IMMUNE RESPONSES TO AAV VECTORS, FROM BENCH TO BEDSIDE

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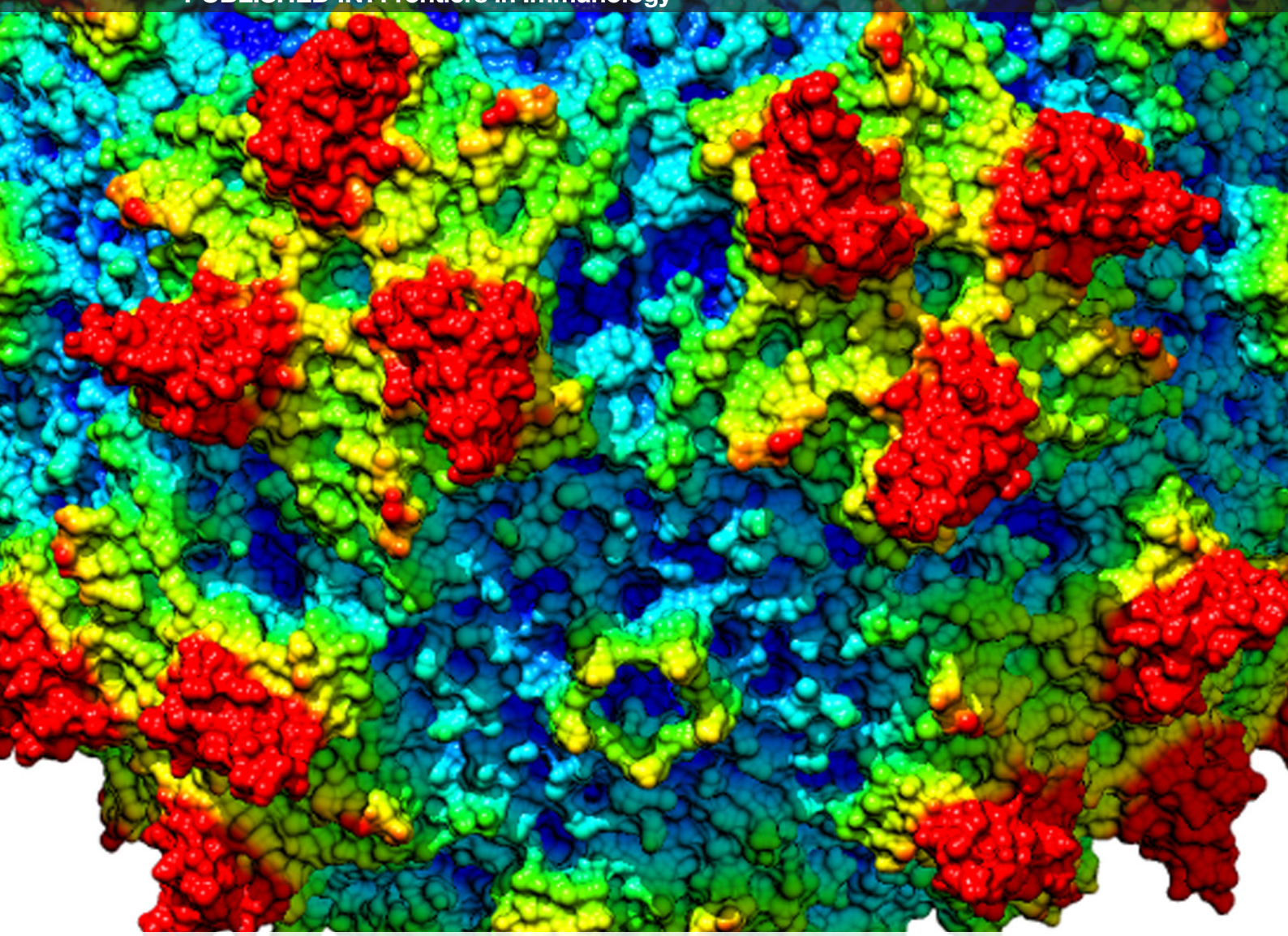

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IMMUNE RESPONSES TO AAV VECTORS, FROM BENCH TO BEDSIDE

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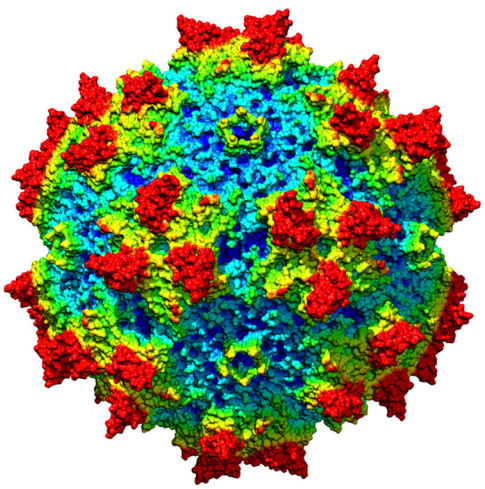

The AAV capsid. Radially color-cued (from capsid center to surface: blue-green-yellow-red; ~110–130 Å) of the AAV1 capsid generated from 60 VP monomers (RCSB PDB # 3NG9). From Tseng YS, Agbandje-McKenna M. Front Immunol. 2014;5:9.

The recent wave of clinical studies demonstrating long-term therapeutic efficacy highlights the enormous potential of gene therapy as an approach to the treatment of inherited disorders and cancer. While in recent years lentiviral vectors have dominated the field of ex vivo gene therapy in man, adeno-associated virus (AAV) vectors have become the platform of choice for the in vivo gene delivery, both local and systemic.

Despite the achievements in the clinic however, a number of hurdles remain to be overcome in gene therapy, these include availability of scalable vector production systems, potential issues associated with insertional mutagenesis, and concerns related to immunogenicity of gene therapeutics.

For AAV vectors, clinical trials showed that immunity directed against the vector could either prevent transduction of a target tissue or limit the duration of therapeutic efficacy. Initial observations in the context of a gene therapy trial for hemophilia spurred over a decade efforts by gene therapists and immunologists to understand the mechanism and identify factors that contribute to AAV's immunogenicity,

including the prevalence of B cell and T cell immunity to wild type AAV in humans and the interaction of AAV vectors with the innate and adaptive immune system.

Despite a number of important contributions in particular in the more recent past, our knowledge on the immunology of gene transfer is still rudimental; this is partly due to the fact that the basic understanding of the complex balance between tolerance and immunity to an antigen, key aspect of gene transfer with AAV, keeps evolving rapidly.

However, continuing work towards a better definition of the interaction of viral vectors with the immune system has led to significant advances in the knowledge of the factors influencing the outcome of gene transfer, such as the vector dose, the immune privilege of certain tissues, and the induction of tolerance to an antigen. A better understanding of the structure-function relationship of the viral capsid has boosted the development of novel immune-escape vector variants. In addition, novel immunomodulatory strategies were established to prevent or reduce anti-capsid immunity have been developed and are being tested in preclinical models and in clinical trials. Together, these advances are bringing us closer to the goal of achieving safe and sustained therapeutic gene transfer in humans.

In this research topic, a collection of Original Research and Review Articles highlights critical aspects of the interaction between gene AAV vectors and the immune system, discussing how these interactions can be either detrimental or constitute an advantage, depending on the context of gene transfer, and providing tools and resources to better understand the issue of immunogenicity of AAV vectors in gene transfer.

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Adeno-associated viral vectors at the frontier between tolerance and immunity

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Keywords: AAV vector, immune response, tolerance, vaccine, gene therapy

In recent years, the field of *in vivo* gene transfer with adeno-associated virus (AAV) vectors has seen an extraordinary expansion of applications and investments. Results emerging from clinical trials (1) and the recent market approval of a gene therapy drug for lipoprotein lipase deficiency (2) contributed to the hype around this vector system (3). Indeed, AAV vectors have several features that make them an ideal tool for gene transfer, for example, parental virions are replication deficient and non-pathogenic (4), and vectors can drive expression of a transgene for several years (5, 6) despite the fact that they do not integrate efficiently into the host genome. In recent years, a portfolio of natural AAV isolates (AAV serotypes) differing in tissue tropism has been developed as vectors. This toolbox has been further expanded with engineered AAV capsids developed to enhance efficiency and specificity of gene delivery, and to escape antibody neutralization (7). At the vector genome level, availability of potent promoter/enhancer sequences, codon-optimization of transgenes, and development of self-complementary AAV vectors (8) further enhanced efficacy of gene transfer. Finally, the availability of scalable processes to produce AAV vectors in GMP contributed significantly to the expansion of the field.

As the AAV vector technology reached a more mature stage, it has become clear that a better understanding of the interactions of viral vectors with the host immune system is needed. In this Research Topic of *Frontiers in Immunology*, the editors present a collection of reviews and research articles discussing the two sides of immune responses triggered by *in vivo* gene transfer. These responses in fact can be desirable when they result in induction of tolerance to the therapeutic transgene (9), or when they are exploited for vaccine development, as discussed by Nieto and Salvetti in their review article (10). Conversely, immunogenicity of the viral capsid or the transgene product can be detrimental, as it may result in lack or loss of efficacy following vector-mediated gene transfer.

Evidence for the critical role of tolerance induction in the achievement of sustained therapeutic efficacy following gene transfer comes from the work of Liao and colleagues, which provides evidence that glucocorticoid-induced TNF receptor (GITR) and its ligand GITR-L are of fundamental importance for the induction of immune regulatory responses in gene transfer and that lack of expression of GITR-L on antigen presenting cells results in impaired induction of regulatory T cells (Tregs) (11). Indeed, evidence of the key function of Tregs for successful *in vivo* gene therapy comes from several studies (12), and Liu and colleagues further demonstrate this concept in a model of plasmid gene transfer for hemophilia A, in which a combination of B cell depleting and Treg-enhancing drugs is used to successfully modulate transgene immunogenicity (13).

INNATE IMMUNE RESPONSES TO AAV VECTORS

The innate immune system constitutes the first line of defense against invading pathogens. It recognizes evolutionarily conserved structures foreign to the host or detects structures known as self, but present in the wrong intracellular compartment, via innate immune sensors termed pathogen recognition receptors (PRRs). Binding of such pathogen-associated molecular patterns (PAMPs) to PRRs activates the intracellular innate immune system, leading to substantial changes in the expression of genes related to host defense, in secretion of cytokines and chemokines, and up-regulation of co-stimulatory molecules, which as a consequence induce or modulate the adaptive arm of the immune system.

Of the four families of cellular PRRs [toll-like-receptors (TLRs), NOD-like receptors, RIG-like receptors, and C-type lectin receptors], as of now only two members of the TLR family, TLR-2 and TLR-9, have been described as sensors for AAV vectors. TLR-2 was identified as a PRR of the viral capsid in studies on cell autonomous immune responses in primary human liver cells (liver sinusoidal endothelial cells, Kupffer cells) and activated endothelial cells (14), while TLR-9 was reported as sensor of AAV vector genomes in plasmacytoid dendritic cells (pDC) isolated from mice and humans (15). Although both PRRs are part of the same family, recognition of the viral capsid caused induction of a Nuclear Factor kB-dependent inflammatory response (14), while activation of TLR-9 induced secretion of type I interferon (IFN) that was found to be enhanced if vectors with self-complementary (sc) AAV vector genomes were used (15, 16). The nature of this enhanced immunogenicity remains to be clarified, but is maybe related to a negative impact of sc vector genomes on capsid stability (16) or to the additional inverted terminal repeat (ITR) sequence present in sc vector genomes (8). The later hypothesis would be in line with a recent study reporting significantly reduced adaptive

immune responses toward the capsid and the transgene product when using AAV vectors with a reduced number of CpG motifs, which are known TLR-9 PAMPs (17). The route of vector delivery appears to be a critical factor in AAV recognition by the innate immune system. The above-described activation of the TLR-9 myeloid differentiation primary response 88 (MyD88) signaling pathway, for example, resulted in humoral and T cell-mediated adaptive immune responses toward the AAV capsid and the transgene product in mice in which AAV vectors were administered intramuscularly. Conversely, following tail vein injection, neither a TLR-2, nor a TLR-9, or a type I IFN dependent induction of AAV specific IgG antibodies could be detected (15, 18).

ADAPTIVE IMMUNE RESPONSES IN AAV VECTOR-MEDIATED GENE TRANSFER

Exposure to wild-type AAV or to AAV vectors, and the consequent activation of innate and adaptive immunity to vector and transgene leads to both antibody and cell-mediated responses. Antibodies directed against the AAV capsid are highly prevalent in humans (up to 60% of healthy individuals) and can efficiently neutralize the vector even when present at low titers, resulting in lack of efficacy, thus posing a significant constrain to patients enrollment in clinical trials. Similarly, vector administration results in long-lasting high-titer anti-AAV neutralizing antibodies (NAb), which prevent vector readministration. Results from human trials and studies conducted in small and large animal models of gene transfer showed that NAb titers as low as 1:5 can completely block AAV vector transduction, and that AAV vectors remain susceptible to antibody-mediated neutralization for several hours after intravascular delivery.

Two contributions on the topic of anti-AAV antibodies can be found in this Research Topic. Calcedo and Wilson reviewed the issue of NAb directed against AAV; in their manuscript, they discussed the prevalence of NAb in various human populations, the issue of antibody cross-reactivity, and finally the assays used to measure antibodies to AAV, and the strategies that could possibly be used to overcome this limitation (19). In the second review article, Tseng and Agbandje-McKenna (20) discuss different approaches to antibody epitope mapping and the relationship of these epitopes with the capsid structure. Furthermore, they suggest how this knowledge can be exploited to drive the efforts toward engineering novel AAV capsid variants resistant to antibodies, and to gain a better understanding on the structure-function-relationship across AAV serotypes when it comes to the interactions with the immune system.

In addition to neutralizing antibodies, natural infection with wild-type AAV also triggers cell-mediated immune responses against the capsid, which results in a reservoir of memory $CD8^+$ T cells that can be reactivated upon vector administration. This can cause the destruction of transduced cells harboring AAV capsid antigen in the context of MHC class I, as it has been observed in subjects enrolled in AAV vector-mediated liver gene transfer trials. Several questions remain on the role of these capsid-specific $CD8^+$ T cells in the outcome of gene transfer, as detection of T cell reactivity to the capsid in PBMCs has not always been associated with detrimental effects on gene transfer in liver and muscle trials. Notably, experience from the AAV8 gene therapy trials in hemophilia B subjects suggests that timely administration of immunosuppression can prevent detrimental effects of capsid-directed T cell immunity.

Three review articles in this Research Topic focus specifically on adaptive immune responses to AAV vectors in the context of gene transfer to different tissues, and discuss the issue of T cell-mediated immunity directed against the vector capsid. Willett and Bennett provide an overview of what it is known about gene transfer in an immune privileged body site, the eye, describing the unique and valuable lessons learned from the preclinical and clinical studies of AAV gene transfer for RPE65 deficiency (21). Ferreira and colleagues describe the experience with AAV vectors in muscle gene transfer in the context of the development of Glybera, the approved drug for the treatment of lipoprotein lipase deficiency (22). Finally, in their manuscript, Basner-Tschakarjan and Mingozzi provide a broad overview on the issue of T cell immunity to AAV vectors focusing on data emerging from gene therapy trials (23). To complete this collection of articles on immune responses in gene transfer, two review articles discuss the tools available to the investigators to study the immunogenicity of AAV vectors. Basner-Tschakarjan and colleagues provide an overview of *in vitro* and *in vivo* preclinical models that have helped to explain the immune responses to AAV vectors observed in human trials (24), while Britten and colleagues address the extremely important issue of immune assay standardization in clinical trials (25).

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Cell-mediated immunity to AAV vectors, evolving concepts and potential solutions

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Adeno-associated virus (AAV) vectors are one of the most efficient *in vivo* gene delivery platforms. Over the past decade, clinical trials of AAV vector-mediated gene transfer led to some of the most exciting results in the field of gene therapy and, recently, to the market approval of an AAV-based drug in Europe. With clinical development, however, it became obvious that the host immune system represents an important obstacle to successful gene transfer with AAV vectors. In this review article, we will discuss the issue of cytotoxic T cell responses directed against the AAV capsid encountered on human studies. While over the past several years the field has acquired a tremendous amount of information on the interactions of AAV vectors with the immune system, a lot of questions are still unanswered. Novel concepts are emerging, such as the relationship between the total capsid dose and the T cell-mediated clearance of transduced cells, the potential role of innate immunity in vector immunogenicity highlighted in preclinical studies, and the cross talk between regulatory and effector T cells in the determination of the outcome of gene transfer. There is still a lot to learn about immune responses in AAV gene transfer, for example, it is not well understood what are the determinants of the kinetics of activation of T cells in response to vector administration, why not all subjects develop detrimental T cell responses following gene transfer, and whether the intervention strategies currently in use to block T cell-mediated clearance of transduced cells will be safe and effective for all gene therapy indications. Results from novel preclinical models and clinical studies will help to address these points and to reach the important goal of developing safe and effective gene therapy protocols to treat human diseases.

Keywords: AAV vectors, T cell responses, gene therapy, immunogenicity, immune modulation

INTRODUCTION

Several clinical studies have shown long-term correction of the disease phenotype following gene transfer (1–9). To attain this goal, two main approaches have been used, one using an integrating viral vector (typically retroviral or lentiviral) to introduce the therapeutic gene *ex vivo* into an autologous stem cell (10), the other transferring the gene into a post-mitotic cell *in vivo* (11).

Viral vectors derived from adeno-associated virus (AAV) have become the tool of choice for *in vivo* gene transfer, mainly because of their superior efficiency *in vivo* (11), their tropism for a broad variety of tissues, and their excellent safety profile. Therapeutic efficacy following AAV vector gene transfer was documented in several preclinical studies and, over the past decade, some of these results were successfully translated to the clinic, leading to some of the most exciting results in the field of gene therapy (11). The recent market approval of the first AAV-based gene therapy product in Europe (12, 13) constitutes additional evidence that the field is progressing from proof-of-concept studies toward clinical development.

However, human studies also highlighted some of the limitations of *in vivo* gene transfer with AAV vectors, which were not entirely identified in preclinical studies. In particular, it has been shown that immune responses triggered by AAV vector-mediated gene transfer may constitute an important obstacle to long-term therapeutic efficacy and a safety concern.

Over the past 10 years, gene therapists have struggled with the issue of immunogenicity of AAV vectors. The initial lack of animal models recapitulating the findings in human trials (14–16) has made clinical observation crucial to understand the interactions between AAV vectors and the immune system. While recent work shows that it is possible to model human T cell responses to the AAV capsid in mice (17), it is likely that human studies will remain the main source of knowledge on immune responses in gene transfer. In this review article, we will summarize the progress that has been made in the understanding of T cell responses in AAV vector-mediated gene transfer focusing on human studies, we will discuss the current state of knowledge, and we will describe some of the proposed strategies to modulate AAV vector immunogenicity.

WILD-TYPE AAV AND RECOMBINANT AAV VECTORS

Adeno-associated virus are small non-enveloped viruses with a single-stranded DNA genome of ~4.7 kb composed by the *rep* and the *cap* genes, which encode for the proteins involved in the life cycle of the virus. The *rep* gene is involved in the virus replication,

while the *cap* gene encodes for the three structural proteins, VP1, VP2, and VP3, which form the viral capsid with a stoichiometry of 1:1:10, respectively (18), and for the assembly activating protein, which targets newly synthesized capsid proteins to the nucleolus and participates to their assembly into an icosahedral capsid (19). The *rep* and *cap* genes are flanked by two inverted terminal repeats (ITRs) that are needed as signals for packaging of the genome into the viral capsid (20). AAV is not autonomously replicating as its replication cycle depends on the coinfection with a helper virus such as adenovirus or herpes simplex; this explains why AAV was first isolated as a contaminant of an adenovirus preparation (21). The exposure to AAV and helper virus may account for the generation of both antibody and memory T cell response to AAV. AAV has never been associated with any known illnesses in humans with the exception of reports of association between spontaneous abortions and AAV infection (22). AAV vectors are derived from wild-type AAV by replacing all the viral coding sequences in the genome with an expression cassette for the transgene of interest. The ITRs are the only viral sequences retained in AAV vectors.

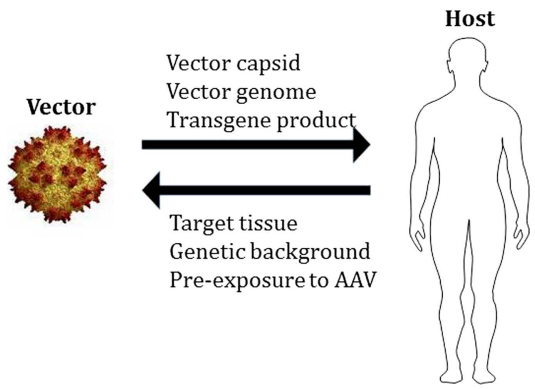

FIGURE 1 | Adeno-associated virus vector:host interactions. The components of AAV vectors interact with the host in determining the outcome of gene transfer. For the AAV vector, the total capsid dose, the genome (single-stranded vs. self-complementary, CpG content, etc.), and the transgene product (to which the host may not be tolerant), represent some of the elements that could contribute to vector immunogenicity. On the host side, the characteristics of the target tissue (immunoprivilege, presence of inflammation, tolerogenic properties, etc.), the genetic background of the host (underlying disease-causing mutation, HLA haplotype, etc.), and the pre-exposure to the wild-type virus, can contribute to shape the overall response to vector delivery.

AAV VECTOR:HOST INTERACTIONS

Like other viral gene transfer vectors, AAV vectors are complex biological therapeutics, and the outcome of gene transfer is dependent on the interactions between vector- and host-related components (**Figure 1**). These interactions occur at multiple levels, starting from the innate recognition of the vector capsid and DNA genome (23), to the development of humoral (24) and cell-mediated adaptive responses to the capsid and/or the transgene product.

AAV VECTOR CAPSID

At the immunological levels, the AAV vector capsid is a replica of the wild-type virion, therefore, the vector capsid antigen is expected to be recognized by T cells like the wild-type AAV capsid antigen. Despite this important similarity, however, vector administration differs from natural infection as it involves the introduction of large numbers of viral particles into an organism, several logs more than in a natural infection (25), via routes substantially different from the natural route of exposure to AAV (i.e., mainly via the airways for wild-type AAV), and does not involve active replication of the virus or presence of a helper virus, as AAV vectors are delivered as preformed particles. These differences are also characteristic of other viral gene transfer platforms, however, unique features of AAV (e.g., their lower immunogenicity compared to adenovirus) may account for the outcome of AAV gene transfer in humans.

Because AAV vectors are non-replicating, it is generally assumed that the AAV capsid remains immunologically detectable within a transduced cell only for a defined period of time, which may vary from few weeks to several months, depending on the target tissue. Experiments in primates in the context of liver gene transfer suggest that the capsid is still detectable by the immune system several weeks following administration (26), indicated by the fact that interruption of immunosuppression (IS) 8 weeks after gene transfer results in a spike in anti-AAV antibodies. In dogs, intact capsid was detectable by electron microscopy in the retina up to 6 years following gene transfer (27), and a similar result was recently obtained in human muscle, in which immunostaining for capsid particles showed a positive signal 12 months following vector administration (28). Finally, results in humans undergoing AAV8 gene transfer for hemophilia B suggest that the capsid antigen in transduced hepatocytes is still recognizable by capsid-specific T cells 8–9 weeks following gene transfer (9).

Why the capsid persists for such a long period of time is not clear at this point. There are several factors that may influence the persistence of viral particles in a tissue, among them the vector serotype and dose administered, the degree of vascularization of the tissue target of transduction, the number of particles introduced in a single injection site, and the possible immune response to the vector itself, which may increase the rate of clearance of the antigen. *In vitro* studies attempting to determine the half-life of the capsid in transduced cells have been largely unsuccessful in predicting the fate of the capsid, mainly because of the poor infectivity of most serotypes in cell lines (29).

VECTOR DNA GENOME

Poorly defined until recently, the *in vitro* and *in vivo* interactions of both the DNA genome and the capsid of AAV vectors with the innate immune system were recently described by several groups (30–35). Additionally, recently published results indicate that the removal of CpG from the vector genome may contribute to reduce the potential immunogenicity of the transgene product (36). While these studies suggest that innate immunity to AAV vectors may trigger adaptive immune responses to the capsid or to the transgene product, there is no evidence that AAV vector administration in humans result in acute inflammatory reactions, although detailed studies in subjects undergoing AAV gene transfer remain to be performed.

TRANSGENE PRODUCT

Unlike the AAV vector capsid, which is not synthesized by infected cells, the transgene product is expressed for a long time after target tissue transduction (37–39). Vector-encoded transgene product may be recognized as a foreign antigen, especially if the recipient of gene transfer is not tolerant to the protein encoded by the vector, thus triggering immune responses that can result in production of transgene-specific neutralizing antibodies (40) or triggering of T cell responses directed against transgene-expressing transduced cells (41).

Preclinical studies suggest that the tissue target of transduction plays a fundamental role as determinant of transgene immune responses in gene transfer. For example, preclinical studies of intramuscular delivery of AAV vectors suggest that this approach carries a higher risk of triggering immune responses to the transgene (42) compared to other tissues such as the liver (43), and that the underlying disease-causing mutation is a major determinant of the risk of developing an immune response to the therapeutic transgene product following AAV-mediated gene transfer to the muscle (44). Conversely, delivery of AAV vectors to the muscle via the vasculature seem to reduce considerably the immunogenicity of the therapeutic transgene (45–47), suggesting that the more widespread and uniform transduction of muscle achieved with this route of vector delivery lowers transgene immunogenicity. Clinical results accumulated thus far on intramuscular delivery of AAV vectors seem to indicate that the approach is safe, as no subject enrolled in trials of intramuscular gene transfer developed anti-transgene immunity (13, 48, 49). Transgene-specific cell-mediated immunity was documented in only one study in which pre-existing immunity to dystrophin seemed to trigger the expansion of dystrophin-specific $CD8^+$ T cell clones (41).

Differently form muscle, a number of studies showed that expression of a transgene in the liver is associated with induction of antigen-specific tolerance (43, 50–59). This was demonstrated for several antigens and in various animal models, including animals that were first immunized and then tolerized against the same antigen used for immunization using liver gene transfer (52, 55, 56).

Results from clinical gene transfer studies seem to support the hypothesis that AAV vector-mediated liver gene transfer is associated with tolerance, as no subject dosed with AAV vectors in the liver developed an immune response directed against the transgene product, including individuals carrying *null* mutations in the disease-causing gene (9, 60).

Lastly, one important point to keep in mind when discussing transgene immunity is that most of the clinical experience to date derives from studies in which cross-reactive immunologic material (CRIM) positive subjects and subjects with prior exposure to the therapeutic protein [e.g., recombinant or plasma-derived factor IX (FIX) for hemophilia B patients] were enrolled (9, 48, 60, 61). These categories of subjects are at lower risk of developing immune responses to the donated therapeutic gene, thus a careful assessment of the immunogenicity of the transgene in gene transfer will be necessary before enrolling previously untreated patients in gene transfer trials. Finally, also the disease state of the organ targeted with gene transfer can influence the magnitude of the immune responses observed following AAV vector-mediated gene transfer (62–64).

CAPSID ANTIGEN INTRACELLULAR PROCESSING AND MHC CLASS I PRESENTATION

Cell transduction with AAV vectors begins with binding of the virion to the cell surface proteins and carbohydrates, an event that is followed by endocytosis. The receptor and co-receptors used for cell entry vary among serotypes, although the receptors for all serotypes have not been identified yet. The steps of endosomal escape, nuclear transport, and vector uncoating are not completely understood in terms of timing or mechanism, this in part due to their complexity and the large number of pathways and compartments potentially involved in the process of cell transduction. The intracellular fate of AAV2 has been the most extensively investigated, because several cell lines are readily transduced by this serotype and because monoclonal antibodies recognizing AAV2 intact particles and capsid proteins were the first available. Of interest to this review article is the ability of the capsid antigen to be processed and cross-presented on MHC class I (MHC I) by transduced cells (e.g., hepatocytes). Since the initial findings on the immunogenicity of AAV2 vectors in human trials (60), the ability of replication-deficient AAV vectors to gain access to MHC I via cross-presentation has been object of debate, and several alternative hypothesis were formulated to explain why AAV2 vectors were immunogenic in humans but not in animal models. These included the possibility of preferential uptake of certain AAV serotypes by dendritic cells, the expression of *Cap* sequences packaged into AAV vectors, and the expression of alternative open reading frames within the transgene cDNA, which would generate aberrant proteins recognized as offending antigens by the host immune system [reviewed in Ref. (65)]. Several years of studies in preclinical models and, most importantly, in human trials (9) helped developing a better understanding of immune responses to AAV vectors, supporting the idea that capsid-specific T cell responses were responsible for clearance of transduced cells.

At the intracellular level, it has been shown that the capsid is substrate for ubiquitination (66, 67), and that proteasome inhibitors, or mutation of surface exposed tyrosine residues that normally undergo phosphorylation and ubiquitination, enhance transduction by enhancing nuclear uptake of virus (68, 69). Data supporting the hypothesis that the AAV capsid antigen is processed by the proteasome and presented on MHC I come from CTL assays performed using AAV-transduced human hepatocytes as targets, and HLA-matched capsid-specific $CD8^+$ T cells as effectors (70). The fact that target cell lysis can be inhibited specifically using a capsid antigen-specific soluble T cell receptor (TCR) to block T cell access to the target cell (70) further confirms that the AAV capsid antigen is processed by transduced cells and presented on MHC I. Presentation of antigen in the context of MHC I was also demonstrated using a T cell line engineered to express luciferase when recognizing the capsid antigen (71). In this study, levels of antigen presentation were directly correlated with the multiplicity of infection used in the assay, and the proteasome inhibitor bortezomib blocked antigen presentation. These results are in agreement with results from clinical studies, which suggest the existence of a correlation between vector dose and magnitude of T cell responses to the capsid.

Recent work from Li et al. (72) indicates that the endosomal escape of vector is not only the pivotal step in cell transduction

but is also fundamental for capsid antigen presentation. While this is not completely surprising, the study also indicates that empty capsids, AAV particles that fail to incapsidate a DNA genome, are less likely to be presented onto MHC I. This result is somewhat in contrast with previous data showing that empty capsids do flag transduced cells for T cell recognition (70). One possible explanation for this difference is that the two studies used different strategies to detect antigen presentation: ovalbumin TCR transgenic T cells and a capsid carrying the ovalbumin SIINFEKL epitope in one study (72), or human peripheral blood mononuclear cells (PBMCs) expanded against a the native AAV epitope VPQYGYLTL to track presentation of native AAV2 capsid antigen (70). Immunogenicity of empty vs. full AAV capsids is an important pending question for the field, particularly given the fact that empty capsids may play an important beneficial role in allowing for vector transduction in the presence of neutralizing antibodies (73).

Although there is no direct evidence that intracellular processing and presentation of AAV onto MHC I differ among serotypes, anecdotal evidence supporting this hypothesis comes, for example, from the observation that the administration of AAV2 and AAV8 vectors to the liver of humans at similar doses results in different kinetics of activation cytotoxic T cell immunity against the transduced hepatocytes (9, 60, 74). Whether these differences can be ascribed to the vector serotype only is unknown, as *in vitro* studies comparing AAV serotypes have been challenging due to differences in efficiency of AAV transduction among serotypes. Conversely, *in vivo* preclinical studies show somewhat contrasting results, some suggesting for example that AAV2 and AAV8 have identical kinetics of T cell induction (75), and others showing that AAV2 and AAV8 do differ in their ability of triggering T cell proliferation (76). Serotype-specific differences are also supported by data generated with an adoptive T cell transfer model in mice, indicating that AAV8 remains immunologically detectable longer than AAV2 following systemic gene transfer to target the liver (17, 76).

In conclusion, several studies identified the key intracellular AAV trafficking steps that are involved in cell transduction and capsid antigen presentation. Whether there is one leading pathway for antigen cross-presentation or rather multiple alternative pathways concur to MHC I presentation (77) of capsid remains to be defined. Most importantly, the significance of *in vitro* findings has to be proven *in vivo*, as kinetics and pathways might differ substantially in living organisms compared to cell lines.

T CELL RESPONSES TO AAV IN HUMAN STUDIES

Several studies on the seroprevalence of AAV in humans suggest that exposure to the wild-type virus mostly occurs early in life (78–80). Similarly, monitoring of T cell reactivity to the AAV2 capsid conducted in humans undergoing splenectomy for non-malignant indications shows that about two-thirds of adults >25 year-old carry a pool of T cells that can produce IFN-γ in response to AAV2 capsid peptides, while only a small proportion of subjects <5 years old present T cell reactivity to the capsid (81). Veron et al. (82) conducted a similar survey for AAV1 in PBMCs from healthy donors. In this study, an overall frequency of respondents of about 30% was documented. The difference in frequency of subjects carrying capsid-specific T cells in the two studies may be due to the fact that reactive T cells fail to circulate in peripheral blood at high frequency [as previously suggested in Ref. (74)], or to the different restimulation protocols used (a peptide library was used in the AAV2 study vs. lentiviral vectors expressing capsid were used in the AAV1 study), or else it may reflect inherent differences in the frequency of subjects exposed to AAV2 vs. AAV1.

Two aspects of capsid T cell reactivity are worth noting, the first is that the high degree of conservation of the AAV capsid amino acid sequence (83) results in a high degree of cross-reactivity of T cell responses across serotypes (74). The second aspect is that B and T cell responses to AAV seem to be uncoupled, as subjects positive for anti-AAV antibodies may not present detectable T cell reactivity to the capsid and, vice versa, subjects with detectable T cell reactivity to AAV in PBMC have lower anti-capsid antibody titers (82). This suggests that, following exposure to AAV, certain individuals may develop a Th1 response to the antigen, while others develop a predominantly Th2 response. Future studies will be required to clarify the relationship between B and T cell responses to AAV in the context of the natural infection with the virus.

LIVER-DIRECTED GENE TRANSFER

The importance of T cell immunity to the AAV capsid in terms of both safety and efficacy of AAV gene transfer in humans was initially evidenced in the first clinical trial in which an AAV2 vector was introduced into the liver of severe hemophilia B subjects (60). In this study, upon AAV gene transfer to liver, two subjects developed transient elevation of liver enzymes and loss of FIX transgene expression around week 4 post vector delivery due to the immune rejection of transduced hepatocytes mediated by capsid-specific $CD8^+$ T cells (74).

While conceptually these findings are not surprising, as antiviral immunity is expected to recognize virus infected cells and clear them, this was the first instance in which a cytotoxic immune response directed against the AAV capsid was observed in the context of gene transfer. Preclinical animal studies failed to predict or to recapitulate the findings in humans, and initial attempts to model the induction of destructive T cell responses in mice have been mostly unsuccessful until recently (17).

The recent findings in a clinical trial of AAV8 gene transfer of FIX to the liver of subjects affected by severe hemophilia B (9) confirmed results in the previous AAV2 trial and supported the hypothesis that AAV capsid antigen is processed and presented onto MHC I by vector-transduced hepatocytes where it is recognized by capsid-specific $CD8^+$ T cells, leading to clearance of vector-transduced hepatocytes, transaminase elevation, and loss of FIX transgene expression.

In particular, in the AAV8-FIX study vector administration resulted in the activation of capsid-specific $CD8^+$ T cells, with an increase in liver enzymes that required intervention with corticosteroids detected in four out of six subjects (9, 84) from the high-dose cohort, who received 2×10^{12} vg/kg of vector, ~8–9 weeks after vector delivery. Results from this clinical study suggest that a short course of steroids, administered at the time of liver enzymes elevation and loss of transgene expression, can at least partially rescue transgene expression. This study also highlights differences in kinetics of T cell responses between the AAV2 and the AAV8 liver trials, as liver enzyme elevation in the AAV2 trial was observed

around week 4 following vector delivery (60), as opposed to the 8- to 9-weeks in the AAV8 trial (9). It is not clear at this point what is the likely explanation for this difference.

One important emerging aspect of AAV capsid-driven capsid T cell reactivity in humans is that capsid-specific T cell responses seem to be detected in a dose-dependent fashion, a result consistent with published *in vitro* antigen presentation data (70). Above a certain threshold of capsid antigen dose, the activation of capsid-specific T cells results in loss of transduced hepatocytes. Whether at vector doses higher than those tested thus far all subjects will mount a T cell response that will result in loss of transgene expression, and whether steroids will effectively control capsid T cells at all vector doses, is not clear at this point. Several factors are like to influence the outcome of vector administration in humans in terms of T cell reactivity, thus complicating the interpretation of results, among them the HLA type of the subjects infused, the concomitant presence of inflammation in the target tissue, and other vector-specific features that may enhance immune responses to the vector, the transgene, or both.

GENE TRANSFER TO THE MUSCLE

Adeno-associated virus vector-induced T cell immunity is not unique to liver-directed gene transfer. Monitoring of capsid T cell responses has been performed in the context of several muscle-directed gene transfer clinical studies (41, 85–92) as well. In agreement with the findings in AAV liver gene transfer studies, results accumulated for muscle gene transfer suggest that the magnitude of T cell responses directed against the AAV capsid correlates with the dose of vector administered (65, 92). Following intramuscular AAV vector delivery, an increase in frequency of circulating reactive T cells in PBMC is observed at higher vector doses (91). In some cases, detection of capsid T cell activation in PBMC correlated with lack of transgene expression in vector injected muscle (89, 91); while in other studies the detection of capsid T cells in PBMC seemed to have no effect on transgene persistence (28).

Immunosuppression has been used in some of the muscle gene transfer studies conducted thus far to modulate capsid immunogenicity (13). Whether this helped the persistence of transgene expression is not completely clear due to the lack of readily detectable efficacy endpoints and the fact that IS itself complicates immunomonitoring as it is likely to modify capsid-directed T cell responses.

In a recent study of intramuscular gene transfer for α_1-antitrypsin deficiency (92), vector administration was associated with detection of capsid-specific T cell reactivity and increase in serum creatine kinase in some subjects around day 30 post vector administration. Furthermore, activation of both $CD4^+$ and $CD8^+$ T cells in peripheral blood and T cell infiltrates in muscle biopsies were detected. T cell reactivity against the AAV capsid antigen did not seem to result in clearance of transduced muscle fibers or loss of transgene expression, a finding that may be explained by the fact that significant amounts of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) were found in muscle biopsies (28). Whether the local induction of Tregs is a phenomenon unique to muscle or to the α_1-antitrypsin transgene (93) remains to be established. However, these findings indicate that proinflammatory and tolerogenic signals may be concomitantly elicited by vector administration and may concur in determining the outcome of gene transfer. Similarly, apoptosis of transgene reactive T cells in muscle has been documented in mice (94) and in humans (90), indicating that additional factors are involved in the modulation immunogenicity of vector and transgene other than the target organ alone.

The inability of collecting tissue biopsies in the AAV trials for hemophilia conducted thus far prevented investigators from comparing results between liver and muscle gene transfer, thus leaving open important questions on the relevance of tissue-specific features that may influence vector immunogenicity.

GENE TRANSFER TO IMMUNOPRIVILEGED BODY SITES

Adeno-associated virus vectors have been administered to humans in several eye- (95) and brain- (96–102) targeted gene transfer trials. So far, doses tested in these immunoprivileged body compartments were lower compared to the doses tested in the liver or muscle trials, and they fail to elicit significant antibody or cell-mediated immune responses to capsid or transgene product. While these results strongly support the safety of gene transfer to eye and brain, future studies will help to understand whether the immune privilege is maintained at all vector doses, especially given the fact that some AAV vector serotypes can escape the blood–brain barrier (103), thus resulting in systemic exposure to the vector.

OVERCOMING T CELL IMMUNITY TO AAV

More than 10 years have passed since the initial AAV2 FIX trial. The field of *in vivo* gene transfer with AAV vectors has progressed enormously thank to a multitude of preclinical and clinical studies that clarified the nature of anti-capsid immune responses and tested the efficacy of strategies aimed at preventing or modulating these responses. In this section of our review article, we will discuss some of these strategies, with particular emphasis on those already tested in the clinic.

REDUCE THE TOTAL CAPSID ANTIGEN DOSE

Results from the AAV8 hemophilia trial (9, 84) suggest that lower vector doses may not trigger destructive T cell responses to the capsid. In this study, subjects who received vectors doses up to 6×10^{11} vg/kg did not experience increase in liver enzymes or loss of transgene expression, at the same time levels of FIX transgene were just above the threshold for therapeutic efficacy. Different strategies are being tested to maintain the total capsid dose low while increasing therapeutic efficacy. One is to use hyperactive variants of the therapeutic protein (104–106), or stronger promoter elements (107), or else codon-optimized transgenes (108, 109). All these maneuvers are either being tested in the clinic or about to enter clinical trials and results will be available soon.

Engineering the AAV capsid to prevent its presentation onto MHC I (17, 69) or administration of proteasome inhibitors (71) to block processing of capsid antigen are also strategies that have been proposed to reduce capsid antigen load following vector administration. These strategies are also associated with higher transduction efficiency in animal models (71, 110), thus they could potentially allowing to decrease the therapeutic dose; however, the role of alternative antigen presentation pathways should be taken into account when evaluating this strategy. Furthermore, the use of proteasome inhibitors like bortezomib is not devoid of potentially serious side effects (111).

The use of AAV vector preparations devoid of empty capsids is also being tested as a strategy to reduce the overall antigen load associated with gene transfer. While clearly this results in lower total amounts of capsid being administered, in the negative side it may render vectors more susceptible for antibody-mediated neutralization (73). The jury is still out on the role of empty capsids in gene transfer, with some reports suggesting that these particles are not immunogenic (72), others arguing that they are detrimental for transduction efficiency (112), and recent reports showing that they are beneficial in the presence of anti-AAV neutralizing antibodies (73).

USE TRANSIENT IMMUNOSUPPRESSION

One potential advantage of the use of IS the context of AAV gene transfer is that, differently from organ transplant or autoimmune disease, the duration of the intervention is expected to be relatively short (9). Initial studies of gene transfer with IS favored the idea of treating subjects upfront, starting just before or at the time of vector administration (26, 58, 113–116). The advantage of this approach is that any immune response occurring after vector deliver would be prevented, the main disadvantage consist in the fact that IS would prevent efficient immunomonitoring, thus would not allow to study the nature and kinetics of immunity to AAV. Additionally, treating all subjects with IS may not be necessary, as some may not develop immunity to the vector (9, 84).

Aside from the obvious higher risk of infection, IS may also influence induction of Tregs (58), which are fundamental for the maintenance of transgene tolerance in liver (43) gene transfer and seem to play an important role in muscle gene transfer (28). Recent reports show impaired transduction levels under IS with mycophenolate mofetil (113), others report a longer plasma half-life of vector in non-human primates receiving IS (114). These are all evidences that interactions of IS with viral gene transfer are quite complex and may result in unexpected findings.

The use of transient IS with steroids at the time of liver enzyme elevation is a relatively novel concept in the field of gene transfer that has been successfully adopted in the AAV8 hemophilia trial. In this study, a mild elevation of liver enzymes has been immediately treated with high-dose prednisolone, resulting in the prevention of what appeared to be an immune-mediated clearance of AAV8 transduced hepatocytes. The main advantage of the approach is that not all subjects were exposed to IS, in fact only those who had increased liver enzymes received prednisolone. The challenge of the approach is that endpoints of liver (or other organ) immunotoxicity may not be always obvious, making a targeted intervention hard. In these latter cases, upfront IS may be necessary; however, the key question revolves around the timing and the duration of this approach. Additionally, steroids may not work in some subjects or at high vector doses. These concerns will be addressed in future studies.

In conclusion, one important consideration about IS regimens and timing of intervention is about the immune state of infused subjects. Some individuals may in fact be recently primed by natural infections with wild-type AAV, or else could have been previously enrolled in gene therapy protocols (in case of vector readministration). Immune responses to the vector capsid in these subjects may be expected to be faster and stronger, thus warranting particular attention.

CAN WE TOLERIZE SUBJECTS AGAINST THE AAV CAPSID?

As an alternative to IS, the induction of Tregs specific to the AAV capsid can result in sustained expression of the transgene with no induction of destructive T cells.

MHC class II epitopes found in human IgG (117) have been described to induce proliferation of Tregs and suppress Th1 and Th2 immune responses (117–120). These peptides, named Tregitopes, have been used to modulate $CD8^+$ T cell responses to AAV, resulting in suppression of cytotoxic responses against AAV-transduced cells and expansion of Tregs *in vitro*. Additional studies *in vivo* (118), in which Tregitopes were co-expressed with the capsid antigen, resulted in modulation of $CD8^+$ T cell responses to the capsid antigen itself following adenoviral vector-mediated vaccination. While results *in vitro* suggest that the approach leads to antigen-specific tolerance (118), additional studies are needed to test if antigen-specificity is conserved *in vivo*, whether the approach is safe, and ultimately, how to translate this strategy to the clinic.

