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Hepatic Gene Transfer as a Means of Tolerance Induction to Transgene Products

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Abstract

The liver is a preferred target organ for gene therapy not only for liver-specific diseases but also for disorders that require systemic delivery of a protein. Diseases that could benefit from hepatic gene transfer include hemophilia, metabolic disorders, lysosomal storage disorders, and others. For a successful delivery of the transgene and sustained expression, the protocol must avoid immune responses in order to be efficacious. A growing number of studies have demonstrated that liver-directed transfer can induce transgene product-specific immune tolerance. Tolerance obtained via this route requires optimal engineering of the vector to eliminate transgene expression in antigen presenting cells while restricting high levels of therapeutic expression to hepatocytes. Innate immune responses may prevent tolerance induction, cause toxicity, and have to be minimized. Discussed in our review is the crucial role of CD4⁺CD25⁺ regulatory T cells in tolerance to the hepatocyte-derived gene product, the immunobiology of the liver and our current understanding of its tolerogenic properties, current and proposed research as to the mechanisms behind the liver's unique cellular environment, as well as development of the tools for tolerance induction such as advanced vector systems.

INTRODUCTION

The liver is an important target for many gene therapies that rely on systemic delivery of therapeutic proteins or that attempt to treat liver disease. This complex organ has shown great promise in the ability to accomplish its metabolic and synthetic roles, while ever mounting evidence points to an important immunologic role. Examples for the application of hepatic gene transfer include treatment of hemophilia, lysosomal storage disorders, metabolic disorders, hepatitis, α_1 -antitrypsin deficiency, and others. In order to successfully use the liver as the target tissue for gene transfer, the protocol must avoid immune responses from the host while safely delivering a therapeutic transgene to the liver's hepatocytes with sufficient efficacy. If the body directs a response to the vector, to the transgene product, or to both, the chances for the therapy to be successful are severely reduced. Additionally, formation of neutralizing antibodies (NAB) against viral vectors prevents re-administration. However, there is now substantial evidence that hepatic gene transfer can induce transgene product-specific immune tolerance [1]. Tolerance induction by this route requires optimal engineering of the vector and efficient expression of the transgene [2]. The purpose of this review is to provide background information on liver immunobiology, to examine the reasons why its immunologic role is crucial, and to illustrate how the choice of vector, its dosing and transgene expression are key elements for tolerance induction by gene transfer. Furthermore, the mechanism of

tolerance will be discussed with particular emphasis on CD4⁺ Treg, which apparently play a critical role in tolerance induction and maintenance.

INTERACTIONS BETWEEN THE LIVER AND THE IMMUNE SYSTEM

Within the last decade, the view of liver function has substantially expanded beyond its synthetic and detoxifying abilities. The liver is in fact an extremely valuable asset to the body's immune system and an integral player in the body's ability to protect itself, while managing the enormous amount of foreign antigens via blood from the digestive tract. The liver's unique cellular environment is the key to the appropriate recognition of self versus non-self molecules and pathogens [3,4]. The immunologic impact of antigen presentation in the liver should be apparent from the large amount of blood flow the liver manages. The hepatic cellular machine in the processing of antigen often favors tolerance over an immune response [5]. This is of importance when directing gene transfer and transgene expression to the liver. On the other hand, the liver contains a large population of immune cells, which facilitate both innate and adaptive responses, and has been shown to be an integral player in the activation of naïve T cells, which are at the backbone of specific immune response to foreign antigens.

THE ROLE OF THE PARENCHYMAL CELLS

The cells of the liver consist of hepatocytes, hepatic stellate cells, Kupffer cells, dendritic cells, sinusoidal endothelial cells and lymphocytes. Several of these cell types are involved in antigen presentation and immune responses. Hepatocytes account for approximately three quarter of the parenchymal cells of the liver. These cells are primarily tasked with the synthesis and secretion of numerous biological molecules. In addition to these roles, it has been shown that T cells within the sinusoids of the liver can interact with hepatocytes through cytoplasmic extensions. Hepatocytes have been showed to play an integral role in immune response as well as immune tolerance [6–8]. Hepatocytes also express a number of immunomodulatory markers on the perisinusoidal membrane, including major histocompatibility complex class (MHC)-I and molecules CD-1 and ICAM-1, which would imply an antigen presentation role for CD-1 and MHC class-I restricted NK-T and CD8⁺ T-cells. Hepatocytes lack constitutively expressed CD40 and other co-stimulatory molecules, but these may be up regulated under periods of stress. During inflammation, hepatocytes may be stimulated by certain cytokines, namely interleukin (IL)-2, IL-6, IL-12 and interferon (IFN)- γ , to produce pro-inflammatory cytokines and acute phase proteins such as C-reactive protein (CRP), which in turn increases their potential to recruit and activate inflammatory cells [9–12]. Additionally, it has been suggested that hepatocytes are capable of activating naïve CD8⁺ T-cells without any prior lymph node priming [6,13].

