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Advances in Overcoming Immune Responses following Hemophilia Gene Therapy

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Abstract

Both Clinical trials and pre-clinical experiments for hemophilia gene therapy showed that it is important to overcome potential immune responses against gene transfer vectors and/or transgene products to ensure the success of gene therapy. Recently various approaches have been investigated to prevent or modulate such responses. Gene transfer vectors have been specifically engineered and immunosuppressive regimens have been administered to avoid or manipulate the immune responses against the vectors. In order to prevent cytotoxic lymphocyte or antibody formation induced by transgene expression, novel approaches have been developed, including methods to manipulate antigen presentation, development of variant genes encoding less immunogenic proteins or gene transfer protocols to evade immune responses, as well as immunosuppressive strategies to target either T and/or B cell responses. Most of these successful protocols involve the induction of activated regulatory T cells to create a regulatory immune environment during tolerance induction. Recent development of these strategies to evade vector-specific immune responses and induce long-term immune tolerance specific to the transgene product will be discussed.

Keywords: Hemophilia; Gene therapy; Immune modulation; Regulatory T cells; Immunotherapy; Tolerance induction; Innate and adaptive immune responses

Introduction

Hemophilia A or B is congenital bleeding disorders caused by a deficiency of the blood clotting protein, factor VIII (FVIII) or factor IX (FIX), respectively; in its severe form, hemophilia is a life-threatening, crippling hemorrhagic disease. Recent development of efficient gene transfer technologies has promoted their application in hemophilia treatment. However from both pre-clinical experiments in animal models and human clinical trials, it is realized that immune responses against gene transfer vectors or transgene products can become major obstacles to the success of gene therapy [1-3].

Hemophilia patients have long been considered as an excellent candidate population for developing gene therapy approaches. This is due to the fact that current protein replacement therapy is very costly, and repeated infusions are required for both acute and prophylactic treatment. In addition, high risk of bleeding exists for the hemophilia patients and their disease resulting from a single factor deficiency can be corrected by a single gene addition. Previously, several phase I clinical trials have been attempted to treat hemophilia A patients [4-6]. However, only transient, low-level FVIII protein expression has been achieved, owing to inefficient gene transfer or impaired survival of transferred cells along with the possible development of immune responses against FVIII and/or associated gene transfer vectors. Similarly, phase I clinical trials for hemophilia B patients using retroviral vectors only produced transient FIX gene expression [7,8]. Subsequently, a study using adeno-associated viral (AAV) gene transfer of FIX into muscles cells was carried out in hemophilia B patients [9]. Long term gene expression was detected in the injection sites of the skeletal muscles, however, circulating FIX levels was generally below the therapeutic range [10]. In this case, gene transfer treatment was well tolerated and no antibodies to FIX were detected. To increase the efficacy of gene transfer, next trial was carried out using liver-directed, AAV-mediated gene transfer of FIX in hemophilia B patients [11]. Therapeutic levels of FIX was achieved initially, however, transgene expression declined at 6 weeks post hepatic gene transfer which correlated with a transient elevation of transaminase levels, indicating CTL responses against the transduced hepatic cells. T cell mediated immunity was also confirmed by the detection of AAV capsid specific CD8+ T cells in a treated patient with low titer of pre-existing antibodies to AAV-2. Most recently, gene therapy clinical trial for hemophilia using AAV serotype 8 (AAV8) vectors to deliver FIX gene into the liver achieved persistently 2 to 11% of normal FIX levels in six treated patients (2 patients each in 3 cohorts treated with high, intermediate, or low dose of vector) for 6 to 16 months [12]. Four patients did not require factor supplements; the other two required less prophylactic injections. However, of the other two patients in the high dose group, one had a transient, asymptomatic elevation of serum transaminase levels and did not achieve an initial therapeutic response. Low-titer neutralizing antibodies against AAV capsids were detected. The other one had a slight increase in liver enzyme levels, and the cause of which is unclear. Interestingly, following subsequent treatment of glucocorticoid therapy, the transaminase levels in both patients returned to normal and 3 to 11% of normal FIX levels were maintained [12]. In addition, no inhibitory antibody against FIX was detected in any of these patients. The long term responses from these patients are currently being followed [13].

From these clinical studies, it is clear that devising safe and effective methods to modulate immune responses is essential to ensure the success of hemophilia treatment. The development of new novel immunomodulation strategies in animal models is discussed below.

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Overcoming vector induced immune responses following hemophilia gene therapy

Following gene transfer, inflammation and innate immune responses against the gene delivery vectors (viral glycoproteins, RNA, and CpG DNAs, etc) and ensuing adaptive immune responses against specific viral proteins and/or transgene products can occur, resulting in induction of humoral responses and CTLs and killing of vector transduced cells. Various strategies have been investigated to avoid or manipulate the immune responses against specific gene transfer vectors.

Adeno-Associated viral vectors

As mentioned in the introduction, multiple clinical hemophilia B gene therapy trials have been carried out using AAV vectors. AAV vectors are nonpathogenic, replication-deficient, and elicit very weak innate immune responses. Long-term gene expression of FIX has been extensively demonstrated in mouse and dog models after muscle- or liver-directed gene transfer [14-17]. Nevertheless CTL responses to AAV capsid antigen especially in patients with pre-existing neutralizing antibodies against AAV remain a major road block to achieve persistent therapeutic correction for clinical application [11].

Recently multiple serotypes of AAV in addition to AAV2 have been developed; these serotypes carry different capsid proteins and exhibit different tropism towards different organs [18]. However these capsid proteins share high percentage of homology and may have significant crossreactivity with neutralizing antibodies raised against a particular serotype. Since AAV vectors remain mostly in episomal forms in transduced cells, it is uncertain if AAV will produce life-long transgene expression *in vivo*. If transgene expression declines over time, whether repeated treatment by AAV using different serotypes is feasible due to memory T cell immunity is currently unknown.

New strategies have been developed to combat these problems by modifying the capsid proteins. Alternative serotypes that do not contain a heparin binding site are less reactive with dendritic cells, thus activate CD8+ T cells less efficiently [19]. Domain shuffling of AAV capsids has been carried out to re-construct more efficient and less immunogenic capsid proteins [20,21]. Another strategy is to produce AAV capsids that can more rapidly uncoat/degrade inside the cells to reduce its immunogenicity. Proteasome inhibitor, bortezomib has been used to simultaneously enhance gene expression and decrease capsid antigen presentation on hepatocytes, thus decreasing the extent of immune activation of antigen-specific immune responses [22]. Furthermore, immunosuppressive protocol using mycophenolate mofetil (MMF) and cyclosporine A (CSA) was successfully applied to suppress T cell responses against the capsids at low vector dosages [23].

Recent reports by Herzog and others indicate that innate immune system also plays important roles in activation of immunity by AAV-mediated gene transfer, both in inducing the initial response to the vector and in promoting a deleterious adaptive immune responses [24,25]. The initial innate immune responses were mediated by the TLR9-MyD88 pathway via a traditional NF-κB pathway to induce type 1 IFN production. Subsequently, alternative NF-κB pathway was triggered, prompting adaptive immune responses. It was demonstrated that NF-κB inhibitor, Bay11 was able to block both NF-κB pathways and eliminate proinflammatory cytokine expression, leading to persistent transgene expression [26]. These studies suggest that transient immunosuppression to block potentially detrimental signaling pathways mediating innate and adaptive immune systems may significantly improve the safety and efficacy of AAV-mediated gene therapy.