CONCLUSION

Since the initial proof-of-concept studies, the field of *in vivo* gene transfer has progressed enormously. Experience from the clinical translation of AAV vector-based gene transfer strategies has highlighted the challenges and helped optimize the choice of the AAV vector serotype, the delivery methods, and has highlighted some of the limitations of the approaches tested. In particular, the interactions between the human immune system and all the components of gene therapy vectors seem to represent one of the major limitations to long-lasting therapeutic efficacy.

The small scale of gene transfer trials for rare diseases, the heterogeneity of vector serotypes, transgenes, and doses tested, and the ability of measuring the endpoints of therapeutic efficacy have been an obstacle to the advancement of knowledge. Furthermore, individual variability associated for example with HLA haplotype and with the underlying disease state also represents an added layer of complexity to data interpretation.

Gene therapists should not forget that immune responses triggered by gene transfer must be understood and studied as a complex network of interactions; as such, the outcome of gene transfer in immunological terms is influenced by both innate and adaptive immunity, which are influenced by the nature of the vector, transgene, route, etc. Future immunosurveillance studies conducted in clinical gene transfer studies will provide the basis for a better understanding of the determinants of T cell responses in AAV-mediated gene transfer, for example, the role of target tissue inflammation due to the underlying disease. Standardization of technologies used for monitoring of T cell responses (121, 122) will help to correlate them with clinical outcomes and, eventually, devise novel strategies around the issue.

Despite these challenges, the field has acquired critical information from the studies conducted thus far, which allowed to address the issue of vector immunogenicity. The results of these efforts are evident, long-term follow up data from AAV gene transfer

trials show many examples of long-lasting therapeutic efficacy. And results accumulating suggest that once transgene expression is established, and immune responses avoided, multi-year therapeutic efficacy is a goal attainable in humans.

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Immunology of AAV-mediated gene transfer in the eye

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The eye has been at the forefront of translational gene therapy largely owing to suitable disease targets, anatomic accessibility, and well-studied immunologic privilege. These advantages have fostered research culminating in several clinical trials and adeno-associated virus (AAV) has emerged as the vector of choice for many ocular therapies. Pre-clinical and clinical investigations have assessed the humoral and cellular immune responses to a variety of naturally occurring and engineered AAV serotypes as well as their delivered transgenes and these data have been correlated to potential clinical sequelae. Encouragingly, AAV appears safe and effective with clinical follow-up surpassing 5 years in some studies. As disease targets continue to expand for AAV in the eye, thorough and deliberate assessment of immunologic safety is critical. With careful study, the development of these technologies should concurrently inform the biology of the ocular immune response.

Keywords: adeno-associated virus, RPE65, gene therapy for rare diseases, immune privilege, translational medical research, sub-retinal injection

INTRODUCTION

Visual impairment is a considerable burden to society. By the estimates of disability-adjusted life years, visual disorders and related diseases are comparable to diarrheal illness and HIV/AIDS when measured globally (1). While the majority of blindness in the world is avoidable by either prevention or therapy, little progress has been made for the remaining etiologies, many of which stem from well described genetic lesions. Gene transfer therapy has advanced tremendously in recent decades, and achieved a milestone success with the clinical efficacy of adeno-associated virus (AAV) mediated gene augmentation in the eye for Leber Congenital Amaurosis type 2 (2–4).

Other viruses such as lentivirus and adenovirus have been or are currently under investigation for ocular gene delivery. Compared to these viral and also non-viral modes of gene transfer, recombinant AAV continues to be a popular vector used in the eye both in basic science and translational studies (**Figure 1**). At present, clinical trials involving ocular administration of AAV are ongoing on four continents with an aggregate enrollment of over 200 participants (**Figure 2**). AAV, a helper-dependent single-stranded DNA parvovirus has never been shown to cause disease in humans or animals. It is appealing as a vector because it can stably and efficiently induce gene expression in dividing or terminally differentiated cells, has a favorable toxicity profile and benign immune response. Also, manipulation of the AAV capsid as well as promoters in the cDNA transgene effectively modulate cellular tropism which is critical to the cell-specific pathophysiology of many eye diseases (5).

Anatomically, the eye is highly compartmentalized and many routes of AAV administration have been studied to target either anterior or posterior tissues (**Figure 3**). For example, lacrimal gland injection (6), topical eye drops (7, 8), intra-stromal corneal injection (9), and intra-cameral injection (10) provide access to the ocular surface, cornea, and anterior chamber which are implicated in dry eye disease, corneal dystrophies, and glaucoma. Intravitreal and sub-retinal injections access the neurosensory retina and the underlying retinal pigment epithelium (RPE). Systemic administration of gene therapy reagents is a theoretical alternative to intraocular surgery that avoids the potential complications of sub-retinal injection. Intravenous or intramuscular administration during the neonatal period in animals has been shown to diffusely transduce the retina (11). A major limitation of this approach, however, is the very large increase in vector dose compared to intraocular delivery, although certain technical improvements could be investigated – for example, adaptation of chemotherapy delivery methods via super selective cannulation of the ophthalmic artery (12). The systemic strategy is further limited by the mature blood brain barrier, the many avascular regions of the eye, and the potential for a detrimental inflammation in the setting of a necessarily large antigen load. By contrast, each of the intraocular compartments requires a relatively small volume of injection and thus highly purified vector is effective in small doses (13). The transparency of the eye is also advantageous in that it affords non-invasive direct visualization of neural and vascular tissue as well as other critical eye structures – facilitating research in animal models and close follow-up in the clinic. Furthermore, the symmetry of disease progression in most hereditary retinal diseases allows one eye to be used as the experimental target and the other as a control in research studies.

Thus far, diseases of the retina have garnered the most interest among ocular targets for gene therapy and will be the focus of this review.

IMMUNITY IN THE EYE

To maintain the transparent structures required for vision, the eye has conserved a number of adaptations that selectively diminish a maximal immune response. For example, complement-fixing antibodies, neutrophils, and macrophages are generally excluded

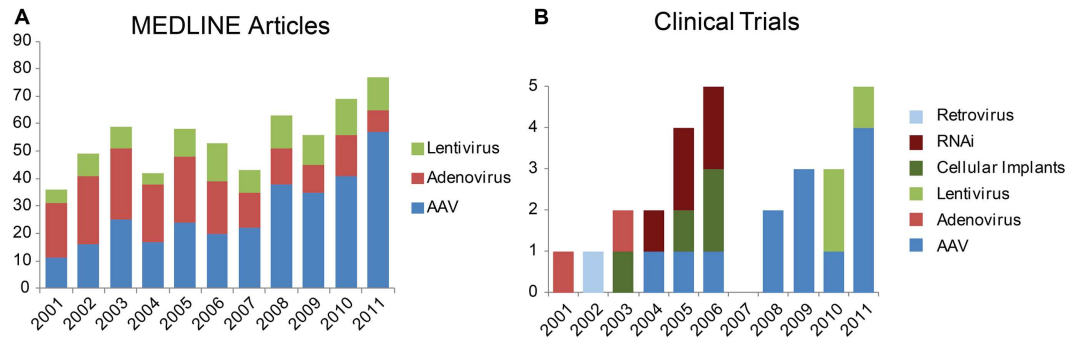

FIGURE 1 | Trends in vectors studied for gene therapy in the eye. (A) Number of results returned when the MEDLINE database was queried via PubMed for "aav eye 2001" etc. **(B)** Newly registered clinical trials by year from the database Gene Therapy Clinical Trials Online from the Journal of Gene Medicine. Results restricted to "ocular diseases" and sorted by date approved.

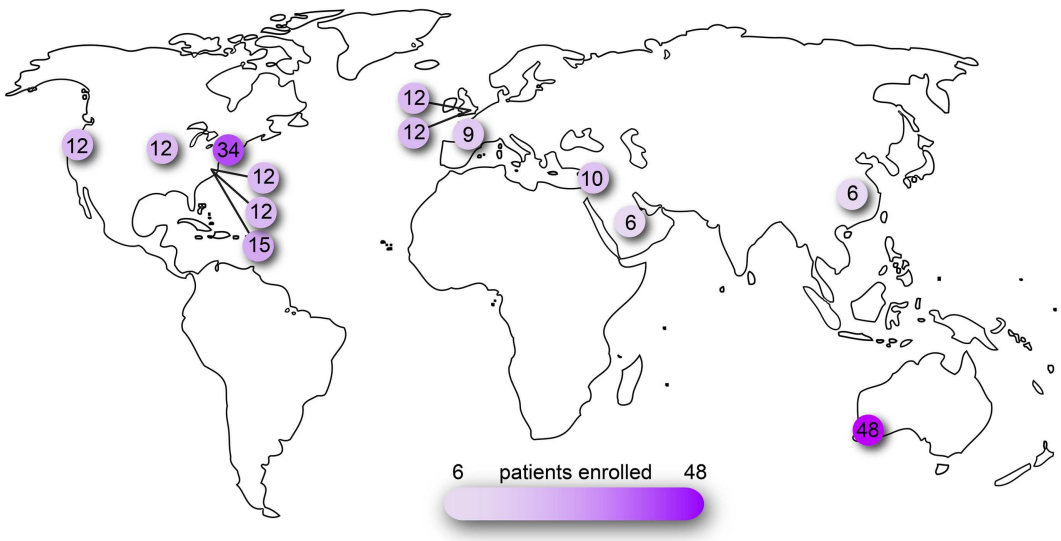

FIGURE 2 | Clinical trials of ocular AAV registered on www.clinicaltrials.gov. Searched as search term = eye and interventions = adeno-associated virus OR AAV OR rAAV. Number of patients reported as actual or planned numbers for enrollment.

from the eye as potential collateral damage could lead to lesions and opacities. Anatomically, the retina is an extension of the central nervous system and is protected by a selective blood-retinal barrier (BRB) established by non-fenestrated capillaries in the retinal vasculature and tight junctions in the RPE. Also, the avascular nature of much of the eye and the lack of lymphatics draining the anterior chamber, vitreous cavity, or sub-retinal space further limit classical antigen presentation and immune response (14). If soluble or cell-associated molecules bypass these obstacles, antigens are subject to a so called "deviant" immune response in the eye. The anterior chamber is the most studied entry point of these responses, and was originally described by Nobel Laureate Peter Medawar in his study of corneal transplants and their survival without immunosuppression (15). Termed anterior-chamber associated immune deviation (ACAID), this phenomenon is classically characterized by the elimination of the delayed-type hypersensitivity (DTH) response to the introduced antigen. This response is mediated by a population of antigen-specific regulatory T cells (Treg) that are elaborated in a multi-stage process involving the eye as well as the spleen, and these mechanisms are thoroughly reviewed by Streilein (14). This population of Treg cells can be transferred to naïve animals which adopt suppression of the DTH. Not surprisingly, this mechanism of immunosuppression is of considerable interest for treatment of autoimmune diseases and reproducing the necessary cytokine environment *in vivo* or *in vitro* has been shown to effectively cultivate similar Treg populations that mitigate autoimmune encephalomyelitis in animal models (16). Similar to the anterior chamber, antigens introduced to the vitreous and sub-retinal space exhibit an analogous immune deviant response (14, 17).

The cytokine environment in each of these ocular compartments is thought to be critical for a deviant response. For example, transforming growth factor β 2 (TGFβ2) was the first such

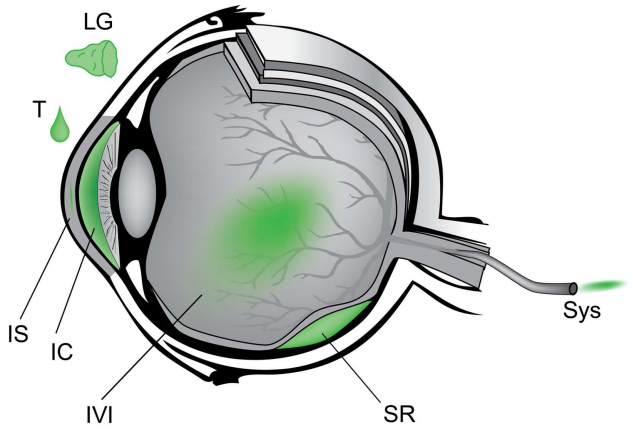

FIGURE 3 | Schematic of anatomic approach to administration of gene therapy vectors in the eye. Counterclockwise from upper left – LG, lacrimal gland; T, topical eye drop; IS, intra-stromal of cornea; IC, intra-cameral, i.e., anterior chamber; IVI, intravitreal; SR, sub-retinal; Sys, systemic.

cytokine described and is present in the vitreous, retina, and aqueous humor. The retina additionally contains vasoactive intestinal peptide (VIP), and somatostatin (SOM) while the aqueous humor contains these as well as α-melanocyte-stimulating hormone (α-MSH) and calcitonin gene-related peptide (CGRP). While all of these molecules are thought to contribute to immunosuppression, their various combinations in separated ocular compartments may underlie the subtle differences in immune deviation between them (18).

Following antigen exposure, the cellular immune response in the eye is similarly modulated by cytokines. The T cell helper Type 1 (Th1) response, for example, is generally injurious to the ocular tissues. Th1 cells secrete Interferon γ (Ifn-γ) which in turn activates phagocytes and stimulates production of IgG2a – a complement-fixing class of antibodies which incur collateral damage. By contrast, Th2 responses are thought to be better tolerated in the eye and can suppress macrophage activation. They are characterized by the anti-inflammatory cytokines IL-4, IL-10, and IL-13 – a milieu that favors IgG1, IgG3, and IgG2b which do not fix complement proteins. It is unclear in the literature if this sometimes termed "cross regulatory" population of Th2 cells in fact overlaps with the Treg population. Both are thought to be elaborated in the environment of TGFβ and IL-10 and both are known to suppress phagocyte activation. Treg cells are $CD25^+$ and $FoxP3^+$ and contain sub-populations of $CD4^+$ "afferent" cells that suppress initiation of Th1 cells and $CD8^+$ "efferent" cells that suppress Th1 action (18).

The regulation of these immune responses is critical to the safety of gene transfer in the eye. An unchecked inflammatory response could potentially damage neural tissue such as the photoreceptors which are post-mitotic at birth and do not regenerate throughout life. The efficacy of gene transfer events similarly depends on immune tolerance to a viral vector as well as the products of a transgene that may be novel to the host organism. Research into gene transfer therapies, therefore, has attempted to describe both the cellular and humoral immune responses to gene augmentation in the eye in animal models and ongoing human trials.

ASSAYS FOR MEASURING IMMUNE RESPONSE TO AAV

Several techniques have been commonly adopted to characterize the ocular immune response to AAV technologies. Humoral responses are typically assayed by enzyme-linked immunosorbent assay (ELISA) which provides quantitation of specific antibody production and can also be refined to sub-type classes of antibodies characteristic of certain immune pathways (19). These antibodies can be measured in sera as well as vitreous and aqueous humor, and a comparison known as the Goldmann–Witmer (GW) coefficient has historically been used to localize antibody production either inside or outside the eye (19, 20). In addition, an *in vitro* functional test of AAV transduction can be conducted in the presence of test sera, commonly called a neutralizing antibody (NAb) assay. In this experiment, serum dilutions that inhibit transduction can be compared, and reflect the presence of neutralizing factors – presumably antibodies – that inhibit AAV transduction.

Cellular immune responses can also be precipitated by AAV or transgene products. An enzyme-linked immunosorbent spot (ELISPOT) is often used to quantitate populations of leukocytes that are activated in response to epitopes of interest. Commonly, Ifn-γ production is used to define activation and generally reflects a Th1 type response, although other markers can be used (21). Recent studies have also monitored cellular immune responses by flow cytometry detecting activation markers within the $CD4^+$ and $CD8^+$ compartments, such as Ki67, HLA-DR, and Bcl-2 (22, 23), however this method does not allow epitope characterization using specific peptide pools.

Finally, in animal studies, histological changes also reflect local immune responses in tissues of interest. Glial cell proliferation is one reaction to CNS insult and can be visualized by immunohistochemical probes detecting Glial fibrillary acidic protein (GFAP) (24, 25). Also, infiltrating leukocytes including activated macrophages can be visualized with antibodies against CD45, CD68, Iba1, and others (19, 24).

CLINICAL TRIALS FOR RETINAL DEGENERATION

Four independent groups have published results of safety and efficacy in clinical trials of gene augmentation with AAV2 for patients deficient in the isomerase RPE-specific 65 kDa protein (RPE65) (2–4, 26). The gene encoding RPE65 is one of at least 18 genes, when mutated, known to cause the rapid retinal degeneration known as Leber Congenital Amaurosis[1]. Some of the initial trials excluded patients with null mutations to remove the possibility of the introduced RPE65 protein being recognized as non-self (4), however some did not make this exclusion (2). Safety from an immunologic standpoint was assessed clinically as well as through laboratory evaluation.

Follow-up has surpassed 5 years for the first three groups and to date no major adverse events have been reported (27–29). By clinical exam, no significant inflammatory response has been

[1]http://www.sph.uth.tmc.edu/RetNet

attributed to the AAV or transgene product. Biodistribution studies have been part of each of the three initial trials and utilized polymerase chain reaction of AAV sequences in various compartments including tears, serum, saliva, and semen. Results have generally been negative with the exception of transient positivity in serum and tears which resolved within a few post-operative days.

To assess adaptive humoral response to the AAV2 capsid, functional assays of NAbs were performed. Generally, NAb assays were negative with the exception of one patient in the study by Maguire et al. (2) who experienced an increase in NAb titer which then decreased to a level slightly above baseline. In the Hauswirth et al. study (3), NAb titers were not measured functionally, but antibodies as assessed by ELISA were positive in one subject transiently. In the eye these responses have been much less than those induced by systemically injected AAV by several log units (30). Adaptive humoral responses to the transgene were assessed by serum ELISA in the Maguire and Bainbridge studies and results were negative (2, 4, 31).

Adaptive cellular immunity to the AAV2 capsid measured via ELISPOT was negative in each of the three seminal studies (2–4). Additionally, Hauswirth et al. assayed antigen-specific lymphocyte proliferation response (ASR) by thymidine uptake following exposure to AAV2 antigen, which was negative initially and at 1 year of follow-up (3, 32). Finally, adaptive cellular immunity against the delivered RPE65 gene product was assayed by Bennett et al. via ELISPOT and were generally negative with two exceptions which are presumed to be artifact (2, 31). The benign immunologic results of sub-retinal re-administration of AAV2.hRPE65 are discussed later in this report (19).

EXPANDING RETINAL DEGENERATION TARGETS

Many inherited retinal degenerations (RD) that were first characterized clinically now have a described genetic etiology and relevant testing is becoming cheaper and more available to clinicians. The national eye institute (NEI) now provides a program called eyeGENE that tests for known heritable eye diseases and compiles results into a database that is freely available to researchers and clinicians (33). Perhaps the most amenable of these genetic targets are autosomal recessive (AR) diseases that confer a loss-of-function mutation which can be potentially compensated by gene augmentation. As discussed above, the first of these specific mutations to be targeted for gene therapy in humans was the AR LCA2. This was an appealing initial target because (1) the 1.6 kB transgene is small enough to fit in the AAV2 capsid (<4.7 kB), (2) mouse and canine models of the disease are available, (3) the degenerative component in this particular disease is slow, thereby providing a wide therapeutic window, and (4) the primary cells effected are RPE which are efficiently transduced by sub-retinal injection of AAV2. LCA2 is a rare disease, however, with an incidence of ∼1:200,000, which amounts to an estimated 500 cases in the United States.

Proof-of-concept of gene augmentation therapy has been demonstrated in animals without significant inflammatory response in a number of other recessive somatic and X-linked RD targets using recombinant viruses including oculocutaneous albinism (34), x-linked juvenile retinoschisis (XLRS) (35, 36), and achromatopsia (37, 38). The retinitis pigmentosa (RP) phenotype encompasses >100 mutations, some of which include the LCA phenotype by certain nomenclatures. In this category, several AR gene targets have been similarly validated in animal models for possible gene augmentation: RPGR (39), GC1 (40), RPGRIP1 (41), MERTK (42), and AIPL1 (43). Furthermore, analogous studies have been done for Stargardt disease with mutations in ABCA4 using lentivirus (44) as well as Usher Syndrome Ib caused by mutations in MYO7A using AAV (45).

Also, toxic gain-of-function mutations characteristic of autosomal dominant disease can be targeted in a two-step approach – first by knocking down the defective gene with RNA interference (RNAi) then supplying a replacement molecule resistant to the introduced RNAi. This approach is being studied in mutations of rhodopsin (46, 47) and rds/peripherin (48). Alternatively, delivery of a wild-type molecule may be sufficient in some instances (49). So far there has not been any clear toxic effect of delivering rhodopsin or rds/peripherin in animals deficient in these proteins.

Delivery of generic pro-survival and anti-apoptotic factors has been investigated as a generalized treatment for a diverse set of retinal diseases, ranging from RP to achromatopsia, to macular degeneration[2]. AAV-mediated delivery of one such factor, ciliary-derived neurotrophic factor (CNTF) has been carried out in animal models with excellent success (50). In certain circumstances, however, it appears that the anatomical protective effect of CNTF can simultaneously diminish retinal function as measured by electrophysiology. Glial cell line-derived neurotrophic factor (GDNF) is a potential alternative that provides structural neuroprotection without adverse electrophysiologic effects at the same dose (51). Additional studies in cell and animal models have identified alternative neuroprotective agents, such as erythropoietin (EPO) (52), rod-derived ciliary neurotrophic factor (RdCVF) (53), and X-linked inhibitor of apoptosis (XIAP) (54). So far, there have not been significant toxic immune-related responses in animal models to these native proteins.

Retinoblastoma and other ocular neoplasms are also potential targets for gene therapy using AAV to deliver therapies that are not well tolerated systemically, such as the anti-cancer signaling protein interferon beta (Ifn-β) (55) or cytotoxic compounds (56). In mouse models, intravitreal injection of AAV2.Ifn-β showed antitumor effects and transgene expression was limited to the eye. No overt immune response was reported, and in these situations, some degree of immune activation could improve tumor regression.

ANTI-ANGIOGENESIS

Retinopathies involving the vasculature are the leading cause of blindness in working-age and elderly adults in developed countries, comprising diabetic retinopathy (57) and the neovascular form of age-related macular degeneration (58), respectively. Efficacious treatment is now available in the form of molecules that inhibit vascular endothelial growth factor (VEGF), but their effects are temporary and require repeated intravitreal injections that can be inconvenient for patients and providers. The use of gene therapy

[2]www.clinicaltrials.gov

to induce the production of anti-angiogenic molecules by endogenous cells could represent a durable solution for these common conditions.

Pigment epithelium derived factor (PEDF) is an attractive anti-angiogenic target because it opposes the action of VEGF and is also a pro-survival factor for retinal neurons. Extensive animal studies led to a clinical trial of adenovirus to deliver PEDF (59), and some mild to moderate inflammation was detected in 25% of patients but otherwise no adverse effects were noted. While adenovirus appears to have an acceptable safety profile in this study, the limited durability of the response favors AAV for future studies. Two additional trials currently underway employ AAV2 to deliver the soluble VEGF receptor sFlt intravitreally (in one study) and sub-retinally (in the other) (see text footnote 2). Non-human primate data supporting the intravitreal AAV2.sFlt trial (60) assessed immunologic responses and found mild to moderate effects evident on clinical exam as well as laboratory testing including induced antibodies to AAV2 in all animals. Time will tell if such effects are identified in humans.

METHODS TO ENHANCE EFFICIENCY

Several technical methods of enhancing the efficiency and specificity of AAV transduction in the eye have been investigated, such as use of cell-specific transgene promoters (61), engineered serotypes of AAV (24, 25), ultrasound micro bubbles (62), and co-administration of either chemo-therapeutic drugs (63) or adenovirus (64). It is unknown how these adjunctive techniques could affect the immune response in humans, but it could be speculated that additional antigens and disruption of cellular barriers could influence antigen presentation and immune infiltration. At present, gene transfer therapy in the human eye for LCA2 is not clearly limited by transduction efficiency, but as more indications are developed, the need for an adjuvant may become relevant. In situations where the dose of transgene is limiting, it could be preferable to increase the efficiency of viral transduction or modulate promoters to increase transgene protein expression, rather than increasing the dose of potentially immunogenic viral vectors. Similarly, it could be advantageous to use transgene constructs that are pharmacologically inducible, so that initial immune responses to surgical injury and viral capsids do not lead to bystander-induced immunity against therapeutic transgene products (65).

REPEAT ADMINISTRATION OF AAV

A practical and ethical consideration for clinical trials of AAV in the eye is the timing of treatment of the contralateral eye. There is a theoretical concern that following exposure to AAV capsid or transgene during the initial treatment, the immune system could adopt memory. Thus, a subsequent injection in the contralateral eye could result in a primed immune system response that diminishes the efficacy of therapy, or worse, triggers destructive inflammation. An alternative theory supported by current data is that ocular gene therapy induces an immune deviant response analogous to ACAID as discussed earlier. In this model, the cytokine milieu of antigen presentation induces a systemic population of Treg which inhibit the cellular immune response to a second presentation of AAV or transgene. This ACAID-like response, however, varies with the ocular compartment injected. While sub-retinal injection seems to mirror the anterior chamber with respect to an immunosuppressive deviant response (17, 66), it has been shown that intravitreal injection of one eye can stimulate NAbs that diminish transduction events in the contralateral eye in animal models (67) – including novel AAV serotypes engineered to transduce the outer retina via an intravitreal injection (24).

Similarly, systemic re-administration of gene therapy vectors for other disease targets have demonstrated neutralization of transduction events due to preformed antibodies in several animal models including cystic fibrosis (68) and hemophilia B (69). In initial clinical trials for hemophilia B patients, therapeutic levels of the deficient coagulation factor IX were achieved, but only persisted ∼8 weeks (21). In this case, it seems that although patients with pre-existing neutralizing antibodies were excluded, a cellular response to AAV capsid incurred selective removal of transduced cells, and recent studies suggest that this limitation can be circumvented with certain immunosuppressive regimes (70). These immune responses to systemic administration in the presence of NAbs appear to differ from sub-retinal repeat administration possibly due to the immune-privileged and enclosed space of the sub-retinal compartment. Yet, other studies in non-ocular tissues have shown that despite the presence of NAbs, transduction events at systemic sites can still occur (71), underscoring the variability of response in different tissues and disease states.

In the eye, sub-retinal injection is the most thoroughly studied route for gene therapy and re-administration of AAV in this manner has been shown to be efficacious in affected dogs and safe in dogs and non-human primates (19, 72), as well as for three patients with 1 year of follow-up after re-administration (13). An alternative clinical strategy to avoid adaptation by the immune system would be to inject both eyes simultaneously. Bilateral surgery, however, incurs an increased risk to the patient's residual vision in the event of a surgical complication. As a compromise, current bilateral studies in humans aim to operate on each eye 7–14 days apart, which is a short enough time to be considered one "event" by the immune system (see text footnote 2; NCT00999609).

CONCLUSION

The eye has played a leading role in the clinical translation of gene transfer therapies. As the range of therapeutic targets increases in the eye, the immune response to these vectors and transgenes will continue to shape both efficacy and safety. The greater variety of tissues targeted as well as the sheer number of treated patients will likely reveal the diversity of immune responses possible in the eye which can further inform the way we study and execute these therapies.

While it is certainly advantageous that many parameters of these technologies can be engineered – surgical delivery, viral capsids, transgene cassettes etc. – it also complicates efforts to aggregate the safety data. For example, optogenetic therapy has been proposed for end-stage retinal disease (73). In this technique, simplified light-sensitive ion channels borrowed from *Archea* and plants could potentially be expressed in human retinal tissues. Clearly, the introduction of such foreign molecular patterns merits thorough study, even though the AAV vector has been shown to be generally safe.

Testing each technical modification for immune safety in animal models can be exhaustive and taxing on resources. However, given the capacity of the immune system for sensitive pattern detection as well as a potentially dangerous inflammatory response, it is critical that researchers remain vigilant in understanding the biology of the immune system and how it interfaces with these novel therapies.

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Immune responses to AAV-vectors, the Glybera example from bench to bedside

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Alipogene tiparvovec (Glybera®) is an adeno-associated virus serotype 1 (AAV1)-based gene therapy that has been developed for the treatment of patients with lipoprotein lipase (LPL) deficiency. Alipogene tiparvovec contains the human LPL naturally occurring gene variant LPL^{S447X} in a non-replicating viral vector based on AAV1. Such virus-derived vectors administered to humans elicit immune responses against the viral capsid protein and immune responses, especially cellular, mounted against the protein expressed from the administered gene have been linked to attenuated transgene expression and loss of efficacy. Therefore, a potential concern about the use of AAV-based vectors for gene therapy is that they may induce humoral and cellular immune responses in the recipient that may impact on efficacy and safety. In this paper, we review the current understanding of immune responses against AAV-based vectors and their impact on clinical efficacy and safety. In particular, the immunogenicity findings from the clinical development of alipogene tiparvovec up to licensing in Europe will be discussed demonstrating that systemic and local immune responses induced by intra-muscular injection of alipogene tiparvovec have no deleterious effects on clinical efficacy and safety. These findings show that muscle-directed AAV-based gene therapy remains a promising approach for the treatment of human diseases.

Keywords: adeno-associated viral vectors, gene therapy, alipogene tiparvovec, immune responses, clinical safety, clinical efficacy

INTRODUCTION

Adeno-associated virus (AAV) is a naturally occurring virus that is known to infect humans and other primates. It is expected to interact at multiple levels with the innate and adaptive immune system and elicit immune responses when injected in man. AAV-based vectors are nowadays often chosen for the development of new, promising gene therapy approaches because of several interesting features such as their inability to self-replicate. However, a potential concern about the use of such virus-derived vectors is the potential to induce humoral and cellular immune responses in the recipient that may impact on efficacy and safety. In this report, we review the current understanding of immune responses against AAV-based vectors and their impact on clinical efficacy and safety using alipogene tiparvovec (Glybera®) as an example. Glybera® has received marketing authorization under exceptional circumstances in Europe in 2012. Alipogene tiparvovec is an AAV-based gene therapy vector that has been developed for the treatment of patients with lipoprotein lipase deficiency (LPLD). It contains the gene of the naturally occurring gain-of-function variant LPL^{S447X} of the human lipoprotein lipase (LPL) in a non-replicating viral vector based on adeno-associated virus serotype 1 (AAV1). The immunogenicity findings from the clinical development of alipogene tiparvovec will be discussed, demonstrating that systemic and local immune responses induced by intra-muscular injection of alipogene tiparvovec have not shown deleterious effects on clinical efficacy and safety. Alipogene tiparvovec is an example that muscle-directed AAV-based gene therapy is a promising approach for the treatment of human diseases.

PARTICULAR FEATURES OF AAV AND AAV-VECTORS

Wild-type AAV is not associated with any known disease or pathology in humans (1, 2). In addition, the virus is naturally replication-defective and requires a helper virus such as adenovirus to replicate (3). Wild-type AAV also has been shown *in vitro* to have the ability to stably integrate into the host cell genome at a specific site (designated AAVS1) in the human chromosome 19 with minimal risk for random incorporations into the genome. For these reasons, AAV has attracted considerable interest because of its potential as a gene therapy vector. The use of AAV as gene therapy vectors has required the elimination of the rep gene from the vector, since it is coding for the protein that is involved in replication of the viral DNA and site-specific integration. In the vector genome, the rep and cap genes are replaced by the transgene, in the case of alipogene tiparvovec the gene for LPL, together with a promoter that is necessary to drive transcription. This cassette is flanked by inverted terminal repeats (ITRs) that are necessary for the formation of so-called concatemers in the nucleus after the single-stranded vector DNA is converted by host cell DNA polymerase complexes into double-stranded DNA. These episomal concatemers remain intact in the nucleus of non-dividing host cells. Hence, transferred genomes tend to persist inside the cells mainly in this episomal, non-integrated form (4, 5). The generation of AAV-vectors currently used for gene therapy in humans has strongly reduced the risk of insertional mutagenesis (6–8). As a result, AAV-vectors are among the simplest gene therapy vectors, containing only the transgene expression cassette flanked by two non-coding viral ITRs, and enclosed in a capsid composed of

three structural proteins, VP1, 2, and 3 (9). Alipogene tiparvovec indeed is such an AAV-vector and contains the transgene coding for LPL^{S447X}.

Another important feature of AAV and also of AAV-based vectors is their very low immunogenic potential. Immune responses against AAV in general seem restricted and mainly consist in the generation of neutralizing antibodies, while well-defined cytotoxic responses seem minimal (10). This feature, along with the ability to infect quiescent cells, is another important advantage for AAV for their use as vectors for human gene therapy. Presumably several features of AAV contribute to this low immunogenicity, including the simplicity of AAV-vectors and their low efficiency in transducing professional antigen presenting cells such as macrophages or dendritic cells, and their lacking capacity to express viral proteins (11, 12).

NON-CLINICAL INVESTIGATIONS ON THE IMMUNOGENICITY OF AAV-VECTORS

A large number of studies in various animal species have demonstrated the potential of AAV-vectors as a therapeutic platform for gene delivery (13–22). However, the AAV capsid protein as well as the transgene product can interact at multiple levels with the innate and adaptive immune system. Consistent with current concepts in immunology, the immune response can vary substantially depending upon the tissue which is targeted, with outcomes ranging from almost unresponsiveness (gene transfer in the eye or in the brain) to responsiveness (gene transfer in the muscle, liver, or lung). Humoral immune responses to AAV capsid proteins were reported in all animal studies in which AAV-vectors were used to target muscle or liver. While cellular and humoral immune responses to AAV were reported to be modest in intensity in mouse models (23–25), cytotoxic T-cell responses to AAV-vector and transgene product in muscle of large animal models have been recently reported, which emphasizes the importance of appropriate animal models to address safety and efficacy of the approach and predict clinical outcomes (26).

CLINICAL STUDIES WITH AAV-GENE THERAPY VECTORS IN HUMANS

Over the last two decades, numerous clinical studies were performed using AAV to deliver therapeutic genes to different organs and tissues including muscle, liver, and the CNS. Those studies included hundreds of patients and indicate an excellent safety record of AAV-vectors for gene therapy in humans. The different safety aspects of AAV for the use in humans have recently been summarized elsewhere [see for review Ref. (23, 26, 27)].

Immune responses have been assessed in clinical trials by measuring systemic and local cytotoxic reactions as well as (neutralizing) antibodies against AAV and/or the expressed therapeutic protein (24, 25, 28–37). Results from these measurements in these clinical studies indicate that the immune responses measured could impact on the efficacy of the product rather than the overall safety profile. The immunogenicity data reported so far show that immune responses against AAV capsid proteins can vary widely and amongst others are influenced by the target organ, route of delivery, and dosing schedule.

The eye and central nervous system are known to be immune-privileged compartments of the body due to adaptations that limit immune responses. Delivery of AAV-vectors directly into the brain has been tested in a number of studies (31, 38–40). Similarly, subretinal vector delivery has been performed in a number of clinical studies (28, 33). In all these studies, AAV-vector administration was associated with little or no detectable immune response to the capsid or the transgene protein in serum and peripheral blood mononuclear cell (PBMC). In contrast, humoral immune responses to AAV capsid proteins were reported in all trials targeting AAV-based vectors to muscle or liver.

Cellular immune responses against the AAV-vector have been found in only some of the clinical trials performed. The first observation of a cellular immune response induced by AAV-gene therapy to our knowledge was in patients with hemophilia B who were treated with an AAV-vector to deliver human coagulation factor IX (24, 27, 41, 42). In this study, a cell-mediated immune response to AAV2 capsid was reported, which was measured in parallel with a loss of transgene expression. In a more recent clinical study in patients with hemophilia B, using the capsid of AAV8 to deliver FIX to the liver, similar reactions were observed in some of the patients treated with the highest dose (37). Whereas both studies in patients with hemophilia B indicate a direct correlation between the induction of a CD8 T-cell response toward the AAV capsid proteins and a loss of transgene expression, this seems not to be the case after intra-muscular administration of an AAV-vector. In a clinical study in patients with α-1 antitrypsin (AAT) deficiency in which the gene for AAT was delivered by an AAV1 capsid, cellular immune responses were found against the capsid proteins from day 14 in all subjects. However, the influence of those T-cells is not clear since the expression of the transgene was sustained at sub-therapeutic levels in all subjects. These data suggest that the cellular immune responses to the AAV capsid did not eliminate the transduced cells (25). Similarly, systemic and local cellular immune responses induced by intra-muscular injection of alipogene tiparvovec did not appear to have an impact on safety and did not prevent clinical efficacy (43). However, cellular host immune responses to both AAV capsid and transgene products have been shown in the context of muscular dystrophy (26).

CLINICAL STUDIES WITH ALIPOGENE TIPARVOVEC

Alipogene tiparvovec (called AAV1–LPL^{S447X} in the early phases of clinical development) is an AAV1 vector expressing a naturally occurring variant of the LPL transgene, LPL^{S447X}, associated with improved lipid profile and carried by approximately 20% of the general population (44). Building on successful proof-of-concept studies in animal models (45, 46), three interventional clinical studies have been conducted with alipogene tiparvovec in patients with LPL-deficiency (CT-AMT-010, CT-AMT-011-01, CT-AMT-011-02) (**Figure 1**). In all studies, alipogene tiparvovec was administered via multiple direct intra-muscular injections into the lower extremities in the subjects with LPL-deficiency. Alipogene tiparvovec was administered with a 22 gage needle as multiple injections of 0.5 ml volume (maximum) with a distance of 2.5–3 cm between each site. The total number of injections was divided equally between the vastus lateralis and vastus medialis of both the left and right musculus femoralis. The calf muscles were also injected if the number of injections exceeded 40 injection sites. Both the muscle and major blood vessels were identified prior to

Dose (gc/kg)	Number of subjects	Immune suppression
Study CT-AMT-010		
1×10^{11}	4	no
3×10^{11}	4	no
Study CT-AMT-011-01		
3×10^{11}	2	no
3×10^{11}	4	yes
1×10^{12}	8	yes
Study CT-AMT-011-02		
1×10^{12}	5	yes

FIGURE 1 | Summary of the clinical studies with alipogene tiparvovec.

injection using ultrasound to ensure intra-muscular administration, and to avoid intra-vascular injection. Two injection sites were labeled with permanent skin tattoos so that injection sites could be identified for subsequent biopsy. The total dose delivered to subjects was calculated based upon the subject's body weight and his/her allocation to a specific dosing cohort.

CLINICAL DEVELOPMENT PROGRAM

Study CT-AMT-010

Eight patients with LPL-deficiency were first monitored for several months, and then treated once with multiple intra-muscular injections of AAV1–LPL^{S447X} (predecessor of alipogene tiparvovec containing the same construct but manufactured in another cell system). The patients did not receive immunosuppressants pre- or post-exposure to AAV1–LPL^{S447X}, and were followed-up for up to 18 months after administration in the active phase of the study.

Study CT-AMT-011-01

After an observation period of a few months, 14 patients with LPL-deficiency were treated with alipogene tiparvovec. Twelve of these patients received immunosuppressants. The immunosuppressant regimen consisted of cyclosporine A at a dose of 3 mg/kg/day and mycophenolate mofetil at a dose of 2 g/day and was maintained until 12 weeks after administration of alipogene tiparvovec. The patients were followed-up for 5 years after administration.

Study CT-AMT-011-02

After a run-in phase of a few months, five patients with LPL-deficiency were treated with alipogene tiparvovec. All patients have received immunosuppressants, starting shortly before exposure to alipogene tiparvovec. The immunosuppressant regimen consisted of 3 mg/kg/day cyclosporine A, 2 g/day mycophenolate mofetil, and a bolus injection of methylprednisolone was given 30 min prior to alipogene tiparvovec administration. The immunosuppressant regimen with cyclosporine A and mycophenolate mofetil was maintained until 12 weeks after exposure. The patients were followed-up for a year after administration in the active phase of the study.

A summary of the clinical analyses schedule with alipogene tiparvovec is given in **Figure 2**. The follow-up scheme included routine hematology and biochemistry up to 3 months, immunology monitoring up to 1.5 years, and a biopsy of the injected muscle. No hematology visits were planned after week 12. Antibodies as well as cellular responses against AAV1 and LPL were monitored in the long-term follow-up at 2, 3, 4, and 5 years after administration of alipogene tiparvovec. Blood samples were obtained from all subjects enrolled in the clinical trials pre- and up to 5-year post-administration of alipogene tiparvovec, and analyzed for the presence of total antibodies against AAV1 capsid proteins and LPL by ELISA-based assay. In addition, all blood samples were tested for presence of T-cells specific for AAV with an enzyme-linked immunospot (ELISpot) assay. Of note, patients with pre-existing total antibodies against AAV1 were included in the clinical trials. Biopsy specimens of the injected muscle as well as specimens from the non-injected muscle (control) were taken between 10 and 52 weeks after injection for immunohistochemical studies. The specimens were analyzed for the presence and the nature of any cellular infiltrates. In addition, blood samples were tested at regular intervals for inflammation markers such as C-reactive protein (CRP) and neutrophil counts, as well as for parameters reflecting local (inflammatory) muscle damage such as creatine phosphokinase (CPK).

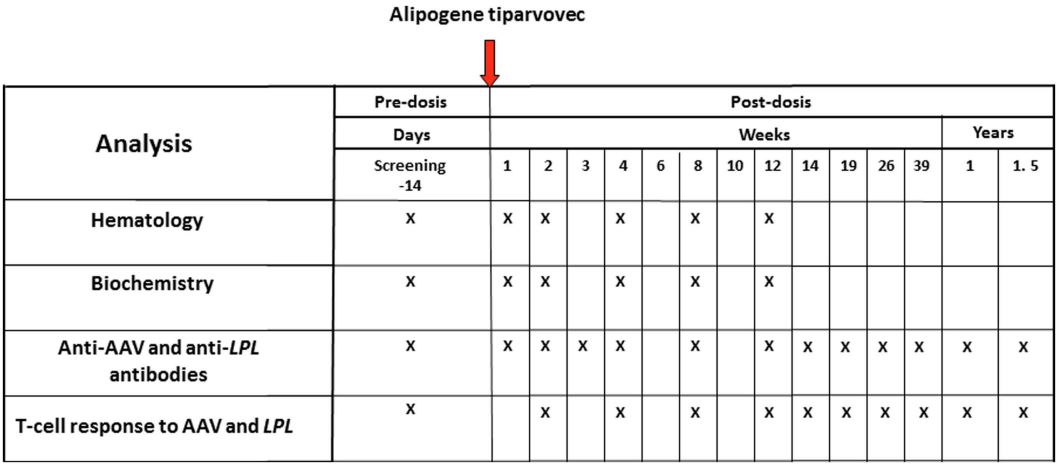
IMMUNOLOGICAL MEASUREMENTS IN THE CLINICAL PROGRAM OF ALIPOGENE TIPARVOVEC

Antibodies against AAV1 capsid proteins

Humoral (total antibodies) responses against AAV1 capsid proteins were measured with an ELISA assay. Briefly, AAV1 capsid proteins were fixed to polystyrene ELISA plates and incubated with the serum samples to be tested. Bound antibodies were detected by a subsequent incubation with conjugated antibodies against human immunoglobulins. The ELISA did not discriminate between IgG subclass antibodies. To discriminate between samples with normal and with elevated levels of anti-AAV antibodies, a cut-off level was established using serum samples from 30 healthy volunteers. The test results of the samples to be tested were scored by comparison with those of the negative control, which was a serum sample from a healthy human control. To this end, algorithms were developed to convey the optical density results into a semi-quantitative scoring system. Based on the algorithms, samples were said to be strongly positive (++), weakly positive (+), or negative (−) for AAV1 total antibodies.

Antibodies against LPL

Antibody responses against LPL^{S447X} were assessed by measuring total antibody levels in pre- and post-exposure serum samples with an ELISA assay. This ELISA was similar to that described above for antibodies against AAV1 proteins, except that recombinant LPL was used to coat plates. The recombinant LPL^{S447X}



Analysis	Pre-dosis	Post-dosis												
	Days	Weeks											Years	
	Screening -14	1	2	3	4	6	8	10	12	14	19	26	39	1	1. 5
Hematology	X	X	X		X		X		X						
Biochemistry	X	X	X		X		X		X						
Anti-AAV and anti-*LPL* antibodies	X	X	X	X	X		X		X	X	X	X	X	X	X
T-cell response to AAV and *LPL*	X		X		X		X		X	X	X	X	X	X	X

FIGURE 2 | Clinical analyses schedule of the studies with alipogene tiparvovec until 1.5 years after drug delivery.

was isolated from medium of stably transfected CHO cells that express LPL^{S447X}. The ELISA did not discriminate between IgG subclass. A cut-off level of the assay was established in a similar way as described in the previous paragraph for anti-AAV1 antibody ELISA. Also for this ELISA, an algorithm was developed to convey the optical density results into a semi-quantitative scoring system. Based on the algorithms, samples were said to be positive (+) or negative (−) for anti-LPL antibodies.