NON-PARENCHYMAL CELLS

Kupffer cells (KCs) are the next most numerous cells in the liver, accounting for approximately 20% of the liver's non-parenchymal cells and approximately 80% of the body's macrophage population [14]. KCs are important for the liver's cellular defense, and, as the local macrophages, are responsible for the clearance of pathogens, including viral particles. Distinguishable from the extrahepatic macrophages by their expression of MHC class-II molecules, ICAM-1 and CD80 and CD86, KCs are restricted to the lumen of the sinusoids and are in close contact with the endothelium, where they reach into the space of Disse [15–17]. Once KCs phagocytose material, they initiate and mediate an immune response by producing and releasing proinflammatory cytokines, which act in a paracrine function to stimulate neutrophil recruitment and chemotaxis [9,18].

KCs are also involved in down-regulating the immune response and promoting tolerance. There are several different mechanisms, by which they accomplish this. When stimulated by

lipopolysaccharide (LPS), they secrete IL-10, transforming growth factor (TGF)- β , and prostanoids, that promote tolerance. KCs have also been shown to suppress T-cell activation *in vitro* and to be involved in cross-presentation of foreign antigens to dendritic cells (DC) and in the synthesis of nitric oxide [19–21]. These observations would predict that KCs play a role in immune modulation. In fact, a recent report implicates IL-10 expression by KC in tolerance induction by AAV-mediated gene transfer to the liver (Breous *et al*, European Society of Gene and Cell Therapy annual meeting, Brugge, Belgium, 2008, abstract # Or 39).

A more traditional view would assume the hepatic DCs are the main player in liver-induced tolerance. Mature DCs are important APCs in the liver, even though they account for the smallest percentage of cells. For example, they are a key component for induction of tolerance following transplantation [22,23]. In the non-inflamed liver, DCs occupy the areas around the portal triads and the central veins. Similar to their peripheral counterparts, hepatic DCs are able to capture, process and transport antigens to the regional lymphoid tissues [24]. There are three distinct subsets of DCs in the murine liver, and they each have dual roles in induction of tolerance or promoting an immune response. These subsets are myeloid, lymphoid, and plasmacytoid DCs. The plasmacytoid DCs essentially occupy the liver parenchyma, where the myeloid and lymphoid subsets locate to the periportal fields.

Another key aspect of the capacity for DCs to trigger immune response is the maturation state of the cell. In the non-inflamed liver, the hepatic DCs play a major role in response to antigens and favor tolerance over immune response [23]. This non-inflamed state also effects how the APCs function in relation to the T-cells and favor regulatory versus effector roles. This is in sharp contrast to the peripheral DCs of the spleen and skin, which more typically favor immunogenic over tolerogenic response. However, under times of stress, hepatic resident DCs may become activated, leave the liver to carry antigen to the regional lymph nodes, and prime effector T cells.

Liver sinusoidal endothelial cells (LSEC) are the first contact for any antigens that enter through the sinusoids. These cells form a fenestrated barrier, which facilitates direct contact with the hepatocytes and the extravasation of T-cells. This lack of a proper basement membrane is unique to the liver. LSEC also exhibit highly efficient antigen uptake and may surpass KCs in this role [5]. LSEC are equipped to present antigen to both CD4⁺ and CD8⁺ T-cells, but the most common consequence of T cell priming is antigen-specific tolerance instead of immunity [19,25,26].

THE ROLE OF INTRA-HEPATIC LYMPHOCYTES

Distributed throughout the sinusoidal spaces in the healthy liver are numerous intrahepatic lymphocytes grouped in small lymphoid aggregates, as well as scattered throughout the parenchyma [20,27]. CD8⁺ T-cells are more abundant than CD4⁺ cells. In mice, the liver contains a high percentage of activated cells of both subsets [28]. With regard to the innate immune system, the liver also contains a high number of NK cells, both classical and non-classical. These cells are activated in response to various chemokines and help to contain viral infections; while the adaptive system utilizes antigen specific CTLs [27]. Another unique aspect of the hepatic immune system is the abundance of NK-T cells. These T cells express a limited diversity of $\alpha\beta$ TCRs but also markers typically associated with NK cells. NK-T cells recognize lipids presented by the MHC-like CD1d molecule rather than MHC-peptide complexes. Upon activation, NK-T cells produce large amounts of IFN- γ , IL-4, and GM-CSF in addition to other cytokines and chemokines. These cells are thought to have immune regulatory functions.

THE HEPATIC RESPONSE TO VIRAL VECTORS

The immune response following administration of the viral vector is a central challenge in the field of gene replacement therapy. The significant risk of using viral vehicles to effect hepatic gene transfer carries a significant risk for inducing a counterproductive immune response. Critical to getting gene replacement therapy as a viable treatment is the ability to circumvent, redirect, or suppress the immune response. Following administration of the virus, the body reacts with two mechanisms; a rapid less specific innate response followed by the adaptive antigen-specific response, which is also responsible for induction of long-term immunity (memory). Both of these mechanisms can hinder efficiency of the vector and can target the transgene product or transduced cells, and must be blocked for effective therapy.