Adenoviral vectors

Adenoviral vectors have been studied intensively in gene delivery for hemophilia. Adenoviral vector particles can elicit both strong innate and adaptive immune responses. The interplay of both systems activates CD4+ and CD8+ T cells, and B cells, as well as facilitates the induction of transgene-specific immune responses; for example, administration of a FIX-adenoviral vector efficiently activated FIX-specific cytotoxic T lymphocytes (CTL) and generated FIX-specific antibodies [27,28]. Recent efforts have been made to reduce the immunogenicity of the vectors by development of new large capacity or gutted (devoid of all viral genes) vectors [29] or modification of capsid sequences [30,31]. However, even using a gutted vector, one major disadvantage is that adenoviral vectors can efficiently transduce APCs [32,33], which readily triggered innate immune responses and further augmented the induction of adaptive immune responses to the transgene product. This problem led to the introduction of tissue-specific promoters in gutted adenoviral vectors to restrict transgene expression in target cells but not in APCs [34]. Therapeutic levels of FVIII or FIX can be achieved using gutted adenoviral vectors with expression cassettes driven by a liver–specific promoter with limited toxicity in hemophilia mice [32,35] and dogs [36,37]. However, antibodies against transgene products eventually developed in several animals and transient hematologic and hepatic toxicities were observed in high-dose animal groups [37,38]. Subsequent phase I clinical trial achieved very low (~1%) levels of FVIII, which was stopped upon the detection of transient inflammatory response with hematologic and liver abnormality [6]. Strategies to further reduce the immunogenicity of adenoviral vectors are needed to evade the induction of strong immune responses against the transgene product. Localized injection of low vector doses into the liver by balloon occlusion catheter delivery helped further reduce the inflammatory and immune responses elicited by adenoviral vectors and led to long-term gene expression in nonhuman primates [39]. Transient non-specific immune suppressive regimens may also aid in reduction of the immunotoxicity of adenoviral vectors.

Retroviral/lentiviral vectors

Retroviral/lentiviral vectors are attractive for gene therapy since these vectors are devoid of all viral genes, integrate into the genome to yield persistent gene expression, and can accommodate relatively large genes [40]. Lentiviral vectors also had the added advantage of infecting non-dividing cells, which is superior than retroviral vectors for *in vivo* transduction of quiescent hepatocytes for hemophilia gene therapy. Transient therapeutic levels of FVIII or FIX were achieved following LV-mediated gene delivery into the liver [41,42]. Nevertheless, lentiviral vectors can efficiently interact with APCs and stimulate a rapid proinflammatory response. Rossetti and colleagues [43] found that delivery of lentiviral vectors induced strong innate immune responses via activation of pDCs and secretion of IFNαβ and TNF-α, leading to elimination of LV transduced cells. In another study [44], the innate responses induced in the cell culture system by LV transduction leading to activation of pDCs and IFNα secretion was found to be elicited not by LVs but by a particular tubulovesicular structures (TVS) derived from VSV-G transfected producer cells. Further *in vivo* testing of VSV-G pseudotyped vectors and elucidation of mechanisms triggering the innate host responses are needed for future human applications. LV pseudotyped with the GP64/Sendai envelope proteins may reduce macrophage transduction and help

evade immune activation [45]. A feline immunodeficiency virus (FIV)-based vector pseudotyped with GP64, which is resistant to human or mouse complement and has high tropism towards liver cells, delivered the B-domain-deleted FVIII gene driven by a liver-specific promoter efficiently into the hemophilia A mouse liver and achieved sustained FVIII gene expression for several months [46]. More recently, LV pseudotyped with the GP64 glycoprotein, in conjunction with a liver-restricted promoter and a microRNA (miRNA)-regulated FVIII transgene also effectively transduced hepatocytes and produced long-term FVIII gene expression [47].

Nonviral vectors

Nonviral gene delivery can be divided into two general catagories: gene delivery using chemical or physical methods. Chemical methods include encapsulating the plasmid DNA into chemical conjugates or nanoparticles made of lipids or polyplexes to facilitate gene transfer [48]. Physical methods involve delivery of genes into cells facilitated by various physical means including hydrodynamics-based delivery [49-51], electroporation [52], and sonoporation [53], etc. In general, DNA carriers and cargo DNAs of nonviral gene delivery are less immunogenic compared to viral vectors. However, the efficiency of nonviral gene delivery is relatively lower than viral vectors mediated gene delivery.

Synthetic carriers such as chitosan and PEIs have been shown to successfully deliver FVIII gene without eliciting much side effects such as immune responses to the carriers [54-56]. Furthermore, one of the major advantages to perform nonviral gene transfer using either chemical or physical methods is that the cargo delivered is naked DNA which is easy to prepare, non-pathogenic, and less immunogenic. However, although delivery of naked plasmids encoding FIX or FVIII into the liver which is a somewhat immune privileged site did not elicit any immune responses against the gene transfer vectors [57], CpG motifs of plasmids can be quite immunogenic in certain cases. Recently, modification of CpG sequences [58] or generation of minicircle constructs [59] using optimized codon sequences can now be easily achieved to reduce/eliminate potential immune responses elicited by the naked DNA.

Overcoming transgene-specific immune responses following gene therapy

The problem of immune responses against exogenously introduced transgene product has been encountered in many gene therapy animal model systems. In many preclinical experiments using immunocompetetent hemophilia A mice and dogs, strong immune responses against factor VIII (FVIII) have completely inhibited circulating FVIII activity, and subverted the effect of gene therapy. With less immunogenic vectors including adenoassociated viral (AAV) [60] and nonviral naked plasmid vectors [61], only transgene-specific humoral responses were detected in the absence of cellular immune responses. Inhibitory antibodies were also observed following gene transfer of a VSV-G pseudotyped, oncoretroviral vector encoding human BDD FVIII [62,63] and a FIV-based lentiviral-factor VIII vector [41] into hemophilia A mice. However, in the presence of other strong signals such as vector induced immunity, both cellular and humoral responses against transgene have been observed. Administration of an E1/E3 deleted adenoviral vector encoding FVIII activated both cytotoxic and humoral responses in hemophilia A mice [64,65]. Similarly, an FIX-adenoviral vector mediated gene delivery efficiently activated FIX-specific cytotoxic T lymphocytes (CTL) and generated FIX-specific antibodies [27,28]. These results underline the importance of establishment of transgene-specific tolerance for achieving effective gene therapy treatment to treat specific diseases. Recently, many new approaches have been investigated to modulate transgene-specific immune responses. These new strategies are summarized in several categories.