Assay for AAV1-specific T-lymphocytes

In order to monitor the T-cell-mediated immune response in the patients treated with alipogene tiparvovec, an ELISpot assay was developed. This assay is based on the detection and quantification of interferon gamma (IFN-γ) secreting cells upon stimulation with AAV1 capsid antigens. To this end, PBMCs were obtained from the patients and incubated with AAV1 capsid antigens. A one color ELISpot assay was used for this purpose as previously described (23, 24) and further validated by a contract research laboratory, SeraCare Life sciences (Milford, MA, USA), according to predefined QA/QC specifications.

Two criteria are widely used to evaluate test results of ELISpot assays, which are the number of spot forming unit (SFU) per million cells upon stimulation with antigen and the increase of SFU number compared to that in medium only. Generally, samples are said to be positive for T-cells when upon stimulation with antigen they contain >50 SFU per million cells, and when that number is at least threefold higher than that of the medium control. These criteria were also used to assess T-cells against AAV1.

Immunohistochemical analysis

Open muscle biopsies were collected between 10 and 52 weeks after intra-muscular injection of AAV1–LPL from both an injected (tattooed) muscle (vastus lateralis) and a non-injected control muscle site (not tattooed musculus tibialis anterior). The specimens were analyzed according to routine evaluations, including muscle histology and immunohistochemical characterization of infiltrating cells when present. The biopsy procedures were performed at variable time after injection mainly dependent on the availability of the patients. The predefined criteria for the collection of tissue specimens in the protocol were 14 weeks for the first biopsy and 52 weeks for the follow-up biopsy, independently of any clinical indication. However, due to the availability of the patients, deviations from the protocol occurred and the real time of collection of the biopsy specimens is reported for each patient in **Figure 3**. Specimens from the injected muscle were compared to those from the contralateral non-injected muscles from each subject. The histological assessments were carried out according to routine procedures at the Department of Neuropathology, Academic Medical Centre (AMC), Amsterdam, The Netherlands, by Dr. Dirk Troost and Dr. Eleonora Aronica, both specialized in the histopathology of human muscle. Tissues' scoring was expressed as negative to 3+ positive.

ANALYSIS OF THE TREATMENT-EMERGENT IMMUNE RESPONSES IN THE PATIENTS TREATED WITH ALIPOGENE TIPARVOVEC

An overview of the systemic as well as local humoral and cellular immune responses observed in all patients participating in the clinical studies is shown in **Figures 3A–C**. It should be noted that only 19 patients gave their consent to have muscle biopsies taken, 7 patients from study CT-AMT-010, 7 from study CT-AMT-011-01, and 5 from study CT-AMT-011-02.

HUMORAL IMMUNE RESPONSES UPON TREATMENT WITH ALIPOGENE TIPARVOVEC

Fifteen of the 26 subject had pre-existing antibodies against AAV1. Among the 19 patients of whom a post-exposure biopsy was available, 11 had pre-existing anti-AAV antibodies. No apparent relationship was found between pre-existing AAV1 antibodies and LPL-expression after administration of alipogene tiparvovec: 7 of the 11 patients with pre-existing anti-AAV1 antibodies had LPL-expression in the biopsy versus 4 of the 7 patients with no such antibodies (**Figure 3**). As expected, and in line with published data observed with other AAV-vectors all subjects, whether exhibiting

pre-existing antibodies or not, showed a treatment-emergent anti-AAV1 total antibody response, which became detectable at 1 or 2 weeks after the admginistration of alipogene tiparvovec. The anti-AAV1 total antibody titers measured at those early time points remained stable over the whole observation period. The responses of circulating antibodies against AAV1 observed in the clinical development program with alipogene tiparvovec are consistent with data reported for other published gene therapy trials with AAV-based vectors [among others, Ref. (25, 41, 42, 47)]. In each patient, an increase in the level of anti-AAV antibody titers was observed upon administration of alipogene tiparvovec, which was sustained overtime. There was no apparent difference in anti-AAV1 antibody response between studies and dose cohorts suggestive that the dose and/or the immunosuppressive regime did not influence anti-AAV1 total antibody formation.

None of the patients had anti-LPL antibodies prior to the administration of alipogene tiparvovec nor developed those after delivery of the product. The baseline levels of anti-LPL antibodies were below the cut-off level of detection in all patients prior to administration of alipogene tiparvovec. Post-administration levels

A

CT-AMT-010

Subject	Dose (gc/kg)	Biopsy: weeks post injection	Biopsy: LPL expression In injected muscle Tissue	Biopsy: T cells infiltrates In injected muscle tissue	Systemic T cells response Against LPL	Systemic T cells response Against AAV1	Ab against LPL: Pre-ad	Ab against LPL: Post-ad	Ab against AAV1: Pre-ad	Ab against AAV1: Post-ad
#1	$1x10^{11}$ gc/kg	36	Yes (2+)	Yes (3+)	no	transient	-	-	+	++
#2		32	Not detected	Yes (1+)	no	no	-	-	-	++
#4		28	Yes (1+)	Yes (2+)	no	no	-	-	-	++
#5		*No biopsy*			no	transient	-	-	-	++
#6	$3x10^{11}$ gc/kg	10	Not detected	Yes (2+)	no	transient	-	-	+	++
#7		32	Yes (3+)	Yes (3+)	no	no	-	-	+	++
#8		26	Yes (3+)	Yes (3+)	no	no	-	-	+	++
#9		27	Not detected	No (0)	no	transient	-	-	+	++

B1

CT-AMT-011-01

Subject	Dose (gc/kg)	Biopsy: weeks post injection	Biopsy: LPL expression In injected muscle Tissue	Biopsy: T cells infiltrates In injected muscle tissue	Systemic T cells response Against LPL	Systemic T cells response Against AAV1	Ab against LPL: Pre-ad	Ab against LPL: Post-ad	Ab against AAV1: Pre-ad	Ab against AAV1: Post-ad
#004	$3x10^{11}$ gc/kg	26	Yes (2+)	Yes (2+)	no	no	-	-	ND	++
#006		25	Not detected	no	no	transient	-	-	-	++
#001	$3x10^{11}$ gc/kg + IS	27	Not detected	no	no	transient	-	-	++	++
#010		25	Yes (1+)	Yes (2+)	no	no	-	-	-	++
#007		*No biopsy*			no	no	-	-	+	++
#014		*No biopsy*			no	no	-	-	++	++

FIGURE 3 | Continued

B2

AMT-011-01

Subject	Dose (gc/kg)	Biopsy: weeks post injection	Biopsy: LPL expression In injected muscle Tissue	Biopsy: T cells infiltrats In injected muscle tissue	Systemic T cells response Against LPL	Systemic T cells response Against AAV1	Ab against LPL: Pre-ad	Ab against LPL: Post-ad	Ab against AAV1: Pre-ad	Ab against AAV1: Post-ad
#015	$1x10^{12}$ gc/kg +IS	*No biopsy*			no	no	-	-	+	++
#008		*No biopsy*			no	transient	-	-	-	++
#019		*No biopsy*			no	transient	-	-	-	++
#018		*No biopsy*			no	transient	-	-	+	++
#020		*No biopsy*			no	transient	-	-	+	++
#011		25	Yes (3+)	Yes (3+)	no	transient	-	-	+	++
#013		26	Not detected	no	no	transient	-	-	+	++
#009		26	Yes (2+)	Yes (2+)	no	transient	-	-	-	++

C

CT-AMT-011-02

Subject	Dose (gc/kg)	Biopsy: weeks post injection	Biopsy: LPL expression In injected muscle Tissue	Biopsy: T cells infiltrates In injected muscle tissue	Systemic T cells response Against LPL	Systemic T cells response Against AAV1	Ab against LPL: Pre-ad	Ab against LPL: Post-ad	Ab against AAV1: Pre-ad	Ab against AAV1: Post-ad
#01-001	$1x10^{12}$ gc/kg +IS	18	Not detected	Yes (1+)	no	no	-	-	+	++
		52	Yes (3+)	Yes (3+)						
#01-003		14	Yes (1+)	Yes (1+)	no	no	-	-	-	++
#01-002		30	Yes (1+)	Yes (1+)	no	sporadic	-	-	+	++
#02-001		36	Yes (3+)	Yes (2+)	no	transient	-	-	+	++
#02-002		36	Not detected	no	no	no	-	-	-	++

FIGURE 3 | A per-patient summary of the immunological data obtained in study CT-AMT-010 (A), CT-AMT-011-01 (B), and CT-AMT-011-02 (C). Biopsies: the scoring of LPL-expression in injected muscle tissue reflect the number of cells positive for lipid deposits, as identified using oil red O staining of cross-sections: 0, −, 1+, rare; 2+, moderate; 3+, high number. The scoring of T-cells infiltrates in injected muscle tissue reflect the number of infiltrates observed, as identified using staining of cross-sections with cell-specific markers: 0, −, 1+, rare; 2+, moderate; 3+, high number. It should be noted that the scores given are arbitrary, simply providing a semi-quantitative or relative means to distinguish between patients in terms of amount of inflammatory cells observed. As such, a score of 3+ represents the highest score observed in the study. Systemic T-cells responses: samples were said to be positive for T-cells when upon stimulation with antigen they contained >50 SFU per million cells, and when that number was at least threefold higher than that of the medium control. A T-cell response to the antigen was reported transient positive (transient) when at least two consecutive sampling time points were measured positive in the ELISpot assay. The patient reported "sporadic" present recurrent non-consecutive T-cell response over time. When none or only one sampling time was measured positive, the T-cell response was reported negative (−). Antibodies: the test results of the samples were scored by comparison with those of a negative control (a serum sample from a healthy human control). To this end, algorithms were developed to convey the optical density results into a semi-quantitative scoring system. Based on the algorithms, samples were said to be strongly positive (++), weakly positive (+), or negative for AAV1 antibodies and positive (+) or negative (−) for anti-LPL antibodies.

remained below the cut-off level, demonstrating that no antibody responses were mounted against the expressed LPL protein after administration of alipogene tiparvovec even in the long-term.

CELLULAR IMMUNE RESPONSES UPON TREATMENT WITH ALIPOGENE TIPARVOVEC

Enzyme-linked immunospot assays were used to assess the course of numbers of T-cells in peripheral blood that were directed against AAV1 epitopes. The results obtained across studies and per subjects are given in **Figures 4A–C**. In study AMT-010 (48), the intramuscular administration without immune suppression of the AAV1 vector in humans resulted in a transient T-cell activation in four of eight subjects. In study AMT-011-01 (49), cellular immune responses against AAV1 capsid proteins were also observed despite immune suppression. On the basis of the data obtained from PBMCs of adequate quality, a moderate and non-persistent T-cell response was observed directed against the AAV1 capsid in 9 out of the 14 subjects. Therefore, the immunosuppressive regimen was further optimized in study CT-AMT-011-02 (50) by starting administration of cyclosporine and MMF administration earlier before alipogene tiparvovec administration, and by adding a bolus injection of methylprednisolone at the time of alipogene tiparvovec administration. However, in study CT-AMT-011-02, comparable cellular immune responses against AAV1 capsid proteins were observed as in study CT-AMT-011-01. Overall, transient cellular immune responses did not have clinical consequences as they were not associated with any clinical signs or symptoms such as persistent elevation of blood levels of CRP, CPK, or other inflammation markers (see Adverse Events).

Enzyme-linked immunospot assays were also used to evaluate whether there was any T-cell responses against the expressed LPL protein product. In none of the patients, such immune responses were observed upon administration of alipogene tiparvovec at any dose.

LOCAL T-CELL RESPONSES

Biopsy specimens of the injected muscle from 19 patients were available for immunohistochemical analyses. As described previously, these specimens were assessed for the presence of inflammatory cell infiltrate by qualified histopathologists. Infiltrates of varying intensity consisting of B cells, macrophages, and mainly T-cells, were found in 15 patients whereas in the other 4, no inflammatory cell infiltrate was observed. However, as illustrated in study AMT-011-02 with subject 02-002 for which no infiltrates in the injected muscle was detected, the biopsy of some subjects was taken weeks after the peak of systemic T-cells response reached baseline, compared to a few weeks for others subjects. Therefore, the time of the biopsy should be taken into consideration when putting in parallel local and systemic T-cell responses.

As none of the patients developed a T-cell response against LPL, the relation between an immune response to the transgene and the presence of a local T-cell infiltrate was not further investigated. The presence of cytotoxic T-lymphocytes (CTL) in the inflammatory cell infiltrates was investigated in the muscle tissues injected with alipogene tiparvovec as well as in muscle biopsies from non-injected muscle. As a parameter for cellular cytotoxicity, granzyme B and Fas ligand expression by the cells was measured. CD8-positive T-cells in the infiltrates were negative for granzyme B and Fas ligand expression, which suggest that the majority of the T-lymphocytes present in the injected muscle tissue lack cytotoxic properties. Furthermore, CD4-positive T-cells observed in muscle biopsies from LPLD subjects injected with alipogene tiparvovec

A **CT-AMT-010**

Patients	Pre-dosis	Alipogene tiparvovec	Post-dosis									Systemic T cells responses against AAV1
			Weeks									
	Screening pre-dose	Dose (gc/kg)	2	4	6	8	12	19	26	39	52	Overall assesment
1	-	$1x10^{11}$ gc/kg	-	-	-	-	+	+	-	-	-	transient
2	-		-	-	-	-	-	-	-	-	-	negative
4	-		-	-	-	-	-	-	-	-	-	negative
5	-		-	-	-	-	+	+	-	+	+	transient
6	-	$3x10^{11}$ gc/kg	-	+	+	+	+	-	-	ND	-	transient
7	-		-	-	-	-	-	-	-	-	-	negative
8	-		-	-	-	-	-	-	-	-	-	negative
9	-		-	-	+	+	+	-	-	-	-	transient

FIGURE 4 | Continued

B

CT-AMT-011-01

Patients	Pre-dosis	Alipogene tiparvovec	Post-dosis									Systemic T cells responses against AAV1
			Weeks									
	Screening pre-dose	Dose (gc/kg)	2	4	6	8	12	19	26	39	52	Overall assesment
004	-	$3x10^{11}$ gc/kg	-	-	-	-	-	ND	-	ND	-	negative
006	-		-	+	+	+	+	ND	-	ND	-	transient
001	-	$3x10^{11}$ gc/kg + IS	-	-	-	ND	-	-	-	+	+	transient
010	-		-	+	-	-	ND	-	ND	-	-	negative
007	-		+	-	-	ND	-	ND	ND	-	ND	negative
014	-		-	ND	ND	-	-	-	ND	-	-	negative
008	-	$1x10^{12}$ gc/kg + IS	ND	ND	ND	ND	ND	ND	+	+	+	transient
019	-		ND	ND	ND	ND	ND	+	+	ND	ND	transient
018	-		-	ND	ND	ND	+	+	-	-	-	transient
020	-		ND	ND	ND	+	-	+	ND	ND	+	transient
015	-		-	-	-	-	-	-	-	+	-	transient
011	-		-	ND	+	+	+	ND	ND	ND	-	transient
013	-		-	-	-	-	+	ND	+	ND	ND	transient
009	-		-	ND	+	+	+	ND	ND	-	-	transient

C

CT-AMT-011-02

Patients	Pre-dosis	Alipogene tiparvovec	Post-dosis									Systemic T cells responses against AAV1
			Weeks									
	Screening pre-dose	Dose (gc/kg)	2	4	6	8	12	14	26	39	52	Overall assesment
01-001	-	$1x10^{12}$ gc/kg + IS	-	-	-	-	-	-	-	-	+	negative
01-002	-		+	-	-	+	-	+	-	+	ND	Transient/ sporadic
01-003	-		-	-	-	-	+	-	-	-	ND	negative
02-001	-		-	-	-	-	-	+	-	+	ND	transient
02-002	-		-	-	-	-	-	+	-	-	ND	negative

FIGURE 4 | Systemic cellular immune responses following alipogene tiparvovec administration. The tables below provides an overview of the individual patient systemic T-cell response against AAV1 overtime for study CT-AMT-010 **(A)**, study AMT-011-01 **(B)**, and study AMT-011-02 **(C)**. We considered that a subject developed a T-cell-mediated immune response to AAV1 capsid proteins when at least two of the eight to nine sampling time points were measured positive (+) in the ELISpot assay. When only one sampling time was reported positive (+), the T-cell response was considered negative.

were further assessed for the expression of the transcription factor FoxP3, as a marker for regulatory T-cells. FoxP3-positive/CD4-positive T-cells were also found in the infiltrates suggesting that multiple mechanisms contribute to the local immune tolerance to alipogene tiparvovec administration (50).

IMMUNOGENICITY OF ALIPOGENE TIPARVOVEC AND EFFICACY

The presence of LPL protein in the muscle biopsies and the improved clearance of post-prandial chylomicrons levels in plasma were used to measure local and systemic activity of alipogene tiparvovec and were considered as efficacy markers (49, 51). However, the use of muscle biopsies has several hindrances. At first, out of the 27 patients treated with alipogene tiparvovec, 19 patients gave their consent for a muscle biopsy once. Only one patient allowed the procedure to be done twice. Second, the results were influenced by the limited spread of the product in the muscle tissue and the variability in the procedure, as not all biopsies were consistently taken in close proximity to the actual injection site. Therefore, the circulating chylomicrons plasma levels measurement was developed in parallel to the clinical study CT-AMT-011-01 and used as a primary end point only in the study CT-AMT-011-02, as the most reliable endpoint to determine the systemic activity of the LPL enzyme.

Fifteen of the 26 patients, with registered data for presence of pre-administration anti-AAV1 antibodies, had pre-existing antibodies against AAV1. Among the 15 patients with pre-existing antibodies against AAV1, biopsies were obtained from 11 patients. Among those 11 patients, 7 had LPL-expression in the biopsy. In comparison, from the 11 patients without pre-existing antibodies, 8 muscle biopsies were obtained; and from those, 5 were stained positive for LPL-expression. This distribution was very similar across the three studies CT-AMT-010, CT-AMT-011-01, and CT-AMT-011-02. Our results strongly indicate that there was no apparent relationship between the presence of pre-existing AAV1 antibodies in LPLD patients and LPL-expression after administration of alipogene tiparvovec.

After the administration of alipogene tiparvovec in all 27 patients, the development of treatment-emergent antibodies against AAV1 capsid proteins was observed, independently of whether pre-existing antibodies were present or not. In relation with efficacy, those treatment-emergent antibody responses against the AAV1 capsid proteins upon treatment with alipogene tiparvovec, did not seem to affect expression of the transgene.

A similar conclusion as drawn for the presence and development of AAV1-specific antibodies can be drawn for the development and presence of AAV-specific T-cells after administration of alipogene tiparvovec. The percentage of patients with treatment-emergent T-cell response across studies and among the three dose groups was 50% (2/4) of the subjects treated with 1×10^{11} gc/kg having a positive response, 40% (4/10) of the patients treated with 3×10^{11} gc/kg, and 69% (9/13) of the patients treated with 1×10^{12} gc/kg, which suggest an AAV1-dose-dependent kinetics of T-cell response appearance (48–50). Of the 19 patients who consented to the biopsy procedure, 10 developed AAV1-specific T-cells. Of those 10 patients, 5 were tested positive for the expression of LPL in the biopsies. Of the 9 patients with no detectable T-cell response against AAV1, 7 had detectable LPL-expression in their biopsy. In relation with efficacy, those transient T-cell responses against the AAV1 capsid proteins upon treatment with alipogene tiparvovec, did not seem to directly influence the expression of the transgene.

However, as mentioned previously, the variability of the biopsy procedure and by consequence, the difficulty for quantification and comparison between patients has to be considered in the interpretation of the data. The differences in the results obtained with the biopsies of patient 01-001 (in study CT-AMT-011-02), collected at 18 and 52 weeks after administration of alipogene tiparvovec, illustrate this issue. The biopsy taken at week 18 yielded no detectable LPL-expression, whereas the biopsy taken at week 52 yielded a strong expression of LPL.

The administration of alipogene tiparvovec resulted in functional LPL activity levels sufficient to achieve a beneficial clinical effect in patients with LPLD. This conclusion is supported by the evidence that levels of plasma TG decreased in LPLD patients after administration of alipogene tiparvovec. The data obtained in studies CT-AMT-010, without immunosuppression, and CT-AMT-011-01 and AMT-011-02, with immunosuppression, are considered comparable in terms of the decrease in TG levels (**Figure 5**). However, plasma TG levels subsequently increased in most patients around 12–14 weeks post-administration of alipogene tiparvovec. This was at a time interval when immune suppression had already been discontinued. This phenomenon was observed across the three studies and showed that fasting TG levels are not a sufficiently sensitive marker to monitor the long-term therapeutic effects of alipogene tiparvovec. Post-prandial chylomicron clearance kinetics has been recognized as the most relevant biological marker for the systemic activity of LPL during the clinical development of alipogene tiparvovec (50, 51). However, post-prandial chylomicron level measurements were included as endpoint only in the last of the three interventional studies, CT-AMT-011-02. The results have been reported (51) and show that the post-prandial chylomicron plasma levels are significantly reduced in all patients included in the study, independently of the presence of humoral (all patients) or cellular systemic (two on five patients) or local (three on five patients) immune responses against AAV1 (**Figure 3**), thus indicating that these responses had no influence on the efficacy of alipogene tiparvovec.

Furthermore, an ongoing study has shown a reduction in acute pancreatitis events in a series of more than 25 affected subjects (43). The analysis for a treatment-effect of Glybera taking into account exposure time demonstrated a significant and clinically relevant reduction in the risk of definite acute pancreatitis during various periods ranging from 2.5 to 10 years pre-treatment to post-treatment (median 2.9 years) (52).

EFFECT OF IMMUNOSUPPRESSANTS ON TREATMENT-EMERGENT IMMUNE RESPONSES

Study CT-AMT-010, the first clinical study with AAV1–LPL^{S447X}, was performed without treating the patients with immunosuppressants. In this study, no antibody or T-cells responses against LPL were found. However, antibodies against AAV1 capsid epitopes were observed in all patients whereas a T-cell response against AAV1 was detected only in four of the eight subjects.

	Dose	Subject ID	% TG reduction (main)	Pre-exposure Ab against AAV	Post-exposure Ab against AAV	Systemic T cell Response against AAV
CT-AMT-010-01	$1x10^{11}$ gc/kg	1	19.35	+	++	transient
		2	50.90	-	++	negative
		4	18.53	-	++	negative
		5	20.44	-	++	transient
	$3x10^{11}$ gc/kg	6	23.08	+	++	transient
		7	47.03	+	++	negative
		8	43.37	+	++	negative
		9	31.72	+	++	transient
CT-AMT-011-01	$3x10^{11}$ gc/kg	4	38.07	nd	++	negative
		6	72.48	-	++	transient
	$3x10^{11}$ gc/kg + IS	1	5.14	++	++	transient
		7	71.12	+	++	negative
		10	-32.65	-	++	negative
		14	34.05	++	++	negative
	$1x10^{12}$ gc/kg + IS	8	18.25	-	++	negative
		9	77.40	-	++	transient
		11	13.88	+	++	transient
		13	70.27	+	++	transient
		15	61.56	+	++	transient
		18	42.13	+	++	transient
		19	-7.14	-	++	transient
		20	48.62	+	++	transient
CT-AMT-011-02	$1x10^{12}$ gc/kg + IS	01-001	70.91	+	++	negative
		01-002	-21.61	+	++	negative
		01-003	57.81	-	++	Transient/sporadic
		02-001	20.47	+	++	transient
		02-002	7.32	-	++	negative

FIGURE 5 | TG responder status in relation with humoral and systemic T-cell response against AAV. The table below provides an overview of the individual patient cellular and humoral immune responses in relation with the clinical end point (fasting) total plasma TG. A T-cell response to the antigen was reported transient positive (transient) when at least two consecutive sampling time points were measured positive in the ELISpot assay. The patient reported "sporadic" present recurrent non-consecutive T-cell response over time. When none, or only one sampling time was reported positive, the T-cell response was reported negative (–).

As discussed, none of these immune responses raised specific safety concerns. However, they triggered more a concern about the efficacy of the product; especially the development of any AAV-specific T-cells (48) that were thought at the time to possibly hamper the expression of LPL. A similar T-cell response was observed in gene therapy trial for hemophilia B, in which an AAV2 vector was used to deliver the human coagulation factor IX (24, 41, 42). In this trial, two patients developed a T-cell response to AAV2 capsid proteins, which was not predicted from pre-clinical studies. In those two patients, transgene expression declined subsequently to pre-treatment levels. Based on these observations, it was concluded by the investigators that a cytotoxic T-lymphocyte response to the capsid may have contributed to a loss of transgene-expressing cells.

These discussions heavily influenced the decision to include an immunosuppressant regimen in the clinical study protocols for CT-AMT-011-01 as well as CT-AMT-011-02.

As a result of these discussions, 17 patients (12 in CT-AMT-011-01 and 5 in CT-AMT-011-02) treated with alipogene tiparvovec received a concomitant administration of immunosuppressants. Treatment-emergent anti-AAV1 antibody responses were observed in all the patients and were not affected by the immunosuppressants, neither during the time of administration nor after cessation of the administration. The AAV1–LPL^{S447X} vector used cannot induce expression of viral proteins in host cells. Hence, AAV1 capsid proteins are expected to be only transiently presented to the immune system of the recipient after injection of AAV1–LPL^{S447X} (3, 19, 53). Therefore, the immune suppressants drugs were given for a period of 12 weeks. A 12-week-period was considered to be sufficient for prevention of capsid immunogenicity, based on observations in monkeys (19) and the investigators early observations in humans that indicated TG levels started to rise after an initial decrease usually at some time between 4 and 12 weeks post-dosing. A combination of cyclosporine and MMF was chosen because this combination is widely used to prevent cytotoxic T-cell responses in transplant rejection. The doses of the immune suppressants proposed to co-administer with alipogene tiparvovec were according to approved doses for transplant rejection.

Patients with	No immune suppression	Immune suppression
LPL expression in biopsy		
Detectable expression	5	7
Undetectable expression	4	3
Anti-AAV1 antibody response		
Response	10	17
No response	0	0
Anti-LPL antibody response		
Response	0	0
No response	10	17
Anti-AAV1 T-cell response		
Response	5	13
No response	5	4
Anti-LPL T-cell response		
Response	0	0
No response	10	17
T cell infiltrate at site of injection		
Infiltrate	7	6
No infiltrate	2	4

FIGURE 6 | Effect of immune suppression on LPL-expression and immune responses upon administration of alipogene tiparvovec.

In study CT-AMT-011-01, humoral and cellular immune responses against AAV1 capsid proteins were observed. These responses were similar to those observed in study CT-AMT-010 although in study CT-AMT-011-01, a higher dose of AAV1–LPL^{S447X} was used (**Figure 4**). Therefore, the immunosuppressant regimen was further optimized in study CT-AMT-011-02 by starting administration of cyclosporine and MMF administration earlier before alipogene tiparvovec administration, and by adding a bolus injection of methylprednisolone at the time of alipogene tiparvovec administration. However, in study CT-AMT-011-02, comparable humoral and cellular immune responses against AAV1 capsid proteins were observed as in study CT-AMT-011-01 (**Figure 4**).

The effect of immunosuppressants on LPL-expression and immune responses upon administration of alipogene tiparvovec is summarized in **Figure 6**. In the clinical development of alipogene tiparvovec, no untoward side effects were observed that could be assigned to the use of prednisolone or one of the other immunosuppressants.

CLINICAL DATA

CHEMISTRY AND HEMATOLOGICAL VALUES

Patients were monitored for up to 12 weeks post alipogene tiparvovec administration regarding routine hematology and biochemistry including CPK and CRP, increases of which are expected in case of local inflammatory damage at the injection site. In addition, other inflammatory markers such as neutrophil counts were also determined at several pre- and post-exposure occasions in the patients. None of the patients had neutrophil counts outside the normal range. A per-patient summary of the CRP and CPK data is given in **Figure 7**. The majority of the patients had normal CRP and CPK levels pre- and post-exposure to alipogene tiparvovec, suggesting that inflammatory reactions in the injected muscle were mild, if present, and of little clinical significance.

ADVERSE EVENTS

Alipogene tiparvovec was administered via a one-time set of intramuscular injections in the lower limbs. No consistent change in any laboratory parameter, linked to the administered product, was observed, including CPK. Injections were well tolerated with mild–moderate local injection site reactions lasting for a few days relating to the injection sites, and no change in muscle function. None of the patients showed clinical signs of persistent local inflammation at injection sites such as redness, swelling, warmth, pain, or dysfunction, upon administration of alipogene tiparvovec. There are to date no reports of muscle dysfunction in LPLD patients administered with alipogene tiparvovec.

The adverse reactions observed during the clinical development of alipogene tiparvovec are summarized in Figure S1 in Supplementary Material. Most of the adverse reactions were related to the administration procedure. All were of transient nature and resolved within days after the administration of the product. Only one serious adverse event involving muscle was seen, that was considered to be at least possibly related to alipogene tiparvovec by the Investigator (#01-002 in CT-AMT-011-02). In this subject, at 15 weeks post-administration, a transient rise in CPK, accompanied by a rise in CRP (**Figure 7**), was correlated with a low positive AAV cellular response associated with no LPL-related cellular or humoral significant response was seen in a complex of clinical symptoms and signs indicated as "polyarthralgia of imprecise origin." The subject show sporadic systemic T-cell responses against AAV across the observation period at weeks 2, 8, 14, 39. Despite those T-cell responses, the muscle biopsy of this subject which was taken 30 weeks post alipogene tiparvovec showed robust LPL-expression. In addition, the subject did not show any anti-LPL cellular or humoral response (50). No adverse effect was observed that could have been related to the immune responses discussed in the previous paragraphs.

From the clinical data obtained across studies, we conclude that there is no clinical untoward impact of the delivery of alipogene tiparvovec.

DISCUSSION

To evaluate the immunogenicity of alipogene tiparvovec, an extensive testing program was performed that included the analyses of antibody and T-cell responses to LPL as well as to AAV1. Antibody and cellular responses were measured pre-exposure to alipogene tiparvovec, and at various occasions post-exposure. In addition, biopsies were taken from the injected muscle and non-injected muscle as control, to evaluate local immune and inflammatory processes. Finally, CPK and CRP levels and neutrophil counts were measured, and patients were clinically evaluated for local and systemic symptoms indicative for inflammatory or immune reactions. Expression of LPL in the injected muscle as well as the improvement of post-prandial chylomicrons clearance in plasma was used as biochemical markers for efficacy.

The success of *in vivo* gene therapy not only depends on the ability to control the immune response toward the vector, but

Patients	Elevated CRP pre-exposure	Elevated CRP post-exposure	Elevated CPK pre-exposure	Elevated CPK post-exposure
AMT-010				
#1	0/2*	0/6	0/2	0/6
#2	0/2	0/6	0/2	0/6
#4	0/2	0/6	0/2	0/6
#5	0/2	0/7	0/2	0/7
#6	0/2	0/7	0/2	0/7
#7	0/2	0/7	0/2	0/7
#8	0/2	0/6	0/2	0/6
#9	0/2	0/6	0/2	0/6
AMT-011-01				
#004	0/2	0/6	0/3	0/6
#006	0/2	0/6	0/3	0/6
#001	0/2	0/6	0/3	0/6
#010	0/2	0/6	0/3	0/6
#007	0/2	0/6	0/3	0/6
#014	0/2	2/6	0/3	0/6
#015	0/2	0/6	0/3	0/6
#008	0/2	0/6	0/3	0/6
#019	0/2	0/6	0/3	0/6
#018	0/2	0/6	0/3	0/6
#020	0/2	0/6	0/3	0/6
#011	0/2	0/6	0/3	0/6
#013	0/2	0/6	0/3	0/6
#009	0/2	0/6	0/3	0/6
AMT-011-02				
#01-001	0/5	1/10	0/5	0/10
#01-003	0/5	0/10	0/5	0/10
#01-002	0/5	1/10	0/5	1/10
#02-001	0/5	0/10	0/5	0/10
#02-001	0/5	0/10	0/5	0/10

FIGURE 7 | A per-patient summary of CRP and CPK values pre- and post-exposure to alipogene tiparvovec. Scheduled visits pre-treatment and post-treatment have been included. The post-treatment visits were at day 1 and weeks 1, 2, 4, 8, and 12 for studies CT-AMT-010 and CT-AMT-011-01. Weeks 14, 26, 39, and 52 were added for study CT-AMT-011-02. In the study AMT-011-01, the isolated elevation of CRP for subject 014 was concomitant with a transient medical condition. In the study AMT-011-02, the isolated elevation of CRP for subject 01-001 was concomitant with a transient medical condition. The isolated elevation of CRP and CPK for subject 01-002 was related to the general clinical condition of the subject.

also to monitor any potential reaction to the therapeutic protein expressed from the transgene. The data obtained from patients with LPL-deficiency who received a single treatment with multiple injections of alipogene tiparvovec, support the initial expectation that the protein product is minimally immunogenic, if at all: neither treatment-emergent antibody responses against LPL nor T-cells responses against LPL were found. Thus, the expressed protein itself appears to be non-immunogenic.

Concerning anti-AAV immunity, the majority of healthy individuals are exposed to AAV during lifetime (2, 54). Hence it is not surprising that 15 of 27 patients had pre-existing antibodies against AAV1. There was no difference among patients with or without these antibodies with regard to detectable LPL-expression in the biopsies or improvement of post-prandial chylomicron clearance, suggesting that pre-existing anti-AAV1 antibodies did not appear to prevent LPL transgene expression and clinical efficacy. Therefore, pre-existing anti-AAV1 antibodies likely have no effect on the efficacy of alipogene tiparvovec following intramuscular administration. All patients showed treatment-emergent antibody responses to AAV1 and there was no difference in anti-AAV1 antibody response between the various dosing cohorts.

The markers that were used to demonstrate LPL-expression and functionality after the delivery of alipogene tiparvovec are complex. Therefore, the present report is focusing on LPL-expression in muscle biopsies and post-prandial chylomicron clearance as marker for systemic LPL activity. For the direct measurement of LPL-expression, we made use of biopsies that were obtained at different time points up to 12 months after drug administration. However, and as described above, not all patients gave their consent to this procedure which can present some variability in the execution. Nineteen patients provided their consent and in total 20 biopsies were obtained, which all were examined for LPL-expression. In 12 of the 20 biopsies, LPL-expression was found irrespective of the presence of antibodies against AAV1. The second biochemical marker for successful gene delivery in this review is the level of clearance of post-prandial chylomicrons in plasma, which reflect the systemic activity of LPL (49). This marker was however, included only in the study CT-AMT-011-02. The post-prandial chylomicrons plasma levels were shown to be significantly reduced in all five patients included in this study, independently of the presence of anti-AAV1 antibodies.

Altogether, these observations demonstrate that anti-AAV1 antibody responses did not exclude sustained transgene expression nor did impair the systemic biological activity of the expressed protein. Our results further support that treatment-emergent anti-AAV1 antibody responses do not necessarily have any influence on the long-term efficacy and safety of AAV-based gene therapy.

Treatment-emergent T-cell responses against AAV1 capsids were measured with the ELISpot assay and were observed in 18 of the 27 patients. However, it has to be noted that the patients treated with the higher dose, 1×10^{12} gc/kg, were more prompt to develop an AAV1-specific T-cells responses. Somewhat higher T-cell responses were noticed in some patients upon cessation of the immunosuppressants, pointing to a suppressive effect on the T-cell responses. It is therefore not possible to make definitive conclusions regarding the effect of immunosuppressants on T-cell responses to AAV1. The immunosuppressants did not affect antibody levels as was expected since the regimen was mainly aiming at reducing potential T-cell responses. MMF and cyclosporine are both described to be effective in suppressing cytotoxic T-cell responses. MMF has an effect on B-cell proliferation, because it inhibits *de novo* guanosine nucleotide synthesis, a pathway commonly required for both T- and B-cell proliferation. Cyclosporine specifically inhibits T-cell activation by inhibiting IL-2 production and exerts limited, if any, effect on B-cell proliferation. Thus, the increased anti-AAV1 titers are not surprising. Similar observations were made in a study in monkeys (52) when administrating AAV8-hFIX together with immunosuppressants consisting of MMF and tacrolimus. In the third study of the three interventional studies for alipogene tiparvovec, CT-AMT-011-02, prednisolone was administered in the form of a bolus injection to prevent the release of substances that mediate inflammation and to enhance the potency of the other immunosuppressants used.

Our studies should be considered in the context of the growing body of clinical and pre-clinical studies evaluating the role of capsid-specific T-cells in AAV gene. The immunogenicity data found in the clinical studies conducted with AAV-based vectors in human, show that immune responses against AAV capsid proteins can vary widely and amongst others are influenced by the target organ, route of delivery, and dosing schedule. When targeting the muscle in humans, T-cell responses directed to the capsid antigen were documented in AAT-deficient subjects receiving intra-muscular injection of an AAV1–AAT vector (25, 55), in study on AAV1-α-sarcoglycan in limb-girdle muscular dystrophy subjects (35) and in our own LPL-deficient subjects. The controversial aspect of the capsid-specific T-cell hypothesis is whether the vector sensitizes transduced cells to become targets for CTL-mediated clearance by virtue of MHC presentation of peptides from the input capsid protein. Also, immune modulation was used which could have impacted on the AAV-specific immune responses, our study provides the most direct and extensive test of this hypothesis because we observed transgene expression until 52 weeks (long-term follow-up) after injection of AAV1–LPL^{S447X} in the muscle, despite the detection of circulating T-cell specific for AAV capsid peptides in some subjects and persistent focal infiltrates in all subjects for whom transgene expression was detected. These data clearly demonstrate that transgene expression can persist, despite the presence of capsid-specific T-cells and cellular infiltrates. Sustained transgene expression in the presence of T-lymphocyte responses have been reported in the literature in experimental animals and in different tissues (56, 57) and humans (25, 35, 37). However, whereas attention has been focused initially on the AAV capsid as target of an undesirable T-cell response (24, 41, 42), observations made by the groups of Dr C. Walker (58) and supported by observations of others (59) suggest that loss of function and programed death by most tissue-infiltrating AAV-primed T-cells seem to argue against their direct participation in clearance of AAV-vector-transduced target cells. It has been described that T-cells in AAV-vectors related infiltrates present characteristics of anergy (55). Such T-cell infiltrates are therefore generally considered as unable to initiate cellular self-destruction and therefore do not impact on efficacy of transgene expression.

Another mechanism via which T-cells may affect LPL-expression is stimulating the proliferation and differentiation of B cells that subsequently form antibodies against AAV1. However, as is discussed above, anti-AAV1 antibodies seem to have no impact on LPL-expression and there is no evidence whether antibody-dependent cell-mediated cytotoxicity played a role. As mentioned before, a sustained long-term transgene expression was observed after intra-muscular injection, despite the presence of circulating antibodies directed against the AAV1 capsid peptides. The results obtained with alipogene tiparvovec demonstrate that the presence of the anti-AAV1 humoral immune response had no apparent influence on the long-term efficacy of the therapy. The same observation has been reported in other clinical studies (25, 35, 37, 47, 55).

Multiple intra-muscular injections of the vector and supposed inflammatory and immune reactions ensuing at the injection sites raise concerns about inflammatory damage in the injected muscle. However, except for transient mild local procedural symptoms at the injection sites, no clinical symptoms such as swelling, pain, or dysfunction pointing at inflammatory damage were observed in the patients. In addition, serial monitoring of CRP and CPK revealed normal levels of these markers in most patients. Occasional elevations of CRP and CPK were seen in two and in one patient, respectively, without any clinical correlation. In addition, though a mild mononuclear infiltrate was observed in 14 of the 19 patients of whom a biopsy was obtained, this infiltrate lacked substantial cytotoxic T-cells activity. Hence, no clinical, biochemical, histochemical, or immunological evidence for inflammatory muscle damage at the injections sites was found.

All together, the data collected on systemic and local immune responses induced by intra-muscular injection of alipogene tiparvovec demonstrate the absence of impact on safety and did not compromise LPL transgene expression. These findings indicate that muscle-directed AAV-based gene therapy through the intravascular route remains a promising approach for the treatment of human diseases.

REGULATORY PERSPECTIVE

During the assessment of the Glybera marketing authorization dossier, the fact that no responses had been seen against the expressed LPL protein was considered as a positive safety asset and no material concerns were expressed.

The necessity of using immunosuppressants was not proven during the clinical development of Glybera since the regimen seemed not to improve efficacy whilst having a major negative impact on the safety aspects. It remains questionable whether it makes clinical sense to co-administer immunosuppressants with any AAV-based vector. However, in the case of Glybera, since LPLD is such a rare disease, it will not be possible to further assess long-term clinical efficacy in absence of any immunosuppressants within the present indication.

During the regulatory review by the European Medicines Agency, multiple questions arose on whether the cellular responses to the viral capsid proteins could have any meaningful negative effect on the long-term safety or efficacy of alipogene tiparvovec. Our data clearly demonstrate that transgene expression can persist, despite the presence of capsid-specific T-cells and cellular infiltrates and without apparent toxicity or attenuation of transgene expression. Furthermore, the purity of the vector preparations (in terms of total amount of viral proteins injected versus dose in genome copies) and impurities profile of the vectors used in the various clinical trials described in the literature may be very divergent and therefore may lead to very different immune responses. Nonetheless, the scientific debate has been powerfully influenced by previous findings with other vectors and by the hypothesis that the vector sensitizes transduced cells to become targets for CTL-mediated clearance. Therefore, since the safety data on alipogene tiparvovec have been collected in a small number of patients, their clinical relevance and possible interpretations were considered not fully unequivocal and further data collection has been requested by the European Medicine Agency post-approval of Glybera®. Such data will be collected from all treated patients in future in a LPLD registry, thus allowing for long-term data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00082/abstract

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Immunological monitoring to rationally guide AAV gene therapy

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Recent successes with adeno-associated virus (AAV)-based gene therapies fuel the hope for new treatments for hereditary diseases. Pre-existing as well as therapy-induced immune responses against both AAV and the encoded transgenes have been described and may impact on safety and efficacy of gene therapy approaches. Consequently, monitoring of vector- and transgene-specific immunity is mandated and may rationally guide clinical development. Next to the humoral immune response, the cellular response is central in our understanding of the host reaction in gene therapy. But in contrast to the monitoring of antibodies, which has matured over many decades, sensitive and robust monitoring of T cells is a relatively new development. To make cellular immune assessments fit for purpose, investigators need to know, control and report the critical assay variables that influence the results. In addition, the quality of immune assays needs to be continuously adjusted to allow for exploratory hypothesis generation in early stages and confirmatory hypothesis validation in later stages of clinical development. The concept of immune assay harmonization which includes use of field-wide benchmarks, harmonization guidelines, and external quality control can support the context-specific evolution of immune assays. Multi-center studies pose particular challenges to sample logistics and quality control of sample specimens. Cooperative groups need to define if immune assessments should be performed in one central facility, in peripheral labs or including a combination of both. Finally, engineered reference samples that contain a defined number of antigen-specific T cells may become broadly applicable tools to control assay performance over time or across institutions.

Keywords: immunological monitoring, assay harmonization, gene therapy, adeno-associated virus, biomarkers

INTRODUCTION

Inherited diseases account for a substantial number of hospital admissions of children but also affect adults. Despite progress in science, efficient treatments that go beyond supportive care are not available for most of these diseases. Gene replacement therapies bear a high potential to address the medical need in affected patients and have been introduced to clinical testing about 20 years ago (1). After many draw-backs in the past an increasing number of reports of successful gene therapies in patients with hemophilia (2), Leber's congential amaurosis (LCA) (3), or X-linked adrenoleukodystrophy (4) were recently published. In November 2012, Glybera® (alipogene tiparvovec) was the first human gene therapy to receive a market approval from the European Medicines Agency (EMA) suggesting that the field has reached a turning point which may give raise to additional approved gene therapies in the future (5).

Most strategies applied for gene replacement focus on two types of vectors to reach stable transfer of functional gene products into selected target tissues, namely lentiviral vectors for *ex vivo* gene transfer into hematopoietic cells or other stem cells, or adeno-associated virus (AAV) for *in vivo* gene transfer to various target tissues such as muscle, the liver, the retina, lung, or the brain. Despite successes reported for AAV gene therapy approaches it has become clear that humoral and cellular immune responses against both the vector as well as the transgene may negatively impact on safety and efficacy of gene therapy approaches (6). Humoral responses against AAV frequently occur in the population, increase by age and are efficiently induced following a single administration of AAV-based therapies. Neutralizing antibodies against AAV have been shown to negatively influence transduction rates and may counter-act therapy approaches with systemic delivery of AAV in particular. More recently, pre-existing antigen-specific T cells recognizing AAV capsid proteins have been suggested to be an independent factor leading to reduced transduction rates on one hand and immune-mediated clearance of transduced cells expressing capsid proteins on the other hand, which led to immune-related adverse events in patients (7, 8). The first results showing that human $CD8^+$ T cells to AAV capsid could limit the efficacy of the gene transfer originated form an initial trial of AAV gene transfer to the liver for treatment of hemophilia (7). Here it could be shown that expression of the initially expressed factor IX (F.IX) disappeared after several weeks, accompanied by a transient elevation of transaminases of the liver that were shown to be mediated by antigen-specific T cells directed against AAV but not the

therapeutic F.IX that eliminated vector-transduced hepatocytes. In addition, extensive T cell monitoring from multiple studies performed in gene transfer to the muscle indicate that there is a dose dependent increase in treated patients showing T cell reactivity against the AVV capsid. Interestingly, currently available data suggest that gene transfer to immune-privileged body compartments such as the eye and central nervous system so far did not lead to detectable immune responses to the capsid or the transgene. In summary, the immunogenicity data found in the clinical studies conducted with AAV-based vectors in human and available in literature, confirm that immune responses against AAV capsid proteins can vary widely and amongst others are influenced by the target organ, route of delivery, and dosing schedule (9). Also antigen-specific T cells against the encoded therapeutic gene have been described and may reduce efficacy of gene therapy (10). All these findings explain why approaches to better understand or block immune responses following gene transfer with viral vectors have recently come into focus in the field (11).