ADENOVIRAL VECTORS

Advantages and disadvantages of three commonly used viral vectors in hepatic gene transfer (adenovirus, adeno-associated viruses and lenti viruses) are discussed in the following. Adenoviral vectors have been extensively used for their high transduction efficiency, ability to transduce both quiescent and dividing cells, and their high tropism for hepatocytes [29,30]. There has been, however, limited success with these vectors for long-term gene expression due to rapid and potent innate responses by the host, which results in significant inflammation, rapid elimination, short-lived transgene expression, and adaptive immune response against viral capsid proteins [31–33]. Ad vectors are rapidly endocytosed by KCs, with only a small amount of viable vector for transduction [34,35]. This sequestering is rapid and is only overcome with an increased dose [12,31]. In addition, there is a “spill-over” effect, which leads to vector being delivered to draining lymph nodes and to a systemic innate response [10]. The death of a patient receiving gene replacement therapy utilizing Ad-vector for ornithine transcarbamylase (OTC) deficiency from an acute inflammatory response underscores the potentially severe systemic innate response that adenovirus may cause [36]. The mechanisms behind the innate immune response to adenovirus are complex but are becoming better understood. The adenoviral DNA activates the inflammasome upon infection of macrophages in a process that is dependent on the cytoplasmic receptor NALP3 and the adaptor protein ASC. This pathway leads to caspase-1 dependent activation of interleukin (IL)-1 β , a potent activator of the innate immune response [37]. In addition, sensing of adenoviral particles by TLR-2 and TLR-2 dependent as well as independent mechanism leads to release of proinflammatory cytokines and an anti-viral IFN- α/β response [38–41]. The latter is required for NK cell activation [42]. Furthermore, adenoviral vector directly or indirectly activate endothelial cells, platelets (leading to dysregulation of coagulation), and complement [43,44]. Depending on vector dose, all these mechanisms may interact in the liver and drive a cascade of innate responses at and beyond the site of gene transfer [45].

KCs have been shown to play an active role in elimination of adenoviral vector from the liver. KC can present antigen to CD4⁺ and CD8⁺ T cells and thus mediate an adaptive response. Following KC depletion, there is significantly attenuated vector elimination, prolonged persistence of vector DNA and transgene expression in the liver [32,46]. The elimination of viral coding sequences in gutted Ad vector and restriction of transgene expression to hepatocytes by appropriate choice of the promoter have reduced adaptive immune responses, resulting in long-term expression in the liver in some examples of pre-clinical studies and even in tolerance induction to the transgene product [47–49]. Tightly regulated hepatocyte-specific promoters reduce expression in professional APCs in liver (such as Kupffer cells) and spleen (macrophages and DCs), which are efficiently transduced *in vivo* by Ad vectors [45]. However, the innate immune response remains a significant obstacle for human treatment. Additionally, inflammation caused by innate immunity may still impact responses to the transgene product even if no viral proteins are expressed. Limited vector doses may solve these issues, which

could become feasible with novel local vector delivery methods for hepatic gene transfer. Beaudet and colleagues have used a surgically isolated liver to deliver low vector doses directly into the liver to reduce the systemic vector dissemination with good results in non-human primates [50–52].

ADENO-ASSOCIATED VIRAL (AAV) VECTORS

Adeno-associated viral vectors on the other hand, do not initiate a potent inflammatory response [11,53]. This has been attributed to low innate immunity to AAV and to the inefficiency of the virus to infect DC or macrophages [54]. The limited inflammatory response is transient and does not result in any significant elevation in serum cytokines or chemokines. Intrahepatic delivery in mice, dogs and non-human primates often results in long-term hepatic expression of therapeutic gene [55–58]. Transduction is achieved without any noticeable vector related toxicity or immunotoxicity. For these reasons, AAV has quickly become the vector of choice for liver-directed gene therapy. Nonetheless, there are some problems with this delivery vector; most notably limited packaging, relatively low efficiency of gene transfer, and a high prevalence of pre-existing immunity to viral capsid proteins (which approaches 80% in some demographics), in particular against serotype 2. Pre-existing NAB to the AAV serotype 2 viral capsid blocked hepatic gene transfer in a Phase I/II clinical trial on coagulation factor IX (F.IX) gene transfer to the liver for treatment of hemophilia B [59]. Multi-year transgene expression documented in animal models has not yet been seen in humans. For example, in the same clinical trial, 2 patients with low pre-existing NAB titers developed transaminitis. This resulted in the loss of F.IX transgene expression despite lack of an immune response against F.IX [59]. Vector administration correlated with a CTL response against viral capsid proteins, which has been hypothesized to have resulted in the killing of hepatocytes harboring input capsid antigen, which may be cross-presented by MHC I to CD8⁺ T cells [60]. This problem may be related to humans being a natural host for AAV infections in contrast to animal models that are typically not natural hosts. It should be noted that AAV is a naturally replication deficient virus that relies on a helper virus to progress through an infectious cycle. Current efforts to avoid immunity against AAV capsid are focusing on developing alternative serotypes, modifying capsid sequences, and developing a transient immune suppression protocol [61–63].