Tolerance induction by recurrent exposures to antigens

Efforts have been made to induce tolerance by recurrent exposure to antigens before or at the onset of gene therapy. Hepatic gene transfer by AAV vectors resulting in persistent gene expression has been successfully used to induce immune tolerance to FVIII or FIX with or without transient immune suppression [66,67]. Recently AAV8-mediated hepatic gene transfer prevented antibody formation against FIX in the easily immune-activated C3H/HeJ hemophilia B mice [68]. Moreover, liver-directed, AAV-mediated gene delivery of canine FVIII into hemophilia B dogs with pre-existing inhibitors completely eliminated the inhibitory antibody titers within several weeks post gene transfer, resulting in persistent cFVIII gene expression [69]. Another strategy is to induce tolerance before gene transfer such as recurrent oral or nasal administration of FIX proteins [70]. Similar strategies used to induce tolerance against FVIII in protein replacement hemophilia A mouse model may be also useful in gene transfer studies; these include repeated nasal or oral administration of FVIII-C2 domain or peptide(s) [71], infusion of lipopolysaccharide (LPS)-activated B-cell blasts transduced with a fusion IgG containing the C2 or A2 domains [72], FVIII pulsed immature dendritic cells [73,74], and apoptotic syngeneic fibroblast cells modified by FVIII expressing foamy vectors [75].

Methods to evade immune responses

Incorporation of tissue-specific promoters in gene transfer vectors: Efficient transduction of APCs can significantly promote the induction of transgene-specific immune responses. This has been observed particularly in adenoviral and lentiviral vector-mediated gene delivery since both vectors transduce APCs quite efficiently. Incorporation of tissue-specific promoters to restrict transgene expression in the target tissue and reduce its expression in APCs can significantly minimize the occurrence of adaptive immune responses. Therefore, it is highly desirable to incorporate highly efficient, tissue-specific promoters in gene therapy vectors aiming at achieving high-level gene expression without undesired side effects and immune responses [16,32,50,57,76,77] .

Incorporation of mciroRNA sequence in gene transfer vectors: MicroRNA (miRNA) is a newly discovered RNA group composed of ~21-23 nucleotide RNAs that negatively regulate gene expression at the post-transcriptional level. Since miRNAs have distinct expression profiles in different tissues, specific miRNA sequence was incorporated in gene transfer expression cassettes to further reduce transgene expression in APCs in combination with tissue-specific promoters. Recently a liver-specific lentiviral-FIX construct incorporating miRNA binding sites (mir-142-3p) specific for hematopoietic stem cells effectively suppressed residual transgene expression in APCs, leading to long-term FIX gene expression in hepatocytes without inducing anti-FIX immune responses [78]. Moreover, gene transfer by a miRNA regulated and GP64 psudotyped lenti-FVIII vector driven by a liver-specific promoter prevented FVIII-specific antibody production after liver-directed gene therapy [47].

Generation of less immunogenic FVIII variants: It will also be desirable to generate a less immunogenic FVIII variant molecule.

However, any modifications on the FVIII molecule should not compromise the functional activity of FVIII. A BDD-FVIII/N6 variant [79] was found to produce lower anti-FVIII antibody titers than BDD-FVIII when delivered by either an adenoviral vector [80] or a plasmid vector [81]. Modifications of immunodominant T cell epitopes are also being investigated to reduce the immunogenicity of FVIII while maintaining its full functional activity [82].

Neonatal gene transfer: Some success have been achieved to evade antibody production and achieve persistent transgene expression following neonatal gene transfer of FVIII [63,77,83] and FIX [84,85]. Tolerance induction is found to be associated with antigen-specific $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs).

Gene therapy targeting specific tissue or cell types: Ex vivo transduction of hematopoietic stem cells by retroviral vectors were shown to induce partial tolerance to FVIII in some animals [86,87]. Recent improvement in the transduction protocol in combination with the use of a vector encoding a B-domain deleted porcine FVIII cDNA and immunosuppressive agents of CTLA4-Ig and anti-CD40L or anti-(murine)thymocyte serum (ATS), achieved therapeutic levels of FVIII long-term in a hemophilia A mouse model under a nonmyeloblative conditioning regimen for the establishment of a mixed chimerism [88]. Such a strategy was also effective even in mice with inhibitors to FVIII prior to HSC transplantation [89]. Ex vivo gene therapy using a lentiviral vector carrying the FVIII gene driven by platelet-specific alphaIIb promoter conferred FVIII gene expression in megakaryocytes [90,91]. The FVIII expressed was stored in platelets and only released at the site of platelet activation. The treated mice survived tail clipping assay and no anti-FVIII antibodies were detected. Platelet specific gene expression may be a way to evade the inhibitory antibody responses for treating hemophilia A. The current major obstacle for this protocol is the low level transgene expression and functional activity which need to be improved to ensure a therapeutic effect.

In addition, ex vivo gene therapy has been performed by implanting lentiviral vector transduced endothelial progenitor cells subcutaneously in a Matrigel scaffold in hemophilia A mice pretreated with tolerizing doses of FVIII and transient immunosuppression [92]. Therapeutic FVIII gene expression persisted for 27 weeks following transplantation before dropping to baseline levels due to the loss of implanted cells. None of the treated mice developed inhibitory antibodies to FVIII. Another group used a nanocapsule carrying the sleeping Beauty transposon system to direct FVIII gene into liver sinusoidal endothelial cells using [56]. Long-term (50 weeks) and high level (equal to normal level) gene expression of FVIII was achieved without apparent antibody formation along with improvement of hemophilia A phenotype in treated mice. Recently murine induced pluripotent stem (iPS) cells were generated and subsequently differentiated to both FVIII-secreting endothelial cells and endothelial progenitor cells and injected directly to the liver of irradiated hemophilia A mice. The recipient mice produced 8-12% FVIII levels and corrected the phenotype correction of murine hemophilia A for at least 3 months [93]. Moreover, lineage negative murine bone marrow cells were transplanted directly into the host mouse livers. These cells subsequently transdifferentiated into liver cells including both hepatocytes and endothelial cells and produced approximately 20% normal levels of FVIII activities which persisted for more than a year without detection of anti-FVIII inhibitory antibodies [94].

Immunosuppressive therapy targeting T cells

Tolerance induction protocols involving antigen presentation and the methods to evade anti-FVIII immune responses often are successful in only a fraction of the treated patients or animals, and/or in combination with immunosuppressive agents. Therefore transient immunomodulation regimens to reduce or eliminate anti-FVIII inhibitory antibodies are highly desirable. Peripheral tolerance to a specific antigen can be induced through several different mechanisms by which to disrupt T cell activation or T and B cell interaction, or inhibit T or B cell function. In addition, dominant tolerance can be established through active suppression of activated effector T cells by Tregs.

Common immunosuppressive drugs: Common immunosuppressive drugs nonspecifically targeting T cell activation, clonal expansion or differentiation into effector T cells have been used to prevent antigen-specific responses. Cyclophosphamide has been used to suppress the immune response following FIX [76,95,96] and FVIII gene transfer [67,92,97]. In addition, cyclosporin A (CSA) suppresses the early stages of lymphocyte activation. Rapamycin (RAP) selectively suppresses activated T cells and favors expansion of Tregs. Mycophenolate mophetil (MMF) is an anti-proliferative agent for both B and T cells. Short-term immunosuppression with CSA in hemophilia B dogs prevented inhibitors following AAV-mediated gene delivery to the skeletal muscle [98]. Furthermore, a two drug therapy consisting of MMF and RAP [99] or MMF and FK-506 (tacrolimus) [100] prevented antibody response in AAV-mediated gene transfer of FIX into the liver of non-human primates. In addition, administration of 4 weeks, repeated dosing of FIX combined with RPA and interleukin (IL)-10 successfully prevented antibody formation and induced FIX-specific tolerance in hemophilia B mice following AAV-mediated gene therapyThis regimen induced substantial reductions in effector T cells (Teffs) and increases in Tregs. Nevertheless, in the FVIII plasmid gene therapy treated hemophilia A mice, application of either single-agent, or combined therapy with MMF, CSA , and RPA only resulted in delayed immune responses [97]. Inhibitory antibodies appeared quickly upon withdrawal of the drug(s).