Adeno-associated virus-specific T cells have been shown to be directed against AAV capsid proteins inducing transient hepatitis following i.v administration of AAVs targeting the liver (7). Although transient toxicity may be acceptable for regenerating tissues such as the liver in regard of safety, immune-mediated removal of AAV-transduced cells may become a major obstacle for gene transfer into toxicity relevant organs such as the brain or the heart. AAV-specific immunity may also lead to safety concerns as well as efficacy loss in replacement of the retinal pigment epithelium-specific 65-kDa protein gene in the retina in patients with LCA as re-therapy in the other eye may be mandated (12).

The currently available data on AAV-therapy induced immunity supports the notion that detailed knowledge of the presence of antigen-specific T cells prior to gene therapy as well as the occurrence of vector- and transgene-specific immunity following therapy may guide clinical decision making in the future (6, 9). Examples for proactive use of immunological data for the benefit of patients could be: (i) the selection of patients that have a high likelihood to exert the wanted effects, (ii) identification of patients that may need an adaption of the therapy (e.g., lower doses, improved vector), or (iii) receive concomitant immune-suppression until immunogenic capsid proteins are cleared. It may also be used to (iv) identify patients at risk for adverse immune reactions following second administration of the vector, or (v) be early indicators of loss of function of the encoded gene product.

In order to confirm the hypothesis that data generated by immunological monitoring can indeed impact on clinical decision making to enhance AAV-mediated gene therapy, more systematic analyses of AAV-specific T cell immunity are mandated and have even been recommend by NIH Recombinant DNA Advisory Committee (13). The fact that T cell assays may have a higher complexity and variability as compared to assays to quantify soluble analytes explains why all investigators that perform correlative biomarker studies should know, control and report on the variables of assay conduct that are known to critically affecting immune assay performance. The critical variables were identified by large scale proficiency programs conducted by the Cancer Research Institute's Cancer Immunotherapy consortium (CRI-CIC), the Cancer Immunotherapy Immunoguiding Program (CIMT-CIP) (14), and the Human Immunophenotyping Consortium (15). In addition analytical labs are advised to optimize and standardize sample logistics and assay conduct prior to testing specimens from clinical trials even at the earliest stage of clinical development in which hypothesis generation represents the primary aim of immune assay use. Assay qualification and validation become mandatory requirements when a hypothesis generated in exploratory research from early clinical development has to be confirmed in advanced clinical development (16). This gradual evolution of immune assays from first exploratory use toward validated assays performed under Good Clinical Laboratory Practice (GCLP) standards may be supported by assay harmonization at any stage (**Figure 1**).

IMMUNE ASSAY HARMONIZATION AND VARIABLES THAT IMPACT ON ASSAY PERFORMANCE

A plethora of assays exists to evaluate specific T cell responses, wanted or unwanted. These assays differ in their sensitivity to detect low frequency T cells, quantity of information obtainable, ability to determine structural and/or functional features of T cells, and their complexity in qualification and validation demands (17). For years the field of T cell monitoring has been plagued by a seemingly inherent variability in assay results, even between assays evaluating the same sample in the same laboratories or by different laboratories. A divergence of Standard Operating Procedures (SOPs), the introduction of new reagents and tools by manufacturers, preferences by the assay performing scientist, varying availability of equipment or the qualification, and training status of personnel, has further complicated the issue. Due to the lack of stringent QC procedures as required for clinical diagnostic tests, many assay "cooks" brewed their own results "soups" following their recipe without knowledge of how good or how bad that soup

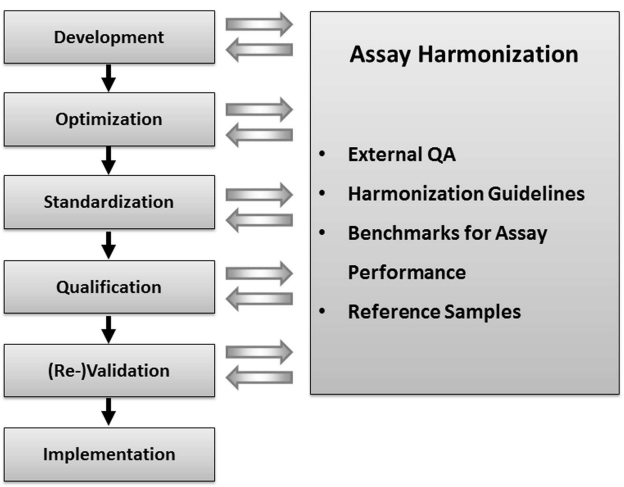

FIGURE 1 | Concept of immune assay harmonization: external quality assurance, harmonization guidelines, benchmarks for assay performance, and reference samples with defined numbers of functional antigen-specific T cells can be used to control the performance of immune assay in one lab. Immune assay harmonization can support quality of immune assays at any step of assay development and use, starting from the initial assay development until assay validation.

"tasted." An additionally complicating fact is the limited availability of test samples, and the intrinsic biological variability in performance of such samples, a factor well known today that has caused a shift in considerations concerning sample handling for T cell monitoring (18–20).

This heterogeneous landscape of assays and protocols exists in cancer immunology, infectious diseases, transplantation immunology, and gene therapy. As outlined earlier a multitude of gene therapy approaches exist that (i) address several unrelated diseases, (ii) apply various viral vectors and serotypes, (iii) target different tissues (e.g., liver, muscle, retina, central nervous system), (iv) are applied using different administration routes and (v) deliver various therapeutic gene products. The fact the gene therapy comes in different flavors mandates a product specific adaption of the immunological monitoring that acknowledges therapy-related peculiarities and integrates available knowledge on immunogenicity and epitope hierarchy of both the applied vectors and therapeutic transgenes. The fact that there is not one single assay format that is applicable for all gene therapy trials poses a challenge on the gene therapy community to agree on some standards that may be used across a variety of clinical products and studies to generate results that may be comparable across institutions.

First activities examining the differences in T cell assay performance between laboratories evolved more than 10 years ago from cancer immunologists and researchers in the HIV field that initiated proficiency panel projects for ELISPOT (21, 22). This concept was soon adopted by two non-profit organizations from the cancer research field, the CIMT-CIP and CRI-CIC, and elevated to highly efficient programs that address proficiency testing of assay performance of individual labs and the identification of variables influencing the assay outcome at large (14). By sending out pretested samples in a blinded fashion to a large number of laboratories which had to test those sample with a given T cell assay following their own protocol, and report the results back to a central site including details about how they performed the assay, CRI-CIC and CIMT-CIP were able to not only give feedback on the performance of each individual laboratory in comparison to the other participating laboratories, but also identified critical protocol variables that influence the results reported back. These studies indicated that independent of SOPs applied, the results of a significant proportion of labs accumulated around an overall median value in results, while others were outliers. With the help of protocol information provided, initial recommendations for SOP adaptations were deduced and introduced to the field for the next testing round, where it could be confirmed whether these recommendation can indeed improve performance. This iterative testing process allowed the identification of protocol steps that, independent of the SOP applied, could elevate the assay performance of labs (23–26). These findings are summarized in harmonization guidelines which are now increasingly used by laboratories (**Figure 2**). The introduction of such harmonization guidelines has led to significant improvement in response detection across laboratories (27) and decreased the overall variability in results between laboratories. Further investigations of specific recommendations have led to even more detailed guidance in the harmonization process, for example by investigating the use of serum-free freezing and assay media in ELISPOT (28, 29), the implementation of a dump channel and viability marker in MHC-peptide multimer staining (26) and obtaining gating recommendations for flow-based experiments (30, 31). Such assay harmonization activities, consisting of: (i) a mechanism for regular external proficiency testing with fast feed-back loops to participants, (ii) dynamic harmonization guidelines and (iii) benchmarks for assay performance, can support investigators to improve and maintain quality of their assays at any stage of development (**Figure 1**).

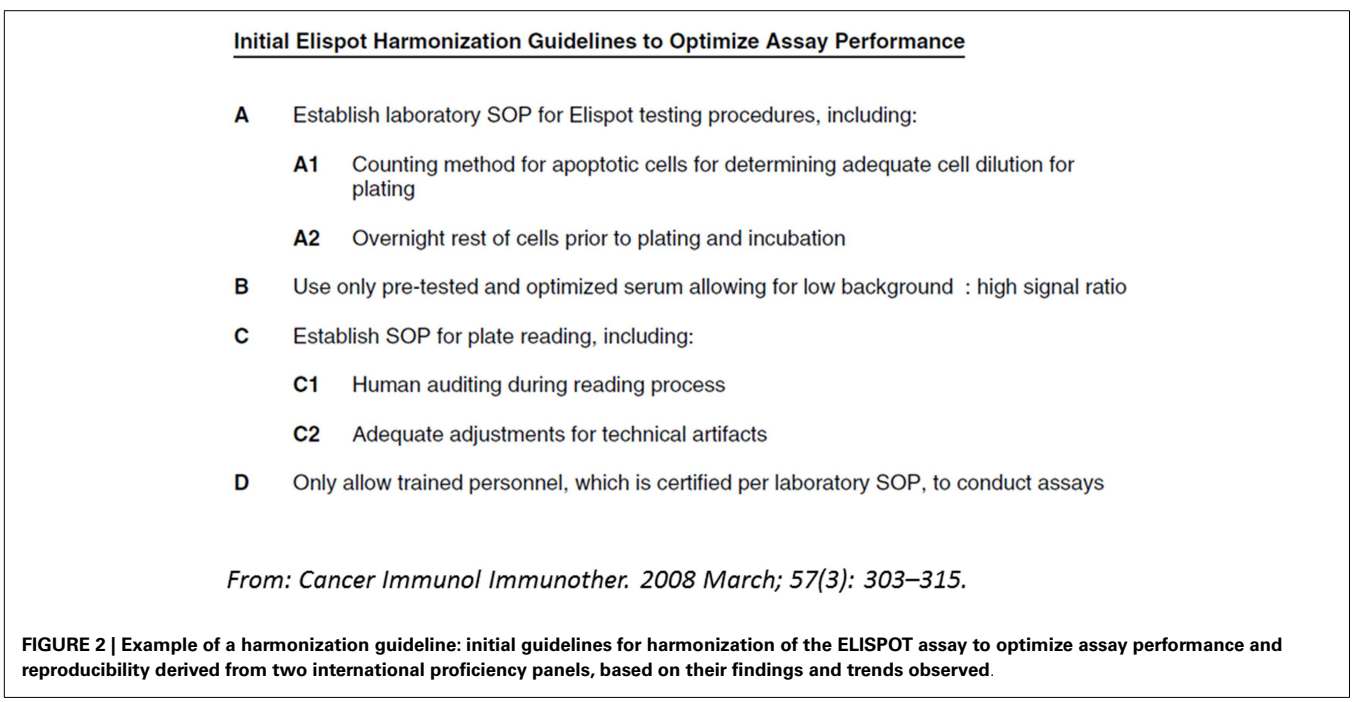

FIGURE 2 | Example of a harmonization guideline: initial guidelines for harmonization of the ELISPOT assay to optimize assay performance and reproducibility derived from two international proficiency panels, based on their findings and trends observed.

While this assay harmonization process is ongoing and an increasing number of labs apply its results, there exists a discrepancy between what is being done in laboratories, and what is reported when publishing results from T cell assays. To address the lack of transparency often observed in publications that hinders the accurate interpretation and replication of results, as well as the comparison of results from different laboratories, the Minimal Information about T cell assays (MIATA) project was introduced (32). A 3-year extensive public consensus process involving more than 100 scientists from the different fields of translational immunology and regulatory agencies as well as editors from scientific journals resulted in a final reporting framework for results of T cell assays (33) that summarizes the minimal information to report about assay conduct. Easy implementation tools are available online (miataproject.org), and MIATA-compliant papers are listed with a link to the original publication.

These activities, together with available harmonization guidelines, if followed, elevate the T cell immune monitoring field to a level that allows the creation of high quality data that support immunotherapeutic developments and advances, and hence deserve the embracement by the translational science community at large.

IMMUNE ASSAYS IN MULTI-CENTER CLINICAL TRIALS

A further complicating factor for performing T cell immunomonitoring is the complexity and fragility of the analyte. In most cases, analysis is focused on T cells obtained from peripheral blood; less often (as more invasive sampling is required) T cells infiltrating other tissues such as bone marrow or skin are analyzed.

Importantly, functional T cells assays are sensitive to the time passed since the sample has been taken on a typical scale of hours (18, 19, 34). In terms of assay validation language, the sample stability is low. This can be both influenced by the inherent instability of the T cells and by the instability of the sample matrix such as granulocytes that become activated over time (35). For some assay parameters, this time window may be prolonged by adding stabilizers or by isolating the T cells without cryopreservation prior to shipping the sample (36). More generally, there are two known solutions to this logistical challenge: (i) all measurements are being performed within short time using fresh samples, or (ii) all T cells are isolated from the biological samples and cryopreserved before the assay.

The first solution may be especially attractive in the case of monocentric studies. A major drawback of such a scenario is that no retrospective analyses will be possible, longitudinal samples of a patient cannot be analyzed within the same assay, and in the case of multi-centric clinical trials this requires all immune assay parameters to be fully standardized at each site where patients are recruited. Therefore, this solution that has been termed as "peripheral analysis" (37, 38) poses high demands on assay standardization, which should be completed before a trial is initiated. Nevertheless, using fresh samples does not control inter-assay variability, and hence can introduce variability in measurements of samples obtained from different time points from a patient, which can confound the response determination between time points. Determining the precision between measurements of samples obtained from different time points is logistically challenging, hence the introduced degree of variability by testing fresh samples cannot be easily defined.

The second solution requires isolation and cryopreservation of the T cells shortly after the sample has been taken. Once the cells are frozen and kept at cryogenic temperatures, they are stable for very long time periods and can be shipped over great distances to enable batch-wise analysis within one central lab, thereby reducing assay variation. This approach does control variability by allowing the simultaneous testing of samples obtained from different time points in one assay. However, isolating and cryopreservation of T cells from biological samples is a relatively laborious process and is not performed as part of the tests for clinical routine. Therefore, if it has to be implemented within a multi-centric clinical trial, all aspects of blood collection, transport to the laboratory isolating T cells, conduct of the isolation and cryopreservation process at the lab and shipping of the frozen sample should be standardized between labs using fully comparable processes and materials (39). This solution has been termed as "central analysis" (37, 38) and poses high demands on standardized sample collection, while allowing more flexibility on the assay that are later being performed with the collected samples. Recent harmonization efforts have addressed cryopreservation challenges, and provide some guidance (28), while others are still ongoing. The performance of cell isolation and cryopreservation may be monitored by counting viable cell numbers at the time of isolation and after thawing the samples and in the case of blood samples the yield can be additionally monitored by comparing the numbers of isolated cells to the numbers of lymphocytes and monocytes from a routine hematology assessment.

To demonstrate the reproducible performance of immunoassays over time in one lab during the conduct of a clinical trial, and between labs as in the case of peripheral analysis, a stable source of reference samples that can be repeatedly analyzed is of central importance. For T cell assays, the generation of a stable reference sample is a challenging task compared to chemical assays, for example, where a small molecule can be spiked into a sample matrix at pre-defined concentrations. In the past, labs have often prepared local reference samples by aliquoting and cryopreserving cells from one large blood draw that contained T cells of a known reactivity. Such reference samples are limited in size, not comparable between different batches, and do not contain pre-defined "known" numbers of T cells. Recently, a first generation of reference samples that contain a defined number of functional antigen-specific T cells have been developed by CIP (40). The technology is based on transfer of antigen-specific T cell receptors (TCRs) into primary lymphocytes using viral gene transfer. Second generations of reference samples that are based on the use of *in vitro* translated RNA for TCR gene transfer are under development and has been shown to be simple to manufacture, robust, and sensitive, stable of at least 1 year and shown to be suitable for ELISPOT assays, HLA-peptide multimer staining as well as cytokine flow cytometry applications (unpublished data). A completed proficiency panel with 12 labs in Europe and the USA showed that the technology is easily transferable to other labs and across protocols. Given the currently available data on T cell reactivity against the AAV capsid, a set of reference samples engineered with capsid-specific TCRs for the most prevalent

HLA-restrictions may become a useful tool for the gene therapy community and help to control assay performance and increase comparability across different studies using AAV gene transfer. Thus RNA-based TCR-engineered reference samples may become a standard tool to control immune assay performance over time and help destigmatizing T cell assay use in clinical trials an become yet another component of immune assay harmonization.

CONCLUSION/SUMMARY

Systematic immune assessments in gene therapy trials using quality controlled immune assays will help to understand the immunological interactions between AAV-based therapies and the adaptive immune system. Associating immunological biomarker data with data on clinical safety and efficacy will enable the field to use immunomonitoring data for clinical decision making to improve gene therapy approaches. Prior to use of immune assays investigators need to know and control those assay variables that determine the quality of sample specimens and assay results and should provide structured reporting on the assay setup in publications and reports. By providing a fast feedback of assay performance over time or across institutions, assay harmonization can support immune assay development and use and thus may be considered as a tool to enhance biomarker research and development of new immune- and gene-therapies.

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Pre-clinical assessment of immune responses to adeno-associated virus (AAV) vectors

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Transitioning to human trials from pre-clinical models resulted in the emergence of inhibitory AAV vector immune responses which has become a hurdle for sustained correction. Early animal studies did not predict the full range of host immunity to the AAV vector in human studies. While pre-existing antibody titers against AAV vectors has been a lingering concern, cytotoxic T-cell (CTL) responses against the input capsid can prevent long-term therapy in humans. These discoveries spawned more thorough profiling of immune response to rAAV in pre-clinical models, which have assessed both innate and adaptive immunity and explored methods for bypassing these responses. Many efforts toward measuring innate immunity have utilized Toll-like receptor deficient models and have focused on differential responses to viral capsid and genome. From adaptive studies, it is clear that humoral responses are relevant for initial vector transduction efficiency while cellular responses impact long-term outcomes of gene transfer. Measuring humoral responses to AAV vectors has utilized *in vitro* neutralizing antibody assays and transfer of seropositive serum to immunodeficient mice. Overcoming antibodies using CD20 inhibitors, plasmapheresis, altering route of delivery and using different capsids have been explored. CTL responses were measured using *in vitro* and *in vivo* models. In *in vitro* assays expansion of antigen-specific T-cells as well as cytotoxicity toward AAV transduced cells can be shown. Many groups have successfully mimicked antigen-specific T-cell proliferation, but actual transgene level reduction and parameters of cytotoxicity toward transduced target cells have only been shown in one model. The model utilized adoptive transfer of capsid-specific *in vitro* expanded T-cells isolated from immunized mice with LPS as an adjuvant. Finally, the development of immune tolerance to AAV vectors by enriching regulatory T-cells as well as modulating the response pharmacologically has also been explored.

Keywords: immune responses, adeno-associated virus, pre-clinical models, AAV, inhibitory AAV

INTRODUCTION

Over the past 20 years, a full spectrum of immune responses to AAV has been assessed to include innate responses, humoral responses, and T-cell responses. In addition to the emphasis on pre-existing antibodies to AAV, cytotoxic T-cell (CTL) responses to AAV have emerged as a challenge for clinical applications. Roughly 70% of the human population has neutralizing antibodies (NAb) titers to AAV2 that can interfere with gene therapy (1–4). The use of different AAV capsids may overcome this challenge yet lower antibody titers to alternative capsids as well as cross-reactivity of AAV2 antibodies is concerning (3, 4).

Since mice and dogs do not have pre-existing immunity to AAV, correction in coagulation actor IX (FIX) deficient animal studies have been sustained without concern of immune responses (5–7). Unfortunately, the lack of immunity in pre-clinical studies did not fully reflect clinical results. In patient studies using AAV2 or AAV8 expressing normal or self-complimentary (sc) hFIX respectively, AAV1 expressing α1-antityrpsin (AAT), and Lipoprotein Lipase (LPL) correction, capsid-specific CTL responses have emerged, particularly at high doses (8–11). In some cases, existence of capsid-specific CTLs did not significantly impact correction as seen in AAT studies involving intramuscular (IM) injections (10). In hemophilia B (HB) liver studies, correction at 10% of normal was achieved in high dose cohorts. This level was only sustained 4–8 weeks and a spike in liver transaminases coincided with a drop in hFIX levels (8, 9), which was coordinated by capsid-specific CTLs. With the occurrence of CTL responses and presence of pre-existing antibodies to AAV capsid there has been a concerted effort to develop suitable pre-clinical approaches for complete assessment of immune responses and to develop intervention techniques.

CHARACTERIZATION OF INNATE IMMUNITY TO AAV

Pattern recognition receptors (PRRs) on monocytes recognize AAV particles and trigger innate immunity (12–15). TLR2 and TLR9 have been identified as the PRRs for AAV. TLR2 triggers responses to the capsid while TLR9 detects the viral genome. Studies have been performed in myeloid differentiation primary response 88 (*myd88*−/−), *tlr9*−/− and *tlr2*−/− deficient mice to pinpoint TLR pathways associated with AAV recognition. Additionally, adaptive responses have been assessed in these mice. Moreover, studies were performed in interferon β receptor of KO mice (*ifnr*−/−) to establish a cytokine for developing adaptive

responses (13, 14). Measuring cytokine secretion from differentiated monocytes isolated from these KO mice was used to segregate responses to AAV2. Absence of type I interferon (IFN) responses in AAV-activated plasmacytoid dendritic cells (pDCs) isolated from *tlr9*−/− mice as well as *myd88*−/− but not *trl2*−/− mice suggests that IFN responses are due to the vector genome, not the capsid (12–14).

To further assess the immune responses to AAV in KO mice, cellular and humoral responses were measured. Assessing NAbs, serum IgG2a or CTL responses to AAV capsid in mice deficient in innate immunity was used to determine a pathogen recognition link between innate and adaptive response. Both *tlr9*−/− and *myd88*−/− mice showed absence of adaptive responses to AAV (13, 14). AAV antibody titers were assessed by enzyme-linked immunosorbent assay (ELISA) while CTL responses were assessed using enzyme-linked immunosorbent spot (ELISpot) analysis of IFN-γ secretion from isolated splenocytes.

Efforts to determine if genome or capsid modifications alter innate responses have been pursued. Gene expression in liver of AAV2-infected mice showed an increase in inflammatory mediators and TLR9 transcripts, but not TLR2. Additionally, the responses remained the same between capsid variants but the sc genome resulted in a significant increase in transcription of inflammatory mediators. Determining cellular recruitment also indicated the sc-genome significantly increased inflammation. This was measured by staining liver sections for neutrophils, Mac-1+ macrophages and cd335+ natural killer cells. The use of *tlr9*−/− mice as well as TLR9 antagonists prevented innate immune responses, showed a reduction in adaptive immunity, and demonstrated a plausible connection between TLR9 and development of antibodies and CTLs (14).

Efforts to isolate immune responses to AAV input capsid have also been performed. TLR2 and TLR4 are highly associated with recognition of viral structural proteins (16). The production of empty capsids devoid of genomes and the use of HEK293 reporter cell lines, which secrete IL-8 due to activation of stably expressed TLR2 or TLR9, have aided in pinpointing PRRs associated with capsid. HEK293/TLR2 but not HEK293/TLR9 showed activation by empty vectors (15).

HUMORAL IMMUNITY

Epidemiology studies have predicted that ~70% of the population is seropositive for AAV2 (1–4). Further studies have estimated a lower degree of seroprevalence for other AAV capsids (4). Moreover, 20–40% of the population has NAb titers (1:80) against AAV2 capable of reducing transduction of Huh7 cultured cells by 50% using a Multiplicity of Infection of 10^4. Additionally, the ability of AAV2 NAb to reduce transduction efficiency against alternative serotypes has developed concern for cross-reactivity.

CHARACTERIZING NAb INHIBITION OF AAV

Dot Blot studies, NAb assays and cross administration of AAV vectors in animals have been used to determine cross-reactivity of AAV antibodies. The cross-reactivity of αAAV1 or αAAV5 antibodies was tested against a dot blot containing AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 capsid. AAV1 antibodies showed strong adherence to only AAV6, while AAV5 antibodies had minimal adherence to AAV1 (17) and only at high concentrations of monoclonal antibodies.

In NAb *in vitro* assays, AAV vectors are pre-incubated with serum containing NAbs prior to infection of cultured cells and can be used for assessing cross-reactivity when alternative capsids are selected. Using this assay, it was determined that NAbs against AAV1 or AAV5 had limited impact on transduction from the other capsid. Additionally, engineered capsid modifications to AAV2 or a chimeric AAV capsid overcame antibody neutralization based on parental AAV antigens (4, 17–19).

Neutralizing antibody in mice also determined the potential of AAV antibodies to cross react with a different capsid using *in vivo* approaches. Mice were pre-immunized by IM injection of AAV2/GFP then challenged with AAV1, AAV3, AAV4, and AAV5 (20). Data showed that AAV2 antibodies only neutralized AAV3. Another model used an intravenous (IV) infusion in mice of AAV2 immunoglobulin from a pool of individual human donors prior to liver delivery of AAV2, AAV6 or AAV8 expressing hFIX. AAV2 administration was completely blocked while AAV6 and AAV8 were partially blocked (18). Given that the immunoglobulin pool was not assessed for AAV6 or AAV8 antibodies the results seen may not have been due to cross-reactivity.

OVERCOMING HUMORAL RESPONSES TO AAV VECTORS

A comparative approach to test the ideal route of administration to overcome pre-existing NAbs in mice involved an initial IV infusion of AAV2 immunoglobulin from a collection of AAV2 seropostive serum prior to administrating AAV2 via different routes. Delivery of AAV2 to liver by portal vein or direct injection into the liver parenchyma showed successful transduction in the face of pre-existing AAV2 antibodies prophylaxis, while IV injection did not (21). It should be noted that even direct administration of vector to the liver resulted in lower expression levels in the presence of NAb, indicating that even this approach is not able to completely bypass Nab inhibition.

Manipulating the capsid is another approach to overcome NAbs. Generation of alternative capsid libraries by rational mutations in antigenic regions (22), error prone PCR (23), and DNA shuffling (24) have demonstrated the potential. PEGylation is a chemical modification which involves pre-coating the AAV capsid with polyethylene glycol (PEG) prior to administration and has shown promise at limiting but not preventing neutralization (25, 26). Another promising strategy involves artificially encapsulating the AAV vector in biomaterial prior to administration which has the potential to shield the AAV capsid (27). This may be useful provided the biomaterial cloak can be degraded after delivery. While these approaches are encouraging they may limit transduction efficiency.

Temporary immunosuppresion (IS) is emerging as a preferred approach for overcoming humoral responses and has been tested in pre-clinical studies. The inhibition of helper T-cells using CD4 antibodies or cyclosporine A prevents NAb formation and facilitates vector readministration (28). Additionally, transient B-cell depletion using Rituximab (CD20 antibody) can reduce pre-existing antibodies (29). Plasmapheresis can eliminate pre-existing humoral responses but requires multiple cycles of blood exchange to reduce NAbs to negligible levels (30). Similarly, flushing the

macaque liver with saline prior to delivery of vector limited NAb inhibition (31). In an ongoing clinical trial, empty capsids were added to the vector with the rational that they will bind away Nabs and thus increase transgene expression (32). However this approach involves increasing vector load, which may not be favorable for trying to prevent the development of CTL responses.

CHARACTERIZING INHIBITORY CTL RESPONSES TO AAV IN PRE-CLINICAL STUDIES

The first CTL response with parenchymal damage, loss of transgene expression, and expansion of capsid-specific CTLs were observed during a liver directed clinical trial for HB. This was unexpected since it was not observed in pre-clinical studies including non-human primates. Early attempts to replicate this immune response in animals were unsuccessful (33, 34). Even dog models have shown consistent transgene expression without an immune response (5). Therefore, efforts were made to determine variations in immunity between humans and pre-clinical animals. The absence of CTL responses in non-human primates, which are natural hosts of AAV8, ruled out the expansion of memory T-cells as the contributing factor (35, 36). Varki and colleagues reported the loss of siglec receptor expression in T lymphocytes during human evolution (37). Siglec receptors have inhibitory function on the immune system. Exploring the loss of these inhibitory receptors as a possible reason revealed enhanced T-cell function in a mouse KO model which mimics human T-cells, suggesting that human T-cells respond with more activation and proliferation (38). However, the response to AAV also could not be induced in these KO mouse.

In trying to develop CTL responses in pre-clinical animal models, one approach delivered a rAAV expressing IL-12 to the liver (39). Despite the induction of liver inflammation and generation of capsid-specific CTLs, transduced hepatocytes were not eliminated. Another model used an immunogenic OVA/SIINFEKL mouse system. The OT-1 mouse line has an increased frequency of OVA SIINFEKL epitope-specific CTLs. The model requires immunization of OT-1 mice with Ad-AAV. Splenocytes were harvested, labeled with carboxyfluorescein succinimidyl ester (cfse), and adoptively transferred into mice injected with an AAV vector with the SIINFEKL epitope incorporated into the capsid (40). Prolonged proliferation of OT-1 transferred cells (at least 10 weeks after transfer) was observed compared to transfer of wt splenocytes from Ad-AAV immunized mice, however no parenchymal cytotoxicity was reported. The Samulski lab also used the SIINFEKL mouse system. They discovered reduced transgene expression after adoptive transfer of OT-1 splenocytes however only in AAV-treated mice pre-immunized with SIINFEKL-pulsed DCs. Unfortunately, this approach is not ideal given the high immunogenicity, which might not fully reflect actual immunity (41).

IN VITRO ASSAYS FOR CHARACTERIZING CTLs

In vitro assays have had more success at characterizing AAV capsid CTL development and involve monitoring CTL activation and cytotoxicity in response to peptide presentation of AAV capsids in target cells. It is possible to detect AAV capsid-specific CTLs by staining with a multimeric antibody. However staining with these multimers only detects single T-cell clones and is HLA restrictive. As a result, T-cells only from individuals who express a specific HLA type can be visualized. An engineered cell line, which expresses luciferase upon engagement of a specific T-cell receptor was used to monitor T-cells activation (42). Again, this assay is restricted to one HLA type (HLAB0702) and AAV2 recognition. A more flexible assay measures cytotoxicity toward AAV-transduced hepatocytes. In this assay, different serotypes can be tested as long as they transduce the target cells (43, 44). Briefly, CTLs are generated by several rounds of stimulation and proliferation of target cell HLA matched peripheral blood mononuclear cells (PBMCs) or spenocytes with the AAV serotype of interest or dominant epitope and then co-cultured with target cells that are transduced with that AAV and cytotoxicity is measured. In general, a major limitation of *in vitro* assays is the fact that transduction efficiency of alternate AAV serotypes differs significantly and hinders comparative studies between alternate capsid variants and serotypes. Still these assays are helpful in monitoring CTLs responses in mice and in PBMCs obtained during clinical trials.

VECTOR CAPSID VARIATIONS FOR AVOIDING CTL RESPONSES

There is a growing field of vector alterations to increase efficiency and avoid immunity. The rational for creating capsid mutants differs among groups, however most target tyrosine and serine residues on the AAV capsid to enhance transduction efficiency. The Srivastava lab (45) has reported a triple tyrosine capsid mutant with increased transduction efficiency *in vitro* and *in vivo*. Other groups also report increased transduction efficiency by altering tyrosine residues on the AAV capsid (46). Initial data suggests that increased transgene expression is likely due to reduced proteasomal degradation which will shuttle more vector to the nucleus, but the intracellular trafficking of vector has not been fully characterized.

From an immunological perspective, avoiding proteasomal degradation may also lead to reduced CTL recognition. Tyrosine mutants were tested in an immunological mouse model and the group reported increased transgene expression and showed reduced T-cell immunogenicity and toxicity within the liver (44). To mimic the cellular immune reaction in humans, the group adoptively transferred *in vitro* expanded CTLs from AAV immunized mice. In this setting, they were able to demonstrate cytotoxicity in the liver by reduction of transgene levels and increased liver enzymes, both of which were reversed when using the capsid mutant.

PHARMACOLOGICAL INTERVENTION

General IS (taken from treatment of autoimmune disorders or transplantations) (9) and more specific interventions for AAV have been proposed and tested in pre-clinical models, such as proteasomal inhibitors (PI) (e.g., bortezomib, MG 132, carfilzomib). Many groups focus on increasing AAV transduction levels in various tissues using PI (47–50), and other compounds like arsenic trioxide (51), rather than their influence on the immune response. At the same time, another group observed reduced CTL responses as well (42). The mechanism is not completely understood yet it is hypothesized that AAV proteasomal capsid degradation is disrupted which leads to diminished capsid–peptide presentation by

MHC class I molecules. This intervention has only been tested in pre-clinical systems for mechanistic value, given that the later fate of undigested capsids is unknown and could present the danger of prolonged or delayed capsid–peptide presentation.

DEVELOPING TOLERANCE

Multiple groups have demonstrated induced tolerance to the transgene for hepatic delivery of AAV vectors with the involvement of regulatory T-cells (Tregs) (52–55). Attempts to induce tolerance to the AAV capsid through the enrichment of Tregs have been published and show promising results. Initially, De Groot and colleagues were able to show reduced immune responses to common immunogens *in vitro* and *in vivo* using MHC class II epitopes (Tregitopes) that favor Treg development (56). These observations were extended to AAV capsid tolerance in a subsequent study (57). They show AAV capsid CTL suppression using these Tregitopes.

CONCLUSION

Due to the combined efforts of multiple groups working on the various aspects of immune responses to gene delivery with a rAAV vector, many parameters have been deciphered. For humoral immune response models, various models have been established to characterize pre-existing humoral immunity and cross-reactivity and assess the impact of potential interventions *in vitro* and *in vivo*. Using pre-existing KO mice, the influence of the innate immune system is also being rapidly investigated. In contrast, finding models to mimic and investigate the cellular immune response toward AAV capsids has caused more difficulty, only robust *in vitro* models have been well established but recently some features of the response could be recapitulated in animal models. It remains to be seen if these approaches will be able to provide a sufficient platform for further deciphering of cellular immune response and testing interventions. While characterizing immune responses in animal models have been informative, it is evident that greater emphasis should be placed on developing CTL models in immunocompetent animals that are more reflective of the clinical scenario. Until reliable models are established, investigators can use the valuable published data gathered in human clinical trials about cellular immune responses and their management (8, 9).

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Humoral immune response to AAV

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Adeno-associated virus (AAV) is a member of the family *Parvoviridae* that has been widely used as a vector for gene therapy because of its safety profile, its ability to transduce both dividing and non-dividing cells, and its low immunogenicity. AAV has been detected in many different tissues of several animal species but has not been associated with any disease. As a result of natural infections, antibodies to AAV can be found in many animals including humans. It has been shown that pre-existing AAV antibodies can modulate the safety and efficacy of AAV vector-mediated gene therapy by blocking vector transduction or by redirecting distribution of AAV vectors to tissues other than the target organ. This review will summarize antibody responses against natural AAV infections, as well as AAV gene therapy vectors and their impact in the clinical development of AAV vectors for gene therapy. We will also review and discuss the various methods used for AAV antibody detection and strategies to overcome neutralizing antibodies in AAV-mediated gene therapy.

Keywords: adeno-associated virus, AAV, neutralizing antibody, immune response, gene therapy

INTRODUCTION

Adeno-associated virus (AAVs) is a member of the family *Parvoviridae* that has been widely used as a vector for gene therapy because of its safety profile, its ability to transduce both dividing and non-dividing cells, and its low immunogenicity. AAV is a small, non-enveloped single-stranded DNA virus that has been detected in many different tissues of several animal species (1, 2) but has not been associated with any disease (3, 4). In the past decade the discovery and development of new AAV types with dramatically improved *in vivo* performance and with unique seroreactivity and tissue tropisms (5–9) has situated AAV in the forefront of vector development for gene therapy trials. One of the most important aspects of the development of AAV as a clinical product is the impact of the host humoral immune response against its capsid. Several studies have shown that the induction of antibodies by natural exposure to AAV early in life can compromise the subsequent use of AAV as a gene therapy vector (10–14). Moreover administration of an AAV vector induces a potent and long term humoral response to AAV that may compromise the use of the same vector if a second administration is required (15, 16). Humoral immune responses to AAV can be of two types: neutralizing or binding (non-neutralizing). Neutralizing antibodies (NAbs) bind to AAV and through several mechanisms (17) inhibit AAV transduction of target cells. Non-NAbs bind to AAV and "flag" the virus without blocking AAV transduction. AAV NAbs have been the focus of many studies because of their significant deleterious effect on the efficacy of AAV-mediated gene therapy. Recent studies have shown that AAV binding antibodies may also have an impact on AAV vector distribution and safety (18).

In this review we will provide an overview of humoral responses to natural infection with AAV and to therapeutic AAV vectors in small and large animal models including humans. We will also discuss the best method to detect these antibody responses and summarize strategies that have been proposed to avoid or overcome NAbs to allow for AAV gene therapy in a wide spectrum of subjects or to patients that already received AAV-mediated gene therapy but need to be re-treated.

DETECTION OF ANTI-AAV ANTIBODIES

Several methods have been developed to detect antibodies to different AAV serotypes. Some of these methods detect total binding antibodies to AAV capsid and other methods detect antibodies that neutralize *in vitro* or *in vivo* transduction of AAV vectors. The first reports in the early 1970s evaluated total antibodies responses to AAV vector as measured by ELISA and Western blot (19–24). These studies focused on AAV1 and AAV2, as these were the AAV serotypes available at that time. The development in the last decade of new AAV types as delivery vectors for gene therapy required more sophisticated assays to evaluate the level of not only binding but NAbs specific to each AAV serotype.

The *in vitro* transduction inhibition assay became the standard assay to evaluate these NAbs to AAV. The assay is usually carried out in a 48- or a 96-well plate format allowing a high throughput sample analysis. Several cell lines have been used as targets for AAV vector transduction: HeLa, 2V6.11, 293, and Huh7 (25–29). Typically an AAV vector expressing a reporter gene is mixed with serial dilutions of the test sample and the vector-serum mixture is incubated with the cell line of choice that is subsequently analyzed for reporter gene expression. The starting dilution of the test sample, which defines the sensitivity of the assay, varies between studies ranging from 1/2 to 1/20 (29–33). In some cases pre-infection of target cells with wild type adenovirus is included to increase AAV transduction. At the same time the AAV vector expressing the reporter gene is mixed with serial dilutions of naïve serum. The purpose of incubating the vector with naïve serum is to evaluate the enhancement in transduction observed at high concentration of serum $\leq$1/5 (18). The NAb50 titer is reported as the highest serum dilution that inhibits transduction by 50%. *In vitro* transduction efficiency is AAV serotype dependent. The high level of *in vitro* transduction observed with AAV2 combined

with the use of very sensitive luminescence reporter genes allows the use of an AAV particle/cell ratio as low as 100 (18). On the other hand, the low levels of *in vitro* transduction observed in some of the new AAV serotypes, like AAV8, demands the use of a higher AAV particle/ratio which for AAV8 is between 1×10^3 and 1×10^4 (10, 18, 27, 30). The use of the minimum amount of AAV particles per cell for each AAV serotype allows the determination of the AAV NAb titer using the most sensitive assay but compromises the comparison of NAb titers between all AAV serotypes. Moreover, the high number of AAV particles/cell in addition to the full/empty AAV particle ratio of the AAV vector preparation used compromises sensitivity and the reproducibility of the results obtained between different laboratories. It should be noted that this assay does not consider the potential antibody-dependent cellular phagocytosis (20) as a blocking mechanism of vector neutralization or the possible change in tissue targeting of AAV (18).

To overcome the above mentioned problems, an *in vivo* NAb assay has been developed by various investigators (10, 18, 34). In this assay, mice are infused with the serum sample immediately prior to intravenous infusion of the AAV vector expressing a secreted reporter gene. Since the level of transduction by the new AAV serotypes is superior to AAV2 in most *in vivo* applications a dose as low as 10^9 GC/animal can be used. Several secreted transgenes have been used to evaluate transduction including FIX and α-galactosidase (10, 18). A reduction in transgene expression of 50% or more when compared to control mice injected only with AAV vector is reported as positive for AAV NAbs. This assay is more sensitive than the *in vitro* assay as up to 29% of samples that were negative by the *in vitro* NAb assay scored positive for AAV NAbs (10, 18). The problem with this assay is that some of the monkeys that tested positive for AAV NAbs did not show a reduction in AAV-mediated gene transfer, suggesting the *in vivo* NAb assay is too sensitive (18).

The conclusions of these studies suggested that the *in vitro* NAb assay provides a better correlation with *in vivo* transduction in macaques than the *in vivo* NAb assay. Therefore the *in vitro* NAb assay has become the standard assay to evaluate clinical samples for the presence of AAV NAbs prior vector administration.

ANTI-AAV ANTIBODIES IN SEVERAL NON-PRIMATE SPECIES

Adeno-associated virus has been isolated from several tissues of non-primate animal species including rat, mice, sheep, bird, snake, cows, goat, and pig (2, 7, 24, 35–43), suggesting a natural exposure to AAV. Pre-existing Abs to AAV in these non-primate species were not thought to be a problem because endogenous parvoviruses were believed to be structurally distinct from primate AAVs. Analysis of serum from small and large non-primate species used as pre-clinical animal models has shown high rates of detectable levels of NAbs to several AAV serotypes found in both monkeys and human. Interestingly the prevalence of AAV NAbs is both AAV serotype and species specific (44). In horses, AAV5 is the most seroprevalent serotype with 100% of the samples testing positive for NAbs. In dogs, AAV6 is the most seroprevalent serotype with 100% of the samples positive for NAbs (28, 45). Interestingly, high levels of AAV6 NAbs are found in newborn puppies suggesting passive immunization from colostrum and breast milk (45). A limitation of this study is that the NAb analysis was performed in the same dog breed and colony and no confirmation of AAV6-specific antibodies by ELISA or Western blot was performed. Rapti et al. (28), using mainly pooled sera also found high levels of NAbs to AAV6. In this study, IgG was purified from pooled sera and blocking of AAV transduction was demonstrated, although at much lower titer than whole serum, suggesting that some other factors may play a role in AAV neutralization. A recent study, also in dogs, showed the high binding capacity of serum protein human galectin 3 binding protein to AAV6 inducing the formation of aggregates and hampering vector transduction (46). In pigs, AAV5 is the most seroprevalent AAV serotype with 100% of the samples testing positive for NAbs; seroprevalence of NAbs to other serotypes like AAV1, AAV2, and AAV8 was close to 50%. AAV6 was the least seroprevalent serotype in pigs. *In vivo* testing of serum from these species confirmed the neutralizing activity of these antibodies (28, 44). These studies highlighted the importance of AAV NAb screening of all animal models used to evaluate *in vivo* AAV performance for pre-clinical gene therapy.

ANTI-AAV ANTIBODIES IN PRIMATES AND HUMANS

Prevalence of antibodies to AAV in humans was first reported in early 60s and 70s and mainly focused on AAV1 and AAV2, the only serotypes available at that time. Frequencies of antibodies ranged from 30 to 80% among human populations (19, 21–23). Recently, more than 100 natural AAV variants have been isolated from human and non-human primates tissue specimens (2, 6, 41). In pre-clinical models AAV7, AAV8, AAV9, and AAVrh.10 have emerged as promising candidates for gene therapy quickly becoming the most commonly used AAV serotypes in pre-clinical research. Several studies addressed the prevalence of NAbs to these new serotypes and compared them to previously described serotypes (25, 29, 30, 32, 47). In all human populations studied, which included samples from 10 countries and four continents (America, Europe, Africa, and Australia), prevalence of NAbs to AAV2 ranged from 60 to 30% and was significantly higher than the prevalence of NAbs to AAV7, AAV8, and AAV9 serotypes which ranged from 30 to 15%. Although the seroprevalence of NAbs to AAV1 was lower than that for AAV2, it was still higher than AAV7, AAV8, and AAV9 in most regions. It is worth noting that significantly higher frequencies of NAbs to all AAV serotypes were observed in Africa (25). Interestingly, the closely related AAV4 and AAVrh32.33 serotypes showed the lowest seroprevalence with less than 2% of the population testing positive worldwide (25, 35). Although these properties were encouraging for the development of AAVrh32.33 as vector for gene therapy, recent studies have shown this serotype induces a remarkable strong T cell mediated immune responses to the transgene product, similar to that induced by adenovirus, considerably limiting gene expression duration (48). Interestingly, when the seroprevalence to AAV serotypes was analyzed in non-human primates, including rhesus macaques, cynomolgus macaques, Japanese macaques, pig tail macaques, squirrel monkeys, chimpanzees, and baboons, AAV7, AAV8, AAV9, and AAVrh10 were the serotypes with the highest seroprevalence, with NAb frequencies up to 100% (6, 49, 50). Contrary to the situation in humans, AAV2 became the AAV serotype with the lowest seroprevalence (51). This finding is supported by

the fact that AAV7, AAV8, and AAVrh10 were originally isolated from rhesus macaque tissues (2, 7). The high seroprevalence of these AAV serotypes in non-human primates presents an important challenge in the evaluation of vector performance in this animal model and an exhaustive screening of monkey colonies for pre-existing NAbs is required.