LENTIVIRAL VECTORS

Another virus that has been used successfully is the lentiviral vector (LV) derived from proviral DNA of human immunodeficiency virus (HIV) or other related viruses. These vectors are also capable of delivering transgenes to non-dividing cells such as hepatocytes. Unlike the problems associated with pre-existing neutralizing antibodies or a memory response in AAV or Ad-vectors, LV are unlikely to encounter these problems in HIV negative subjects. One of the major obstacles for liver-directed gene therapy with LV is the robust adaptive response to the transgene product [64,65]. This response is the consequence of two events, an early innate response to the RNA genome of the enveloped virus, which leads to plasmacytoid DC activation and interferon- α/β secretion, and a subsequent specific response to the transgene product [66–69]. The type I interferon response in the liver limits efficacy of LV gene transfer to hepatocytes and, secondly, increases effectiveness of a transgene product-specific CTL response, presumably by enhancing activation and differentiation of CD8⁺ T cells to become fully functional CTLs [67]. The robust transgene-specific immune response is a major problem for *in vivo* gene transfer with LV vectors. T cell activation is caused by transgene expression in bone marrow derived APCs in the liver and in lymphoid organs including the spleen [64]. The resulting expression directs antigen presentation to and activation of CD4⁺ and CD8⁺ T-cells, which leads to an inflammatory response and destruction of LV-transduced hepatocytes by CTL. In addition, antibodies are formed against the transgene product [65,67]. Use of a

hepatocyte-specific promoter was not adequate in effectively preventing this immune response. An alternate strategy to de-target expression from APCs is to incorporate a sequence target for a hematopoietic lineage micro-RNA into the transcript of the transgene that results in degradation of the transgene specifically in hematopoietic cells such as professional APCs, thus effectively preventing transgene expression in these cells [64]. This method proved to be very powerful in eliminating adaptive immune responses against transgene products in LV gene transfer to the liver and resulted in sustained transgene expression and immune tolerance [64,67].

THE LIVER AS TARGET FOR GENE THERAPY AND TOLERANCE INDUCTION

The liver's tolerogenic nature was first reported in 1969, with the successful transplant of an MHC mismatched organ in a pig [70]. Depending on how the antigen arrives at the liver, whether by systemic means or via the portal circulation, there can be marked differences in final response [25,71]. The key to tolerance induction may be the processing of antigens by liver APCs independently of the surrounding lymphoid organs [14].

In recent years, several studies have documented tolerance induction to different proteins following *in vivo* gene transfer and hepatocyte-restricted transgene expression in adult animals [47,67,72–79]. The list of antigens expressed in a tolerogenic fashion upon hepatic gene transfer is ever growing and includes, among others, therapeutic proteins for treatment of hemophilia (coagulation factors VIII and IX), apolipoprotein A-I, α_1 -antitrypsin, ovalbumin, GFP, erythropoietin, myelin basic protein, and enzymes used to treat lysosomal storage disorders (Table 1). The latter include acid α -glucosidase (Pompe disease), α -galactosidase (Fabry disease), and acid sphingomyelinase (Niemann-Pick disease). As mentioned above, several types of optimally engineered vectors are capable of delivering these genes to the liver for tolerance induction, albeit that AAV vectors have been most extensively utilized. The majority of the transgene products listed here are used in gene replacement therapy for treatment of genetic disease. In this regard, hepatic gene transfer should be an ideal form of therapy, because it offers a potential solution for two problems. First, efficient gene transfer provides therapy by supplying the functional protein that is missing in a subject who inherited the genetic defect. Second, hepatocyte-derived expression could tolerize the immune system to the therapeutic protein. This is of particular importance for subjects with underlying mutations that represent a severe loss of coding information for the missing protein function such as a gene deletion or nonsense mutation. As opposed to missense mutations, these genetic defects may result in a loss of tolerance to the functional wild-type protein [80,81].

Multi-year expression of canine F.IX in a canine model of hemophilia B characterized by a F.IX null mutation (an early stop codon and an unstable mRNA), and long-term expression of canine factor VIII in hemophilia A dogs and of human F.IX in non-human primates suggest that the hepatic tolerance phenomenon can also be attributed to species other than mice, including large animal models [55,57,82,83]. However, AAV-mediated hepatic arylsulfatase B gene transfer to rats with a null mutation in this gene (causing in MPS VI, mucopolysaccharidosis type VI) results in immune responses rather than tolerance, and experiments with neonatal retroviral gene transfer to the liver also suggest species-specific differences in immune responses (Cotugno *et al.*, European Society of Gene Therapy annual meeting, Brugge, Belgium, 2008, abstract # Or 7) [84–86]. More large animal studies will be required to address these issues. At the same time, there are differences in the response even between different strains of mice because of genetic effects that modify immune functions [76,87,88]. Certainly, hepatic gene transfer protocols and vectors can be further improved with regard to efficacy and tolerance induction. For example, novel AAV serotypes such as AAV-8 have been superior in liver transduction and tolerance induction in mice with lysosomal storage disorders [77,78,89,90]. Similar improvements in large animals would be encouraging for

translation to human application. MicroRNA-regulated lentiviral vectors are also on track for evaluation in large animal models.

It is likely that ongoing inflammation or innate immune responses to pathogens could alter immune responses in the liver. For example, it has been shown that TLR3 activation can break immune tolerance in the liver [91]. However, we found that TLR3 signaling did not alter responses to F.IX in hepatic AAV gene transfer [92].