Blockade of costimulatory pathways: Recently, monoclonal antibodies (mAbs) have emerged as a new class of immunosuppressive agents which appear to be both more effective and selective in facilitating immune tolerance induction; and are generally very well-tolerated by recipients. These agents targeting specific pathways also make them less toxic than non-specific, traditional immunosuppressive agents, and can induce antigen-specific tolerance in the presence of antigens. Monoclonal antibodies can be used to block the costimulatory pathway to induce T cell apoptosis or anergy. Multiple T cell costimulatory pathways, including CD28 and B7 molecules (CD80, CD86), ICOS and ICOSL, CD-40L and CD40, OX40 (CD134) and OX40-L, provide a functional network to ensure robust T cell activation to mount immune responses against foreign antigens.

Several agents to interrupt costimulatory pathways have been used to induce tolerance to FVIII. CTLA4-immunoglobulin (CTLA4-Ig) blocks the costimulatory interaction of B7 and CD28. It has been shown to transiently block the inhibitor formation in hemophilia A mice [102]. In relation to CTLA4-Ig, the part of its action at least in certain models is through the induction of indoleamine 2,3-dioxygenase (IDO), a tryptophan degrading enzyme. Co-delivery of FVIII and IDO genes in a transposon system yielded long-term therapeutic FVIII expression and significantly reduced anti-FVIII antibody titers [103]. Similar to CTLA4-Ig, anti-CD40L has been shown to block the re-stimulation and differentiation of FVIII-specific memory B cells in the presence of FVIII antigen [104,105]. Dual blockade of CD40/CD40L and B7/CD28

pathways using a combination regimen of anti-CD40L and CTLA4-Ig have been demonstrated to act synergistically to prevent antigen-specific immune responses and induce long-term tolerance to FVIII in FVIII plasmid treated hemophilia A mice [97].

Anti-inducible co-stimulatory molecule (ICOS) blocks the third costimulatory pathway of interaction between ICOS and ICOS-ligand (ICOS-L). In the plasmid-treated hemophilia A mice, inhibitory antibody formation was successfully prevented following nonviral gene transfer of FVIII and anti-ICOS treatment [106]. Multiple phases of changes were observed including transient depletion of $CD4^+$ T cells during initial treatment period, reduction of T effector cells, up-regulation of $CD4^+CD25^+Foxp3^+$Tregs in terms of both percentage and activity, up-regulation of regulatory cytokines including interleukin-10 and transforming growth factor-β. These results indicated the involvement of antigen-specific regulatory T cells in tolerance induction. Furthermore, Anti-ICOS-treated mice tolerized to hFVIII generated normal primary and secondary antibody responses after immunization with the T-dependent antigen, bacteriophage Φ x174, indicating maintenance of immune competency of the treated mice. Anti-ICOS which is currently under development for clinical applications represents a highly effective agent for modulating antigen-specific immune responses.

T-cell depletion therapy: Depletion of T cells and sometimes in combination of B cells can significantly reduce the number of effector T cells capable of mounting an immune response at the time of initial exposure to foreign antigens. Five consecutive anti-CD3 treatments concomitant with factor VIII plasmid injection prevented the formation of inhibitory antibodies against FVIII and achieved persistent, therapeutic levels of FVIII gene expression in treated hemophilia A mice [107]. Repeated plasmid gene transfer is applicable to tolerized mice without eliciting immune responses. It was demonstrated that FVIII-specific tolerance was induced by anti-CD3 treatment via a mechanism involving the increase in TGF-β levels and the generation of adaptive FVIII-specific $CD4^+Foxp3^+$ regulatory T cells in the periphery [107]. Depletion of effector T cells following anti-CD3 administration generated a suitable window for antigen-specific Tregs to proliferate and be activated.

Adoptive regulatory T cell therapy: A dominant tolerance can be induced by suppression of activated T cells by regulatory T cells. T cell homeostasis was achieved by balancing the $CD4^+CD25^+$ Tregs and effector T (Teff) cells; tolerance induction can be accomplished by inducing a balance shift between Treg and Teff cells. Recently, $CD4^+CD25^+$ Treg cell populations have been successfully induced and/or expanded, and shown to suppress autoimmune and alloimmune responses [108-111]. Adoptive transfer of Tregs isolated from FVIII-exposed HemA/Foxp3-Tg mice produced significantly reduced antibody titers in Treg recipient hemophilia A mice compared to untreated control mice after initial challenge with FVIII plasmid and second challenge 16 weeks post first plasmid treatment [112]. It was suggested that the adoptively transferred Tregs induced significant activation of endogenous Tregs in the recipient mice via an infectious tolerance mechanism, which was responsible for the long-term protective effect *in vivo* against FVIII-specific immune responses and limit recall responses induced by a second challenge. Adoptive transfer therapy of Tregs to modulate antigen-specific responses has many advantages over conventional treatments. Some of these benefits include: protection through antigen-specific activity without general immunosuppression, the possibility of inducing physiological long-lasting regulation *in vivo*, and customized therapy for individual patient with limited or no side effects.

In vivo expansion of regulatory T cells: Many successful approaches developed towards induction of tolerance to factor VIII (FVIII) and other antigens [113] involve suppressive function of Tregs. An *in vivo* approach for inducing selective expansion of Treg cells by injecting hemophilia A mice with IL-2 mixed with a particular IL-2 monoclonal antibody (mAb, JES6-1) [114] was used to modulate FVIII-specific immune responses. The mice treated with IL2/IL2mAb complex generated no inhibitory antibodies against FVIII and achieved therapeutic-level of FVIII gene expression in FVIII plasmid-treated mice [115]. The treatment led to a marked 5-7-fold increase in total numbers of Treg cells in the peripheral blood on the peak day (day 6 following the last IL2-IL2mAb complex treatment) and these levels gradually returned to normal within the next 7-14 days. These short-lived expanded Tregs are highly activated and display superior suppressive function. Little or no change in other cell populations was observed. Furthermore, IL2/IL2 mAb complexes can transiently reduce inhibitory antibody titers in mice with pre-existing inhibitory antibodies following gene transfer [115]. These results demonstrate the important role of Tregs in suppressing anti-FVIII immune responses and the potential of developing *in vivo* Treg expansion therapy to induce long-term tolerance to FVIII.

Immunosuppressive therapy targeting B cells

Tolerogenic B-cell therapy: David Scott's laboratory has investigated a B cell therapy by using tolerogenic B cells transduced with a retroviral vector carrying the cDNA encoding the A2 or C2 domains of FVIII fused to the heavy chain of murine IgG to induce FVIII tolerance [72,116]. Specific tolerance to A2 or C2 domain was induced by this protocol. Furthermore, a combination of A2-IgG and C2-IgG expressing B cells induced tolerance to the full-length FVIII protein. This therapy successfully prevented antibody production in naive mice without inhibitors or reduced the inhibitor titers in FVIII-primed hemophilia A mice (mice with pre-existing inhibitors)[72]. Significant increase of Tregs was detected in B cell- transduced animals and depletion of $CD4^+CD25^+$ Tregs completely abolished the tolerance [117].