Binding AAV antibodies may also play an important role in the clinical application of AAV vectors. AAV particles opsonized by non-NAbs may be taken up by cells of the immune system such as dendritic cells and macrophages through Fc receptors which may lead to the development of inflammatory responses. Frequencies of binding antibodies in humans were close to 70% for both AAV1 and AAV2, 45% to both AAV6 and AAV9, and 38% for AAV8 (30). Although the frequencies to binding antibodies were higher than frequencies of NAbs, the relative frequency between AAV serotypes remained the same with AAV1 and AAV2 being the most prevalent AAV serotypes.

Several studies have tried to associate binding antibodies to NAbs with the goal of using binding antibodies as an indirect measure of NAbs (10, 18). Although these studies found a correlation between both types of antibodies, almost 20% of the samples with high binding antibodies had very low or no detectable NAbs. The significance of the Fc interactions to host cells/proteins has been demonstrated in a large animal study in which AAV8 was administered systemically in a non-human primate model with pre-existing AAV8 antibodies. The study showed that AAV vectors were redirected from the liver to the spleen where they were stably sequestered by follicular dendritic cells (18). Although pre-existing humoral immune responses to the AAV capsid do not always correlate with the presence of AAV capsid deposition in the spleen this finding raised concerns of the safety profile of systemic AAV administration.

CROSS-REACTIVITY OF THE ANTIBODY RESPONSE

An important feature of the humoral response against AAV is the breadth of the response. If a subject is positive for antibodies to a specific AAV serotype, what is the likelihood that this subject will also be positive for other AAV serotypes? Several studies have analyzed the specificity of this response and found a strong link in seropositivity toward distinct AAV serotypes. The majority of the subjects with NAbs and/or binding antibodies to AAV7, AAV8, and AAV9 also had antibodies to AAV2. Conversely, only a few subjects with NAbs and/or binding antibodies to AAV2 had also antibodies to AAV7, AAV8, and AAV9 (25, 30).

Most subjects enrolled in the recent phase 2 clinical trial of AAV1 vector expressing α1-antitrypsin developed a NAb response specific to AAV1 with minimal or no cross-reactivity to AAV2, AAV7, and AAV8 serotypes (15, 52). Only those subjects with low pre-existing NAbs to AAV1, AAV2, AAV7, and AAV8 developed a highly cross-reactive NAb response to AAV2, AAV7, and AAV8 when injected with AAV1. One hypothesis to explain the difference in the breath of the AAV NAb response between naturally exposed and gene therapy-treated subjects is that subjects with co-occurrence of NAbs against multiple AAV serotypes may be the result of multiple infections with various AAV types. Moreover the propensity of AAVs to evolve through various mechanisms of molecular evolution (6) would lead to the generation of NAbs directed against a wide spectrum of homologous antigens. The fact that AAV2 is the serotype with the highest prevalence in the human population and also with the highest NAb titer indicates that the initial and most frequent exposure to AAV in humans occurs with an AAV2 or an AAV2 like serotype (31).

This data would also indicate that subjects naive for AAV NAbs would be the preferred candidates for gene therapy if a second administration of AAV vector is required. These subjects may develop a NAb response specific to the first AAV serotype injected, and a second administration with a different AAV serotype should not pose a problem because of the narrow breath of the AAV NAb response generated. Subjects with pre-existing NAbs to multiple serotypes with a AAV NAb titer low enough ($\leq$1/10) not to interfere with the first vector administration would develop a strong and broadly cross-reactive AAV NAb response that may block a second vector administration with another AAV serotype.

AGE, GENDER, HEALTH STATUS, AND GEOGRAPHICAL REGION DEPENDENT PREVALENCE OF NAbs

When and how humans are exposed to AAV for the first time is still not clear, although several studies suggest that this may happen early in life as serum-circulating binding and NAbs have been reported in children (44, 53). This is especially important for the treatment of many genetic diseases that manifest early in infancy and therefore early gene therapy treatment with AAV is beneficial. NAbs to AAV2 were detected in almost 60% of the infants and to AAV8 in 36% of the infants right after birth. Prevalence of NAbs to both AAV serotypes declined during the first year of life due to the drop in maternal antibody levels. A continuous increase in the prevalence of AAV NAbs after 1 year of age with a peak at 3 years of age suggests this age window is the time of the first exposure to natural AAV infections, and closely models that of adenovirus infection (22). Therefore the best age for an early gene therapy intervention with an AAV vector may be right prior to 1 year of age. The higher prevalence of AAV2 NAbs over AAV8 in early childhood also confirms the hypothesis that AAV2 or an AAV2 like serotype is the first AAV to which humans are exposed.

Prevalence of AAV NAbs can vary depending on the geographical origin of the population studied. While the prevalence of AAV1 NAbs in Africa and China is close to 50–70%, in other countries like Belgium, Greece, Italy, and USA it is only 20–30% (25, 54). Overall the prevalence of AAV NAbs seems to be higher in developing countries. It remains unclear whether living conditions, population density, hygienic conditions, different level of health care, MHC background, or method of detecting AAV NAbs are involved in this phenomenon. Interestingly, gender is another factor that influences the prevalence of AAV NAbs. Women have a significantly higher prevalence of AAV1 NAbs than men (54). The health status of the target population may also impact the prevalence of AAV NAbs, especially in those subjects with a compromised immune system. These subjects had a lower prevalence of AAV NAbs when compared to the healthy population (32, 55).

STRATEGIES TO OVERCOME AAV NAbs IN AAV-MEDIATED GENE THERAPY

The presence of AAV NAbs in both animals and human subjects has necessitated the development of strategies to generate new

AAV variants with limited or reduced recognition by NAbs. One approach has focused on the identification of the immunogenic domains on the AAV capsid and their subsequent modification to avoid recognition by NAbs. Such targeted engineering of AAV capsid requires knowledge of the antibody recognition site, the epitope. At present, only epitopes to AAV2 and AAV8 serotypes have been identified (56, 57). In this regard, it should be noted that strategies to determine epitopes that are conformational are likely to be more challenging. In spite of these difficulties, it has been demonstrated that amino acid substitutions at sites 459, 493, 551, and 587 of AAV2 capsid confer escape from neutralizing human antisera. While modification of these epitopes provided a reduction in neutralization, it did not completely avoid it. Naturally occurring variants with different amino acid composition at these sites outperformed these mutants in terms of eluding NAbs (8).

An alternate approach is the development of an AAV capsid library generated by error-prone PCR that is subsequently screened in the presence of NAbs to select AAV variants resistant to neutralization. This technique, called directed evolution of AAV vectors, has been employed by Maheshri et al. to identify an AAV2 variant that carried the E12A, K258N, T567S, N587I, and T716A mutations which was 96-fold more resistant to neutralization by anti-AAV2 polyclonal serum than wild type AAV2 (9). A similar approach, but using human serum positive for AAV2 NAbs, identified an AAV2 variant containing R459K and N551D point mutations which was 5.5-fold more resistant to neutralization than wild type AAV2 (58).

The creation of these new AAV variants and the discovery new AAV serotypes (2, 7) with impressive transduction capabilities and distinct serological properties have provided scientists with the tools necessary to avoid undesirable NAbs to one AAV serotype by using a different AAV vector type. This approach has been shown to be effective in both small and large animal models (16, 59). Although this approach is viable for subjects with an AAV NAb response restricted to one serotype or with a cross-NAb titer equal or less than 1/10 (18) it may not be in those subjects with high titers of cross-reactive NAbs. To overcome this problem several strategies have been proposed:

(a) *Plasmapheresis.* Some investigators have explored the use of plasmapheresis to reduce the overall levels of AAV NAbs right before vector administration (10, 27). In this technique, blood is removed and separated into plasma and blood cells. Blood cells are returned to the body and plasma is disposed. An albumin solution is infused into the patient to replace the plasma volume removed (60). Studies using this technique have shown a two to threefold reduction in AAV binding and NAb titers after each sequential apheresis session (10, 18, 27). Although this approach may be very useful for subjects with low levels of broadly cross-reactive AAV NAbs it may still not be enough for those subjects with high levels of NAbs.

(b) Minimizing contact of AAV vector with NAb. Intravenous administration of AAV to target the liver exposes the vector to NAbs present in blood reducing significantly transgene copy number in liver resulting in undetectable levels of transgene expression (10, 11, 18). An approach focused on delivering the vector in the target organ and minimizing the exposure of the vector to circulating NAbs has been shown to be efficient with AAV8 in non-human primate studies targeting the liver via the portal vein (26). In this study investigators flushed the liver with saline, to remove blood and NAbs with it, before injection of the vector into the portal vein. Their results showed no significant impact on gene expression when animals had an AAV NAb titer of up to 1/28 and only partial reduction of gene transfer when the NAb titer was 1/56.

Another approach to minimize the contact of AAV vector with NAb includes targeting a tissue or organ via direct infiltration rather than via the circulation. This organ/tissue should have a proven record of stable and long term AAV-mediated gene expression. Muscle has been one of the candidate sites to test this approach. Investigators using non-human primates and an AAV8 vector have shown minimal impact on systemic gene expression in the presence of AAV8 NAbs as high as 1/320 when monkeys were injected directly into muscle (61). A similar approach has been used in clinical trials for A1AT deficiency and hemophilia B using AAV1 and AAV2 vectors respectively, demonstrating that high titers of AAV NAb did not prevent gene transfer (15, 52, 62). The retina has also been used as a target organ for AAV-mediated gene therapy for localized systemic expression of therapeutic proteins such us erythropoietin (63). As into the muscle, the subretinal space into which the vector is injected has reduced contact with blood; consequently pre-existing AAV NAbs in blood had a minimal impact of AAV-mediated gene transfer (64, 65). Intrathecal administration (i.e., direct injection into the cerebral spinal fluid) is another route of administration that minimizes contact of the AAV vector with blood. In recent mouse studies, AAV2 and AAV5 were injected intrathecally to transduce the central nervous system (66). These studies showed that the potentially debilitating effect of AAV NAbs could be partially overcome by direct administration of AAV to the target organ. The caveat associated with these alternative routes of administration is the immune response that is induced in these organs/tissues. For example, the muscle is a very immunogenic tissue and intramuscular administration, unlike intravenous administration, induces a strong humoral and cellular immune response to the AAV capsid and in some instances to the transgene product (15). Humoral and cellular immune responses to the AAV capsid and transgene product using these alternative routes of administration need to be carefully assessed to ensure that the lack of AAV interference with pre-existing NAbs is not eclipsed by the induction of a stronger immune response to the therapeutic product.

(c) Immunosuppression has been proposed by several groups as a method to lowering NAbs by reducing the number of cells producing antibodies. In these studies rituximab, a B-cell depleting antibody that targets the CD20 antigen and is used clinically, was able to reduce AAV2 and AAV5 NAbs in ∼30% of subjects to levels that for some subjects were under limit of detection (32). These data are consistent with results obtained in non-human primates using rituximab and cyclosporine, although in this study rhesus

macaques were injected systemically with an AAV vector and then NAb response were monitored (67). The caveat with pharmacological immunomodulation of the immune response is that the tolerogenic properties of AAV can be altered after this treatment and undesirable immune response to the transgene may be induced, as was reported for monkeys injected with AAV2 and carrying FIX as transgene (68). An immediate antibody response to the FIX was observed when the immunosuppression regimen was stopped after 10 weeks of vector administration. An antibody response to FIX was never observed in monkeys that did not receive immunosuppression.

In conclusion, a combination of alternative AAV types, route of vector administration with minimum contact with blood and techniques directed to lower AAV NAb by physical methods or pharmacological modulation of the humoral immune response may ultimately overcome the impact of pre-existing AAV NAbs in subjects who would otherwise not be eligible for AAV-mediated gene transfer therapy.

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Mapping the AAV capsid host antibody response toward the development of second generation gene delivery vectors

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The recombinant adeno-associated virus (rAAV) gene delivery system is entering a crucial and exciting phase with the promise of more than 20 years of intense research now realized in a number of successful human clinical trials. However, as a natural host to AAV infection, anti-AAV antibodies are prevalent in the human population. For example, ~70% of human sera samples are positive for AAV serotype 2 (AAV2). Furthermore, low levels of pre-existing neutralizing antibodies in the circulation are detrimental to the efficacy of corrective therapeutic AAV gene delivery. A key component to overcoming this obstacle is the identification of regions of the AAV capsid that participate in interactions with host immunity, especially neutralizing antibodies, to be modified for neutralization escape. Three main approaches have been utilized to map antigenic epitopes on AAV capsids. The first is directed evolution in which AAV variants are selected in the presence of monoclonal antibodies (MAbs) or pooled human sera. This results in AAV variants with mutations on important neutralizing epitopes. The second is epitope searching, achieved by peptide scanning, peptide insertion, or site-directed mutagenesis. The third, a structure biology-based approach, utilizes cryo-electron microscopy and image reconstruction of AAV capsids complexed to fragment antibodies, which are generated from MAbs, to directly visualize the epitopes. In this review, the contribution of these three approaches to the current knowledge of AAV epitopes and success in their use to create second generation vectors will be discussed.

Keywords: AAV vectors, antibody response, AAV capsid structure, antigenic epitopes, parvoviruses

INTRODUCTION

Adeno-associated viruses (AAVs) are a promising gene delivery vector system. They are small (~26 nm) non-enveloped viruses belonging to the *Parvoviridae*, are assembled with $T = 1$ icosahedral capsid symmetry, and package a 4.7 kb single-stranded (ss) DNA genome (1). There are over 100 AAV genomic isolates and 13 human and non-human serotypes described. These viruses have different transduction efficiencies in different tissues dictated by the capsid sequence (2). To date, no diseases have been associated with wild-type AAV infection. Further, AAVs can transduce both dividing and non-dividing cells and sustain long-term gene expression in non-dividing cells (3). All these properties make them desirable vectors for therapeutic gene delivery.

Recombinant AAV (rAAV) vectors, used in clinical trials, contain a desired transgene cassette flanked by two inverted terminal repeats (ITRs) instead of the wild-type viral genome flanked by these elements (4). In recent years, the AAV gene delivery system has been successfully utilized in several animal and human clinical trials. In an ongoing hemophilia B trial, therapeutic levels of Factor IX protein has been maintained in patients for over 2 years with only one infusion of an rAAV8 vector packaging this gene (5). In addition, rAAV2 vectors, encoding the retinal pigment epithelium-specific 65 kDa protein, improved vision in Leber's congenital amaurosis (LCA) patients, without any significant side effects (6–9). rAAV vectors have also been developed for the transduction of a variety of other cells in addition to liver and the eye, including, as examples: brain cells for the treatment of Parkinson's disease (10) and Canavan disease (11); skeletal muscle for the treatment of emphysema, lipoprotein lipase deficiency, and muscular dystrophy (12–14); and heart muscle for the treatment of heart failure (15). Significantly, in 2012, an AAV1 vector, encoding the lipoprotein lipase, was approved as a gene therapy treatment in Europe (16), heralding a new era for this vector system.

However, despite the above successes, several obstacles must still be overcome for full realization of the AAV vector system in patient care and treatment. One of the most important of these is pre-existing immunity. Serologic studies have shown that the majority of the human population has been exposed to wild-type AAVs (17–19). For example, the prevalence of anti-AAV antibodies in the human population has been reported to be ~40–70%, with the most reactivity against AAV2, the most studied and best characterized of the AAV serotypes. Although rAAV vectors used for gene delivery do not carry viral genes and are unable to drive viral protein synthesis, they are assembled from wild-type viral capsid shells; thus, the host immune response to the vector can be influenced by prior exposure to wild-type AAV. A pre-existing

antibody response against AAV can initiate an immune memory response, which could impede gene delivery. For example, neutralization effects from pre-existing antibodies have been reported to decrease transduction efficiency, even at low antibody titers (20–22). For this reason, in the recent gene delivery trial for hemophilia B, individuals with evidence of pre-existing AAV antibody immunity were excluded from participation (5). Thus, to develop the AAV vector system into a more practical and efficacious gene transfer system, it is important to understand how antibodies interact with the AAV capsid, especially to map dominant epitopes, both neutralizing and non-neutralizing. With sufficient information on the AAV antigenic structure, combined with data on capsid determinants of tissue tropism and transduction, it would be feasible to design a neutralization-escaping vector, which can evade the host antibody immune response while retaining desired tissue tropism and transduction efficiency.

Here we will briefly review the AAV capsid structure and what is known about the effects of its interaction with antibodies, and then discuss example approaches utilized for mapping capsid antigenic epitopes. The three most common approaches include: directed evolution, an indirect method for obtaining the antigenic information through the selection of AAV variants under antibody pressure; epitope searching, which utilizes peptide scanning, peptide insertion, or site-directed mutagenesis; structural biology, namely cryo-electron microscopy and three-dimensional (3D) image reconstruction (cryo-reconstruction), which directly visualizes the antigenic sites on the capsid by 3D reconstruction of capsid-fragment antigen binding (Fab) complexes.

AAV CAPSID STRUCTURE

The ~4.7 kb genome of the AAVs contains three open reading frames (ORFs), *rep*, *cap*, and *aap*, flanked by two ITRs (~145 kb). This genome is packaged into $T = 1$ icosahedral capsids that are ~26 nm in diameter. The *cap* ORF encodes three overlapping structural capsid viral proteins (VPs): VP1, VP2, and VP3, in a ratio of 1:1:10, which assemble the capsid. A total of 60 VPs assemble the capsid by 2-, 3-, and 5-fold symmetry-related interactions (**Figure 1**). The VP3, the major capsid component, is able to assemble the capsid as long as the assembly activating protein (AAP) encoded by the *aap* ORF, is present (23, 24). Three-dimensional structures have been determined for AAV1-AAV9, the clade and clonal isolate representatives of the over 100 genomic sequences known for the human and non-human primate AAVs (2), by either X-ray crystallography and/or cryo-reconstruction (25–32). In all these structures only the VP3 common sequence is ordered. While the AAVs have a sequence similarity that ranges from ~55 to 99%, they are structurally very similar (33). The VP topology consists of a conserved alpha helix (αA) and a eight stranded anti-parallel (βB-βI) β-barrel core with large inter-strand loops (**Figure 2A**) that form the exterior surface of the capsids. A comparison of the AAV2 and AAV4 structures, two of the most distantly related, identified nine common variable regions (VRs), designated VR-I to VR-IX (**Figures 2A,B**), located on the capsid surface at the top of the inter-strand loops (26, 34). The AAV capsid surface is characterized by depressions at the 2-fold axes (dimple), surrounding a cylindrical channel at the 5-fold axes (canyon), and protrusions surrounding the 3-fold axes (**Figure 1**). A wall or plateau is located between the depression at the 2-fold axis and surrounding the 5-fold channel, the "2/5-fold wall" (**Figure 1**) (35). The VRs contribute to local topological differences between the AAV capsid surfaces. For example, VR-II forms the top of the 5-fold channel; VR-IV, V, and VIII form the top of the 3-fold protrusion and VR-VI and VR-VII form their base; and VR-I, III, VII, and IX contribute to the 2/5-fold wall (**Figure 2B**). The VRs also dictate functional differences, including receptor attachment, transduction efficiency, and antigenic reactivity between the AAVs (26, 28, 30, 36–39).

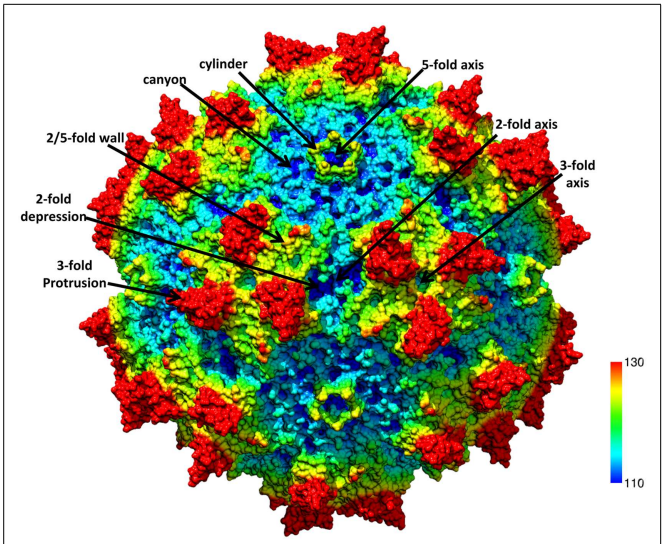

FIGURE 1 | The AAV capsid. Radially color-cued (from capsid center to surface: blue-green-yellow-red; ~110–130 Å) of the AAV1 capsid generated from 60 VP monomers (RCSB PDB # 3NG9). The approximate icosahedral 2-, 3-, and 5-fold symmetry axes are as well as the AAV capsid surface features are indicated by the arrows and labeled. This image was generated using the Chimera program (40).

AAV AND ANTIBODIES

All viral vectors are susceptible to the immune response from the host (41). The most detrimental immune threat that AAV vectors encounter soon after administration is the B-cell mediated antibody response (42, 43). Antibodies use their complementarity determining region (CDR), located on the end of Fab region, to interact with antigens by specific surface complementarities (44). This binding site, or "epitope," is generally located on the capsid surface of viruses. Antibodies against viruses may neutralize infectivity prior to viral attachment to host cell receptors, or post attachment; interfering with internalization or fusion at the cell surface, or during endosomal trafficking (45–48). Other antibody neutralization mechanisms include antibody-mediated phagocytosis, complement binding and activation, opsonization, and antibody-dependent cellular cytotoxicity (ADCC) of infected cells (49–52). As already stated above, in humans the prevalence of AAV antibodies in healthy individuals is high and ranges from ~40–70% depending on serotype. Previous reports indicate that the highest prevalence of anti-AAV immunoglobulin G antibodies in humans was for AAV2 (~60–70%) and AAV1 (~35–70%), followed by AAV9 (~50%), AAV6 (~50%), AAV5 (~40%), and

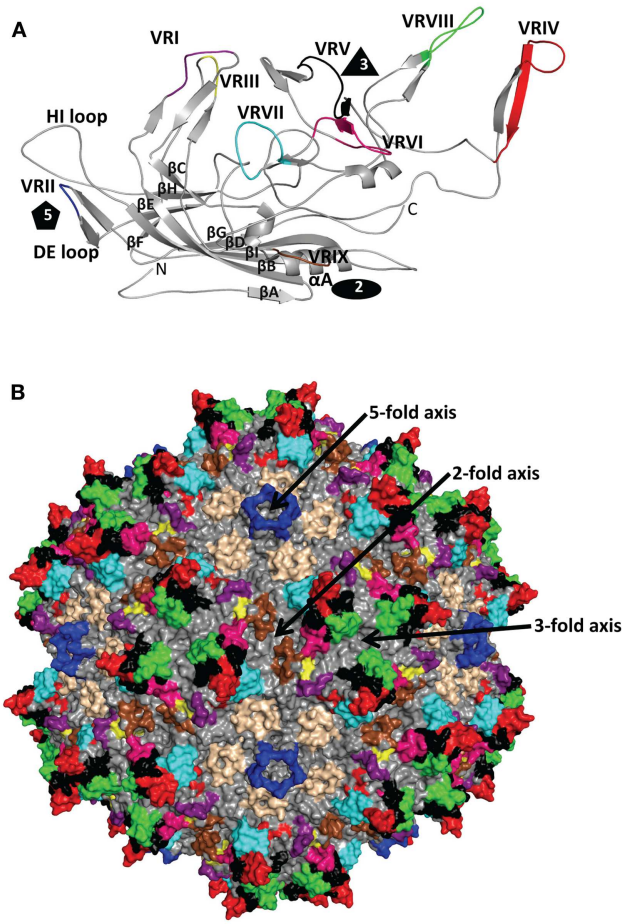

FIGURE 2 | AAV variable regions. **(A)** A ribbon diagram representation of the ordered overlapping VP3 monomer region of AAV1. The conserved β-barrel core motif (βBIDG-βCHEF, gray), conserved αA helix, DE loop (between βD and βE), HI loop (between βH and βI), VR-I to VR-IX [defined (26)] are colored; I: purple, II: blue, III: yellow, IV: red, V: black, VI: hot pink, VII: cyan, VIII: green, and IX: brown; and labeled. The approximate positions of the 2-, 3-, and 5-fold axes are indicated by the filled oval, triangle, and pentagon, respectively. The N and C labels are the N- and C-terminal ends of the ordered VP region, respectively. **(B)** The capsid surface of AAV2 with VR-I to VR-IX colored as in **(A)**. The approximate icosahedral 2-, 3-, and 5-fold symmetry axes are indicated and labeled as in **Figure 1**. Both **(A)** and **(B)** were generated with the PyMOL program (http://www.pymol.org).

AAV8 (~40%) [e.g., Ref. (17, 53)]. For the AAVs, neutralization mechanisms have been described for only three monoclonal antibodies (MAbs), A20 and C37-B against AAV2, which act at the post-entry steps and receptor attachment, respectively (54, 55), and ADK8 against AAV8, which acts at a post cell/pre-nuclear entry step (37).

Given the reported detrimental effects of anti-AAV antibodies on transgene expression, it is not surprising that the most successful applications of the AAV vector system have been in the eye and brain, immuno-privileged sites when vector is directly injected, or in patients with low or no anti-AAV antibodies titers prior to vector administration. Furthermore, since the AAV capsid structures are similar, different anti-AAV antibodies may cross-react, as has been observed, for example, between AAV2 and AAV3 that are highly similar at the sequence level (55), and recently for AAV1 and AAV5 which are disparate in at the sequence level (56). Boutin et al. (17) showed that patients with a positive AAV2 serum response were also seropositive for AAV1, AAV5, AAV6, AAV8, and AAV9 in 93, 52, 59, 57, and 58%, respectively, of the samples tested. Thus a new generation of AAV vectors is needed to circumvent the neutralization effects from pre-existing antibodies. The development of these new vectors will largely depend on available AAV antigenic and structure information. To obtain the antigenic information, three main approaches have been used. These are discussed below.

DIRECTED EVOLUTION

Without knowledge of AAV capsid biology, structure, or immunogenic sites, directed evolution serves as a strategy to generate neutralization-escaping AAV variants. It is a high-throughput molecular engineering procedure, which mimics natural evolution through iterations of genetic diversification under artificial selection pressure (57). For generating neutralization-escaping AAV variants, wild-type *cap* genes, from one or several AAV serotypes, are mutated to generate a large genetic plasmid library, which can generate numerous capsid variants through recombination during plasmid transfection. During viral infection with viruses arising from the recombination process, a selective pressure, in this case antibodies, is applied. Only the variants that can circumvent the antibody barrier presented to infect the desired cells will transduce and drive progeny virus synthesis. The successful variants are then recovered and amplified for the next round of selection. After several cycles of selection, additional mutagenesis can also be introduced before further selection to increase viral fitness (58). Comparison of the capsid sequences of the final resulting variants and the wild-type input viruses provides the antigenic information and the effect of the changes on tropism and transduction efficiency. Generally, there are two strategies to create the genetically diverse library, one is using error-prone mutagenesis to randomize the capsid DNA sequences; the other is to shuffle the DNA sequences of different AAV serotypes.

ERROR-PRONE MUTAGENESIS

By a "sloppy" polymerase chain reaction (PCR), random point mutations can be introduced into an ORF at a certain rate. Tuning the PCR conditions can also introduce a different number of mutations into the target gene sequence (59). Perabo et al. (60) used this approach to generate an AAV2 *cap* mutant library for directed evolution under human sera selection. Approximately, 70% of the neutralization-escaping variants obtained contained point mutations clustered on the external face of the capsid at the 3-fold protrusion (**Figure 1**). The two most frequently selected mutations were amino acids 459 and 551 (**Table 1**) located in AAV VR-IV and VR-VII, respectively (**Figure 2**). The variant with the best neutralization escape capability carried the double mutation R459K/N551D. The single (R459G or N551D) and the double (R459K/N551D) mutant variants had comparable genome packaging, infectivity, and particle titers to wild-type virus. However, the N_{50} value, the amount of serum required to decrease

Table 1 | Antigenic sites identified by polyclonal antibodies.

Antibody sample	Method	Residues	Reference
Rabbit anti-AAV2 serum	Directed evolution	12, 42, 117, 152, 180, 258, 418, 493, 567, 587, 713, 716	Maheshri et al. (61)
Human serum	Directed evolution	459, 551	Perabo et al. (60)
Human serum	Peptide scanning	17–28, 113–124, 241–260, 305–356, 401–420, 443–460, 473–484, 697–716	Moskalenko et al. (62)
Human serum	Peptide insertion	534, 573, 587	Huttner et al. (63)
Human serum	Site-directed mutagenesis	471, 497, 498, 531, 548, 550, 586, 587, 705, 708	Lochrie et al. (38)
Human IVIG	Site-directed mutagenesis	264, 265, 269, 471, 491, 497, 498, 502, 527, 531, 532, 544, 550, 574, 586, 705, 706, 708	Lochrie et al. (38)

transduction by 50%, for R459G, N551D, and R459K/N551D were 4.1-, 3.3-, and 5.5-fold higher, respectively, than the corresponding N_{50} value obtained for the wild-type AAV2.

Maheshri et al. (61) improved this strategy by combining the error-prone PCR with a staggered extension process, which utilized a short time polymerase-catalyzed extension (64) to produce AAV2 variants. Mutant capsids were next selected for infectivity in HEK293 cells in the presence of a neutralizing rabbit anti-AAV2 serum. Nine mutants, which contained different combinations of mutations at amino acid positions 12, 42, 117, 152, 180, 258, 418, 493, 567, 587, 713, or 716, had neutralizing titers that were 3-fold higher than the wild-type virus (**Table 1**) (61). All the successful variants carried a T716A mutation. This residue is located on the capsid surface at the 2/5-fold wall next to VR-IX (**Figures 1** and **2B**). One variant, r2.15, had a 96-fold improvement in transduction compared to wild-type AAV2 and the ability to mediate moderate gene delivery at a low 1:2 serum dilution. This variant has two mutations not present in the others: T567S and N587I. T567S is in a minor epitope of the A20 MAb previously identified by pepscan and peptide competition (55). Residue 587 is in VR-VIII (**Figure 2**) and it is located proximal to residues involved in heparan sulfate proteoglycan (HSPG) receptor binding in AAV2 (65, 66).

Maersch and colleagues (67) also used error-prone PCR to establish an AAV2 *cap* library with the mutations focused only on amino acids located in the 3-fold region previously identified as being immunogenic: 449, 458, 459, and 551 (60), and 493 (61). The directed evolution was carried in HEK293 cells under the selection of neutralizing human serum. The resistance to neutralization of six resulting variants was compared to those of wild-type AAV1, AAV2, and the double mutant R459K/N551D generated by Perabo et al. (60). Two of the variants, with substitutions at 459, 493, and 551, outperformed the best variants from the previous selections by Maheshri et al. and Perabo et al. (60, 61). This observation indicated that fine tuning single amino acid types on the capsid surface can dramatically change the immunogenicity of an epitope. Residue 493 is located in VR-V, which as previously mentioned, together with VR-IV and VR-VIII form the top of the protrusions surrounding the 3-fold axis. Thus the data from error-prone mutagenesis provides indications that the 3-fold region is immuno-dominant in the AAVs.

DNA SHUFFLING

DNAse digestion followed by polymerase ligation can generate a chimeric capsid variant from different AAV serotypes. Grimm et al. (68) applied this method to randomly combine the cap sequences from AAV2, AAV5, AAV8, AAV9, caprine AAV, avain AAV, and bovine AAV prior to selection. They first selected capsid variants capable of human liver cell transduction and then selected in the presence of pooled human anti-sera (Intravenous immunoglobulin, IVIG). This strategy created a single chimera named AAV-DJ. The amino acid sequence for AAV-DJ was closely related to AAV2, AAV8, and AAV9 at 92, 88, and 85% identity, respectively. Interestingly, the IVIG selection generated this variant with higher homology to AAV8 compared to variants selected for human liver cell transduction alone, which were mostly similar to AAV2. This implies that the IVIG selection pressure eliminated variants with AAV2 epitope(s) from the resulting clones, consistent with the higher percentage of anti-AAV2 sero-prevalence in the human population compared to other AAV serotypes. AAV-DJ was capable of transducing mice passively infused with IVIG prior to infection at low (4 mg) IVIG dose to similar levels as parental AAV8 and AAV9 while AAV2 transduction was abolished in either high (20 mg) or low (4 mg) IVIG dose. The AAV-DJ was also inhibited at the high IVIG dose.

Another group, Koerber et al. (69), also generated chimeric AAV capsids through DNAse I digestion and polymerase ligation which displayed neutralization escape capability. The library was established from the *cap* ORF of AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, and AAV9. The selection was carried once in HEK293 cells to optimize just for infectivity without any antibody pressure. However, surprisingly, some IVIG neutralization-escaping variants were generated. Four of the final seven chimeric capsids obtained showed improved IVIG neutralization resistance compared to the parental serotypes despite ~90% sequence similarity. Three of the variants that had higher resistance than the parental AAV2 had a ~80 aa stretch from AAV9 or a V709I mutation and the last C-terminal 19 aa from AAV6. The C-terminal stretch contained a previously described AAV2 epitope, 697–716 (AAV2 numbering, see below) (62). One of the chimeras, cB4, was similar to AAV1/6 at the sequence level, and had >400-, 8-, and 2-fold resistance to IVIG neutralization compared to AAV2, AAV1, and AAV6, respectively. While it was difficult to define which region

of this chimera dictated its strong immune evading ability, it contained a Y706H substitution. Both the Y706H and V709I changes would be located in the 2/5-fold wall.

Other examples of chimeras selected for infectivity without antibody selection which display increased antibody neutralization resistance have been reported. Li et al. (70) used iterative cycles of infection to select variants with hamster melanoma cell tropism started from a shuffled library constructed from AAV serotypes 1–9, except serotype 7. In this study, a specific chimeric AAV variant was isolated, containing residues 1–409 from AAV1, 410–450 from AAV8, 451–704 from AAV2, and 705–736 from AAV9. This chimera, chimeric-1829, did not show any cross-reactivity to antisera from mice immunized with AAV1, AAV8, and AAV9. The sera from mice immunized with AAV2 had some cross-reactivity to chimeric-1829, but the neutralizing antibody (Nab) titer was 25-fold lower than the titer for wild-type AAV2 (70). In another study, Yang et al. (71) used DNA shuffling and *in vivo* selection to generate a muscle-cell targeting AAV chimera from serotypes 1–9 with a different antigenic reactivity compared to the parental serotypes. The mutant, M41, assembled from AAV1, AAV6, AAV7, and AAV8 sequences, showed a higher resistance to IVIG neutralization compared to AAV2. At a 1:64 dilution of IVIG, AAV2 infectivity decreased to ~33%, whereas M41 infectivity still remained at 83% compared to controls without IVIG incubation (71). The wild-type AAV8 infectivity remained at ~94% under these conditions, suggesting that the sequence of M41 derived from AAV1, AAV6, and AAV7, not those from AAV8, resulted in the increased susceptibility to IVIG recognition. This observation is consistent with the fact that residues 410–450 contributed to M41 by AAV8 are mostly internal in the capsid and form core β-strand regions of the VP structure.

EPITOPE SEARCHING

Rather than viral evolution in the presence of antibodies to obtain information on the antigenic regions of the capsid, epitope searching focuses directly on the interaction(s) between antibodies and peptides generated from the capsid protein sequence to map possible epitopes. This approach utilizes three main strategies, peptide scanning, peptide insertion, and site-directed mutagenesis.

PEPTIDE SCANNING

Moskalenko et al. (62) scanned the entire AAV2 capsid amino acid sequence for potential epitopes using a total of 91 15-mer peptides that overlapped by five amino acids. The peptides were tested for their ability to inhibit capsid binding by AAV2 neutralizing human serum samples in an ELISA assay in which the peptides were applied to an AAV2 capsid coated ELISA plate. This study identified several overlapping peptides regions, two within the VP1u sequence, 17–28 and 113–124, and six within the common VP3 sequence, 241–260, 305–356, 401–420, 443–460, 473–484, and 697–716 (**Table 1**) (62). As already stated, there is no structure available for the VP1u. The VP3 sequences are localized to βB, strands βD-βE and the intervening loop that forms the 5-fold channel including VR-II, strands βF-βG, the outer finger of the 3-fold protrusion that contains VR-IV, a strand region of the GH loop, and the 2/5-fold wall that contains VR-IX, respectively, in the VP3 crystal structure. The peptides containing residues 305–356, 401–420, 443–460 were considered the core neutralizing peptides. A number of these peptides are located in VP regions that are inside the capsids, and thus their ability to block capsid binding by human serum likely reflects the polyclonal nature of the human serum, which must include antibody responses to denatured VP regions or fragments.

Wobus et al. (55) also used a peptide mapping strategy to determine the epitopes of three mouse MAbs, A1, A69, and B1 previously reported to react against AAV2 (72, 73). An AAV2 *cap* gene fragment phage display library was screened with the antibodies and positive clones were scanned for binding by the antibodies, as overlapping 15-mer peptides on a membrane, and further confirmed by peptide competition in a Western blot. Linear epitopes were mapped for A1 (residues 123–131) within VP1u, A69 (residues 171–182) in the VP1/VP2 region and for B1 (residues 726–733) at the C-terminus of VP3. Consistently, these antibodies react against denatured capsids. The epitopes for three conformational AAV2 directed antibodies, A20, C37-B, and D3 (55) were also identified through a similar strategy. Overlapping 10-mer peptides (covering the AAV2 capsid sequence) detected by the antibodies were confirmed by an ELISA assay. Multiple peptides were identified for these antibodies consistent with their recognition of the assembled capsid, although C37-B and D3 were also capable of recognizing the VPs (55). The epitopes proposed for the three MAbs were: 272–281, 369–378, and 566–575 for A20; 492–503 and 601–610 for C37–B; and 474–483 for D3 (**Table 2**). The A20 epitope residues are mostly located below surface loops containing VR-I (for 272–281), the HI loop (for 369–378), and VR-V (for 566–575); the C37-B epitope peptides are located in VR-V (492–503) and buried at the 3-fold axis (601–610), and the D3 epitope is buried close to the 3-fold axis (474–483) (**Figure 2B**). As previously mentioned, the A20 MAb neutralizes virus infection at a post cell entry step and the C37-B antibody inhibits HSPG receptor attachment by AAV2. The D3 antibody is non-neutralizing, which is consistent with the mostly buried location of its mapped epitope.

PEPTIDE INSERTION

Wobus et al. (55) attempted to further confirm the binding sites for the A20, C37-B, and D3 antibodies as well as another antibody named C24-B using mutant AAV2 capsids onto which a 14 amino acid integrin binding ligand, L14, had been inserted. Prior to the availability of the AAV2 capsid structure, Girod et al. (74) utilized the 3D structure of canine parvovirus (75) to generate a 3D homology model for AAV2, which was used for predicting potential capsid surface sites onto which this peptide could be inserted for re-targeting AAV2 (74). Six such sites, 261, 381, 447, 534, 573, and 587, were identified. Testing of the antibody binding properties of these insertion mutants with the A20, C37-B, D3, and C24-B antibodies showed that the insertions at 261, 381, 534, and 573 decreased A20 binding; at 534, 573, and 587 decreased C37-B binding; at 261, 381, 534, and 573 decreased D3 binding; and at 534, 573, and 587 decreased C24-B binding (**Table 2**). These insertion points did not overlap with the epitopes predicted based on peptide scanning data, except for residue 573 for A20 (55). For the A20 and C37-B MAbs, a number of

Table 2 | Antigenic epitopes identified using monoclonal antibodies.

AAV	MAb	Method	Residues						Reference
			200–299	300–399	400–499	500–599	600–699	700–731	
AAV1	4E4	Cryo-EM			456–459, 492–498				Gurda et al. (36)
AAV1	5H7	Cryo-EM			494, 496–499	582, 583, 588–591, 593–595, 597			Gurda et al. (36)
AAV2	A20	Cryo-EM	253, 254, 258, 261, 262, 264	384, 385		548, 556	658–660	708, 717	McCraw et al. (39)
		Peptide scanning	272–281	369–378		560–573			Wobus et al. (55)
		Peptide insertion	261	381		534, 573			(55, 63)
		Site-direct mutagenesis	263, 264	384, 385		548		708	Lochrie et al. (38)
	C37-B	Cryo-EM			492–498,	585–589			Gurda et al. (36)
		Peptide scanning			493–499	500–502	601–610		Wobus et al. (55)
		Peptide insertion				534, 573, 587			(55, 63)
	D3	Peptide scanning			474–483				Wobus et al. (55)
		Peptide insertion	261	381		534, 573			Wobus et al. (55)
AAV5	3C5 site A	Cryo-EM	254–261	374, 375	483, 485–492, 494, 496, 499	500, 501			Gurda et al. (36)
	3C5 site B	Cryo-EM	246			530, 532–538	653, 654, 656, 657	704–708	Gurda et al. (36)
AAV8	ADK8	Cryo-EM				586–591			Gurda et al. (37)

the sites do overlap with or are close to epitope regions mapped using site-directed mutagenesis and structural biology as discussed below.

Huttner et al. tested the ability of these six insertion mutants to evade binding by human serum samples in an ELISA assay (63). The A20 and C37-B antibodies were used as positive controls. Insertion mutations at amino acid positions 534 and 573 reduced human anti-sera binding, in 19/29 tested samples, by up to 30% compared to the wild-type AAV2. Both of these residues are buried inside the protrusions surround the 3-fold axis. Thus their negative impact on serum binding is likely due to disruption of the surface loops that assembles the protrusions suggested above to be important for antigenic reactivity for the AAVs. The mutant with an insertion at position 587 only slightly impaired serum binding in ELISA assays, but was able to transduce Hela cells in the presence of human serum. The inserted L14 ligand also enabled this mutant to infect B16F10 cells, a cell line which is non-permissive to the parental AAV2, in the presence of human serum. These observations are consistent with the fact that residue 587 of AAV2 is located in a capsid region involved in immunogenicity, e.g., the C37-B epitope, and cellular attachment, e.g., proximity to the HSPG binding site (65, 66).

SITE-DIRECTED MUTAGENESIS

The best example of the use of site-directed mutagenesis for antigenic epitope mapping is provided by the study by Lochrie et al. (38). This group used the crystal structure of AAV2 (32) to identify sites for mutations on the capsid surface at amino acid positions predicted to be potential antigenic sites based on the docking of a murine IgG2a. All the mutants (57 alanine substitutions, 41 non-alanine substitutions) were screened for binding and neutralization ability with A20, three individual human serum samples, and IVIG. The mutated positions that decreased neutralization by A20 were 263, 264, 384, 385, 548, and 708. These residues are different to the amino acid stretches mapped by Wobus et al. (55) and Hunter et al. (63), but are located structurally proximate to these residues and are all clustered on the 2/5-fold wall (**Table 2** and **Figure 1**). The three human sera and IVIG screening identified several epitopes (**Table 1**) due to the complexity of the polyclonal response being tested. Different mutants showed resistance to different serum, and the mutations that resulted in neutralization escape from the individual serum and IVIG were located over a capsid region that was three times larger than an average Fab epitope footprint and spanned the 3-fold protrusions as well as the 2/5-fold wall. In addition, the ability to escape from human sera

and IVIG differed for some mutants. For example, mutant R471A and mutant N587A were both resistant to all three tested human sera, but mutant N587A was not resistant to IVIG neutralization. Significantly, two A20 neutralization escape mutants, E548A and V708A, also escaped neutralization by these sera, showing similarity in the murine and human immune response to AAV2. In addition, AAV2 V708 is positionally equivalent to or proximal to the V709 position in the chimera generated by Koerber et al. which when mutated to an isoleucine (V709I) improves resistance to IVIG neutralization (69).