REQUIREMENTS AND MECHANISM OF TOLERANCE INDUCTION

Upon examination of studies that have optimized tolerance induction by *in vivo* gene transfer to the liver in multiple strains of experimental mice, a picture emerges of the requirements for such a protocol. The vector has to efficiently transfer the therapeutic gene to hepatocytes while avoiding expression in professional APCs. This can be achieved by using a tightly regulated promoter and/or a miRNA-regulated transcript [64]. A minimal level of transgene expression is required for tolerance [65]. Ideally, the vector should also elicit only minimal innate immunity, which may otherwise drive maturation of DCs and subsequent adaptive responses [67]. Once these requirements are met, transgene product-specific CD4⁺ T cell tolerance is achieved, which in turn results in an absence of antibody formation and CTL responses to the transgene product. Even after challenge with exogenous protein in adjuvant or administration of a genetic vaccine expressing the same transgene product, the immune system remains unresponsive, including lack of transgene product-specific T cell proliferation and antibody formation [75–77,93–95]. This unresponsiveness cannot be explained by a shift in cytokine secretion patterns or in isotype switch for antibody production, ruling out an immune deviation mechanism [76].

T cell tolerance may be established by one or a combination of several mechanisms, including elimination of effector T cells (deletion), induction of a state of unresponsiveness (anergy) that is reversible by antigen presentation in the presence of high amounts of IL-2, or by active suppression mediated by regulatory T cells (Treg). In recent years, convincing evidence has been mounting that the mammalian immune system contains a population of “naturally occurring” Treg, which constitutively express the alpha chain of the receptor for IL-2 (CD25, which is only transiently expressed in effector T cell upon activation) and transcription factor FoxP3 and comprise 5–10% of the CD4⁺ T cell population. The latter is believed to be a master switch for the development of these cells, which are potent suppressors of effector CD4⁺ T cells and are required to prevent autoimmunity [96,97]. Presentation of self-antigens likely results in differentiation of T cells into naturally occurring Treg during thymic development or peripherally, for example at a site of tumor growth [98]. There are likely several mechanisms by which Treg suppress, including cell contact-dependent suppression of IL-2 expression (which is required for activation of T_{eff}), *in vivo* secretion of suppressive cytokines such as TGF- β and, in certain tissues such as the gut, IL-10, as well as more “extreme” effects such as killing of T_{eff} or APCs [96–99]. The relative contributions of these mechanisms are subject to an ongoing discussion in the immunology field.

ROLE OF IMMUNE REGULATION IN TOLERANCE

Our laboratory sought to define mechanisms of T cell tolerance to the transgene product induced by hepatic gene transfer. Using mice transgenic for an ovalbumin (ova)-specific T cell receptor (TCR), we found that hepatic AAV-mediated gene transfer of ova (but not of GFP) induced anergy among and caused deletion of ova-specific CD4⁺ T cells [73]. However, ova-specific T cell proliferation was partially restored if CD4⁺ CD25⁺ Treg were depleted prior to the assay. These results pointed toward a role for immune regulation in tolerance to the transgene product. Subsequent experiments in TCR transgenic mice lacking endogenous TCRs (due to targeted deletion of Rag-2) and, consequently, lacking Treg, gave direct evidence for

induction of ova-specific Treg by hepatic AAV gene transfer [100]. The induced Treg expressed transcription factor FoxP3 as well as CD4, CD25, CTLA-4, and GITR, and suppressed CD4⁺CD25⁻ T cells *in vitro*. Therefore, induced Treg were phenotypically similar to naturally occurring Treg. Similar to our results on induction of T cell anergy and clonal deletion, Treg induction and expansion occurred during the first 2 months after gene transfer, which represents the induction phase for tolerance to the transgene product [100,101].

Lohse and colleagues found that liver-restricted transgene expression in a transgenic mouse model induced myelin basic protein (MBP) specific CD4⁺CD25⁺FoxP3⁺ Treg, which prevented experimental autoimmune encephalitis (EAE), an inflammatory T cell response against the brain that is normally observed upon immunization with MBP-specific peptide [75,102]. Using a TCR transgenic model, this study found no evidence for clonal deletion of MBP-specific T cells but demonstrated in serial adoptive transfer studies that Treg induced by hepatic expression were able to convert CD4⁺CD25⁻ cells into Treg. Treg from mice expressing a dominant non-functional TGF- β II receptor did not have this ability, indicating a role for TGF- β signaling in Treg cell function [75]. Interestingly, van Andrian and colleagues found that CD4⁺CD25⁺FoxP3⁺ are capable of suppressing CD8⁺ T cells in a TGF- β dependent process [99]. Suppressed CD8⁺ T cells retained some functionality but were impaired in the ability to release their granules, a state that was reversible in the absence of Treg. Also of importance, there is bidirectional signaling between Treg and APCs, so that Treg can modulate an APC and enhance suppression while APCs, conversely, can turn off suppression by Treg. A model that summarizes changes to the transgene product-specific lymphocyte population, including induction of Treg and subsequent suppression, is summarized in Fig. (1).