B-cell depletion therapy: Scott and colleagues [118] also studied the effect of B cell depletion on tolerance induction to FVIII. In FVIII primed mice, a single dose of IgG1 anti-CD20 pretreatment prevented the increase in inhibitor formation in the majority of treated mice receiving high dose protein replacement therapy. This antibody can selectively deplete follicular B cells while spare marginal zone (MZ) B cells as a potential tolerogenic antigen-presenting cells. Both of these therapies have implication on being potential immunomodulation strategies for regulating transgene-specific immune responses following gene therapy.

In FVIII plasmid-treated hemophilia A mice, it was found that administration of anti-murine CD20 IgG2a into hemophilia A mice significantly reduced $CD19^+$ B cells in blood, spleen, and lymph node[119]. The depletion of B cells lasted for sustained periods of time and gradually returned to normal at ~8 weeks post anti-CD20 treatment. No significant change in the numbers or percentages of $CD4^+$ T cells and $CD4^+CD25^+Foxp3^+$ regulatory T cells was observed during the experimental period. Transient depletion of B cells induced tolerance to FVIII in a portion of the gene therapy treated hemophilia A mice. However, most of the mice eventually developed inhibitory antibodies. In these cases, depletion of B cells following gene transfer did not create a sufficient window for induction of tolerance in the presence of antigen. Anti-CD20 still represents a potential candidate in

therapies combined with other immunomodulation agents, especially in modulating pre-existing immune responses [120].

Perspectives

In order to achieve the goal of long-term transgene expression following gene therapy to treat genetic diseases, it is imperative to prevent or modulate the induction of immune responses to gene transfer vectors, gene modified cells, as well as transgene products. The immune system is complex and its defense can mount several layers of host responses to barricade the persistence of transduced cells and their transgene products. As discussed previously, once innate responses were initiated, it can not only trigger the destruction of transduced cells, but also prime the adaptive immune responses against the viral proteins and transgene products. These responses will ultimately destruct the vector transduced cells and/or neutralize the therapeutic protein. Some studies are finally embarked to delineate the mechanism of each of these individual activation pathways and how they interplay. Some immunomodulation regimens discussed above may have impact on other responses. Effective therapy will need to be carefully evaluated depending on the vector system, the type of transgene, delivery route, target tissue, and other factors. Furthermore, safety profile will need to be considered by the following criteria: [1] require only transient exposure to immunosuppressive protocols; [2] induce minimal cytoablation and other associated toxicity; [3] exhibit minimal effects on immune competence; and [4] mediate antigen-specific long-term tolerance. Recent successes in the preventative therapies to reduce/eliminate immune responses in animal models brought us closer to clinical application of gene therapy for hemophilia, especially in the case of hemophilia A. In particular, transient immunomodulation regimens with minimum side effects and toxicity are highly promising strategies for patients at high risk of inhibitor formation. Some of these reagents are already used in the clinic and some of these are under development for clinical use. Several studies [107,121] also showed that transient immunosuppression did not hamper the immune system for long-term; immune responses to other antigens or pathogens can be successfully induced following subsequent challenges.

Furthermore, although it is best to develop a preventative therapy to suppress immune activation during gene therapy application, novel strategies are needed to achieve successful gene therapy in the presence of pre-existing immunity against viral proteins or transgene product. Only a few studies have addressed this more intricate problem. Combined therapies targeting distinct immune activation pathways may have a better chance to modulate pre-existing immunity and induce long-term trangene-specific tolerance.

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References

1. Herzog RW, Dobrzynski E (2004) Immune implications of gene therapy for hemophilia. Semin Thromb Hemost 30: 215-226.
2. Miao CH (2007) Recent advances in immune modulation. Curr Gene Ther 7: 391-402.
3. Waters B, Lillicrap D (2009) The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. J Thromb Haemost 7: 1446-1456.
4. Powell JS, Ragni MV, White GC 2nd, Lusher JM, Hillman-Wiseman C, et al. (2003) Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. Blood 102: 2038-2045.
5. Roth DA, Tawa NE Jr, O'Brien JM, Treco DA, Selden RF et al. (2001) Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. N Engl J Med 344: 1735-1742.
6. Chuah MK, Collen D, VandenDriessche T (2004) Clinical gene transfer studies for hemophilia A. Semin Thromb Hemost 30: 249-256.
7. Lu DR, Zhou JM, Zheng B, Qiu XF, Xue JL, et al. (1993) Stage I clinical trial of gene therapy for hemophilia B. Sci China B 36: 1342-1351.
8. Qiu X, Lu D, Zhou J, Wang J, Yang J, et al. (1996) Implantation of autologous skin fibroblast genetically modified to secrete clotting factor IX partially corrects the hemorrhagic tendencies in two hemophilia B patients. Chin Med J (Engl) 109: 832-839.
9. Larson PJ, High KA (2001) Gene therapy for hemophilia B: AAV-mediated transfer of the gene for coagulation factor IX to human muscle. Adv Exp Med Biol 489: 45-57.
10. Jiang H, Pierce GF, Ozelo MC, de Paula EV, Vargas JA, et al. (2006) Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. Mol Ther 14: 452-455.
11. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, et al. (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 12: 342-347.
12. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, et al. (2011) Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B. N Engl J Med 365: 357-365.
13. Nathwani AC, Tuddenham EGD, Rangarajan S, Rosales C, McIntosh J, et al. (2011) Adeno-Associated Viral Vector Mediated Gene Transfer for Hemophilia B. Blood Ameriacn Society of Hematology, 53rd Annual Meeting at San Diego: Abs 5.
14. Herzog RW, Hagstrom JN, Kung SH, Tai SJ, Wilson JM, et al. (1997) Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proc Natl Acad Sci U S A 94: 5804-5809.
15. Herzog RW, Yang EY, Couto LB, Hagstrom JN, Elwell D, et al. (1999) Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat Med 5: 56-63.
16. Snyder RO, Miao C, Meuse L, Tubb J, Donahue BA, et al. (1999) Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. Nat Med 5: 64-70.
17. Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, et al. (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet 16: 270-276.
18. Wu Z, Asokan A, Samulski RJ (2006) Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol Ther 14: 316-327.
19. Vandenberghe LH, Wilson JM (2007) AAV as an immunogen. Curr Gene Ther 7: 325-333.
20. Grimm D, Lee JS, Wang L, Desai T, Akache B, et al. (2008) In vitro and in vivo gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. J Virol 82: 5887-5911.
21. Li W, Asokan A, Wu Z, Van Dyke T, DiPrimio N, et al. (2008) Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. Mol Ther 16: 1252-1260.
22. Finn JD, Hui D, Downey HD, Dunn D, Pien GC, et al. (2010) Proteasome inhibitors decrease AAV2 capsid derived peptide epitope presentation on MHC class I following transduction. Mol Ther 18: 135-142.
23. Wang Z, Kuhr CS, Allen JM, Blankinship M, Gregorevic P, et al. (2007) Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. Mol Ther 15: 1160-1166.
24. Zhu J, Huang X, Yang Y (2009) The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. J Clin Invest 119: 2388-2398.
25. Martino AT, Suzuki M, Markusic DM, Zolotukhin I, Ryals RC, et al. (2011) The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver. Blood 117: 6459-6468.
26. Jayandharan GR, Aslanidi G, Martino AT, Jahn SC, Perrin GQ, et al. (2011)

Activation of the NF-kappaB pathway by adeno-associated virus (AAV) vectors and its implications in immune response and gene therapy. Proc Natl Acad Sci U S A 108: 3743-3748.

