted in allergic reactions and death of all treated mice within 2 weeks after the beginning of the “tolerization” protocol. The mice likely

died due to an antibody mediated hypersensitivity response due to their prior exposure to the F.IX protein, which occurred in spite of the presence of rapamycin. Work by others in the laboratory showed that repeated IV delivery of F.IX results in an IgE formation in addition to IgG1. In a different experiment, Hemophilia B mice with an ongoing immune response were given an injection of AAV8-hAAT-hF.IX, these mice did not undergo hypersensitivity responses and remained alive. The difference in mortality rate compared with the mice that underwent rapamycin/whole protein tolerization suggests that liver delivered protein antigen did not systemically react with antibody in the way that IV delivered protein did, this aspect requires further study.

With regard to rapamycin/IL-10/specific peptide protocol in combination with liver gene therapy, the results were surprising as the effect of the treatment was to increase the overall antibody response slightly in treated animals with an actual decrease in hF.IX production compared to the gene transfer controls. This phenomenon was further studied in Rag-/- mice to eliminate the possibility of reduced transduction of hepatocytes by AAV-hF.IX in the presence of rapamycin. Results showed that rapamycin did not block transduction of hepatocytes in the Rag-/- mice. The strain of mouse used may prove important as the hemophilia B mice were on a C3H/HeJ background but the Rag-/- mice used were on a C57BL/6 background.[105] This experiment also suggests that the immune system was in some way involved, although the mechanism remains unclear.

In conclusion, antigen administration, when combined with a suitable cocktail of immune modulatory drugs, can create a powerful shift from an effector to a regulatory T cell population. This concept can be exploited for the development of prophylactic immune tolerance protocols for treatment of inherited protein deficiencies such as hemophilia

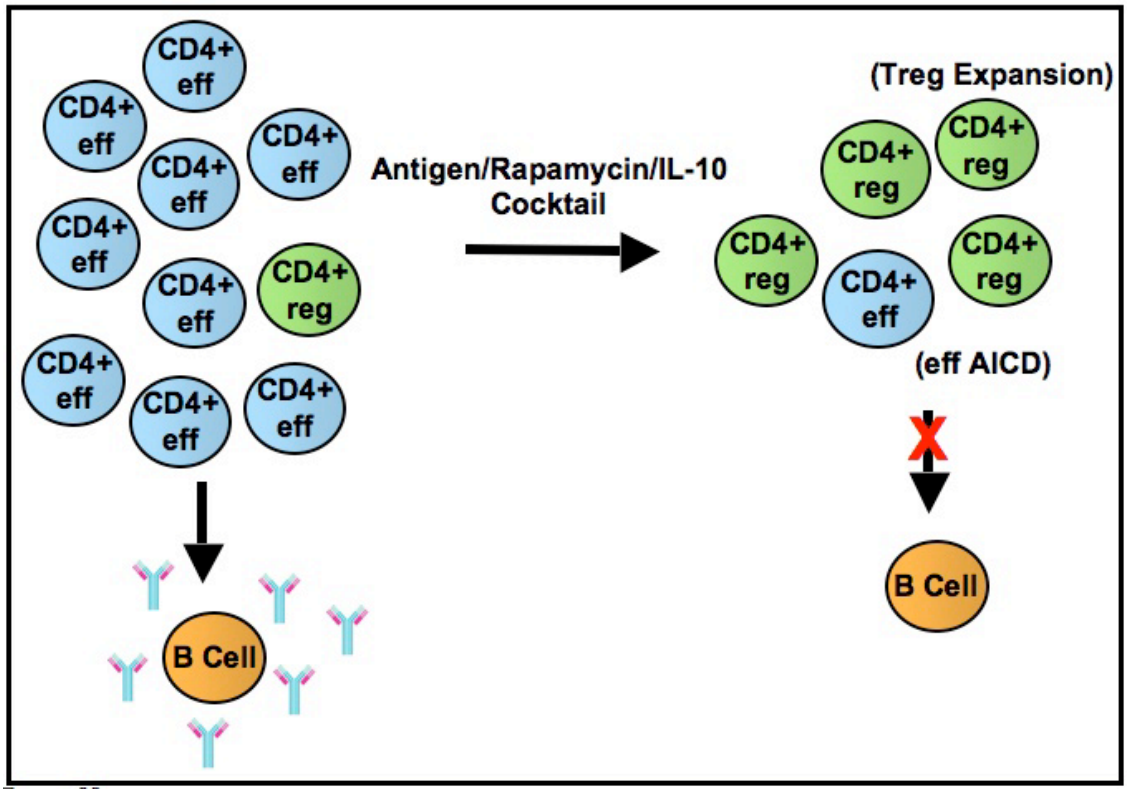


Figure 5-1. Induction of tolerance to therapeutic proteins by shifting the balance from an effector ($CD4^+$ T_{eff}) to a regulatory response ($CD4^+$ T_{reg}) prior to gene or protein replacement therapy in an antigen-specific manner.

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BIOGRAPHICAL SKETCH

Sushrusha Nayak was born in 1981 in Manhattan, New York, USA to Dr. Rabindranath Nayak and Mrs. Sukanti Nayak. She grew up in Bangalore, India within the ramparts of the Indian Institute of Science. The association with a major academic institution in India afforded her the opportunity to test her interest in fields like ethology and biomedical science with Dr. Gadagkar, Dr. Ramananda Rao, Dr. Lakshmi Sita and Dr. R Nayak. She completed her undergraduate degree in Microbiology, Zoology and Chemistry in 2003 and received her Master of Science in biotechnology in 2005 from Bangalore University. During her master's program she was drawn towards the study of immunology, gene therapy and ribosomal RNA based research. She proceeded to join the Interdisciplinary Program in Biomedical Science at the University of Florida in 2005 with ample support from her parents. She trained in the laboratory of Dr. Alfred Lewin, Dr. William W. Hauswirth, Dr. Susan L. Semple Rowland and Dr. Roland Herzog during rotations, before joining the laboratory of Dr. Roland Herzog in 2006. The Herzog lab focused on gene therapy and immunological studies in hemophilia. She received the Medical Guild research incentive award in 2007 and research competition award in 2009. She published her first two first author papers in 2009, including a research and a review article. She undertook an MS degree in management at the Warrington Business School, University of Florida, concurrent with her Ph.D. work in 2008, which gave her an insight into the working of business organizations in the USA.

Sushrusha plans to continue developing her career in translational medicine, gene therapy and immunology with an eye open for collaborations with the industry. She hopes to keep her professional and personal life moving in a positive direction with reasonable harmony.