Immune tolerance to the therapeutic protein is a key requirement in gene therapy for genetic disease, the goal of which is to provide long-term transgene expression. In this regard, we examined hepatic AAV gene transfer of factor IX (F.IX), the coagulation factor that is deficient in the X-linked bleeding disorder hemophilia B [103]. Upon hepatic AAV-2 gene transfer to several strains of wild-type or F.IX-deficient mice, sustained systemic expression without antibody formation against F.IX was observed. Moreover, mice expressing in the therapeutic range (>50 ng/ml) typically remained tolerant to F.IX after challenge with subcutaneous F.IX protein in complete Freund's adjuvant or after secondary F.IX gene transfer with adenoviral vector [76,94,95]. Within a week of such a challenge, a spike in FoxP3 message is detectable in splenic CD4⁺ T cells [94,100]. Control animals had strong B and T cell responses to F.IX upon such immunizations. Hepatic gene transfer with a microRNA-regulated lentiviral vector can achieve a similarly robust level of tolerance (Annoni *et al.*, American Society of Gene and Cell Therapy annual meeting, Boston, MA, 2008, abstract # 1005). Data from a number of gene transfer studies using vectors or transgenic mice and numerous controls show that it is hepatic gene expression rather than an immune suppressive effect by the vector itself that induces tolerance.

Tolerized mice were not only protected from antibody formation against F.IX but also failed to generate CTL responses against F.IX after challenge with adenoviral vector [94]. Despite T cell responses against E1/E3-deleted adenovirus, which expresses viral proteins, CD8⁺ T cell infiltrates and inflammation in general were substantially reduced in tolerized mice compared to controls. Suppression of the inflammatory T cell response against the liver was transferable to naïve mice of the same strain using splenic CD4⁺ T cells. Tolerance induction to F.IX was sufficient to suppress the T cell response to the adenoviral vector in murine liver, resulting in sustained expression from this otherwise highly immunogenic vector [94]. It is possible that bystander suppression by Treg contributes to this outcome.

In a more detailed adoptive transfer study, we found that CD4⁺CD25⁺GITR⁺ T cells were primarily responsible for suppression of antibody formation against F.IX [100]. Tolerance

induction to the transgene product by hepatic AAV-2 or lentiviral gene transfer can be prevented in mice by depletion of CD25⁺ cells with monoclonal antibody (Annoni *et al.*, American Society of Gene and Cell Therapy annual meeting, Boston, MA, 2008, abstract # 1005) [100]. A recent study in non-human primates, using different immune modulatory reagents including anti-CD25, also demonstrated the importance of preserving Treg in hepatic AAV gene transfer in order to maintain tolerance to a F.IX transgene product [82]. The data in their totality indicate that hepatic gene transfer induces transgene product-specific CD4⁺CD25⁺ Treg that are similar to naturally occurring Treg and that are required and play a crucial role in tolerance, including the suppression of B and T cell responses to the transgene product. While the liver in the absence of inflammation provides a microenvironment and cytokine milieu that is favorable for immune tolerance (see above), the critical APC and the process of presentation of hepatocyte-derived antigen that results in Treg activation have not yet been identified or characterized.

INTERACTIONS BETWEEN THE IMMUNE SYSTEM OF THE LIVER AND OTHER ORGANS

Several very recent studies have shown that immune tolerance to a protein initially established by hepatic expression is maintained in other organs or compartments of the body. For example, we demonstrated that tolerance to F.IX induced by hepatic AAV-2 gene transfer was sustained after a second gene transfer to skeletal muscle using AAV serotype-1 or E1/E3-deleted adenoviral vector [95]. However, only the AAV-1 vector directed a sustained increase in F.IX expression. Despite an absence of B and T cell responses against F.IX, inflammatory CD8 cell responses against adenoviral antigens eliminated F.IX expression in skeletal muscle after one month. Interestingly, the AAV-F.IX transduced liver remained protected from CD8⁺ T cell responses and inflammation, which is normally seen because of adenoviral vector dissemination from the site of IM injection to the liver. This difference in muscle and liver may reflect differences in Treg function in these two organs. Nonetheless, with an appropriate choice of vector, muscle can be used successfully for supplementary gene transfer following tolerance induction by the hepatic route, a concept that is not only relevant for systemic protein delivery but also for local treatment of diseases such as Pompe disease or other lysosomal storage disorders. Equally important, Sun *et al.* have shown that Pompe mice, which have a lysosomal storage disorder caused by deficiency of acid α -glucosidase (GAA), fail to form antibodies against intravenously delivered GAA protein following tolerance induction by hepatic gene transfer with AAV-8 [90]. Consequently, the therapeutic effect from the exogenous, intravenously infused protein was improved in this enzyme replacement therapy.

Cheng and colleagues have published another example of combination therapy that took advantage of the tolerogenic effect of hepatic expression [78]. Here, it was found that intracranial administration of an AAV-2 vector expressing acid sphingomyelinase (ASM) for correction of lysosomal storage in the brain in a mouse model of Niemann-Pick disease resulted in antibody formation to ASM despite the immune privilege of this organ. In contrast, if an AAV-8 vector expressing ASM was used for hepatic gene transfer around the same time as the AAV-2 gene transfer to the brain, antibody formation was prevented and optimal therapy was achieved. Experimental groups treated only in one organ showed some improved quality of life, but were short-lived. The combination group, however, had a much improved survival length as well as an improved quality of life.