27. Dai Y, Schwarz EM, Gu D, Zhang WW, Sarvetnick N, et al. (1995) Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. Proc Natl Acad Sci U S A 92: 1401-1405.

28. Kay MA, Landen CN, Rothenberg SR, Taylor LA, Leland F, et al. (1994) In vivo hepatic gene therapy: complete albeit transient correction of factor IX deficiency in hemophilia B dogs. Proc Natl Acad Sci U S A 91: 2353-2357.

29. Thorrez L, VandenDriessche T, Collen D, Chuah MK (2004) Preclinical gene therapy studies for hemophilia using adenoviral vectors. Semin Thromb Hemost 30: 173-183.

30. Kreppel F, Kochanek S (2008) Modification of adenovirus gene transfer vectors with synthetic polymers: a scientific review and technical guide. Mol Ther 16: 16-29.

31. Seregin SS, Amalfitano A (2009) Overcoming pre-existing adenovirus immunity by genetic engineering of adenovirus-based vectors. Expert Opin Biol Ther 9: 1521-1531.

32. Chuah MK, Schiedner G, Thorrez L, Brown B, Johnston M, et al. (2003) Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. Blood 101: 1734-1743.

33. Schiedner G, Hertel S, Johnston M, Dries V, van Rooijen N, et al. (2003) Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. Mol Ther 7: 35-43.

34. Pastore L, Morral N, Zhou H, Garcia R, Parks RJ, et al. (1999) Use of a liver-specific promoter reduces immune response to the transgene in adenoviral vectors. Hum Gene Ther 10: 1773-1781.

35. Ehrhardt A, Kay MA (2002) A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. Blood 99: 3923-3930.

36. Ehrhardt A, Xu H, Dillow AM, Bellinger DA, Nichols TC, et al. (2003) A gene-deleted adenoviral vector results in phenotypic correction of canine hemophilia B without liver toxicity or thrombocytopenia. Blood 102: 2403-2411.

37. McCormack WM, Seiler MP, Bertin TK, Ubhayakar K, Palmer DJ, et al. (2006) Helper-dependent adenoviral gene therapy mediates long-term correction of the clotting defect in the canine hemophilia A model. J Thromb Haemost 4: 1218-1225.

38. Brunetti Pierri N, Nichols TC, McCorquodale S, Merricks E, Palmer DJ, et al. (2005) Sustained phenotypic correction of canine hemophilia B after systemic administration of helper-dependent adenoviral vector. Hum Gene Ther 16: 811-820.

39. Brunetti Pierri N, Stapleton GE, Law M, Breinholt J, Palmer DJ, et al. (2009) Efficient, long-term hepatic gene transfer using clinically relevant HDAd doses by balloon occlusion catheter delivery in nonhuman primates. Mol Ther 17: 327-333.

40. Vigna E, Naldini L (2000) Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J Gene Med 2: 308-316.

41. Stein CS, Kang Y, Sauter SL, Townsend K, Staber P, et al. (2001) In vivo treatment of hemophilia A and mucopolysaccharidosis type VII using nonprimate lentiviral vectors. Mol Ther 3: 850-856.

42. Tsui LV, Kelly M, Zayek N, Rojas V, Ho K, et al. (2002) Production of human clotting Factor IX without toxicity in mice after vascular delivery of a lentiviral vector. Nat Biotechnol 20: 53-57.

43. Rossetti M, Gregori S, Hauben E, Brown BD, Sergi LS, et al. (2011) HIV-1-derived lentiviral vectors directly activate plasmacytoid dendritic cells, which in turn induce the maturation of myeloid dendritic cells. Hum Gene Ther 22: 177-188.

44. Pichlmair A, Diebold SS, Gschmeissner S, Takeuchi Y, Ikeda Y, et al. (2007) Tubulovesicular structures within vesicular stomatitis virus G protein-pseudotyped lentiviral vector preparations carry DNA and stimulate antiviral responses via Toll-like receptor 9. J Virol 81: 539-547.

45. Markusic DM, van Til NP, Hiralall JK, Elferink RP, Seppen J (2009) Reduction of liver macrophage transduction by pseudotyping lentiviral vectors with a fusion envelope from Autographa californica GP64 and Sendai virus F2 domain. BMC Biotechnol 9: 85.

46. Kang Y, Xie L, Tran DT, Stein CS, Hickey M, et al. (2005) Persistent expression of factor VIII in vivo following nonprimate lentiviral gene transfer. Blood 106: 1552-1558.

47. Matsui H, Hegadorn C, Ozelo M, Burnett E, Tuttle A, et al. (2011) A microRNA-regulated and GP64-pseudotyped lentiviral vector mediates stable expression of FVIII in a murine model of Hemophilia A. Mol Ther 19: 723-730.

48. Yu H, Wagner E (2009) Bioresponsive polymers for nonviral gene delivery. Curr Opin Mol Ther 11: 165-178.

49. Kamimura K, Suda T, Xu W, Zhang G, Liu D (2009) Image-guided, lobe-specific hydrodynamic gene delivery to swine liver. Mol Ther 17: 491-499.

50. Miao CH, Ye X, Thompson AR (2003) High-level factor VIII gene expression in vivo achieved by nonviral liver-specific gene therapy vectors. Hum Gene Ther 14: 1297-1305.

51. Ye X, Loeb KR, Stafford DW, Thompson AR, Miao CH (2003) Complete and sustained phenotypic correction of hemophilia B in mice following hepatic gene transfer of a high-expressing human factor IX plasmid. J Thromb Haemost 1: 103-111.

52. Heller LC, Heller R (2006) In vivo electroporation for gene therapy. Hum Gene Ther 17: 890-897.

53. Miao CH, Brayman AA (2011) Ultrasound-mediated gene delivery Non-viral gene therapy, edited by Xubo Yuan, Intech, Rijeka, Croatia: 214-244.

54. Dhadwar SS, Kiernan J, Wen J, Hortelano G (2010) Repeated oral administration of chitosan/DNA nanoparticles delivers functional FVIII with the absence of antibodies in hemophilia A mice. J Thromb Haemost 8: 2743-2750.

55. Bowman K, Sarkar R, Raut S, Leong KW (2008) Gene transfer to hemophilia A mice via oral delivery of FVIII-chitosan nanoparticles. J Control Release 132: 252-259.

56. Kren BT, Unger GM, Sjeklocha L, Trossen AA, Korman V, et al. (2009) Nanocapsule-delivered Sleeping Beauty mediates therapeutic Factor VIII expression in liver sinusoidal endothelial cells of hemophilia A mice. J Clin Invest 119: 2086-2099.