STRUCTURE-BASED APPROACH

Cryo-reconstruction is a powerful technique for studying the structures of macromolecular complexes, including viruses and their complexes with receptors and antibodies. This method has thus been applied for the study of AAV capsids bound to Fabs, generated from MAbs, toward 3D characterization of the AAV capsid antigenic structure. Purified Fabs and AAV samples are mixed and incubated prior to vitrification on an electron microscope grid. For cryo-reconstruction, a large number of 2D projections of the sample vitrified in native state, at different orientations, are combined and processed to generate a 3D image reconstruction. Current resolutions for AAV:Fab complex structures range from subnanometer to ~20 Å. For identification of the antibody footprint, available atomic structures for the AAV capsids (35) and homology models for the Fabs are fitted into the reconstructed density map, in an approach termed pseudo-atomic model building, to provide the information on interacting sites. Below we review the current AAV:Fab complexes and the antigenic sites arising from these studies.

CRYO-RECONSTRUCTION OF AAV1/6:Fab COMPLEXES

AAV1 and AAV6 differ by 6/736 VP1 residues with 5/6 of them located within the VP3 common region, are cross-reactive, and belong to the same antigenic clade A (2), yet they differ in their cellular tropisms pointing to a key role for the specific amino acid differences in dictating these properties. The muscle tropism of AAV1 has made it an attractive vector for several gene delivery applications [see clinicaltrials.gov and (76)] and like AAV2, epidemiological studies show a high level of pre-existing anti-AAV1 immune response in the general population (17, 53). Efforts to structurally map the antigenic structure of AAV1 have included the cryo-reconstruction of AAV1 complexed with Fabs from two neutralizing mouse MAbs, AA4E4.G7 (4E4) and AA5H7.D11 (5H7) (36, 56). These structures were determined to ~12 and ~23 Å resolution, respectively. The proposed epitopes for the Fabs are residues 456–459 (in VR-IV) and 492–498 (in VR-V) for 4E4 and residues 494, 496–499 (on VR-V) and 582, 583, 588–595, and 597 (on VR-VIII) for 5H7 (**Table 2**). A structure of AAV6 complexed with the 5H7 Fab, determined to ~15 Å resolution, identified a similar footprint on the capsid. These epitopes are located on the 3-fold protrusions assembled from VR-IV and VR-VIII from one VP monomer and VRV from a neighboring VP. The binding of the Fabs occur in different orientations. 4E4 binds to the "outer side" of the protrusion, with the long axis of Fab toward and across the 2-fold axis. Steric hindrance limits the binding of this Fab to just one at a time across the 2-fold axis, thus the occupancy is 0.5. 5H7 binds on the "inward facing side" of the 3-fold protrusion with its density centered at the 3-fold axis; hence, on average, only one Fab can bind to a group of three protrusions, resulting in occupancy of 0.3. Binding and transduction studies suggested that these two antibodies neutralize infection by either competing with cell surface receptor attachment or inhibition of a step post cellular entry (56).

CRYO-RECONSTRUCTION OF AAV2:Fab COMPLEXES

For AAV2, which has broad tissue tropism and has been the vector most often used for clinical gene delivery applications (76), the binding sites for the A20 and C37-B antibodies have also been mapped by cryo-reconstruction (36, 39). These structures were determined to 8.5 and ~11 Å resolution, respectively. The A20 footprint, determined by several approaches including pseudo-atomic model building, includes residues 253, 254, 258, 261–264, 384, 385, 548, 556, 658–660, 708, and 717 (**Table 2**) (39). These residues had some overlap with those previously described based on peptide insertion as well as site-directed mutagenesis (see above) but extended the footprint to include additional residues (**Table 2**). As stated above, when discussing the A20 epitope mapped by site-directed mutagenesis, there was no overlap to the footprint predicted by peptide scanning, but the residues are within the same capsid region. Significantly, the binding site included residue contributions from symmetry-related VP monomers, confirming the conformational nature of the A20 epitope. The residues are located in AAV VR-I and VR-III and the HI loop from one VP monomer, and VR-VII and VR-IX from a second VP monomer. The AAV2 C37-B footprint, based on a pseudo-atomic model built into the cryo-reconstructed density map using the AAV2 crystal structure and the structure of a generic Fab, includes residues 492–498 (on VR-V) from one VP monomer and 585–589 (on VR-VIII) from another VP monomer (**Table 2**) (36). As already stated above, this region of the 3-fold protrusion overlaps with the AAV2 HSPG binding site. This epitope overlaps with binding residues determined based on peptide scanning and insertion (**Table 2**).

CRYO-RECONSTRUCTION OF AAV5:Fab COMPLEXES

AAV5 is one of the most divergent AAV serotypes with respect to sequence and structure and is classified as a clonal isolate based on antigenic non-cross-reactivity with other AAVs. As with the other AAVs, efforts are underway to characterize its antigenic structure because human sera also show pre-existing reactivity. While a number of mouse MAbs have now been generated against the AAV5 capsid (56, 77), an antigenic footprint has only been characterized for one, BB3C5.F4 (3C5), based on a structure reconstructed to ~16 Å resolution (36). The 3C5 MAb is non-neutralizing, and the observation that both the variable and constant regions of its Fab contact the capsid was suggested as being possibly due to it being an affinity immature antibody (36). This is because the MAb was generated only 4 days after AAV5 capsid immunization of a mouse that had been previously immunized with AAV1 (56). The Fab density covered the majority of the capsid surface, with the exception of the 3-fold axis. Pseudo-atomic model building into the reconstructed complex density identified two contact regions designated site A and site B with the variable region being better accommodated in site B. This site

B region extended from the 2/5-fold wall toward the 5-fold axis and included residues 246, 530, 532–538, 651, 653, 654, 656, 657, 704–708 (**Table 2**). These residues are structurally located in VR-I, VR-VII, the HI loop, and VR-IX. Interesting, the site B footprint is similar to that mapped for the A20 MAb against AAV2 (**Table 2**) (38, 39, 55), despite AAV2 and AAV5 being highly antigenically divergent (2).

CRYO-RECONSTRUCTION OF AAV8:Fab COMPLEXES

AAV8 has shown great promise as a liver tropic vector and is currently being utilized as the gene delivery vector in a clinical trial for hemophilia B (5). While the patients selected to receive the Factor IX gene had little or no pre-existing antibody response against AAV8, these patients have since developed an antibody response and as such a repeat administration would require the use of an alternative serotype or AAV8 variant with altered antigenic reactivity. The only information on the antigenic structure of AAV8 has been provided by a cryo-construction of this serotype complexed with a neutralizing antibody, ADK8 (23, 37). This structure was determined to 18.7 Å resolution (37). The AAV8 crystal structure and that of a generic Fab were docked into the reconstructed density to create a pseudo-atomic model of the complex. The footprint predicted from this model was confirmed by mutagenesis, biochemical, and *in vitro* assays to be residues 586–591 located in VR-VIII. This region is located on the inner face of the protrusions facing the 3-fold axis. The mechanism of neutralization by the ADK8 antibody is currently unknown but occurs post cellular attachment and pre-nuclear entry (37). Significantly, an AAV8 vector mutated at residues 589–591 is capable of evading neutralization by ADK8 and retains the liver transduction efficiency of the parental AAV8 vector in a mouse model (78). This study thus provides a proof of the concept that AAV antibody binding sites can be engineered to evade recognition while retaining the natural parental transduction properties.

COMMONALITIES IN MAPPED AAV ANTIGENIC EPITOPES

The AAV antigenic epitopes mapped by the structural-based approach show significant overlap among the AAVs despite differences in the amino acid types at these epitopes (**Table 2**). The 4E4 and 5H7 antibodies against AAV1 and C37-B against AAV2 have epitopes that contain residues in the 492–499 peptide stretch while epitopes for C37-B against AAV2 and ADK8 against AAV8 contain the 585–589 sequence stretch. As already mentioned above, these residues are localized to the protrusions that surround the 3-fold axis (**Figure 3**). The epitopes of A20 and 3C5, which cover the 2/5-fold wall and the floor of the depression, surround the 5-fold axis and share residues 254, 258, 261, and 708. With the exception of the HI loop structure, which is conserved in all AAV structures so far determined, the AAV antigenic epitopes mapped by cryo-reconstruction are localized to VRs on the AAV capsid surface (**Figure 3**) (26). This clustering of epitopes suggests a limited number of common antigenic regions on the AAV capsid surface. Significantly, the 3-fold and 2/5-fold regions have been implicated in antibody binding and neutralization for other parvovirus capsids including *Aleutian Mink Disease Virus*, *Human Parvovirus B19*, *Canine Parvovirus*, and *Feline Panleukopenia Virus*

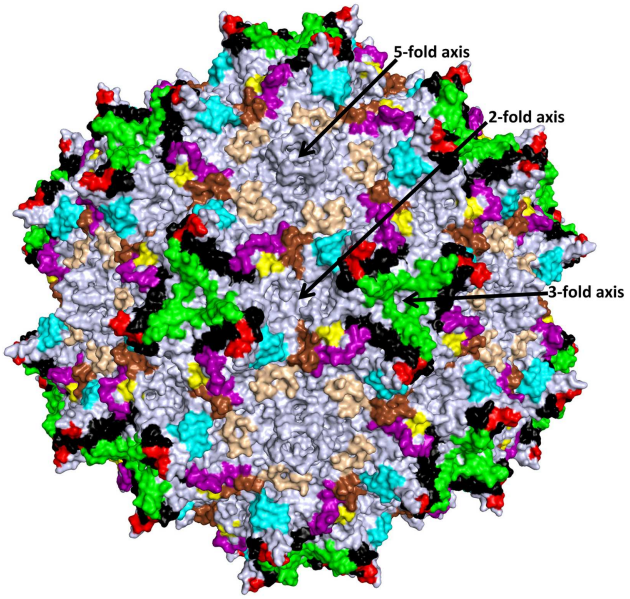

FIGURE 3 | Structurally mapped AAV antigenic epitopes. The epitopes identified on the AAV capsid surface by cryo-reconstruction structure are depicted in the colors used for the VRs in **Figure 2** based on overlap with the VR amino acids; aa253–271: purple; aa383–386: yellow; aa456–459: red; aa492–515: black; aa544–557: cyan; aa582–597: green; aa659–669: wheat; and aa709–720: brown. Amino acids 659–669 (wheat) were not previously described as VR regions. The approximate icosahedral 2-, 3-, and 5-fold symmetry axes are indicated and labeled as in **Figure 1**. This image was generated with the PyMOL program (http://www.pymol.org).

(79–83). These observations suggest a commonality in development of the host humoral response against parvovirus capsids, which can inform on the antigenic regions of the AAVs. Thus the epitopes presented here likely represent the capsid sites that dominate antibody recognition by the AAV capsid.

While single mouse MAb footprints, as mapped by cryo-reconstruction or peptide scanning/insertion may not be enough to predict the antigenic repertoire of the polyclonal antibody response present in human sera, the combined information from several AAV:Fab complexes is proving to be predictive of the antigenic structure of these viruses against the human immune response. A comparison of the AAV MAb footprints identified by cryo-reconstruction or for AAV2 also using peptide scanning, peptide insertion or site-directed mutagenesis with the list of antigenic sites obtained from screening capsid mutants against human sera and IVIGs neutralization shows significant overlap (**Tables 1** and **2**). For example, AAV2 residues 497, 498, 586, and 587, which when mutated enable AAV2 to escape from serum and IVIG neutralization (**Table 1**) (38), are part of the C37-B MAb epitope identified cryo-reconstruction (36) and peptide insertion (63). The A20 MAb against AAV2, which had a expansive footprint, included escape mutant residues also identified by directed evolution against rabbit serum and the screening of site-directed mutants with human IVIG (**Tables 1** and **2**). The commonality between AAV2 antigenic footprints and those for the other AAVs, as mapped by cryo-reconstruction, for example C37-B with 4E4

and 5H7 against AAV1/6, C37-B with ADK8 against AAV8, and A20 with 3C5 against AAV5, suggests that a similar overlap will exist between the footprints of these MAbs and the human polyclonal response to their capsid.

PROS AND CONS OF ANTIGENIC MAPPING APPROACHES

The approaches discussed above have their own pros and cons. Directed evolution, in the presence of human sera or IVIG, has the potential to generate a selected AAV variant with the ability to escape neutralization and retain its genome packaging capacity and infectivity. In addition, a specific/desired tissue tropism can be selected (70, 71, 84). However, this process is time consuming, and unlike the rational approaches, the outcome is much harder to predict. Peptide scanning and the structural-based approaches focus on identifying epitopes first and then designing vectors to escape antibody binding. Peptide scanning has the potential to detect interactions from serum or IVIG which resemble the situation in the natural host. However, the epitopes detected are mostly linear and may not represent the full repertoire of important pre-existing antibody interactions against the capsid unless it has been uncoated, denatured, or digested. Thus this method has limitations on footprint prediction accuracy. For example, peptide scanning identified A20 epitope peptides that were adjacent to the footprint identified by cryo-reconstruction and model building but none overlapped (**Table 2**). In fact the peptides identified by scanning were mostly located under the capsid surface VRs that contained the epitope regions identified by site-directed mutagenesis and cryo-reconstruction. For C37-B, one of the peptides mapped by scanning, residues 493–502, included the residues on one of the amino acid stretches, 492–498, predicted to be within the footprint by the cryo-reconstruction (**Table 2**). The other peptide identified by peptide scanning, 601–610, is buried inside the capsid, and not predicted to interact with the antibody in the reconstructed complex structure. Residues 585–589 predicted to form part of the C37-B epitope by cryo-reconstruction was not identified by peptide scanning. The structural-based approach, either to confirm previously predicted epitopes, e.g., the AAV2:A20 and AAV2:C37-B complex structures, or to identify new epitopes, e.g., the AAV1:4E4, AAV1:5H7, and AAV8:ADK8 complex structures, is able to accurately map antibody footprints. However, to date, all the structures determined are for viruses complexed with Fabs generated from mouse MAbs. This is because cloning MAbs from human B-cells is challenging and structural studies with polyclonal human antibodies could lead to poorly resolved densities due to the variability in the sample. It could be argued that the murine immune response differs from that of humans and that human serum represents a polyclonal population of antibodies, and thus modification of single mouse MAb footprints mapped by cryo-reconstruction studies may not generate vectors that evade neutralization from human serum. To overcome this bottleneck and mimic the polyclonal response, the structure of each clinically relevant AAV complexed with Fabs from several MAbs can be determined to obtain information on dominant antigenic regions.

Given the advantages and disadvantages of the approaches described above, a two pronged-attack, concurrent directed evolution and structural mapping, is likely optimal for defining the capsid surface antigenic properties of AAVs. The available 3D structures for AAV1 to AAV9 provide the platform required for the visualization of epitopes obtained by directed evolution onto the parent serotypes. This can inform further modifications for desired tropisms, on the background of an escape mutant, given information on tropism determinants. Structural mapping will provide information that can enable rational engineering of vectors for escape mutation while retaining natural tropisms. Thus both strategies will delineate dominant epitopes for the AAVs. The current data, using both approaches, point to the protrusions around the icosahedral 3-fold axis and 2/5-fold wall, as the dominant targets for future modification for antibody escape.

Regardless of the method used to obtain the antigenic information, the ultimate goal is to create an AAV variant that can evade the neutralizing effect of a pre-existing immune response and has the capacity to effectively assemble genome packaged vectors and retain efficient cell tropism. The observations described in this review show that minor and local variations on the AAV capsid surface, including those due to single amino acid substitutions, may alter more than one phenotype of AAV vector, including tropism and antigenicity. The antigenic mapping data at hand, combined with efforts at chemical capsid modifications, pharmacological immuno-suppression, plasmapheresis, and saline flushing, point to potential strategies for improving the clinical efficacy of this promising gene delivery system.

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Anti-CD20 as the B-cell targeting agent in a combined therapy to modulate anti-factor VIII immune responses in hemophilia A inhibitor mice

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Neutralizing antibody formation against transgene products can represent a major complication following gene therapy with treatment of genetic diseases, such as hemophilia A. Although successful approaches have been developed to prevent the formation of anti-factor VIII (FVIII) antibodies, innovative strategies to overcome pre-existing anti-FVIII immune responses in FVIII-primed subjects are still lacking. Anti-FVIII neutralizing antibodies circulate for long periods in part due to persistence of memory B-cells. Anti-CD20 targets a variety of B-cells (pre-B-cells to mature/memory cells); therefore, we investigated the impact of B-cell depletion on anti-FVIII immune responses in hemophilia A mice using anti-CD20 combined with regulatory T (T_{reg}) cell expansion using IL-2/IL-2mAb complexes plus rapamycin. We found that anti-CD20 alone can partially modulate anti-FVIII immune responses in both unprimed and FVIII-primed hemophilia A mice. Moreover, in mice treated with anti-CD20+IL-2/IL-2mAb complexes+rapamycin+FVIII, anti-FVIII antibody titers were significantly reduced in comparison to mice treated with regimens targeting only B or T cells. In addition, titers remained low after a second challenge with *FVIII* plasmid. T_{reg} cells and activation markers were transiently and significantly increased in the groups treated with IL-2/IL-2mAb complexes; however, significant B-cell depletion was obtained in anti-CD20-treated groups. Importantly, both FVIII-specific antibody-secreting cells and memory B-cells were significantly reduced in mice treated with combination therapy. This study demonstrates that a combination regimen is highly promising as a treatment option for modulating anti-FVIII antibodies and facilitating induction of long-term tolerance to FVIII in hemophilia A mice.

Keywords: anti-CD20, factor VIII, hemophilia, tolerance induction, immunomodulation, B-cell depletion

INTRODUCTION

Hemophilia A is an X-linked, congenital bleeding disorder resulting from a deficiency of factor VIII (FVIII). Approximately 35% of patients with hemophilia A develop complications of anti-FVIII neutralizing antibodies following FVIII protein replacement therapy (1, 2). In order to overcome anti-FVIII immune responses, we sought transient immunosuppressive strategies that can reduce pre-existing antibodies and induce long-term tolerance to FVIII. CD20 is a 35-kDa transmembrane protein expressed on B-cells from the pre-B-cell stage to mature B lymphocytes, but not plasma cells (3). Monoclonal antibodies (mAbs) against human CD20 (Rituximab) induce rapid B-cell depletion (4) and is currently approved by the Food and Drug Administration (FDA) for treatment of non-Hodgkin B-cell lymphomas (5, 6) and several autoimmune disorders including type 1 diabetes (T1D) (7), rheumatoid arthritis (8), and Sjögren's syndrome (SS) (9). Anti-CD20 depletes B-cells via several mechanisms (10, 11), such as direct induction of apoptosis, antibody-dependent cell-mediated cytotoxicity (ADCC) (12), and complement-dependent lysis (CDC) (13), which are considered to be immediate and comparatively short-acting. Nevertheless, the clinical response to a single course of the anti-CD20 mAb can be late acting and prolonged. This has led to the suggestion that anti-CD20 could also have an immunization effect (14); however, it is unknown whether this correlates with clinical outcome. Recently anti-CD20 IgG_1 (15) and IgG_{2a} (16) molecules have been used successfully to prevent the production of anti-FVIII antibodies.

Webster et al. (17) defined a strategy in which complexes of IL-2/IL-2-specific mAbs (JES6-1A12) can be used to selectively expand $CD4^+CD25^+Foxp3^+$ regulatory T (T_{reg}) cells *in vivo* with little or no change in other cell populations. This approach has been used to successfully treat asthma (18) and experimental myasthenia gravis (MG) (19) in mouse models. In addition, rapamycin is currently used as an immunosuppressive agent to prevent acute graft rejection in humans (20). Rapamycin combines with the intracellular immunophilin FK506-binding protein (FKBP12) to form FKBP12-rapamycin complexes that inhibit the activity of mammalian target of rapamycin (mTOR) and result

in inhibiting effector T-cell (T_{eff}) proliferation (21). Rapamycin not only increased T_{reg}:T_{eff} cell ratios but also improved the suppressive activity of T_{reg} cells (22, 23).

In our previous studies, administration of IL-2/IL-2mAb complexes prevented anti-FVIII immune responses in hemophilia A mice following gene or protein replacement therapy (24, 25). Nevertheless, overcoming pre-existing antibody responses in primed subjects remains challenging. Anti-FVIII neutralizing antibodies persist in part due to memory B-cells (26). Moreover, molecular studies have shown that long-lived plasma cells (LLPCs) can support chronic inflammatory processes by secreting pathogenic antibodies for long periods (27, 28). It is hypothesized that LLPCs may also play an important role in prolonged production of anti-FVIII antibodies in hemophilia A patients. In this study, we developed a treatment strategy of single or combination therapy using agents targeting B-cells (to eliminate memory responses) and those inducing T_{reg} cell expansion (to suppress T helper cell function). By using a combination of anti-CD20+IL-2/IL-2mAb complexes+rapamycin, anti-FVIII immune responses were significantly reduced. Hemophilia A mice treated with combination therapy showed little or no anti-FVIII antibodies titers, and this was also evident after a second challenge with *FVIII* plasmid. This study sought to identify strategies toward induction of immune tolerance to FVIII transgene product following gene therapy and to demonstrate that combination therapy targeting B and T lymphocytes can be a viable option.

RESULTS

ANTI-CD20 TREATMENT CAN REGULATE ANTI-FVIII PRODUCTION IN A NON-VIRAL GENE THERAPY MODEL

To test if B-cell depletion can regulate anti-FVIII immune responses, we utilized anti-CD20 IgG2a antibody (anti-CD20) in a murine model. Hemophilia A mice were divided into two treatment groups (**Figure 1** and Figure S1 in Supplementary Material): *FVIII* plasmid-treated mice were given anti-CD20 (250 μg/mouse) on days 0 and 14 combined with a *FVIII* plasmid (pBS-HCRHPI-FVIIIA; 50 μg/mouse) expressing B domain-deleted hFVIII under the control of the liver-specific hAAT promoter (HP) and the hepatic control region (HCR) on day 0. Control mice were treated with rat IgG2a (250 μg/mouse) on days 0 and 14. Anti-CD20 significantly reduced total $B220^+/CD19^+$ B-cells (80–90% reduction) both in blood (Figures S1A,B in Supplementary Material) and spleen (Figure S1C in Supplementary Material). B-cell depletion was sustained over 4–6 weeks with gradual return to normal levels at 8 weeks following treatment. No reduction in B-cell levels were observed in IgG2a isotype-treated control and naive mice.

In order to investigate the best treatment schedule and therapeutic effects of anti-CD20 treatment, three groups of hemophilia A mice were injected with three different dosages: 100 μg/mouse (on days −2, 0, 3, 6, and 9), 250 μg/mouse (on days 0 and 14), and 500 μg/mouse (on day 0). Control mice were treated with rat IgG2a 250 μg/mouse on days 0 and 14. All mice were injected with *FVIII* plasmid (50 μg/mouse) at day 0. Following treatment, FVIII activities and neutralizing antibody titers were assessed by aPTT and Bethesda assays at different time points. In the rat IgG2a control group, anti-FVIII antibody appeared within 2 weeks post plasmid injection, increased to high-titers at 3–4 weeks, and maintained high-titer levels through 24 weeks. In addition, initially high levels of FVIII activity decreased to low-undetectable levels within 4 weeks (**Figure 1**). In the anti-CD20-treated groups, one mouse from each group of mice had persistent FVIII activity without detectable inhibitory anti-FVIII antibodies (**Figure 1**). The remaining mice displayed delayed immune responses; however, all mice generated moderate to high-titers of neutralizing antibodies with FVIII activity decreasing to undetectable levels at 6–15 weeks. While antibody titers were clearly reduced following anti-CD20 treatment, these titers increased over time. Although mice treated with anti-CD20 were not completely resistant to FVIII immune responses, they all exhibited partial modulatory effects compared to the rat IgG2a treated control mice.

A similar treatment was given to *FVIII* plasmid-primed hemophilia A mice with pre-existing neutralizing antibodies. These mice were developed by hydrodynamic injection of 50 μg of *FVIII*

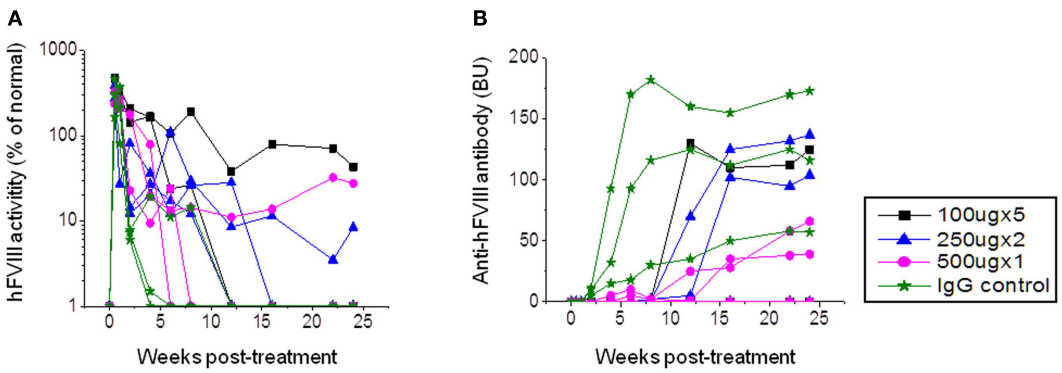

FIGURE 1 | *Factor VIII* gene expression and anti-FVIII antibody formation after *FVIII* plasmid+anti-CD20 treatment in hemophilia A mice. Four groups of hemophilia A mice were treated with *FVIII* plasmid (50 μg/treatment/mouse) at day 0 and i.v. injection of anti-CD20 at various doses and schedules as listed in the following: group 1: 100 μg/treatment/mouse, injected at days −2, 0, 3, 6, and 9. Group 2: 250 μg/treatment/mouse, injected at days 0 and 14. Group 3: 500 μg/treatment/mouse, injected at day 0. Group 4: control rat IgG, 250 μg/treatment/mouse, injected at days 0 and 14. Peripheral blood samples were collected at different time points to examine FVIII activities **(A)** and inhibitor titers **(B)**. Each symbol represents data obtained from an individual mouse. Data shown is representative of two independent experiments.

plasmid via tail vein, and only mice with neutralizing antibody titers >30 Bethesda units (BU) were used. Mice were then treated with anti-CD20 ($n = 5$; 250 μg/mouse) on days −7, −4, and 0. Control mice were treated with IgG2a ($n = 2$; 250 μg/mouse) on days −7, −4, and 0. Plasma samples were collected on day 1 following treatment. As shown in **Figure 2**, neutralizing antibody titers were maintained at high levels in rat IgG2a control-treated mice. In contrast, neutralizing antibody titers were significantly reduced following anti-CD20 treatment in 80% (four of five) treated mice. In particular, 20% (one of five) of anti-CD20-treated mice showed therapeutic FVIII activities levels for 14 weeks (**Figure 2B**). These results indicate that anti-CD20 can partially modulate anti-FVIII immune responses both in the FVIII unprimed and primed hemophilia A mice.

COMBINATION TREATMENT WITH IL-2/IL-2mAb COMPLEXES, RAPAMYCIN, AND ANTI-CD20 ENHANCED *FVIII* PLASMID-MEDIATED GENE THERAPY IN HEMOPHILIA A MICE

Since anti-CD20 treatment can partially modulate anti-FVIII immune responses in hemophilia A mice, we investigated whether a combination therapy using anti-CD20 to deplete B-cells and IL-2/IL-2mAb complexes to expand T_{reg} cells (24) can more consistently reduce anti-FVIII responses. Hemophilia A mice were treated with: IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII ($n = 4$, group 1; **Figure 3A**); IL-2/IL-2mAb complexes+anti-CD20+FVIII ($n = 4$, group 2; **Figure 3B**); IL-2/IL-2mAb complexes+rapamycin+FVIII ($n = 3$, group 3; **Figure 3C**); anti-CD20+FVIII ($n = 4$, group 4; **Figure 3D**); and mock agents (control inhibitor mice; $n = 2$, group 5; **Figure 3E**) weekly for 4 weeks. FVIII protein (1 U/mouse) was given weekly for 4 weeks for induction of FVIII-specific tolerance during the treatment period. *FVIII* plasmid second challenge was applied at 5 weeks following 4 weeks of treatment (week 9). Except for control mice, all treated groups showed decreased antibody titers. The most significant and prolonged reduction of neutralizing antibody titers was observed using the combination treatment of IL-2/IL-2mAb complexes+anti-CD20+rapamycin+FVIII (**Figure 3A**). Neutralizing antibody titers were reduced to 0 in 50% (two of four) treated mice, and a reversion of 8% FVIII gene expression was observed. Additional animals ($n = 3$–5/group) have been treated in repeated experiments with similar results to those shown (**Figures 3A–E**). In addition, treated mice were challenged with non-specific antigen, TNP-ficoll (24), at 16 weeks following treatment (week 20). These animals responded similarly to control/naive mice, demonstrating the tolerance effect was FVIII-antigen-specific.

EFFECTS ON T/B-CELL RESPONSES IN PERIPHERAL BLOOD AND SPLEEN WERE SIGNIFICANT IN TREATED HEMOPHILIA A MICE

Next, we evaluated changes in T- and B-cell populations of hemophilia A mice following treatment. We analyzed peripheral blood in mice treated with IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII ($n = 4$). Mice with neutralizing antibodies and those treated with anti-CD20+FVIII; IL-2/IL-2mAb complexes+rapamycin+FVIII; and IL-2/IL-2mAb complexes+anti-CD20+FVIII were used as control groups. Mice were treated weekly with the indicated regimen for 4 weeks. Flow cytometry analysis showed that the $CD4^+$ T cells in total T-cell populations did not significantly change over time (**Figure 4A**). Interestingly, there was a slight decrease in the percentage and numbers of $CD4^+$ T cells in anti-CD20 treated groups. However, the percentage of $CD4^+CD25^+Foxp3^+$ T cells within the $CD4^+$ T-cell compartment was significantly increased in the IL-2/IL-2mAb complexes treated groups compared to other groups for 4 weeks during treatment period (**Figure 4B**; $P < 0.05$). The expanded T_{reg} cells declined rapidly to baseline levels within 2 weeks post treatment. Similar to our previous studies (24, 25), IL-2/IL-2mAb complex-expanded T_{reg} cells showed considerably higher expression of molecules crucial for the suppressive function of T_{reg} cells, including CD25, glucocorticoid-induced tumor necrosis factor receptor (GITR), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (**Figure 4C**). The substantial increase in T_{reg} cells following injection of IL-2/IL-2mAb complexes occurred not only in blood but also appeared as a fivefold increase in spleen (Figure S2A in

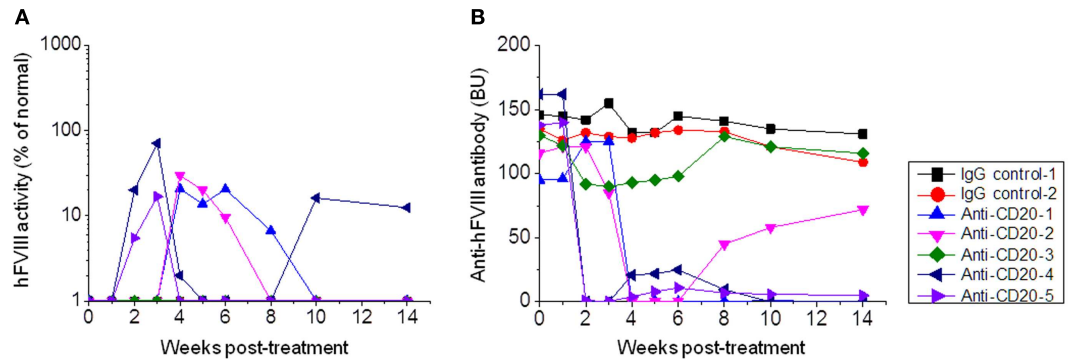

FIGURE 2 | *Factor VIII* gene expression and anti-FVIII antibody titers following anti-CD20 treatment in *FVIII* plasmid-primed hemophilia A mice with pre-existing inhibitors. Mice were primed with *FVIII* plasmid to induce high-titer inhibitory antibodies at 8 weeks before anti-CD20 treatment. The inhibitor mice were then treated with anti-CD20. Group 1: control rat IgG, 250 μg/treatment/mouse, injected at days −7, −4, and 0. Group 2: anti-CD20, 250 μg/treatment/mouse, injected at days −7, −4, and 0. Peripheral blood samples were collected at different time points to evaluate FVIII activities **(A)** and inhibitor titers **(B)**. Each symbol represents data obtained from an individual mouse. Data shown is representative of two independent experiments.

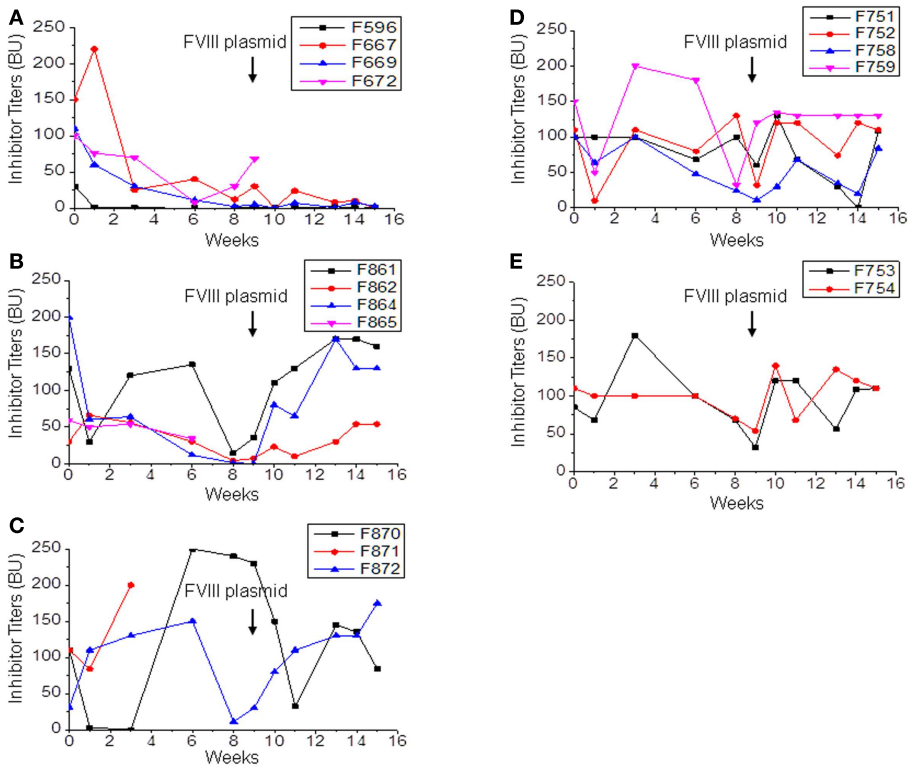

FIGURE 3 | Immunomodulation with separate or combined therapy by IL-2/IL-2mAb complexes, rapamycin, and anti-CD20 in *FVIII* plasmid-primed hemophilia A mice with pre-existing inhibitors. Four groups of hemophilia A inhibitor mice were treated separately with different combined regimens: **(A)** IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII injection ($n = 4$, group 1), **(B)** IL-2/IL-2mAb complexes+anti-CD20+FVIII ($n = 4$, group 2), **(C)** IL-2/IL-2mAb complexes+rapamycin+FVIII injection ($n = 3$, group 3), **(D)** anti-CD20+FVIII injection ($n = 4$, group 4), and **(E)** Control inhibitor mice ($n = 2$, group 5). Each experimental group was treated with indicated immunomodulation regimen weekly for 4 weeks. Anti-FVIII antibody titers were assessed by Bethesda assay over time. Each symbol represents data obtained from an individual mouse. Data shown is representative of three independent experiments.

Supplementary Material; middle panel and Figure S2B in Supplementary Material; upper panel). Almost all expanded T_{reg} cells were Helios$^+$ (Figure S2A in Supplementary Material; right panel and Figure S2B in Supplementary Material; upper panel) natural T_{reg} cells derived from thymus. In contrast, no significant change in the CD4$^+$ T-cell population was observed in the spleen (Figure S2A in Supplementary Material; left panel and Figure S2B in Supplementary Material; upper panel). The expression levels of the activation markers of T_{reg} cells including CD25, GITR, and CTLA-4 after IL-2/IL-2mAb complex treatment also reached high levels in the spleen (Figure S2B in Supplementary Material; lower panel).

Effects of anti-CD20 treatment were also evaluated on B-cell populations in the hemophilia A mice treated with combination therapy. After two treatments of anti-CD20, proportions of B-cell populations were measured by flow cytometry. We observed a significant decrease in proportions in the anti-CD20-treated mouse groups of total B-cells (B220$^+$ cells) (**Figure 5A** and Figure S2C in Supplementary Material); mature B-cells (IgD$^+$IgM^{low}) (**Figure 5B** and Figure S2C in Supplementary Material); transitional B-cells (IgM$^+$IgD^{low}) (**Figure 5C** and Figure S2C in Supplementary Material); memory B-cells (IgM$^-$IgD$^-$) (Figure S2C in Supplementary Material); and plasma B-cells (B220$^-$CD138$^+$) (**Figure 5D**).

COMBINATION THERAPY DEPLETES ANTI-FVIII-SPECIFIC ANTIBODY-SECRETING CELLS (ASCS) AND FVIII-SPECIFIC MEMORY B-CELLS IN MICE

Hemophilia A mice were treated with IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII; IL-2/IL-2mAb complexes+rapamycin+FVIII; anti-CD20+FVIII; and FVIII alone as described previously. CD138$^+$ cells were obtained from spleens of treated mice 2 weeks following treatment. Plasma cells were incubated with FVIII and analyzed for the formation of spots in an ELISPOT assay. Anti-FVIII ASCs correlated with the number of cells plated. Total anti-FVIII ASCs were reduced in mice treated with IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII and IL-2/IL-2mAb complexes+rapamycin+FVIII compared to other groups (**Figure 6A**). To confirm specificity of the assay, naive mice that had not been treated with FVIII were included. No FVIII-specific ASCs were detected in these mice (**Figure 6A**). Furthermore, no background staining in plates without FVIII immobilization was observed using cells obtained from FVIII-treated mice (data not shown). Evaluation of

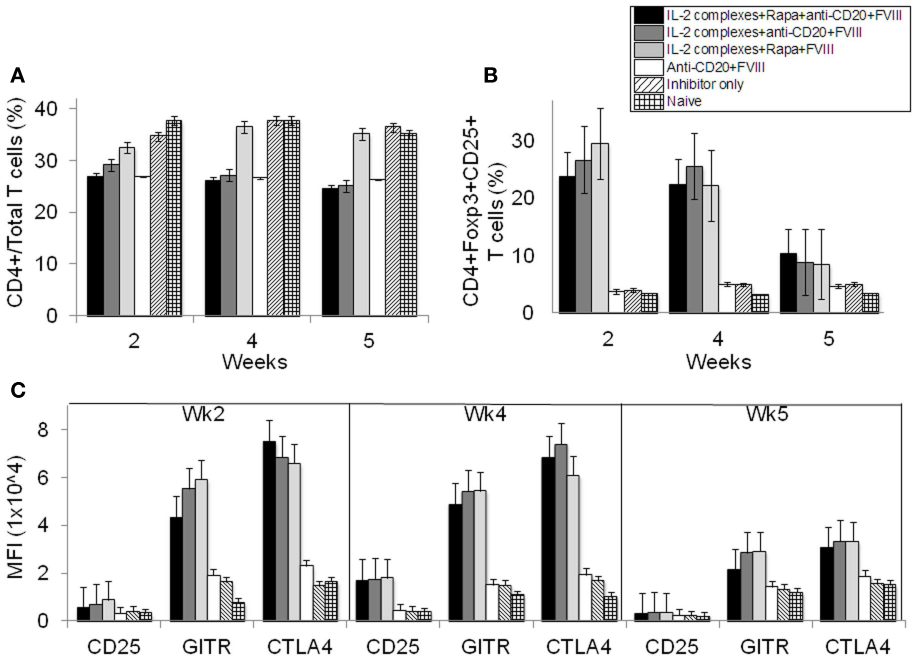

FIGURE 4 | Effects of immunomodulation on CD4⁺ T cells, CD4⁺CD25⁺Foxp3⁺ T_{reg} cells and T_{regs} activation markers in peripheral blood of treated hemophilia A inhibitor mice over time. Lymphocytes were isolated from the blood of naive (light grid), inhibitor only (light slant), anti-CD20+FVIII (white), IL-2/IL-2mAb complexes+rapamycin+FVIII (light gray), IL-2/IL-2mAb complexes+anti-CD20+FVIII (dark gray), and IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII (black) treated mice. **(A)** CD4⁺ in total T cells and **(B)** CD4⁺CD25⁺Foxp3⁺ in CD4⁺ T cells were stained and analyzed by flow cytometry during the treatment. **(C)** Blood cells were also stained and analyzed for T_{reg} cells markers: CD25, GITR, and CTLA-4. Data shown are median fluorescence intensity (MFI) values of the three activation markers. Data shown is representative of two independent experiments.

FVIII-specific ASCs and memory B-cells at later time points (4 and 6 weeks) after treatment showed similar results as those obtained at 2 weeks.

We also assessed whether depletion of FVIII-specific memory B-cells occurred following treatment. We isolated $CD138^{-}$ spleen cells (presumably containing FVIII-specific memory B-cells) from hemophilia A mice treated with different single or combined regimens and re-stimulated these cells with high-dose FVIII (2 U/well). FVIII-specific memory B-cells were detected by ELISPOT. Interestingly, FVIII-specific memory B-cells were significantly reduced only in the IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII and anti-CD20+FVIII-treated mouse groups (**Figure 6B**).

DISCUSSION

Treatment of hemophilia A patients with inhibitors is very challenging and costly. In addition, probability of morbidity is increased in these patients. Although treatments with inhibitors has been successful in hemophilia mice, it is very challenging to decrease pre-existing anti-FVIII neutralizing antibodies in FVIII-primed hemophilia A mice. *In vivo* expansion of activated T_{regs} had profound suppressive effects and was able to prevent formation of anti-FVIII antibodies in both gene therapy and protein replacement therapy treated mice; however, this regimen can only transiently modulate pre-existing anti-FVIII immune responses. Anti-FVIII neutralizing antibodies can circulate for long periods and is thought to be partly due to the persistence of memory B and plasma cells. In a preliminary experiment, we found that either use of bortezomib (an inhibitor of 26S proteasome that can reduce/eliminate plasma cells) alone or in combination with T-cell-regulating agents such as IL-2/IL-2mAb complexes did not help reduce pre-existing neutralizing antibody titers (data not shown). Meslier et al. (29) reported similar results that bortezomib only delayed the onset of FVIII neutralizing antibodies in hemophilia A mice but failed to eliminate established anti-FVIII IgG-producing cells. Anti-CD20 mAbs can deplete pan B lymphocytes, from pre-B-cells to memory B-cells, ranging from 50 to 90%. Rituximab (anti-human CD20) is beneficial in treating patients with acquired hemophilia (30). Limited data have also been described in case reports with respect to the use of Rituximab in children (31, 32) and adults (31, 33) with congenital hemophilia A and neutralizing antibodies. It has been hypothesized that concurrent administration of anti-CD20 and high-dose FVIII might be beneficial to treat hemophilia A patients with neutralizing antibodies. Emerging data suggests that repopulating transitional murine and human B-cells (which increase markedly in numbers following anti-CD20 depletion therapy) exhibited potent regulatory activity (34). Therefore, use of anti-CD20 may promote effects distinct from merely reducing the mature B-cell pool. IL-10 expressing immature B-cells may promote a regulatory environment to aid in tolerance induction (35, 36). As anti-CD20 treatment exhibits a distinct mechanism of action and relatively few side effects, it is an excellent candidate agent for combinational approaches. Thus, we set out

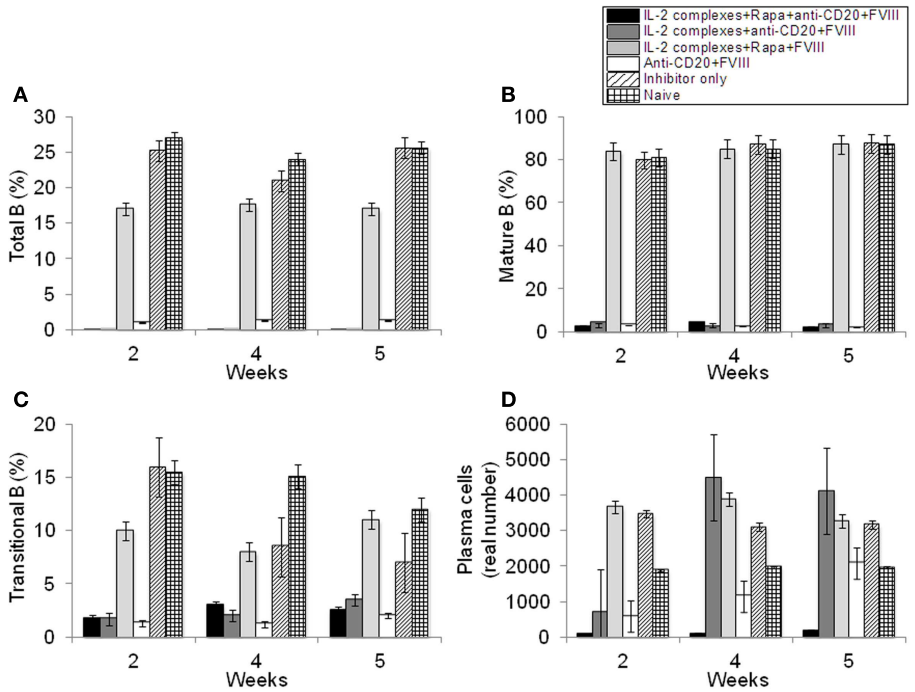

FIGURE 5 | Effects of immunomodulation on total B, mature B, transitional B, and plasma B-cells in peripheral blood of each mouse group. Lymphocytes were isolated from the blood of naive (light grid), Inhibitor only (light slant), anti-CD20+FVIII (white), IL-2/IL-2mAb complexes+rapamycin+FVIII (light gray) IL-2/IL-2mAb complexes+anti-CD20+FVIII (dark gray), and IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII (black) treated mice. **(A)** $B220^{+}$ B-cells, **(B)** $IgM^{+}IgD^{hi}$ B-cells, **(C)** transitional B-cells, and **(D)** plasma cells were stained and analyzed by flow cytometry during the treatment period (week 2, 4, and 5). Data shown are cell percentages **(A–C)** and real numbers **(D)** of the B-cell populations. Data shown is representative of two independent experiments.