As explained above, ectopic MBP expression in livers of transgenic mice was protective of EAE, a model for multiple sclerosis [75]. Importantly, viral or non-viral gene transfer to the liver achieved an identical level of tolerance, and expression in skin had no effect on the onset of EAE. These findings have two significant consequences. First, hepatic gene transfer may be a tool not only for tolerance induction in treatment of genetic disease, but also of autoimmune

diseases. Second, and consistent with findings on gene transfer to other organs, the immune response in liver has unique properties that are more prone to result in tolerance induction than is the case for other target organs and tissues. At the same time, more studies are required to determine whether this approach can not only prevent but also ameliorate or reverse ongoing autoimmunity.

ALTERNATIVE STRATEGIES – IMMUNE MODULATION

Certain proteins may be more immunogenic or more difficult to express at levels sufficient for tolerance induction. In this case, immune modulation is an alternative approach at ensuring successful transgene expression, and much work has been done recently using small molecule drugs or monoclonal antibodies as single agents or in combination [104]. The blockade of selected costimulatory pathways can modulate the immune response in the gene therapy setting thus ablating pathogenic T cell responses or antibody formation and in fact, tip the immune balance toward favoring the induction for Treg cells to predominate.

For example, Miao and colleagues have evaluated a number of standard immunosuppressive drugs, namely cyclosporine (CSA), rapamycin (RAP) and mycophenolate mofetil (MMF) as single agents and combinations (CSA with MMF or RAP and MMF), with the objective to block antibody formation against factor VIII (F.VIII) in liver-directed gene transfer with naked plasmid DNA [105]. In these experiments, the plasmid was delivered by a hydrodynamic injection into the tail veins of hemophilia A mice. Without immune suppression, this protocol leads to inhibitor formation against F.VIII. Interestingly, the most effective reagents for blocking this response were either a combination of anti-CD40L and CTLA4-Ig or single use of anti-ICOS mAb [105,106]. All 3 reagents block co-stimulatory pathways between APCs and T cells, which are required for T activation and T cell-dependent antibody formation (CD40-CD40L, B7.1/2-CD28, and ICOS-ICOSL interactions). In the absence of proper co-stimulation, antigen presentation to T cells may result in tolerance rather than T cell activation. However, neither CTLA-4 nor anti-CD40L alone was able to induce tolerance, while these were effective in combination. Administration of anti-ICOS mAb resulted in 90% depletion of CD4⁺ICOS⁺ T cells and a relative increase in CD4⁺CD25⁺FoxP3⁺ Treg [106]. Additionally, administration of anti-ICOS mAb over an extended period of time (10 mg/kg daily for 1 week followed by 16 more doses over a period of 3 weeks) effectively promoted tolerance to F.VIII. The ICOS blockade seems to effectively suppress a number of pathways that may be involved in immune activation. While these experiments were based on administration of a high dose of mAb, it is nonetheless encouraging that a single agent was effective. Similarly, transient administration of high-dose CTLA4-Ig or continuous vector-derived expression of CTLA4-Ig prevented immune responses to β -galactosidase, resulting in sustained expression, in adult rats that received *in vivo* retroviral gene transfer to the liver following partial hepatectomy [107]. This gene transfer protocol previously resulted in CTL and antibody responses against transgene products [108].

Although this has not been tested yet for gene therapy, another promising reagent is non-Fc-receptor binding anti-CD3 mAb, which has recently been shown to promote tolerance to F.VIII protein infused intravenously to hemophilia A mice [109]. Anti-CD3 was effective at low doses and in a very short regimen (~0.4 mg/kg daily for 5 days). The antibody caused partial depletion of CD4⁺ and CD8⁺ effector T cells, a shift in the cytokine production profile in response to F.VIII (from Th2 to Th1) and induced F.VIII-specific Treg. Suppression of the inhibitory antibody response against F.VIII was dependent on Treg.

An alternative strategy for immune modulation that does not utilize immune suppressive drugs is antigen administration to mucosal surfaces. For example, oral tolerance takes advantage of an immune deviation mechanism that results in induction of CD4⁺ T cell responses that are

suppressive rather than inflammatory. Antigen presentation in gut-associated lymphoid tissues can result in activation of Th2, Th3, and Tr1 cells, which secrete immune suppressive cytokine IL-10 and TGF- β . Similarly, repeated administration of a F.IX-specific peptide (representing a dominant CD4⁺ T cell epitope) caused an immune deviation toward a suppressive response in hemophilia B mice. This response allowed our laboratory to block an immune response against F.IX and to achieve sustained expression following AAV vector administration to a strain that has been more refractory to tolerance induction by hepatic gene transfer than others. Suppression of antibody formation against F.IX was again adoptively transferable with CD4⁺CD25⁺ T cells [110].