57. Miao CH, Thompson AR, Loeb K, Ye X (2001) Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. Mol Ther 3: 947-957.

58. Hodges BL, Taylor KM, Joseph MF, Bourgeois SA, Scheule RK (2004) Long-term transgene expression from plasmid DNA gene therapy vectors is negatively affected by CpG dinucleotides. Mol Ther 10: 269-278.

59. Kay MA, He CY, Chen ZY (2010) A robust system for production of minicircle DNA vectors. Nat Biotechnol 28: 1287-1289.

60. Chao H, Mao L, Bruce AT, Walsh CE (2000) Sustained expression of human factor VIII in mice using a parvovirus-based vector. Blood 95: 1594-1599.

61. Ye P, Thompson AR, Sarkar R, Shen Z, Lillicrap DP, et al. (2004) Naked DNA transfer of Factor VIII induced transgene-specific, species-independent immune response in hemophilia A mice. Mol Ther 10: 117-126.

62. Greengard JS, Jolly PJ (1999) Animal testing of retroviral-mediated gene therapy for factor VIII deficiency. Thromb Haemost 82: 555-561.

63. VandenDriessche T, Vanslembrouck V, Goovaerts I, Zwinnen H, Vanderhaeghen ML, et al. (1999) Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor VIII-deficient mice. Proc Natl Acad Sci U S A 96: 10379-10384.

64. Andrews JL, Kadan MJ, Gorziglia MI, Kaleko M, Connelly S (2001) Generation and characterization of E1/E2a/E3/E4-deficient adenoviral vectors encoding human factor VIII. Mol Ther 3: 329-336.

65. Sarkar R, Gao GP, Chirmule N, Tazelaar J, Kazazian HH Jr (2000) Partial corretion of murine hemophilia A with neo-antigenic murine factor VIII. Hum Gene Ther 11: 881-894.

66. Mingozzi F, Liu YL, Dobrzynski E, Kaufhold A, Liu JH, et al. (2003) Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. J Clin Invest 111: 1347-1356.

67. Sarkar R, Mucci M, Addya S, Tetreault R, Bellinger DA, et al. (2006) Long-term efficacy of adeno-associated virus serotypes 8 and 9 in hemophilia a dogs and mice. Hum Gene Ther 17: 427-439.

68. Cooper M, Nayak S, Hoffman BE, Terhorst C, Cao O, et al. (2009) Improved induction of immune tolerance to factor IX by hepatic AAV-8 gene transfer. Hum Gene Ther 20: 767-776.

69. Finn JD, Ozelo MC, Sabatino DE, Franck HW, Merricks EP, et al. (2010) Eradication of neutralizing antibodies to factor VIII in canine hemophilia A after liver gene therapy. Blood 116: 5842-5848.

70. Cao O, Armstrong E, Schlachterman A, Wang L, Okita DK, et al. (2006) Immune deviation by mucosal antigen administration suppresses gene-transfer-induced inhibitor formation to factor IX. Blood 108: 480-486.

71. Rawle FE, Pratt KP, Labelle A, Weiner HL, Hough C, et al. (2006) Induction of partial immune tolerance to factor VIII through prior mucosal exposure to the factor VIII C2 domain. J Thromb Haemost 4: 2172-2179.

72. Lei TC, Scott DW (2005) Induction of tolerance to factor VIII inhibitors by gene therapy with immunodominant A2 and C2 domains presented by B cells as Ig fusion proteins. Blood 105: 4865-4870.

73. Qadura M, Othman M, Waters B, Chegeni R, Walker K, et al. (2008) Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. J Thromb Haemost 6: 2095-2104.

74. Ragni MV, Wu W, Liang X, Hsieh CC, Cortese-Hassett A, et al. (2009) Factor VIII-pulsed dendritic cells reduce anti-factor VIII antibody formation in the hemophilia A mouse model. Exp Hematol 37: 744-754.

75. Su RJ, Epp A, Latchman Y, Bolgiano D, Pipe SW, et al. (2010) Suppression of FVIII inhibitor formation in hemophilic mice by delivery of transgene modified apoptotic fibroblasts. Mol Ther 18: 214-222.

76. Herzog RW, Mount JD, Arruda VR, High KA, Lothrop CD Jr (2001) Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. Mol Ther 4: 192-200.

77. Matsui H, Shibata M, Brown B, Labelle A, Hegadorn C, et al. (2009) A murine model for induction of long-term immunologic tolerance to factor VIII does not require persistent detectable levels of plasma factor VIII and involves contributions from Foxp3+ T regulatory cells. Blood 114: 677-685.

78. Brown BD, Venneri MA, Zingale A, Sergi Sergi L, Naldini L (2006) Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. Nat Med 12: 585-591.

79. Pipe SW (2009) Functional roles of the factor VIII B domain. Haemophilia 15: 1187-1196.

80. Cerullo V, Seiler MP, Mane V, Cela R, Clarke C, et al. (2007) Correction of murine hemophilia A and immunological differences of factor VIII variants delivered by helper-dependent adenoviral vectors. Mol Ther 15: 2080-2087.

81. Jirovska D, Ye P, Pipe SW, Miao CH (2008) Reduction of inhibitory Anti-FVIII Antibody titer by Using a Domain Variant FVIII/N6 cDNA for Nonviral Gene Therapy in Hemophilia A Mice. American Society of Hematology, 50th Annual Meeting at San Francisco: Abs.3537.

82. Ettinger RA, Lieverman JA, Boigiano DC, Thompson AR, Pratt KP (2009) Reduced immunogenicity of FVIII peptides by rational modifications of an immunodominant T cell epitope. Blood (ASH Annual Meeting Abstracts) 114: 220.

83. Liu L, Mah C, Fletcher BS (2006) Sustained FVIII expression and phenotypic correction of hemophilia A in neonatal mice using an endothelial-targeted sleeping beauty transposon. Mol Ther 13: 1006-1015.

84. Xu L, Gao C, Sands MS, Cai SR, Nichols TC, et al. (2003) Neonatal or hepatocyte growth factor-potentiated adult gene therapy with a retroviral vector results in therapeutic levels of canine factor IX for hemophilia B. Blood 101: 3924-3932.

85. Zhang J, Xu L, Haskins ME, Parker Ponder K (2004) Neonatal gene transfer with a retroviral vector results in tolerance to human factor IX in mice and dogs. Blood 103: 143-151.

86. Evans GL, Morgan RA (1998) Genetic induction of immune tolerance to human clotting factor VIII in a mouse model for hemophilia A. Proc Natl Acad Sci U S A 95: 5734-5739.

87. Moayeri M, Hawley TS, Hawley RG (2005) Correction of murine hemophilia A by hematopoietic stem cell gene therapy. Mol Ther 12: 1034-1042.

88. Ide LM, Gangadharan B, Chiang KY, Doering CB, Spencer HT (2007) Hematopoietic stem-cell gene therapy of hemophilia A incorporating a porcine factor VIII transgene and nonmyeloablative conditioning regimens. Blood 110: 2855-2863.

89. Doering CB, Gangadharan B, Dukart HZ, Spencer HT (2007) Hematopoietic stem cells encoding porcine factor VIII induce pro-coagulant activity in hemophilia A mice with pre-existing factor VIII immunity. Mol Ther 15: 1093-1099.