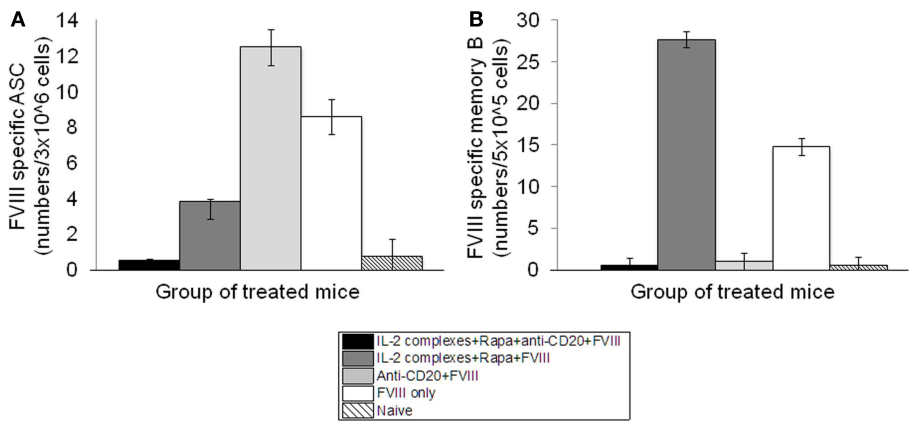

FIGURE 6 | Depletion of FVIII-specific ASCs (Antibody-Secreting Cells) and memory B-cells in the inhibitor mice treated with IL-2/IL-2mAb complexes plus rapamycin and anti-CD20. Cells were isolated by MACS from spleens of naive (light slant), *FVIII* plasmid only (white), anti-CD20+FVIII (light gray), IL-2/IL-2mAb complexes+rapamycin+FVIII (dark gray), and IL-2/IL-2mAb complexes+rapamycin+anti-CD20+FVIII (black) treated mice ($n = 2$, each group) 2 weeks after treatment. **(A)** 3×10^6 cells were used to detect FVIII-specific ASC cells by ELISPOT assay. **(B)** 5×10^5 non-plasma cells were cultured and stimulated with FVIII at 10 U/ml for 6-days, and memory B-cells were detected by ELISPOT assay. Data shown are mean of spot numbers for each treated group ($n = 2$).

to study whether anti-CD20 treatment alone or in combination with other immune tolerance therapies can be more beneficial to treat hemophilia subjects with pre-existing inhibitory antibodies.

With anti-CD20 treatment alone, we found that anti-FVIII neutralizing antibody titers were reduced in both FVIII unprimed and primed hemophilia A mice. However, antibodies were not completely eliminated and FVIII activity did not improve in these

animals. To further improve the therapeutic efficacy, we combined B-cell depletion using anti-CD20 with *in vivo* T_{reg} cell expansion using IL-2/IL-2mAb complexes+rapamycin to treat hemophilia A inhibitor mice. Our previous studies using IL-2/IL-2mAb complexes alone showed five to sevenfold expansion of highly suppressive T_{reg} cells *in vivo*, which induced long-term tolerance to FVIII in unprimed hemophilia A mice following gene therapy (24). IL-2/IL-2mAb complexes that can selectively promote human T_{reg} expansion are currently under development. Although no clinical trials have been initiated so far, IL-2/IL-2mAb complexes have high potential as a clinically feasible strategy; however, IL-2/IL-2mAb complex single treatment in the hemophilia A "inhibitor" mice only transiently reduced neutralizing antibody titers during treatment. It has been shown that rapamycin blocked T-cell-cycle progression from G1 to S phase after activation (37) and promoted TCR-induced T-cell anergy (38), achieving induction of operational tolerance (39). Additionally, rapamycin enriched antigen-specific $Foxp3^+$ T_{reg} cells to promote organ transplant tolerance (40) and inhibited relapsing experimental autoimmune encephalomyelitis (EAE) by modulation of both effector/regulatory T cells (41). Moghimi et al. (42) reported that transient oral delivery of rapamycin combined with repeated injections of low doses of FVIII prevented induction of neutralizing antibody responses in hemophilia A mice. Thus, we adopted a treatment strategy that included rapamycin with IL-2/IL-2mAb complexes to enhance T_{reg} cell function *in vivo* for targeting T-cell-mediated immune responses and induction of FVIII-specific tolerance in hemophilia A mice with pre-existing antibodies.

Our results demonstrate that treatment with IL-2/IL-2mAb complexes+rapamycin had a synergistic effect when combined with anti-CD20 antibody to reduce neutralizing antibody titers. In contrast, mice treated with anti-CD20 only or different combinations only transiently reduced neutralizing antibody titers. In our immunomodulation studies, we also included weekly injection of FVIII protein (1 U/mouse/treatment) for induction of FVIII-specific tolerance. Furthermore, no increase in neutralizing antibody titers was observed following a second *FVIII* plasmid challenge, indicating induction of tolerance to FVIII. The mechanistic studies showed that the combined therapy promoted immune tolerance by increasing $CD4^+CD25^+Foxp3^+$ T_{reg} cells and their activation markers CD25, GITR, and CTLA-4. Furthermore, combined treatment using anti-CD20 showed 98% depletion of total B, mature B, transitional B, and plasma cells. Changes in T/B-cell populations were not only detectable in the peripheral blood but also found in splenocytes isolated from treated mice. Most importantly, FVIII-specific ASCs and memory B-cells were reduced following treatment with combination therapy. It was demonstrated that anti-CD20 predominantly depleted FVIII-specific memory B-cells, and IL-2/IL-2mAb complexes+rapamycin helped reduce FVIII-specific ASCs. In Figure S2C in Supplementary Material, the percentage of total memory T cells (gated as IgM^-IgD^- population) increased due to the more significant depletion (98%) of total B, mature B, and transitional B-cells. However, we found that FVIII-specific memory B-cells were significantly reduced after anti-CD20 treatment (shown in **Figure 6B**) in both mouse groups treated with anti-CD20 combination regimen. Whether the effects of IL-2/IL-2mAb complexes+rapamycin on B-cells were attributed to impaired T_H cell responses or directly mediated by the immune complexes is unclear. Upon second challenge with *FVIII* plasmid, the antibody titer remained low without an increase in FVIII activity, indicating that only partial tolerance against FVIII was achieved with combination therapy. Furthermore, treated mice were challenged with non-specific antigen, TNP-ficoll (24) at 16 weeks following treatment and responded similarly to control mice, demonstrating that this tolerance effect is FVIII-antigen-specific. Based on these results, we hypothesize that residual, long-lived FVIII-specific plasma cells may contribute to sustained low-titer neutralizing antibodies that limit the therapeutic benefits of immunomodulatory regimens.

Zhang et al. showed that a single dose of anti-CD20 IgG1 pretreatment prevented the increase of neutralizing antibodies in hemophilia A mice receiving high-dose protein replacement therapy (15). These antibodies can selectively deplete follicular B-cells while sparing marginal zone (MZ) B-cells as potential tolerogenic antigen-presenting cells. Interestingly, this treatment also led to an increase of T_{reg} cells. In a similar case, Sarikonda et al. showed that transient B-cell depletion with anti-CD20 IgG2a in combination with proinsulin resulted in modest increases in T_{reg} cells and offered limited efficacy in type 1 diabetes (T1D) prevention in NOD mice (7). In rhesus macaques, Mingozzi et al. (43) successfully used Rituximab in combination with cyclosporine to eradicate anti-human factor IX antibody following AAV8-mediated gene therapy. In addition, transient B-cell depletion by anti-CD20 IgG2a prevented FVIII inhibitor formation in hemophilia A mice receiving protein therapy but failed to induce long-term tolerance (16). In our study, administration of anti-murine CD20 IgG2a significantly reduced $CD19^+$ B-cells in blood, spleen, and lymph nodes, as well as neutralizing antibody titers in FVIII plasmid-treated mice. Furthermore, our results showed that combination therapy targeting both B and T cells had better results to more significantly reduce anti-FVIII immune responses. As shown in our previous experiments, FVIII expression persisted in the liver for very long periods following hydrodynamic delivery of FVIII plasmids. With reversion of FVIII expression, FVIII antigen is present continuously during follow-up. Thus, it is concluded that long lasting FVIII-specific partial tolerance has been achieved with the combination therapy. In addition, viral gene transfer with adenoid-associated viral (AAV) vectors has been proposed as a therapeutic strategy for hemophilia A (44, 45). The immunomodulation regimens developed for transgene-specific immune responses may also be helpful in the design of modulatory protocols for immune responses to gene transfer vectors, in particular pre-existing immunity against viral vectors such as AAV. In addition, liver-directed gene transfers with several vectors have been associated with the induction of tolerance to the expressed transgene (46, 47). Combination of the immunomodulatory regimen developed in this study with liver gene transfer should increase the efficacy of tolerization against FVIII transgene. In summary, we demonstrated that IL-2/IL-2mAb complexes plus rapamycin acted synergistically with anti-CD20 to promote induction of immune tolerance to FVIII by increasing the number and function of $CD4^+CD25^+Foxp3^+$ T_{reg} cells as well as eliminating both FVIII-specific ASCs and memory B-cells. These findings provide important preclinical evidence for the safety and enhanced therapeutic efficacy of the combined treatment

for antibody responses in hemophilia A patients with pre-existing inhibitors.

MATERIALS AND METHODS

MICE

All mice were kept according to the National Institutes of Health guidelines for animal care and the guidelines of Seattle Children's Research Institute, and maintained at a specific pathogen-free (SPF) facility. Hemophilia A mice in a 129/SV × C57BL/6 mixed genetic background were generated by targeted disruption of exon 16 of *FVIII* gene (48) and were used at the age of 6–8 weeks.

IMMUNOMODULATION USING IL-2/IL-2mAb COMPLEXES IN HEMOPHILIA A MICE TREATING WITH FVIII PLASMID

Hemophilia A mice were intravenously (i.v.) injected with 50 μg of *FVIII* plasmid [pBS-HCRHPI-FVIIIA (49)] in 2 ml phosphate-buffered saline (PBS) via tail vein in 8–10 s. IL-2/IL-2mAb complexes were prepared as previously described (17). One microgram recombinant mouse IL-2 (PeproTech, Rocky Hill, NJ, USA) was mixed with 5 μg anti-IL-2mAb (JES6-1A12) (eBioscience, San Diego, CA, USA), incubated at 37°C for 30 min, and then injected intraperitoneally (i.p.) into mice according to schedules specified in Results. Groups of IL-2/IL-2mAb complexes only treated mice, *FVIII* plasmid only treated mice, and naive mice were included as controls. Selected mice treated with immunomodulation received a second plasmid challenge at 9 weeks after the first treatment with immunomodulation therapy. Blood samples were taken from the retro-orbital plexus at serial time points and assessed for FVIII activity and anti-FVIII antibody levels.

B-CELLS DEPLETION BY ANTI-CD20 TREATMENT

Mice were depleted of B-cells using anti-murine CD20 IgG2a antibody (clone 18B12; Biogen Idec, Weston, MA, USA) at indicated schedules and dosages. To assess B-cell depletion, mice were given two i.v. doses 14 days apart, and peripheral blood and spleen tissues were collected at different time points after the first and second doses. Peripheral blood was collected in microcapillary tubes with 3.8% sodium citrate solution via retro-orbital plexus, centrifuged to remove plasma, and remaining cells suspended in PBS for staining and flow cytometry.

FLOW CYTOMETRY AND ANTIBODIES

Cell suspensions of peripheral blood, lymph nodes (LNs from superficial cervical), and spleens of each treated mouse group were prepared according to standard protocols. Cell suspensions were stained for FACS analysis using the following antibodies (obtained from eBioscience unless otherwise stated): PE-Cy5-anti-mouse CD25; FITC-anti-mouse CD62L (L-selectin); Alexa Fluor®647-anti-mouse/rat Foxp3; PE-anti-mouse CD152 (CTLA-4); Alexa Fluor®700-anti-mouse CD4 (BD Pharmingen™; San Jose, CA, USA); PE-Cy7-anti-mouse GITR (BD Pharmingen™); Alexa Fluor®700-anti-mouse B220; FITC-anti-mouse IgD; PE-Cy7-anti-mouse IgM and PE-anti-mouse CD138. Cells were first stained for T-cell surface markers CD4, CD25, CD62L, and GITR, and subsequently stained intracellularly with T-cell markers for Foxp3 and CTLA-4 following the company protocol (eBioscience). For B-cell populations, cells were stained with surface markers B220, IgD, IgM, and CD138. Samples were analyzed on an LSRII flow cytometer (Becton Dickinson, Palo Alto, CA, USA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

FVIII ACTIVITIES AND INHIBITOR TITERS ASSAYS

Peripheral blood samples were taken from the experimental mice and collected in a 3.8% sodium citrate solution. FVIII activities were measured by a modified clotting assay using FVIII deficient plasma and reagents to measure activated partial thromboplastin time (APTT) and FVIII deficient plasma (49, 50). FVIII activities were calculated from a standard curve generated by using serially diluted normal human pooled plasma. Anti-FVIII activities were measured by Bethesda assay as previously described (51).

ELISPOT ASSAY

In preparation for the enzyme-linked immunospot (ELISPOT) assay, spleen cells from the treated mice were prepared to isolate the $CD138^+$ (ASCs plasma) cells, 96-well filter plates (Millipore, MAHA N4510) were coated with the ASC cells (1×10^6/well) and incubated overnight at 4°C. To detect the ASC cells, plates were washed and blocked with RPMI-1640 supplemented with 10% preselected fetal calf serum (Hyclone, Logan, Utah), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Life Technologies), and 5×10^5 M β-mercaptoethanol (Sigma-Aldrich) for 1 h at 37°C prior to detection. The restimulation of memory B-cells *in vitro* was achieved as described. (26) Briefly, spleen cells were isolated and depleted of $CD138^+$ ASCs. $CD138^-$ spleen cells were cultured at 3×10^5 cells/well in RPMI-1640 medium at 37°C for 6-days. About 2 U/well of FVIII was added to the cells on day 0. After 6-days culture, newly formed ASCs were detected by ELISPOT assays.

AUTHOR CONTRIBUTIONS

Chao Lien Liu designed and performed research, analyzed data, and wrote the paper. Peiqing Ye performed research. Jacqueline Lin performed research and helped revise the manuscript. Chérie L. Butts provided anti-CD20 antibody and helped revise the manuscript. Carol H. Miao designed the project and research, analyzed data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fimmu.2013.00502/abstract

Figure S1 | B-cell depletion following anti-CD20 treatment in hemophilia A mice. Mice were treated with i.v. injection of *FVIII* plasmid (50 μg at day 0) and anti-CD20 (gray) or IgG2a isotype control (white) at a dose of 250 μg/injection at days 0 and 14. PBMCs and spleen cells isolated from anti-CD20 treated hemophilia A mice were stained with FITC-CD19, and APC-B220 at 0.5, 2, 4, 8, 12, and 16 weeks following plasmid treatment and analyzed by flow cytometry. Naïve (black) and IgG control-treated hemophilia A mice were used as controls. **(A)** Representative plot for blood cells at different time points, **(B)** Total B-cell ($CD19^+B220^+$) depletion in blood over time, **(C)** Total B-cell ($CD19^+B220^+$) depletion in spleen over time. Data shown is representative of two independent experiments.

Figure S2 | Effects of immunomodulation on both T and B-cells isolated from spleens of each treated mouse group. Spleen **(A–C)** cells were collected and isolated at serial time points from naive (light slant), *FVIII* plasmid only (white), anti-CD20+FVIII (light gray), IL-2/IL-2mAb complexes+rapamycin+FVIII (dark gray), and IL-2/IL-2mAb complexes+rapamycin+anti-CD20+*FVIII* (black) treated mice ($n = 2$, each group). Cells were stained and analyzed for T-cell populations **(A,B)** and B-cell populations **(C)**. Data shown is representative of two independent experiments.

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Glucocorticoid-induced TNF receptor family-related protein ligand is requisite for optimal functioning of regulatory CD4$^+$ T cells

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Glucocorticoid-induced tumor necrosis factor receptor family-related protein (TNFRSF18, CD357) is constitutively expressed on regulatory T cells (Tregs) and is inducible on effector T cells. In this report, we examine the role of glucocorticoid-induced TNF receptor family-related protein ligand (GITR-L), which is expressed by antigen presenting cells, on the development and expansion of Tregs. We found that GITR-L is dispensable for the development of naturally occurring FoxP3$^+$ Treg cells in the thymus. However, the expansion of Treg in *GITR-L*$^{-/-}$ mice is impaired after injection of the dendritic cells (DCs) inducing factor Flt3 ligand. Furthermore, DCs from the liver of *GITR-L*$^{-/-}$ mice were less efficient in inducing proliferation of antigen-specific Treg cells *in vitro* than the same cells from *WT* littermates. Upon gene transfer of ovalbumin into hepatocytes of *GITR-L*$^{-/-}$FoxP3(GFP) reporter mice using adeno-associated virus (AAV8-OVA) the number of antigen-specific Treg in liver and spleen is reduced. The reduced number of Tregs resulted in an increase in the number of ovalbumin specific CD8$^+$ T effector cells. This is highly significant because proliferation of antigen-specific CD8$^+$ cells itself is dependent on the presence of GITR-L, as shown by *in vitro* experiments and by adoptive transfers into *GITR-L*$^{-/-}$*Rag*$^{-/-}$ and *Rag*$^{-/-}$ mice that had received AAV8-OVA. Surprisingly, administering αCD3 significantly reduced the numbers of FoxP3$^+$ Treg cells in the liver and spleen of *GITR-L*$^{-/-}$ but not *WT* mice. Because soluble Fc-GITR-L partially rescues αCD3 induced *in vitro* depletion of the CD103$^+$ subset of FoxP3$^+$CD4$^+$ Treg cells, we conclude that expression of GITR-L by antigen presenting cells is requisite for optimal Treg-mediated regulation of immune responses including those in response during gene transfer.

Keywords: GITR-L, TNFSF18, Flt3L, Treg, CX3CR1

INTRODUCTION

CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Treg), which develop in the thymus or can be induced in peripheral organs, control many aspects of the immune response (1–4). Tregs constitutively express glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR, TNFRSF18, CD357), which is inducible on effector T cells (Teffs) (2, 5–8). Using Fc-GITR-L, a soluble form of the natural ligand of GITR, we found recently that glucocorticoid-induced TNF receptor family-related protein ligand (GITR-L) preferentially induces the *in vivo* and *in vitro* expansion of functionally competent Tregs (9). Furthermore, a significantly higher proportion of FoxP3$^+$ Tregs is also found in GITR-L transgenic mouse strains, in which the expression of GITR-L is under control of the CD19- and MHC-II-promoter respectively (10, 11). GITR-L is not expressed by T cells (8), but is found on plasmacytoid dendritic cells (pDCs), Langerhans cells, macrophage subpopulations, and endothelial cells (12–15). Here we use *GITR-L*$^{-/-}$ mice to examine the role of GITR-L in the induction of Tregs and Treg-mediated suppression in response to hepatic gene transfer with the adeno-associated viral vector AAV8.

Tolerance induction to specific foreign protein by hepatic gene transfer may be established in two steps. First, antigen-specific Tregs are *de novo* induced in the hepatic microenvironment. Second, antigen-specific Tregs are expanded systemically. Indeed, we previously found that transgene product-specific Treg actively suppresses antibody and T cell responses thereby ensuring long-term gene expression (16). Recently, studies in hemophilic mouse models have shown that AAV-mediated hepatic gene transfer can not only prevent but also reverse pathogenic antibody responses and desensitize from severe allergic reactions to the therapeutic coagulation factor IX protein (17–20). We have recently shown that the immune suppressive cytokine TGF-β is required for Treg induction in hepatic AAV gene transfer and thus necessary for suppression of antibody and CD8$^+$ T cell responses against the transgene product

(21). TGF-β, a cytokine highly expressed in mucosal tissues and sites of inflammation, plays a role in conversion of conventional peripheral $CD4^+$ T cells into Treg, and TGF-β up-regulates expression of CD103 (Integrin $\alpha_E\beta_7$) (22), which is the primary ligand of E-cadherin, an epithelial adhesion molecule. Expression of CD103 marks a subset of peripheral inducible Tregs (about 20–30% of the $CD4^+FoxP3^+$ Tregs in the spleen), which inhibit graft-versus-host disease more potently than the $CD4^+CD25^+$ Tregs (23, 24).

In this study, we provide evidence in support of the concept that the interactions between GITR and GITR-L are requisite for optimal functioning of Tregs. To this end, we analyze *GITR-L*$^{-/-}$FoxP3(GFP) and *GITR-L*$^{-/-}$CX3CR1(GFP) mice after gene transfer of ovalbumin into hepatocytes with adeno-associated virus (AAV8-OVA). Coordinate expansion of Treg and dendritic cells (DCs) was assessed after injection of Flt3 ligand in *GITR-L*$^{-/-}$ mice. The interactions between antigen presenting cells and Tregs are also evaluated after administering αCD3 in *GITR-L*$^{-/-}$ mice or by co-activation with αCD3 and soluble Fc-GITR-L.

MATERIALS AND METHODS

MICE

B6, OT-II Tg, and CX3CR1(GFP) reporter mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). OT-I × *Rag*$^{-/-}$ mice were purchased from Taconic Labs (Germantown, NY, USA). *GITR-L*$^{-/-}$ and FoxP3-IRES-EGFP-SV40 knock-in [FoxP3(GFP)] B6 mice were described previously (8, 25). *GITR-L*$^{-/-}$ mice were crossed with FoxP3(GFP) and CX3CR1(GFP) mice to generate *GITR-L*$^{-/-}$FoxP3(GFP) and *GITR-L*$^{-/-}$CX3CR1(GFP) B6 mice. All animals were housed in the Center for Life Science animal facility of BIDMC. The Guide for the Care and Use of Laboratory Animals was followed in the conduct of the animal studies of the Institutional Animal Care and Use Committee at BIDMC. Veterinary care was given to any animals requiring medical attention.

ANTIBODIES

Anti-CD11b-PacBlu, αCD11b-FITC, αCD4-PE, αCD4-APC, αCD11c-APC, αCD11c-PE, αTCRvα2-PE, and αCD3ε(145-2C11) were purchased from BioLegend (San Diego, CA, USA). Anti-Ly6C-PerCP and αFoxP3-APC were products of eBioscience (San Jose, CA, USA). Anti-Ly6G-PE, αNK1.1-PE, αCD8α-PacBlu, αCD25-PE, and αCD103-Alexa Fluor 647 were products from BD Biosciences (San Jose, CA, USA). Flt3L-Fc fusion protein was purchased from BioXCell (West Lebanon, NH, USA). Anti-IL-2 was purchased from R&D Systems (Minneapolis, MN, USA). Fc-GITR-L fusion protein was produced as described previously (9).

AAV8-OVA MEDIATED EXPRESSION OF FOREIGN PROTEIN IN HEPATOCYTES

AAV8-OVA vector (containing an ovalbumin expression cassette driven by AAV-EF1α) was packaged into serotype 8 capsid as described previously (16). Vector was injected *i.v.* into FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice at a dose of 10^{10} vector genome/mouse. Five weeks later, leukocytes from liver, spleen, and thymus were stained with TCRvα2.

Also, $Ly6G^-NK1.1^-GFP^+$ cells FACS sorted from the liver of CX3CR1(GFP) mice 7 days after AAV8-OVA injection were incubated with OT-II $CD4^+$ or CFSE-labeled OT-I $CD8^+$ T cells for 3 days. OT-II $CD4^+$ T cell cultures were stained with TCRvα2 and FoxP3. OT-I $CD8^+$ T cell culture was stained with TCRvα2 and proliferating $CD8^+$ cells were evaluated by CFSE dilution.

INDUCTION OF DENDRITIC CELLS AND TREG WITH Flt3L

Flt3L-Fc fusion protein (10 ng/mouse/injection) was *i.p.* injected into FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice for nine consecutive days as described previously (26). Leukocytes from the spleen and liver were analyzed at day 10.

CELLULARITY IN MICE AFTER αCD3-MEDIATED ACTIVATION OF T CELLS BY *IN VIVO*

Anti-CD3ε was *i.p.* injected into CX3CR1(GFP) and *GITR-L*$^{-/-}$CX3CR1(GFP) mice (20 μg/mouse, one injection). After 72 h, leukocytes of the spleen and liver were stained with CD4 and FoxP3. $CX3CR1^+$ cells were evaluated by expression of the reporter gene GFP.

IN VITRO ACTIVATION OF $CD4^+$ T CELLS

$CD4^+$ T cells from the spleen of FoxP3(GFP) mice were negatively selected using a $CD4^+$ T cells isolation kit (Miltenyi, Auburn, CA, USA) and were activated with αCD3-coupled microbeads in a round bottom 96-well plate in the presence or absence of Fc-GITR-L (1 μg/ml) for 2 days as described previously (9). Cells were stained with CD4 and CD103. Expression of FoxP3 was judged by the reporter protein EGFP. Cell numbers were counted with a Countess Automated Cell Counter (Invitrogen, Grand Island, NY, USA).

ISOLATION OF LIVER LEUKOCYTES

Liver leukocytes were isolated as described previously (27). Briefly, liver was mashed and filtered through a 70 μM cell strainer. Hepatocytes and cell debris were removed by spinning at 300 rpm for 10 min. Supernatant was centrifuged at 1500 rpm for 10 min to collect cells. Leukocytes were isolated from the interface of a 40 and 70% Percoll gradient.

Statistical analysis used Prism 4.0c software (GraphPad, San Diego, CA, USA). Statistical comparisons were performed using the two-tailed Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Flt3L-INDUCED EXPANSION OF TREG WAS IMPAIRED IN GITR-L DEFICIENT MICE DUE TO A PARTIALLY REDUCED NUMBER OF DENDRITIC CELL SUBPOPULATIONS

We previously found that after administering a Fc-GITR-L fusion protein to *WT* mice the number of Treg cells increased, which was confirmed by studies with GITR-L transgenic mice (9–11, 28). Surprisingly, we found that GITR-L was dispensable for the development of naturally occurring Treg, as the number of $FoxP3^+$ Treg cells was normal in the thymus and spleen of *GITR-L*$^{-/-}$FoxP3(GFP) mice under resting conditions (**Figure 1A**; Figure S1 in Supplementary Material).

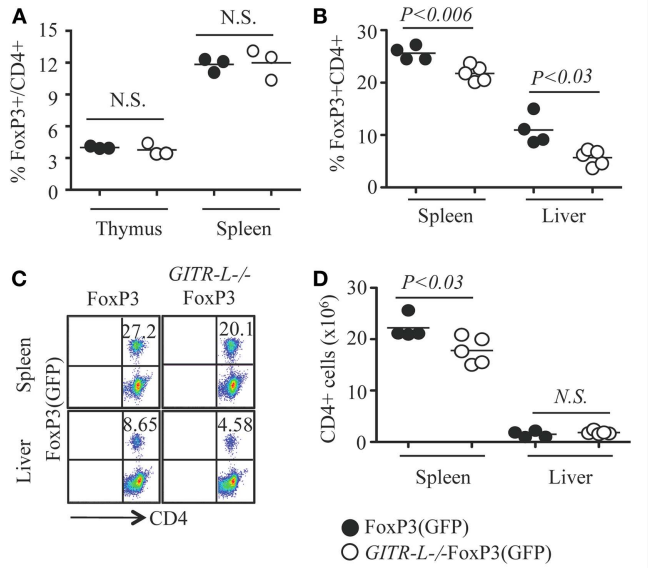

FIGURE 1 | Flt3L-induced expansion of Treg. Flt3L-Fc fusion protein was injected into FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice (10 ng/mouse/injection, nine injections). CD4^{+}CD8^{-} T cells from the thymus, spleen, and/or liver were analyzed by FACS for FoxP3 expression based on the expression of reporter protein EGFP. **(A)** Percentages of CD4^{+}FoxP3^{+} Treg in the thymus and spleen of FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice without stimulation. Percentages **(B)** and representative staining **(C)** of Treg in the spleen and liver after administering Flt3L. **(D)** Number of CD4^{+} T cells in the spleen and liver of FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice after administering Flt3L. Filled circle represents FoxP3(GFP) mouse. Open circle represents *GITR-L*$^{-/-}$FoxP3(GFP) mouse. Each circle represents one mouse.

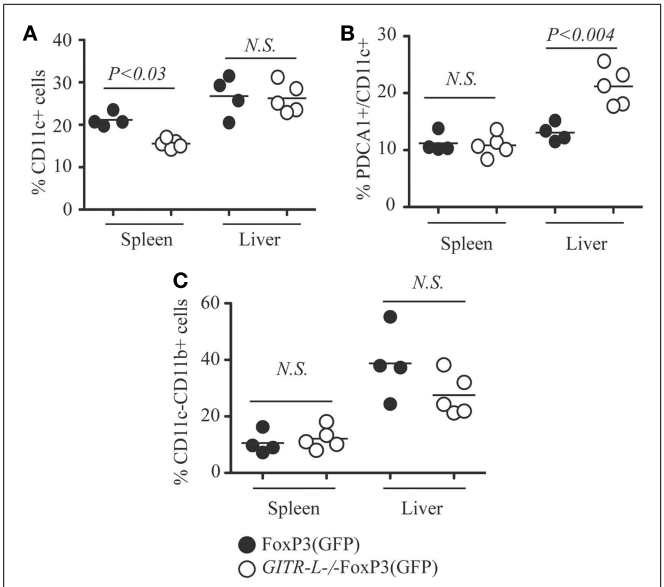

FIGURE 2 | CD11c^{+} DCs after Flt3L induction. Flt3L-Fc fusion protein was injected into FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice as described in **Figure 1**. Different subsets of myeloid cells in the spleen and liver were analyzed. **(A)** Percentages of CD11c^{+} cells in the spleen and liver. **(B)** Percentages of CD11c^{+}PDCA1^{+} cells in the spleen and liver. **(C)** Percentages of CD11c^{-}CD11b^{+} cells. Filled circle represents FoxP3(GFP) mouse. Open circle represents *GITR-L*$^{-/-}$FoxP3(GFP) mouse. Each circle represents one mouse.

To further investigate the role of GITR-L in controlling Treg development, we assessed the consequences of injecting Fms-related tyrosine kinase 3 ligand (Flt3L) into FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice for nine consecutive days. Not only is Flt3L a potent inducer of DC and macrophage proliferation (26, 29), several phagocyte subpopulations express GITR-L (12, 30). After the injection of Fc-Flt3L fusion protein, both the numbers and the frequency of FoxP3^{+} Treg were significantly increased in the spleen and liver. This Fc-Flt3L-induced expansion was, however, significantly reduced in *GITR-L*$^{-/-}$FoxP3(GFP) mice (**Figures 1B,C**). The total number of CD4^{+} T cells in the spleen was also lower in *GITR-L*$^{-/-}$FoxP3(GFP) mice than the *WT* counterparts (**Figure 1D**). Thus, GITR-L plays a significant role in the expansion of Treg in the peripheral tissues.

We next evaluated whether the impaired Flt3L-induced expansion of Treg cells in *GITR-L*$^{-/-}$FoxP3(GFP) mice correlated with reduced numbers of DCs and macrophages (MØ) (31, 32). As shown in **Figure 2A** and Figure S2A in Supplementary Material, the percentage of CD11c^{+}CD11b^{+} and CD11c^{+}CD11b^{-} DCs was reduced in the spleen of *GITR-L*$^{-/-}$FoxP3(GFP) mice as compared to FoxP3(GFP) mice. Although the number of conventional CD11c^{+} DCs in the liver was normal (**Figure 2A**), the percentage of pDCs in *GITR-L*$^{-/-}$FoxP3(GFP) mice was higher than that of their *WT* counterparts (**Figure 2B**; Figure S2B in Supplementary Material and Data not shown). The frequency of CD11c^{-}CD11b^{+} MØ was comparable between these two mice (**Figure 2C**). Taken together, these data indicate that after Flt3L induction, GITR-L affects the expansion and differentiation of subpopulations of DCs, which in turn leads to expansion of Tregs.

GITR-L$^{-/-}$ CX3CR1^{+} DCs ISOLATED FROM THE LIVER ARE LESS EFFICIENT THAN WT CX3CR1^{+} DCs IN THE *IN VITRO* INDUCTION OF OVA-SPECIFIC TREG AND CD8^{+} T CELLS

To directly test whether the absence of GITR-L in DC subpopulations affects proliferation of antigen-specific GITR^{+} Treg and CD8^{+} cells, we immunized *GITR-L*$^{-/-}$CX3CR1(GFP) and *WT* CX3CR1(GFP) mice by gene transfer with AAV8-OVA (**Figure 3A**). One week after injection of AAV8-OVA, liver CX3CR1(GFP)$^{+}$ cells purified by FACS were incubated with OVA-specific OT-II CD4^{+} T cells or OT-I CD8^{+} cells for 3 days. *GITR-L*$^{-/-}$ CX3CR1^{+} cells were less efficient in inducing Treg as compared to the same cells isolated from *WT* mice (**Figures 3B,C**). Since activated CD8^{+} cells carry GITR on their surface, we also evaluated whether *in vitro* proliferation of CD8^{+} T cells would be affected by the absence of GITR-L from the surface of these DCs. Indeed, the proliferation of CD8^{+} OT-I cells was reduced when cocultured with liver CX3CR1^{+} cells from AAV8-OVA-primed *GITR-L*$^{-/-}$CX3CR1(GFP) mice compared to OT-I cells cultured with *WT* CX3CR1^{+} DCs (**Figures 3D,E**).

We conclude that GITR-L on the surface of antigen presenting cells can drive proliferation of both FoxP3^{+}CD4^{+} Treg cells and activated CD8^{+} T cells in an antigen-specific manner.

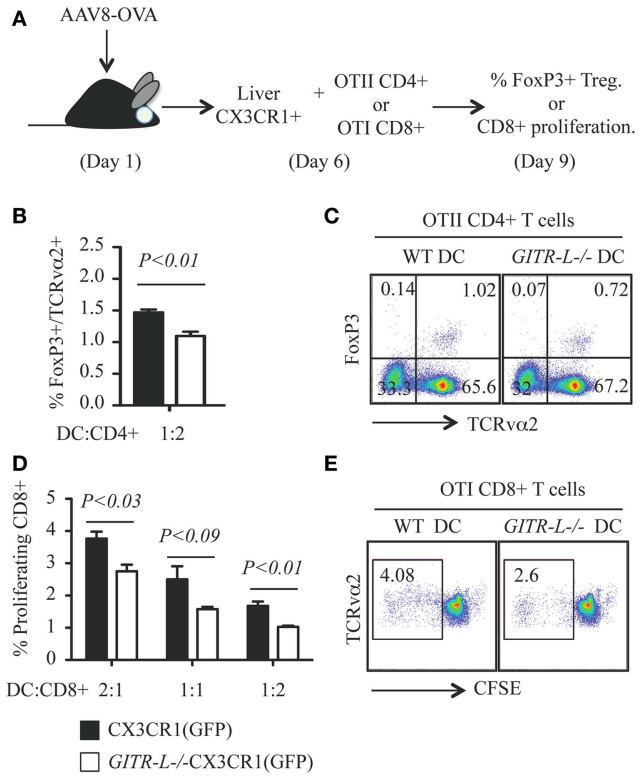

FIGURE 3 | *In vitro* induction of OVA-specific Treg and CD8$^+$ T cells with hepatic CX3CR1$^+$ DCs. **(A)** Schematic for *in vitro* priming of CD8$^+$ OT-I and CD4$^+$ OT-II T cells. Briefly, AAV8-OVA(10^{10} vector genome/mouse) was *i.v.* injected into CX3CR1(GFP) and *GITR-L*$^{-/-}$CX3CR1(GFP) mice. After 7 days, Ly6G-NK1.1-CX3CR1(GFP)$^+$ cells were purified from the liver and incubated with CD8$^+$ OT-I T and CD4$^+$ OT-II T cells at different ratios for 3 days. Divisions of CD8$^+$ OT-I T cells were evaluated by CFSE dilution. **(B)** Percentages of *in vitro* induced of TCRvα2$^+$FoxP3$^+$ Treg. **(C)** Representative stainings of **(B)**. **(D)** Ratios of proliferating TCRvα2$^+$CD8$^+$ OT-I T cells. **(E)** Representative staining of **(D)**. Results represent one of three independent experiments. Error bars indicate mean ± SEM of triplicates.

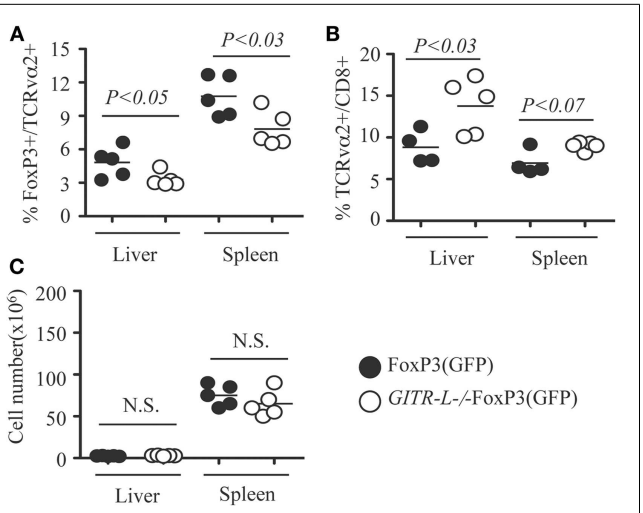

FIGURE 4 | *In vivo* induction of OVA-specific Treg and CD8$^+$ T cells in *GITR-L*$^{-/-}$FoxP3(GFP) mice. AAV8-OVA (10^{10} vector genome/mouse) was *i.v.* injected into FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice. Five weeks later, leukocytes from the liver and spleen were stained with TCRvα2. **(A)** Percentages of FoxP3$^+$/TCRvα2$^+$ cells. **(B)** Percentages of TCRvα2$^+$/CD8$^+$ cells. **(C)** Number of leukocytes. Filled circle represents FoxP3(GFP) mouse. Filled circle represents FoxP3(GFP) mouse. Open circle represents *GITR-L*$^{-/-}$FoxP3(GFP) mouse. Each circle represents one mouse.

AFTER AAV8-OVA GENE TRANSFER, THE NUMBER OF ANTIGEN-SPECIFIC TREG IN GITR-L$^{-/-}$FoxP3 MICE IS REDUCED, WHICH RESULTS IN AN INCREASED NUMBER OF OVA-SPECIFIC CD8$^+$ T CELLS

Because targeted expression of exogenous protein in hepatocytes by AAV8-mediated gene transfer induces a Treg-mediated tolerance (16), we assessed whether this process involves GITR-L. To assess this, we injected an AAV8-OVA vector into in FoxP3(GFP) and *GITR-L*$^{-/-}$FoxP3(GFP) mice and determined the number of OVA-specific Treg and CD8$^+$ T cells. Consistent with the results when administering Flt3L, there was a reduced percentage of OVA-specific FoxP3$^+$TCRvα2$^+$ T cells in the spleen and liver of *GITR-L*$^{-/-}$FoxP3(GFP) mice as compared to that of *WT* mice 5 weeks after vector administration (**Figure 4A**). Conversely, AAV-mediated OVA expression in the hepatocytes induced an increased percentage of OVA-specific CD8$^+$TCRvα2$^+$ T cells in the spleen and liver of *GITR-L*$^{-/-}$FoxP3(GFP) mice (**Figure 4B**). By contrast, the total cell numbers were comparable between these two mouse strains (**Figure 4C**). The data suggest that GITR-L deficiency may impair the induction of antigen-specific Tregs (16–18, 21, 33), which may at least partially compromise their immunosuppressive capability.

As the *in vitro* data suggest that GITR-L expression on DCs causes the expansion of CD8$^+$ cells, this *in vivo* result might underestimate the consequences of the reduced number of the Tregs in the *GITR-L*$^{-/-}$ mice. To test whether GITR-L is implicated in the *in vivo* expansion of antigen-specific CD8$^+$ cells, we used a system in which the Treg-mediated suppression is absent. To this end, we injected AAV8-OVA into *Rag*$^{-/-}$ and *GITR-L*$^{-/-}$*Rag*$^{-/-}$ mice followed by the adoptive transfer of OT-I CD8$^+$ T cells after 1 week (**Figure 5A**). Eight weeks after transfer of OT-I CD8$^+$ T cells, the number of CD8$^+$ T cells in the blood of the *GITR-L*$^{-/-}$*Rag*$^{-/-}$ recipients was significantly lower than that of the *Rag*$^{-/-}$ recipients (**Figure 5B**). This was not due to an inadequate amount of OVA antigen production in the *GITR-L*$^{-/-}$*Rag*$^{-/-}$ recipients (**Figure 5C**). Taken together, the data indicate that GITR-L is required for optimal induction and/or expansion of antigen-specific Treg in the context of hepatic AAV8 gene transfer.

DEPLETION OF CX3CR1$^+$ (GFP) CELLS BY αCD3 IN GITR-L$^{-/-}$ MICE CORRELATES WITH A REDUCED NUMBER OF FoxP3$^+$ TREG CELLS

In vitro expansion of FoxP3$^+$ Treg cells can be achieved by stimulation with a combination of αCD3 and soluble GITR-L (Fc-GITR-L) (9). We then assessed whether injection of αCD3 into *WT* and *GITR-L*$^{-/-}$ mice would affect the Treg population. As shown in **Figures 6A,B**, αCD3 induced a significant reduction of the percentage of FoxP3$^+$ Treg in the spleen and liver of *GITR-L*$^{-/-}$CX3CR1(GFP) mice, but not in *WT* CX3CR1(GFP) mice. In support of our observations in this paper, the reduced number of Tregs coincided with a reduction of CX3CR1$^+$ DCs in the spleen

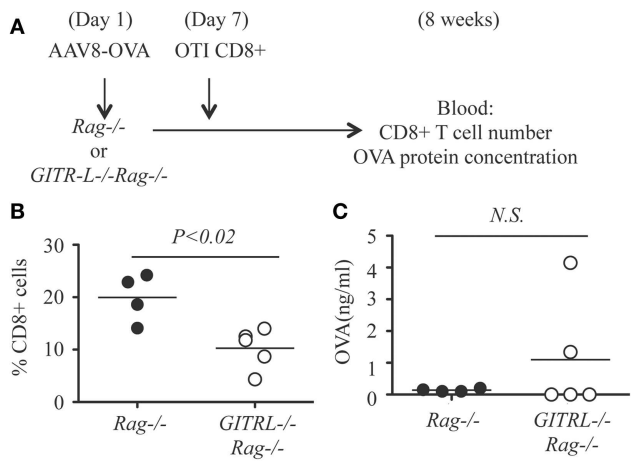

FIGURE 5 | *In vivo* induction of OVA-specific CD8$^+$ T cells in *GITR-L*$^{-/-}$*Rag*$^{-/-}$ mice. **(A)** Schematic for *in vivo* expansion of CD8$^+$ OT-I T cells. Briefly, AAV8-OVA (10^{10} vector genome/mouse) vector was *i.v.* injected to *Rag*$^{-/-}$ or *GITR-L*$^{-/-}$*Rag*$^{-/-}$ mice. Seven days later, CD8$^+$ OT-I T cells (10^6 cells/mouse) were *i.p.* injected to mice with AAV8-OVA gene transfer. Mice were euthanized after 8 weeks. **(B)** Percentages of CD8$^+$ T cells in the blood were evaluated by FACS. **(C)** Concentrations of chicken ovabulmin protein in the plasma were measured by ELISA. Filled circle represents *Rag*$^{-/-}$ mouse. Open circle represents *GITR-L*$^{-/-}$*Rag*$^{-/-}$ mouse. Each circle represents one mouse.