NEONATAL GENE TRANSFER TO THE LIVER

Another approach to coax the immune system into accepting a transgene product is to take advantage of the immature immune system of the neonate [111]. There is impairment in the neonate of all three main antigen-specific response mechanisms, namely the CTL, T-cell dependent antibody and T-cell independent antibody responses. Performing retroviral gene transfer to the liver of neonatal animals, Ponder and colleagues have established the feasibility of this approach for therapeutic and tolerogenic gene delivery [112–115]. However, this group also noticed distinct differences between species such as mice, cats, and dogs in their immunologic response to the therapeutic protein. The range of response was the complete lack of antibody response from cats, which in some cases appear to mount a CTL response, to a low-dose mediated tolerance in dogs, which are tolerized at protein levels significantly less than those required for mice [85,86]. Additionally, using both hemophilia A and B as models, newborn mice that received high doses of retroviral vector had stable expression of protein without antibodies, whereas adult mice that received the same regimen typically had a robust immune response. Similar to data on transduction of adult liver with AAV vectors, a minimal level of expression is required for tolerance induction, which may be lower than that required in adults and therefore represents another advantage of the neonatal protocol [115,116]. Neonatal hepatic gene transfer with retroviral vectors was also successful for long-term therapeutic expression of UDP-glucuronosyl transferase in a rat model of the rare metabolic disorder Crigler-Najjar type 1 disease (absence of this enzyme causes high levels of unconjugated bilirubin) [117]. On the other hand, Lentiviral gene transfer to neonatal rats failed to induce tolerance to UDP-glucuronosyl transferase, again indicating that neonatal gene transfer does not always avoid immune responses to gene products [118]. In adult mice, high-dose retroviral vector co-injected with hepatocyte growth factor was effective in tolerance induction to F.IX (note that retroviruses require dividing target cells for transduction) [86]. Much of this work has been carried out utilizing retrovirus, which does carry a risk of insertion mutagenesis that would need to be addressed as these processes move toward human trials.

CONCLUSIONS

Efficacy of gene transfer has substantially improved in recent years following dramatic advances in vector development and engineering. In this review, we have shown the importance of the liver as a target site for vector delivery and transgene expression. This organ has emerged as a place where antigen presentation often favors immune tolerance over immune-mediated rejection of the delivered vector or gene product. These observations offer many opportunities for development of gene therapies for inherited protein deficiencies such as lysosomal storage diseases, hemophilia, metabolic disorders, as well as autoimmune diseases and others. In addition, the field is starting to define the parameters required for tolerance induction by gene transfer and to design vectors specifically for this purpose. Use of hepatocyte-specific promoters, microRNA regulation of gene expression, and engineering of viral capsids and envelopes are examples for rationale designs based on recent mechanistic dissections of the interactions between vectors and the immune system, in particular in hepatic gene transfer.

Furthermore, advances in immunology, specifically in immune regulation and biology of regulatory T cells, have help shed light onto the mechanisms by which transgene products may be accepted rather than rejected by the immune system.

While hepatic gene transfer, using AAV and micro-RNA regulated Lentiviral vectors, has been shown to induce tolerance to many different proteins, most of these studies have been carried out in mice. One should therefore caution that more studies are needed to address potential species-specific differences. As pointed out in this review, there are examples of immune responses to transgene products with this approach. In addition to optimization of vector design and dose, others factors that have to be considered include genetic modifiers (species, underlying mutation, MHC haplotype, polymorphisms in loci encoding cytokines and other functions of the immune system, pre-existing inflammation/liver pathology in the host, and autoimmunity/immune dysregulation) [56,86–88]. Furthermore, some antigens may be generally more immunogenic or more difficult to express at high level. Whether certain general properties of a transgene product (such as cellular localization or systemic delivery) facilitate tolerance induction also remains to be defined

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Fig. 1.

Proposed model for transgene product-specific immune tolerance induced by hepatic gene transfer. An optimal vector transfers a gene to the liver and restricts expression to hepatocytes. Transgene product-derived peptide are presented by a yet to be identified antigen presenting cell (APC) to naïve CD4⁺. This process, which may occur in the liver or a lymphoid organ, leads to deletion and anergy of transgene product-specific effector T cells (Teff) and to induction of CD4⁺CD25⁺FoxP3⁺ Treg. Either directly, or more likely through interactions with APCs, induced Treg suppress Teff and CD8⁺ T cells and convert Teff to Treg. Suppression of Teff also leads to a lack of T help and therefore suppression of B cell responses/antibody formation and cytotoxic T lymphocyte (CTL) responses.

Table 1

Examples of Tolerance Induction to a Specific Protein by Hepatic Gene Transfer

Vector	Transgene Product	Reference
Adenovirus	Apolipoprotein A-I	<i>Blood</i> , 101: 2551–6
Gutted adenovirus	(α_1 -antitrypsin	<i>Hum Gene Ther</i> , 18: 1215–24
Lentivirus	Factor IX	<i>Blood</i> , 110: 4144–52
Lentivirus	GFP	<i>Nat. Med.</i> , 12: 585–591
Retrovirus*	Factor IX	<i>Blood</i> , 103: 143–51
Retrovirus*	Factor VIII	<i>J Thromb Haemost</i> , 5: 1805–12
Retrovirus*	UDP-glucuronosyl transferase	<i>Hepatology</i> , 42: 431–438
AAV	Factor IX	<i>J. Clin. Invest.</i> 111: 1347–1356
AAV	Alpha-galactosidase	<i>Mol Ther</i> , 9: 231–240
AAV	Ovalbumin	<i>Blood</i> , 104: 969–977
AAV	Acid alpha-glucosidase	<i>Mol Ther</i> , 12: 876–84
Adenovirus or plasmid	Myelin basic protein	<i>J Clin Invest</i> , 118: 3403–10
AAV	Acid sphingomyelinase	<i>Proc Natl Acad Sci U S A</i> , 104: 9505–10

* Neonatal gene transfer.