90. Shi Q, Wilcox DA, Fahs SA, Fang J, Johnson BD, et al. (2007) Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. J Thromb Haemost 5: 352-361.

91. Greene TK, Wang C, Hirsch JD, Zhai L, Gewirtz J, et al. (2010) In vivo efficacy of platelet-delivered, high specific activity factor VIII variants. Blood 116: 6114-6122.

92. Matsui H, Shibata M, Brown B, Labelle A, Hegadorn C, et al. (2007) Ex vivo gene therapy for hemophilia A that enhances safe delivery and sustained in vivo factor VIII expression from lentivirally engineered endothelial progenitors. Stem Cells 25: 2660-2669.

93. Xu D, Alipio Z, Fink LM, Adcock DM, Yang J, et al. (2009) Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. Proc Natl Acad Sci U S A 106: 808-813.

94. Yadav N, Kanjirakkuzhiyil S, Kumar S, Jain M, Halder A, et al. (2009) The therapeutic effect of bone marrow-derived liver cells in the phenotypic correction of murine hemophilia A. Blood 114: 4552-4561.

95. Fields PA, Arruda VR, Armstrong E, Chu K, Mingozzi F, et al. (2001) Risk and prevention of anti-factor IX formation in AAV-mediated gene transfer in the context of a large deletion of F9. Mol Ther 4: 201-210.

96. Arruda VR, Stedman HH, Haurigot V, Buchlis G, Baila S, et al. (2010) Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B. Blood 115: 4678-4688.

97. Miao CH, Ye P, Thompson AR, Rawlings DJ, Ochs HD (2006) Immunomodulation of transgene responses following naked DNA transfer of human factor VIII into hemophilia A mice. Blood 108: 19-27.

98. Arruda VR, Stedman HH, Nichols TC, Haskins ME, Nicholson M, et al. (2005) Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model. Blood 105: 3458-3464.

99. Mingozzi F, Hasbrouck NC, Basner-Tschakarjan E, Edmonson SA, Hui DJ, et al. (2007) Modulation of tolerance to the transgene product in a nonhuman primate model of AAV-mediated gene transfer to liver. Blood 110: 2334-2341.

100. Jiang H, Couto LB, Patarroyo-White S, Liu T, Nagy D, et al. (2006) Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. Blood 108: 3321-3328.

101. Nayak S, Cao O, Hoffman BE, Cooper M, Zhou S, et al. (2009) Prophylactic immune tolerance induced by changing the ratio of antigen-specific effector to regulatory T cells. J Thromb Haemost 7: 1523-1532.

102. Qian J, Burkly LC, Smith EP, Ferrant JL, Hoyer LW, et al. (2000) Role of CD154 in the secondary immune response: the reduction of pre- existing splenic germinal centers and anti-factor VIII inhibitor titer. Eur J Immunol 30: 2548-2554.

103. Liu L, Liu H, Mah C, Fletcher BS (2009) Indoleamine 2,3-dioxygenase attenuates inhibitor development in gene-therapy-treated hemophilia A mice. Gene Ther 16: 724-733.

104. Hausl C, Ahmad RU, Schwarz HP, Muchitsch EM, Turecek PL, et al. (2004) Preventing restimulation of memory B cells in hemophilia A: a potential new strategy for the treatment of antibody-dependent immune disorders. Blood 104: 115-122.

105. Hausl C, Ahmad RU, Sasgary M, Doering CB, Lollar P, et al. (2005) High-dose factor VIII inhibits factor VIII-specific memory B cells in hemophilia A with factor VIII inhibitors. Blood 106: 3415-3422.

106. Peng B, Ye P, Blazar BR, Freeman GJ, Rawlings DJ, et al. (2008) Transient blockade of the inducible costimulator pathway generates long-term tolerance to factor VIII after nonviral gene transfer into hemophilia A mice. Blood 112: 1662-1672.

107. Peng B, Ye P, Rawlings DJ, Ochs HD, Miao CH (2009) Anti-CD3 antibodies modulate anti-factor VIII immune responses in hemophilia A mice after factor VIII plasmid-mediated gene therapy. Blood 114: 4373-4382.

108. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, Lucas PJ, Gress RE, et al. (2004) L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood 104: 3804-3812.

109. Rifle G, Herve P (2004) Regulatory (suppressor) T cells in peripheral allograft tolerance and graft-versus-host reaction. Transplantation 77: S5.

110. Clark FJ, Gregg R, Piper K, Dunnion D, Freeman L, et al. (2004) Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4+CD25high regulatory T cells. Blood 103: 2410-2416.

111. Jaeckel E, von Boehmer H, Manns MP (2005) Antigen-specific FoxP3-transduced T-cells can control established type 1 diabetes. Diabetes 54: 306-310.

112. Miao CH, Harmeling BR, Ziegler SF, Yen BC, Torgerson T, et al. (2009) CD4+FOXP3+ regulatory T cells confer long-term regulation of factor VIII-specific immune responses in plasmid-mediated gene therapy-treated hemophilia mice. Blood 114: 4034-4044.

113. Cao O, Loduca PA, Herzog RW (2009) Role of regulatory T cells in tolerance to coagulation factors. J Thromb Haemost 7 Suppl 1: 88-91.

114. Chai JG, Coe D, Chen D, Simpson E, Dyson J, et al. (2008) In vitro expansion improves in vivo regulation by CD4+CD25+ regulatory T cells. J Immunol 180: 858-869.

115. Liu CL, Ye P, Yen BC, Miao CH (2011) In vivo expansion of regulatory T cells with IL-2/IL-2 mAb complexes prevents anti-factor VIII immune responses in hemophilia A mice treated with factor VIII plasmid-mediated gene therapy. Mol Ther 19: 1511-1520.

116. Su Y, Carey G, Maric M, Scott DW (2008) B cells induce tolerance by presenting endogenous peptide-IgG on MHC class II molecules via an IFN-gamma-inducible lysosomal thiol reductase-dependent pathway. J Immunol 181: 1153-1160.

117. Skupsky J, Zhang AH, Su Y, Scott DW (2010) B-cell-delivered gene therapy induces functional T regulatory cells and leads to a loss of antigen-specific effector cells. Mol Ther 18: 1527-1535.

118. Zhang AH, Skupsky J, Scott DW (2011) Effect of B-cell depletion using anti-CD20 therapy on inhibitory antibody formation to human FVIII in hemophilia A mice. Blood 117: 2223-2226.

119. Ye P, Peng B, Kehry M, Rawlings DJ, Miao CH (2010) Depletion of B cells by anti-CD20 partially regulates anti-factor VIII antibody production in the nonviral gene therapy model. Blood (ASH Annual Meeting Abstracts) 116: 550.

120. Liu CL, Ye P, Lin J, Miao CH (2011) Development of novel strategies to modulate pre-existing anti-factor VIII immune responses in hemophilia A inhibitor mice treated with factor VIII plasmid-mediated gene therapy. Mol Ther (ASGCT Annual Meeting Abstracts) 19: 294.

121. Moghimi B, Sack BK, Nayak S, Markusic DM, Mah CS, et al. (2011) Induction of tolerance to factor VIII by transient co-administration with rapamycin. J Thromb Haemost 9: 1524-1533.

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