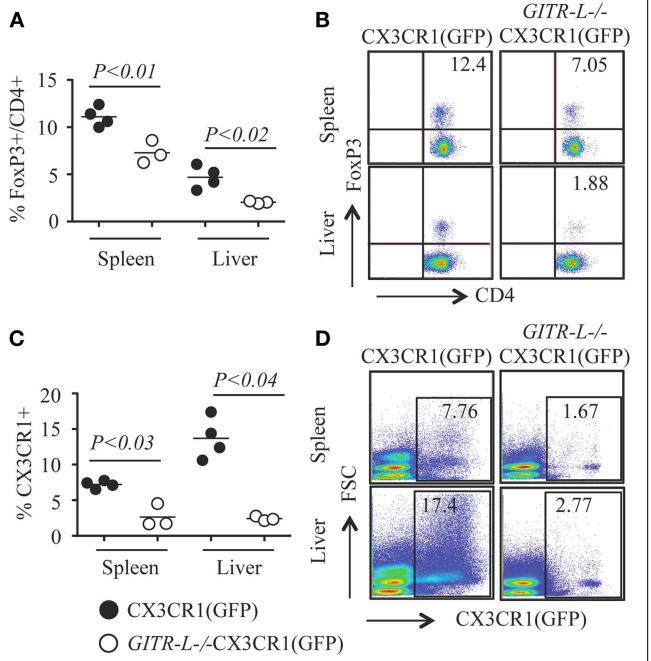

FIGURE 6 | Numbers of Treg and CX3CR1$^+$ DCs after administering αCD3. Anti-CD3ε (20 μg/mouse, one injection) was *i.p.* injected into CX3CR1(GFP) and *GITR-L*$^{-/-}$CX3CR1(GFP) mice. Mice were euthanized at day 3. Splenocytes and liver leukocytes were stained for the expression of CD4 and FoxP3 by FACS. CX3CR1$^+$ cells were assessed by the expression of reporter protein EGFP. Percentages **(A)** and representative staining **(B)** of FoxP3$^+$CD4$^+$ Treg cells in the spleen and liver. Percentages **(C)** and representative staining **(D)** of CX3CR1$^+$ phagocytes in splenocytes and liver leukocytes. Filled circle represents CX3CR1(GFP) mouse. Open circle represents *GITR-L*$^{-/-}$CX3CR1(GFP) mouse. Each circle represents one mouse.

and liver of *GITR-L*$^{-/-}$CX3CR1(GFP) mice (**Figures 6C,D**). In contrast, the numbers of CX3CR1$^+$ cells in the spleen and liver were comparable in the two mouse strains under homeostasis (Figure S3 in Supplementary Material).

To further investigate the role of GITR-L in the expansion of FoxP3$^+$ Treg, CD4$^+$ T cells were purified from the spleen of FoxP3(GFP) mice and stimulated *in vitro* with αCD3 with either Fc-GITR-L or IgG. Forty-eight hours after exposure to αCD3, the number of total CD4$^+$ and FoxP3$^+$CD4$^+$ Treg was significantly higher in the presence of Fc-GITR-L than that of IgG (**Figures 7A,B**). Interestingly, a subset of CD103$^+$ Treg cells, which is induced in epithelium and in sites of inflammation (23, 34) and comprises approximately 20% of all FoxP3$^+$ Treg cells in the spleen, was also expanded by Fc-GITR-L (**Figures 7C,D**).

We conclude that while the induction or expansion of Treg is impaired in the absence of GITR-L, Fc-GITR-L provides a positive signal to GITR$^+$ Treg.

DISCUSSION

The receptor-ligand pair GITR/GITR-L (TNFRSF18/TNFSF18) appears to be involved in the development of a variety of inflammation-related diseases in murine models (6, 8, 12, 35, 36). It was originally thought that the suppressor function of Treg cells, which constitutively express GITR, would be abrogated by anti-GITR thus breaking immune self-tolerance (2). More recent additional evidence shows that GITR engagement by its natural ligand GITR-L causes an extensive expansion of functionally competent Tregs (9–11), although the relative role of GITR on Treg and Teff cells remains only partly understood. In this study we find that in the absence of GITR-L the expansion of FoxP3$^+$ Treg cells is impaired in an antigen-specific manner, which can be mimicked by *in vivo* and *in vitro* activation of CD4$^+$ Treg cells with αCD3. Our results are consistent with the findings of the Chatila group that expansion and contraction of Teff and Treg dynamically control primary immune responses to foreign antigen (25).

Glucocorticoid-induced TNF receptor family-related protein ligand impacts immune regulation in gene replacement therapy at least at three levels. First, the induction/expansion of antigen-specific Treg cells in the liver after AAV-mediated gene therapy is impaired directly by the absence of GITR-L. Second, the expansion of antigen-specific CD8$^+$ T cells is reduced by GITR-L deficiency. However, impaired expansion of Treg cells can on the other hand up-regulate CD8$^+$ T cell expansion indirectly. Third, GITR-L deficiency affects the infiltration of monocyte-derived MØ to the sites where exogenous protein is expressed and/or the sites of inflammation (30), which changes the local function of different immune cells. These GITR-L-expressing, monocyte-derived MØ may provide a microenvironment for the expression of CD103 in Treg cells, an integrin that facilitates the retention of Treg cells in the sites of inflammation or infection.

Surprisingly, we found that administering αCD3 causes the depletion of CX3CR1$^+$ DCs in the spleen and liver of *GITR-L*$^{-/-}$

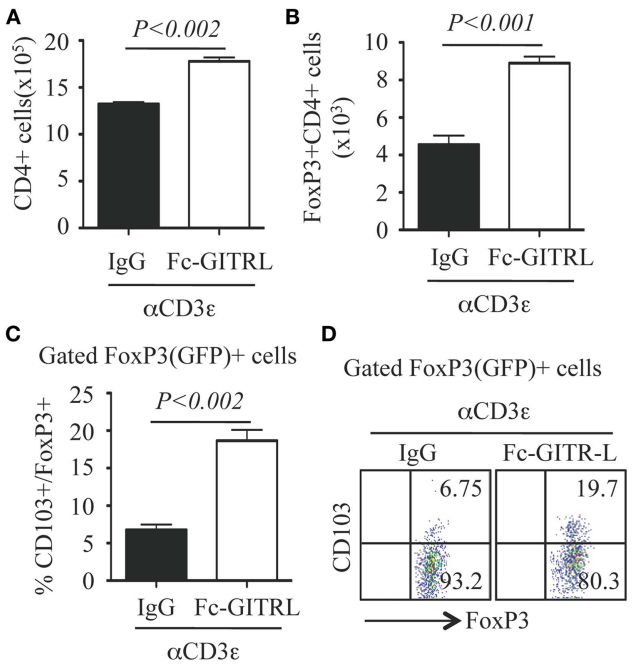

FIGURE 7 | Expansion of FoxP3$^+$ and CD103$^+$ Tregs by Fc-GITR-L *in vitro*. CD4$^+$ T cells (10^5 cells/well) from the spleen of FoxP3(GFP) mice were stimulated with αCD3-beads in the presence of Fc-GITR-L or control IgG for 2 days and then stained with CD4 and CD103. FoxP3 expression was judged according to the expression of reporter protein EGFP. **(A)** Number of total CD4$^+$ cells. **(B)** Number of FoxP3$^+$CD4$^+$ Tregs. **(C)** Percentages of CD103$^+$ cells among gated FoxP3$^+$ Tregs. **(D)** Representative staining of **(C)**. Results were representative of at least three independent experiments. Error bars indicate mean ± SEM of triplicates.

mice, which correlates with a reduced number of FoxP3$^+$ Tregs. It is reported that IL10-secreting GITR$^+$ Tr1 cells may suppress immune responses by granzyme B-mediated killing of myeloid APCs (37, 38). Granzyme B is also important for the ability of Treg, NK cells, and CD8$^+$ T cells to kill their targets (39). It is possible that Tr1, Treg, and CD8$^+$ T cells play a role in the depletion of CX3CR1$^+$ DCs in *GITR-L*$^{-/-}$ mice. In the presence of GITR-L, an increased expansion of Treg may inhibit this self-destructive cytotoxicity. Depletion of CX3CR1$^+$ DCs, which includes the GITR-L-expressing pDCs and MØ (12, 30), may feedback to cause the reduction of Treg number during immune responses.

Ly6C^{hi} monocytes give rise to CX3CR1$^+$ DCs under both steady state and inflammation. Under resting conditions, CX3CR1$^+$ DCs in the intestine is reported to induce a immunosuppressive CD8$^+$ T cells (40). CX3CR1$^+$ DCs isolated from the liver are able to induce Treg *in vitro*. However, during inflammation CX3CR1$^+$ DCs give rise to proinflammatory effector cells (41). The mechanism how this Ly6C^{hi} monocyte-derived DC subpopulation is educated to be either protagonist or antagonist is still not well understood. Anti-CD3-mediated depletion of CX3CR1$^+$ DCs in the liver may provide an important tool for the study of migration, colonization, and education of this special DC subset (30).

In conclusion, our data show that GITR and GITR-L have important implications for gene therapy. Optimal induction of an immune regulatory response, which is crucial for tolerance to the transgene product and for immune modulatory gene therapy, requires co-stimulation by GITR-L, which enhances Treg induction and function. Expression of GITR-L on hepatic APCs may in part explain the tolerogenic/Treg inducing capacity of hepatic gene transfer.

AUTHOR CONTRIBUTIONS

Gongxian Liao performed all the experiments; Michael S. O'Keeffe helped in processing the samples and editing the manuscript; Guoxing Wang and Boaz van Driel helped in processing the samples and discussing the results. Rene de Waal Malefyt generated GITR-L deficient mice; Hans-Christian Reinecker brought deeper insight into αCD3-inducing murine model. Roland W. Herzog helped in discussing and writing the manuscript; Cox Terhorst is the major organizer of this work and designed the experiments with Gongxian Liao.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00035/abstract

Figure S1 | Representative staining of Figure 1A.

Figure S2 | (A,B) Representative staining of Figures 2A,B.

Figure S3 | CX3CR1(GFP)+ phagocytes in spleen and liver leukocytes of CX3CR1(GFP) and *GITR-L*$^{-/-}$CX3CR1(GFP) mice under resting condition.

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AAV vectors vaccines against infectious diseases

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Since their discovery as a tool for gene transfer, vectors derived from the adeno-associated virus (AAV) have been used for gene therapy applications and attracted scientist to this field for their exceptional properties of efficiency of *in vivo* gene transfer and the level and duration of transgene expression. For many years, AAVs have been considered as low immunogenic vectors due to their ability to induce long-term expression of non-self-proteins in contrast to what has been observed with other viral vectors, such as adenovirus, for which strong immune responses against the same transgene products were documented. The perceived low immunogenicity likely explains why the use of AAV vectors for vaccination was not seriously considered before the early 2000s. Indeed, while analyses conducted using a variety of transgenes and animal species slowly changed the vision of immunological properties of AAVs, an increasing number of studies were also performed in the field of vaccination. Even if the comparison with other modes of vaccination was not systemically performed, the analyses conducted so far in the field of active immunotherapy strongly suggest that AAVs possess some interesting features to be used as tools to produce an efficient and sustained antibody response. In addition, recent studies also highlighted the potential of AAVs for passive immunotherapy. This review summarizes the main studies conducted to evaluate the potential of AAV vectors for vaccination against infectious agents and discusses their advantages and drawbacks. Altogether, the variety of studies conducted in this field contributes to the understanding of the immunological properties of this versatile virus and to the definition of its possible future applications.

Keywords: AAV vectors, anti-viral vaccination, humoral responses, cytotoxic responses, antibody gene transfer, immunoadhesins, capsid

INTRODUCTION

Historically, vaccination strategies against infectious agents have mostly used live attenuated pathogens. These vaccines are highly efficient for generating both humoral and cellular immune responses but for many pathogens this approach is too risky even in their attenuated form to be used in humans. Subunit vaccines, usually recombinant proteins, have provided an interesting and safe alternative; however their use is limited to the generation of antibody (Ab) responses and is, therefore, limited to preventive vaccination strategies against pathogens that can be efficiently cleared by a humoral response. In addition, their efficiency frequently requires repeated injections of high doses of the vaccines coupled to adjuvants. In this context, the development of viral vector has provided a very interesting alternative since they can efficiently deliver antigens (Ag) into the antigen processing pathway leading to the stimulation of cytotoxic T cell responses, which are essential to clear intra-cellular pathogens and to develop therapeutic vaccines (1).

Viral vectors are derived from wild type viruses by deleting a part or all of viral genes. The immunogenic properties of a viral vector results not only from that of Ag which is expressed, but also on the intrinsic biological properties of the viral particle which determine its interaction with the cells of the immune system, in particular antigen presenting cells (APC), and with other target tissues. Both contribute to the nature and the potency of the immune response that is induced (1). So far, the most widely evaluated viral vectors for vaccination, in particular, in human clinical trials are those derived from adenovirus (Ad) and the poxvirus family (2). Both of these types of vectors provide several advantages as vaccines because of their efficiency of infection of several cell types including APC. However, when used as a vaccine both types of vectors contain, in addition to the transgene encoding for the Ag, several viral genes whose expression can constitute a safety concern and lower or modify the efficacy of the vaccine by diverting immune responses from the Ag itself. In addition, for both vector types, if the strong immunogenicity of the viral particle itself may be seen as helping to induce strong immune responses, it can also constitute a safety problem, due to the strong inflammatory responses that are induced. Last but not least, as for many other viral vectors, a main issue is that of the pre-existing immunity in the human population, which may constitute a barrier to their use in man.

Adeno-associated virus (AAV) vectors already have a relatively long history in gene therapy but have only recently emerged in the vaccination field. Since the first and unique report by Manning et al. (3) documenting the capacity of AAV to induce a strong humoral and cellular response against the herpes simplex virus (HSV) type 1 glycoprotein B (3), an increasing number of studies

have explored the use of AAV vectors for genetic vaccination, thus contributing to the understanding of their immunological properties. This review is focused on the description of vaccination studies conducted mostly against viral or microbial antigens and using AAV vectors directly *in vivo* to highlight their properties, potential limitations, and future developments. Neither the few studies which used AAV vectors for vaccination against non-infectious diseases nor the use of these vectors for immunotherapy by *ex vivo* gene transfer into dendritic cells (DC) are included. The two first sections summarize the main characteristics of AAV vectors when used in various vaccination settings. The third section presents the results from the most advanced studies, which explored the potential of AAV vaccines against experimental challenge in a relevant animal model and/or have explored the efficacy of AAV-mediated vaccination in non-human primates (NHP). Finally, the last part of this review describes the most likely future developments in this field.

AAV VECTORS FOR ACTIVE IMMUNOTHERAPY

Compared to other viruses used as vectors for vaccination and in particular to Ad and poxviruses, AAV potentially offers a significant number of advantages. First, the vectors are derived from a non-pathogenic virus that is inherently replication defective (4). Accordingly, several preclinical and clinical gene therapy trials have demonstrated their favorable safety profile (5, 6). The vectors are gutless and, therefore do no encode for any viral gene. The vector genome is usually composed of a single-stranded (ss) DNA molecule containing the transgene expression cassette flanked by the viral inverted terminal repeats [for a review, see Ref. (7)]. AAV particles containing a double-stranded, also called self-complementary (sc) AAV genome, can be also developed to improve the kinetics and the level of expression of the transgene (8). AAV vectors possess the capacity to efficiently transduce several tissues *in vivo* and the isolation of several AAV serotypes and of a multitude of capsid variants potentially offers the possibility to develop prime/boost strategies by switching the AAV capsid, thus avoiding the anti-capsid neutralizing humoral responses induced after the first injection. However, as with other viral vector systems, AAVs also have a number of drawbacks, notably the limited transgene capacity, a strong and wide pre-existing immunity in humans, and the technological challenge of producing large and high titter vector stocks. The studies conducted in the field of active vaccination using AAV vectors are very diverse in terms of targets, objectives, and strategies (**Table 1**). However, so far, only a limited number of studies have been conducted directly comparing AAV vectors to other vector vaccines. Despite this diversity and lack of comparative studies, several common conclusions can be extrapolated from these studies which define the advantages and also the pitfalls of AAV vectors for this particular application.

EVALUATION OF DIFFERENT AAV SEROTYPES AND ROUTES OF IMMUNIZATION

Initial analyses conducted in the field of vaccination have been performed using AAV2-derived vectors. Despite their lower efficiency compared to other AAV serotypes (29), in these initial studies, AAV2 vectors already demonstrated their capacity to induce strong immune responses using a variety of injection routes and viral Ag derived from HSV, the human immunodeficiency virus (HIV), the severe acquired respiratory syndrome coronavirus (SARS-CoV), and the human papillomavirus (HPV) (3, 10, 15, 16, 30) (**Table 1**). Notably, a study using AAV2 vectors expressing several simian immunodeficiency virus (SIV) genes indicated that a single intra-muscular administration of the vector was able to elicit SIV-specific T cells and Ab, demonstrating its potential in a macaque NHP model (22). Thereafter, other AAV serotypes rapidly entered the field and out-performed AAV2 in terms of immune responses, as expected from their higher efficiency for gene transfer (13, 17–19, 28). The use of various AAV serotypes also allowed varying the injection routes (**Table 1**). In particular, several studies have used AAV5 or AAV9 vectors for intra-nasal vaccination to induce a mucosal immunity (11, 12). Most notably, nasal application of AAV9 was efficient even in the presence of high levels of pre-existing serum anti-AAV9 neutralizing antibodies (NAb), which

Table 1 | Summary of active immunization studies using AAV vectors.

Antigen	AAV serotype	Injection route	Reference
HSV-1 (gB and gD)	AAV2	IM/mice	Manning et al. (3)
HPV (E7, E7/hsp70 L1)	AAV2	IM/mice	Liu et al. (9)
	AAV2	IM/mice	Liu et al. (10)
	AAV5	IN/mice	Kuck et al. (11)
	AAV5, 8, 9	IN/mice	Nieto et al. (12)
	AAV1, 2	IM/mice	Zhou et al. (13)
	AAV5, 9	IN/macaques	Nieto et al. (14)
HIV (env, tat, rev)	AAV2	IM, SC, IN, IP/mice	Xin et al. (15)
	AAV2	Oral/mice	Xin et al. (16)
	AAV1, 3, 4, 5, 7, 8	IM/mice	Xin et al. (17)
	AAV1, 2, 5, 7, 8, 9	IM/mice	Lin et al. (18), Lin et al. (19)
	AAV8, rh32-33	IM/mice	Lin et al. (20)
	scAAV2, 7, 8	IM/mice	Wu et al. (21)
SIV	AAV2	IM/macaque	Johnson et al. (22)
SARS-CoV (S protein)	AAV2	IM/mice	Du et al. (23)
Malaria (MSP4, 4/5)	AAV1, 3	IM/mice	Logan et al. (24)
Influenza (NP, H1, M1)	AAV8, rh32.33	IM/mice	Lin et al. (20)
	AAV9	IM/mice	Sipo et al. (25)
DEV (Env)	AAV8, rh32.33	IM/mice	Li et al. (26)
TB (Ag85A)	Modified AAV2	IM/mice	Rybniker et al. (27)
NIV (G protein)	AAV1, 8, rh32.33	IM, ID/mice	Ploquin et al. (28)

IM, intra-muscular; IN, intra-nasal; ID, intra-dermal; IP, intra-portal; IPL, intra-pleural; HSV-1, herpes simplex virus type 1; HPV, human papillomavirus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SARS-CoV, severe acquired respiratory syndrome coronavirus; DEV, dengue virus; TB, Mycobacterium tuberculosis, NIV, Nipah virus.

did not prevent re-immunization with the same serotype and transgene expression (14, 31).

INDUCTION OF HUMORAL RESPONSES

A remarkable feature of AAV vector vaccines in most, if not all studies, is their capacity to induce strong and long lasting Ab responses, even after a single administration. Several studies documented the induction of humoral responses lasting for many months and sometimes more than 1 year (3, 11, 12, 26, 28). In addition, in some studies, the AAV-induced Ab response was higher and more sustained than using other vaccination strategies including DNA, recombinant proteins, inactivated virus, or virus-like particles (VLPs) (3, 10–12, 24, 30). Such pronounced Ab response may be linked to the high and sustained expression of the transgene over time achieved with most AAV serotypes (18, 19, 26, 28). The high and long lasting level of Ab responses may also explain why, in some instances, a boost effect was not always observed upon re-injection with the same or an alternative AAV serotype (22, 24, 28). In contrast, some studies have documented the possibility of enhancing the AAV-induced humoral response by using an Ad vector expressing the same Ag for the boost (18, 19). These observations suggest that the enhancement of humoral responses can be achieved only after stimulation of different immune pathways than those used for the initial prime vaccination. This scenario is similar to that described for vaccination strategies using plasmid DNA for the prime injection and an heterologous vaccine for the boost (1).

INDUCTION OF CD8$^+$ T-CELL RESPONSES

The control of several infectious diseases requires, in addition to a strong humoral response, the concomitant induction of cytotoxic cellular responses to not only prevent virus spreading but also eradicate virus-infected cells. Since the first report using AAV for vaccination, several studies have documented the induction of transgene-specific CD8$^+$ T-cell responses following injection of AAV vectors in mice (3, 12, 13, 15, 17, 25) and NHP (22). However, only two studies have thoroughly analyzed such cellular responses quantitatively and qualitatively. Two reports, published in 2007 indicated that CD8$^+$ T-cell responses induced by AAV vectors, derived from a variety of natural AAV serotypes, failed to be successfully recalled and amplified upon a boost with an Ad vector indicating a default in the CD8$^+$ T-cell memory response. A detailed analyses of such responses further showed that CD8$^+$ T cells had markers of exhaustion, which were correlated to a continuous expression of the transgene (18, 19). Whether the persistence of transgene expression was a consequence or a cause of such a functionally impaired CD8$^+$ T-cell response is still unclear. Interestingly however, a functional transgene-specific CD8$^+$ T-cell response could be induced by changing the AAV capsid. A hybrid capsid AAVrh32.33, derived from two natural rhesus macaque isolates, was able to generate a CD8$^+$ T-cell response against the transgene product in mice and NHP which could be successfully amplified following a boost with an Ad vector (20). Further studies conducted in mice demonstrated that intra-muscular administration of the AAVrh32.33 vector was able to induce a strong cellular response even against transgene products, such as LacZ, which are usually tolerated using natural AAV serotypes and resulted in the elimination of AAV-transduced cells within 2 months after AAV administration (32). Altogether, these analyses indicated that the capsid, in addition to the transgene, is a key modulator of immune responses in particular by changing the tropism, and thus the interaction of AAV particles with immune cells. It is worth noting that although natural AAV serotypes are considered to be unable to efficiently transduce APC, in particular DC, it is currently unknown how AAVrh32.33 interacts with these cells. Finally, it is important to highlight that such strong differences among AAV capsids, observed in murine models may not hold true in other animals species, in particular primates, in which even natural AAV vectors were shown to induce immune responses leading to the elimination of transgene-expressing cells (33).

AAV VECTORS FOR PASSIVE IMMUNOTHERAPY

The ability of AAV vectors to efficiently express various transgenes including those coding for soluble proteins has fostered their use for Ab-gene transfer to produce neutralizing Ab (NAb) directly *in vivo* (**Table 2**). Indeed, Ab-based therapies are costly and limited by the half-life of the Ab, with single administrations resulting only in short term protection. Therefore, most of these therapies require frequent administration of relatively high doses of the Ab, often via intravenous administration, since high and persistent serum levels of Ab are frequently required for optimal clinical efficacy. In this scenario, the use of AAV vectors may be of great interest, in particular, to allow a sustained and continuous expression of the Ab after a single administration. In these studies, as in gene therapy, AAV vectors are used only as vehicles to produce high levels of proteins *in vivo* and, in contrast to the previous situation (active immunotherapy), immune responses against the transgene product, here the Ab, are unwanted. Most of the studies performed in this area are recent and have used natural AAV serotypes other than AAV2 (**Table 2**).

One of the challenges in the engineering of an AAV vector coding for a full length Ab was to ensure an efficient and equimolar production of the light and heavy chains. A major advance was achieved by demonstrating the possibility to produce high levels of Ab in mice after intra-portal administration of an AAV8 vector expressing heavy and light chains linked with a

Table 2 | Summary of passive immunization studies using AAV vectors.

Antigen	AAV serotype	Injection route	Reference
HIV	AAV2 AAV8	IM/mice	Lewis et al. (34) Balazs et al. (35)
RSV	AAVrh10	IPL/mice	Skaricic et al. (36)
SIV	AAV1, scAAV1	IM/macaque	Johnson et al. (37)
Influenza	AAV8 AAV9	IM/mice, ferrets IN/mice, ferrets, macaques	Balazs et al. (38) Limberis et al. (39)

*RSV, respiratory syncytial virus. See the legend of **Table 1** for additional abbreviations.*

2A self-processing peptide derived from the foot-and-mouth disease virus (FMDV2A) (40). With this technology, 1 mg/ml of Ab was detected in the serum of the animals several months after AAV administration, a level much superior to that previously observed using an AAV2 vector expressing the two chains using a dual promoter system (34). This initial report fostered a series of studies to validate this strategy in various experimental models. Notably, by using specific AAV serotypes, it was possible to address Ab secretion into tissues such as the lungs, which are the site of entry of several viruses and bacteria. For example, intra-pleural administration of AAVrh10 encoding for a murine Ab against the respiratory syncytial virus (RSV) resulted in the long-term production of anti-RSV NAb in the serum and the lungs and partially protected the animals against a challenge with the virus (36). A similar strategy was developed to express *in vivo* Ab able to protect against the deleterious effects induced by some toxins or compounds such as anthrax, cocaine, or nicotine (41–43).

PROTECTION STUDIES AND PRE-CLINICAL EVALUATIONS

The use of AAV as vehicle for gene delivery to induce immune responses against foreign and self-antigens has been explored in animal models, but only one phase I clinical trial was performed in humans (44). For prophylactic vaccination against infectious agents, there are safety concerns since typically such vaccines are given to healthy children or adolescents with an unknown risk for late consequences. Also, prophylactic vaccines are often targeted to large populations. From this standpoint, AAV vectors are interesting tools since their use for gene therapy has already demonstrated their overall absence of toxicity (6). However, current very high costs for manufacturing AAV vectors and the need for high particle doses are clearly major hurdles for vaccine development. Nevertheless, future studies may help define some niches in which AAV may be particularly advantageous over other vaccination strategies.

This chapter will describe a number of informative pre-clinical vaccination studies, which demonstrated a complete protection against experimental challenge in a relevant animal model and/or have explored the efficiency of AAV-mediated vaccination in NHP.

HENIPAVIRUSES

Nipah virus (NiV) and Hendra virus (HeV) are closely related, recently emerged paramyxoviruses, belonging to the henipavirus genus. Both viruses are capable of causing considerable morbidity and mortality in a number of mammalian species, including humans (45). Infection of humans is characterized by a rapid and extensive spread of the virus in several organs with symptoms including respiratory distress and encephalitis (46). These symptoms can be reproduced by experimental infection of several animal models including hamsters (47). Because of their high pathogenicity in humans, their broad tropism and the absence of any vaccine or treatment, henipaviruses are presently classified as biosafety level 4 (BSL4) agents and considered as potential biothreats (48).

The major vaccination strategy to prevent henipavirus infection has focused on direct administration of soluble forms of the G viral glycoprotein to induce a protective immune response (49–53). This form of vaccination requires several injections of the recombinant protein coupled to adjuvants to achieve a significant immune response. In a recent study, Ploquin et al. evaluated the efficiency of AAV vectors expressing the NiV G protein to induce a protective immune response (28). The evaluation of two routes of vaccination and different prime/boost strategies employing three AAV serotypes indicated that a single IM AAV injection in mice was sufficient to induce a potent and long lasting Ab response consisting of IgG and NAb. Further translational studies in hamsters demonstrated that a single injection of an AAV vector encoding NiV G was sufficient to protect 100% of the animals against a lethal challenge with NiV and 50% of the animals against a challenge with HeV, thus indicating the induction of cross-neutralizing immune responses. Altogether, this study presented a new vaccination approach whereby a single immunization is sufficient for the induction of a protective immunity against henipaviruses and opened new perspectives toward the evaluation of AAV vectors as a vaccine against these emergent infectious diseases.

HUMAN IMMUNODEFICIENCY VIRUS

Controlling the epidemic of HIV infection worldwide remains a major challenge. Indeed, even though significant success in controlling the disease has been achieved by understanding the virus biology and by developing targeted drugs, vaccine-based preventive measures are still needed. Major challenges toward this goal are the antigenic variability and multitude of virus strains (54). Several viral vectors have been tested as vaccines against HIV, most notably poxviruses and Ad, but have met little success in protective efficacy and even adverse effects in terms of protection against HIV infection (1). This was, in particular, the case of the STEP human trial which used Ad vectors encoding HIV proteins (55).

Among several vaccine strategies, AAV vectors have been evaluated in several animal studies (**Table 1**). In an early study, an AAV vector expressing the HIV env, tat, and rev genes was given to BALB/c mice in single applications and by different administration routes including intramuscular and intra-nasal. This resulted in the production of a high level of HIV-specific serum IgG and fecal secretory IgA as well as in the appearance of a cytotoxic T-cell response (15). Non-invasive oral administration of AAV2 vectors expressing the HIV *env* gene was further studied by Xin and colleagues (16) who showed the induction of systemic and mucosal humoral and cellular immune response that partially protected against rectal challenge with a recombinant vaccinia virus expressing the HIV *env* gene. The most advanced studies in the field were conducted by Johnson et al. (22), who investigated the efficacy of AAV2 vectors expressing several SIV genes (rev-gag, rev-env, and RT-IN) injected intramuscularly against a challenge with SIV (22). In this study, the authors demonstrated the induction of robust T cell and Ab responses after a single vaccination. Upon challenge with SIV, complete protection was observed when low doses of SIV were given intravenously. However, only partial protection was observed at higher doses of SIV. Of note, one phase I trial with AAV2 HIVgag-protease-ΔRT demonstrated safety but modest immunogenicity (Gag-specific T cells in 16% of the recipients) (44).

A much more interesting perspective in the field was provided by two recent studies which used AAV vectors for passive immunotherapy (**Table 2**). This concept was first validated by Johnson and colleagues, who used an AAV1 vector to express

imunoadhesins (IA) against SIV in macaques (37). IA are chimeric Ab-like molecules that are generally composed of the Fc region of an immunoglobulin linked to the ligand-binding region of a receptor or adhesion molecule. IA offer the advantage of being small molecules whose sequence can be easily accommodated in an AAV vector and eventually in a scAAV vector, which packages a double-stranded DNA genome enhancing the kinetics and level of transgene expression (8). Johnson and colleagues demonstrated that intra-muscular injection of the scAAV1 vector resulted in a higher level of secretion of the IA than using a conventional ss AAV1 and generated a long lasting neutralizing activity in the serum. Importantly, most vaccinated animals were protected against infection with SIV and all were protected against the disease. However, Ab against the IA were generated in some animals and correlated with partial protection. The potential of AAV-based passive immunotherapy against HIV infection was further demonstrated by Balazs and colleagues, who reported the lifelong expression in mice of monoclonal Ab against HIV after a single intra-muscular injection of an AAV8 vector. Importantly, humanized mice vaccinated with AAV were protected against intravenous injection of replication competent HIV at a very high dose (35).

HUMAN PAPILLOMAVIRUS

Two VLPs-based vaccines are commercially available against HPV 16 and HPV 18, which are the most important risk factors for the development of cervical cancer and other malignant tumors of the anogenital tract and of the head and neck. Clinical trials and first reports after launching vaccination campaigns demonstrated highly efficient protection against persistent infection and precancerous lesions besides excellent safety profiles (56–58) [for review, see Ref. (59)]. Early data on the influence of this vaccination strategy on cancer incidence are expected to arise 15–20 years after initiation of mass immunization. Despite the high costs of the available products, in the near future, an AAV-based HPV vaccine is unlikely to become a serious contender to the existing vaccination scheme, which involves three IM injections of adjuvanted VLPs manufactured by recombinant expression of the HPV major structural protein (L1). Yet, studies with HPV 16 L1 AAVs have yielded interesting results in mice and NHP and make these vectors an interesting option for future developments.

For example, Kuck and colleagues demonstrated a sustained humoral and cellular immune response (>1 year) in mice immunized with a single intra-nasal dose of AAV5-HPV16L1. The responses by far outlasted the ones obtained after three doses of HPV 16 VLPs that had been applied by the same route. Lyophilized and re-dissolved AAV particles remained immunogenic albeit at reduced efficiency (11). Thus, AAV has the potential for a needleless vaccine and – unlike the available vaccines – does not need refrigeration since it can be stored as lyophilized powder. Both features are important in developing countries where cervical cancer is a major public health challenge. Intra-nasal immunization with AAV9-HPV16L1 was also tested in NHP (macaques). L1-specific NAb were elicited and persisted for at least 7 months post AAV administration. As expected from previous studies, the presence of pre-existing high titer AAV9 Ab did not prevent immunization with the same serotype when administered intra-nasally (14, 31).

As mentioned before, the introduction of prophylactic HPV vaccination has the potential to significantly reduce the worldwide burden of HPV-related disease. However, even in countries with sufficient resources there will always be individuals that, for reasons of ignorance about HPV as a human carcinogen or active objection against vaccination *per se*, will not benefit from such programs. Therefore, there is a medical need for the development of HPV-specific therapeutic vaccines against an established infection. Such strategies involve cytotoxic T cells against early proteins that are expressed in persistently infected and HPV-transformed cells [for a review, see Ref. (60)]. One of the intensively studied targets, namely the viral oncoprotein E7 has also been analyzed as the transgene in AAV vectors (**Table 1**). Two studies used AAV vectors coding for a 19 aa HPV 16 E7 peptide, which contains a well-defined mouse (H-2b)-restricted CTL epitope or the complete HPV 16 E7 gene fused to the *Mycobacterium tuberculosis* heat shock protein 70 (hsp70) (9, 13). A single intra-muscular immunization of C57/BL6 mice induced E7-specific $CD4^+$ and $CD8^+$ T lymphocytes and – upon challenge with syngeneic E7-transformed cells – a significant delay or a complete inhibition of tumor growth. In experiments aiming toward an HPV 16 vaccine that combines prophylactic and therapeutic properties, Nieto and colleagues analyzed different AAV serotypes that carried a fusion gene of L1 and the major part of E7 (12) by a single intra-nasal immunization of mice. The AAV5 and the AAV9 (not the AAV8) vectors efficiently induced both humoral and cellular immune responses that were superior to vaccination with HPV16-L1 VLPs or HPV16-L1/E7 chimeric VLPs. In addition, vaccination with the AAV vectors led to a significant protection of animals against a challenge with different HPV tumor cell lines.

INFLUENZA VIRUS

The currently available vaccines are insufficient to keep in check the seasonal influenza outbreaks that affect people of all ages and claim at least one millions of lives in children up to 5 years of age worldwide (61). The reason vaccines are relatively less successful is the high variability of the virus and highly type-specific nature of the vaccines that need to be designed for the actual emerging virus strain. The unavoidable delay between the identification of a new variant and availability of the appropriate vaccine – particularly owing to the laborious manufacturing process of virus replication in chicken eggs – leaves the population unprotected at least against the first wave of a new epidemic.

Earlier studies have demonstrated strain-specific immunization and protection against lethal challenge. Sipo and colleagues generated AAV9 vectors expressing the hemagglutinin (HA), nucleoprotein (NP), or matrix protein (M1) genes of the A/Mexico/4603/2009 (H1N1) isolate, a pandemic influenza of swine origin. After the single injection of a mixture of two or three AAV serotypes, they obtained complete protection against a homologous challenge and partial protection against a heterologous and highly virulent strain (25). The authors argued that, although this vaccine candidate will not induce sterilizing immunity it may mitigate the clinical symptoms, diminish the transmission rate, and, thus, generate a herd immunity before the homologous classical vaccines have been made available. A slightly more recent study also examined the protection level in

mice vaccinated with AAV vectors expressing NP. In particular, the objective of those studies was to evaluate the efficacy of protection in mice receiving high doses of pooled human IgG to mimic a situation in which a pre-existing immunity against AAV may interfere with the vaccine. Interestingly in those studies, the authors showed that an AAVrh32.33 vector-expressing NP could fully protect the animal against a challenge with lethal doses of influenza virus strain PR8 (20). Altogether, these studies demonstrated the efficiency of strain-specific AAV vaccines.

However, as for other RNA viruses, vaccination against seasonal influenza outbreaks is complicated by the continuous emergence of new variants or strains which escape NAb generated by the previous infection. The recent isolation of cross-neutralizing Ab, which bind to a conserved region of HA has recently allowed the evaluation of new vaccination strategies based on passive immunotherapy and aimed at generating a long-term broadly protective humoral activity *in vivo*. Interestingly, two independent groups simultaneously reported the use of AAV vectors to produce, *in vivo*, either a full length human Ab against HA or an IA directed against the same protein (38, 39). In the first study, the authors injected the AAV8 vector intramuscularly in mice and ferrets and demonstrated that the long-term production of the anti-HA Ab in the serum conferred complete protection against five different influenza strains (38). In the second study, an AAV9 vector was administered intranasally to induce production of the IA in the nose and the lungs (39). An advantage of this mode of delivery is that vector expression is localized to the nasal epithelia and is not expected to be widely disseminated in the body. In addition, the natural turnover of the airway epithelial cells may ensure that the vector is not permanently present *in vivo*. As in the first study, the animals were protected against the IN challenge with different influenza strains. Importantly, in this latter example, the authors also showed that the time between AAV injection and challenge could be reduced to 3 days, demonstrating the potency of this expression system (39). However, for both studies, analyses performed in larger animal species, in particular ferrets, resulted in only partial or no protection probably because of the emergence of an immune response against the human Ab or the IA. Of course, an immune response against the human Ab is not expected in humans. Whether the same holds true for IA is still unclear. Even though some important issues, such as the potential immunogenicity of IA and the translation of this approach in larger animals still need improvements, these approaches clearly indicate that AAV vectors are powerful tools for passive immunotherapy which certainly deserve further studies in NHP models.

CONCLUSION AND FUTURE DIRECTIONS

The studies conducted so far have highlighted several important advantages of AAV vectors for vaccination and notably, in the case of active immunotherapy, their capacity to induce sustained levels of Ab responses after a single injection or in passive immunotherapy, as tools for secreting Ab directly into the circulation. These features are linked to the capacity of AAV vectors to persist for long periods in the transduced tissues and clearly distinguish AAV from other viral vectors, such as Ad and poxviruses, with which a rapid elimination of transduced cells is observed *in vivo*. Despite these properties, many drawbacks still hamper the use of these vectors as a vaccine in humans. This last section will review the major problems, which remain to be solved and discuss possible solutions.

IMPROVING AAV IMMUNOGENICITY

The rationale for using AAV vectors for genetic vaccination is mainly based on their intrinsic absence of pathogenicity, their capacity to infect a variety of tissues, and to express transgenes at a high and sustained level. When using AAVs for passive immunotherapy, these properties are sufficient to consider AAV vectors as very promising tools even though safety studies are required to evaluate the effects of a continuous secretion of Ab *in vivo*. However, in the case of active immunotherapy, these properties may not always be sufficient to ensure an efficient vaccination. Indeed, even if shown capable to induce transgene-specific immune responses in large animal models (33) and to activate innate responses at modest but detectable levels (62), AAV vectors are still considered to possess a low immunogenic profile, compared to other viral vectors, in particular Ad vectors. This is notably illustrated by the persistence of transgene expression observed in several vaccination studies and by the reported lack of functional $CD8^+$ T-cell responses observed with natural AAV serotypes (19, 20, 28). This aspect clearly represents a major disadvantage for using AAVs for preventive or therapeutic vaccination trials requiring the induction of robust cytotoxic T-cell responses. Therefore, the use of these vectors for these applications requires enhancement of their intrinsic immunogenic properties. Several studies already indicate that this is possible, notably through the manipulation of the viral genome and the capsid.

Increasing AAV immunogenicity by manipulating the vector backbone

Adeno-associated virus vectors can be composed of a ss or a sc DNA genome. Changing the nature of the DNA genome has a profound impact on the kinetics and the level of transgene expression by bypassing the need for DNA second-strand synthesis before transcription of the vector cassette (8). Accordingly, the comparison of ss and scAAV vectors for passive immunotherapy indicated that the latter produced higher levels of Ab than the former (37). Interestingly, several recent studies indicated that this modification could also impact on the immunogenic properties of the vectors. Indeed, modifying the vector backbone enhanced both innate and transgene-specific adaptive immune responses (21, 63). In particular, using an AAV vector expressing a secreted version of the HIV Gag protein, Wu and colleagues showed that scAAV vectors of different serotypes induced more potent $CD8^+$ T-cell and Ab responses than conventional ssAAV (21). However, as previously observed with conventional ssAAV vectors a progressive loss of function of $CD8^+$ T cell was observed, indicating that the modification of the nature of the vector genome was not sufficient to generate fully functional T-cell responses. In addition, this strategy was applicable only to transgenes that could be accommodated in an expression cassette which was one half that of conventional AAV vectors.

Increasing AAV immunogenicity by manipulating the capsid

Another strategy to enhance transgene-specific immune responses induced by AAVs consists of changing the viral capsid. Obviously,

the level and the nature of the immune response are tightly linked to the nature of the capsids, which determines the tropism of the particle and the efficiency of transduction. Accordingly, several studies documented significant differences in the levels of immune response induced by natural AAV serotypes (12, 18, 19, 28). Presently, more than a 100 AAV variants have been isolated from human and non-human tissues and approximately 13 are used to produce vectors (29). An even greater variety is offered by the possibility of genetically modifying the capsid either by creating artificial variants or by inserting specific immunogenic peptides on the capsid surface as discussed below (64).

Regarding the generation of new artificial AAV variants, the most compelling evidence was provided by studies performed with the AAVrh32.33. This variant is an engineered hybrid between two natural AAV rhesus macaque isolates, which was specifically selected for its immunogenic properties. The analysis of the immune response induced by this variant indicated that it was able to induce a vibrant and functional $CD8^+$ T-cell response directed against the Ag, unlike the other natural AAV serotypes, and exploiting this capacity was beneficial for vaccine development (20, 32). The recent structural analysis of this variant further indicated that the functional T-cell activating domain lies within the VP3 portion of the capsid (65). A future deeper understanding of the immunological properties of the capsid domains of AAV may then lead to the rational design of artificial variants capable of differentially stimulating immune responses.

Other interesting strategies to increase AAV immunogenicity have focused on the generation of capsid exposing selected epitopes. AAV particles are composed of 60 capsid protein subunits, named VP1, VP2, and VP3 (66). Efficient methods to generate genetically modified capsid exposing selected peptides have been developed in the recent years (67). Due to the highly structured and repetitive presentation of epitopes on the capsid, potent B-cell responses against this peptide are expected. Two recent studies illustrate the feasibility of this approach. Nieto and colleagues inserted two neutralizing epitopes from the L2 protein of HPV16 and HPV31 into two different positions of VP3 and used assembled empty AAV particles – AAVLP(HPV16/31L2) – to vaccinate mice and rabbits. AAVLP (HPV16/31L2) empty particles coupled to montanide adjuvant, induced high levels of Ab able to cross neutralize several HPV types (68). In a similar approach, Rybniker and colleagues used genetically modified AAV2 capsids displaying at their surface Ag85A, a well described Ag from *M. tuberculosis*. Using such modified capsids to package a vector over-expressing the same Ag, increased the kinetics of Ab production as well as their avidity, compared with an AAV assembled into a wild type capsid. In addition, insertion of the antigen at the capsid surface was also shown to be sufficient to induce a memory B-cell recall response (27).

Both of these strategies illustrate the potential of capsid modification for manipulating the immune response and it is likely that, in a very near future, new AAV vectors specifically selected for their ability to induce strong transgene-specific immune responses will continue to emerge.

CIRCUMVENTING ANTI-AAV PRE-EXISTING IMMUNITY

Epidemiological studies indicate that anti-AAV Ab can be detected in the majority of the population worldwide and that their seroprevalence varies according to the AAV serotype and the geographical region (69–71) [for a review, see Ref. (72)]. As a result, the efficacy of AAV vectors for *in vivo* gene transfer can be severely reduced. In animal models, the use of an alternative AAV serotype is sufficient to circumvent anti-AAV NAb induced by previous immunization with a different capsid. However, this strategy may not be valid in humans in which potentially wider cross-reacting immune responses exist. Interestingly however, artificial variants such as AAVrh32.33 showed a much lower seroprevalence than natural serotypes, with less than 2% of the population testing positive worldwide, indicating that it could represent a good candidate for vaccination (69). An additional strategy to circumvent pre-existing immunity is provided by the possibility to engineer AAV capsids with mutations targeting key immunogenic amino acids. This sophisticated strategy was developed by Maersch and colleagues, who demonstrated its feasibility by using the *in vitro* directed evolution method to select AAV particles capable of escaping anti-AAV2 NAb (73).

REDUCING AAV VECTOR DOSES

In gene therapy trials, the use of AAV vectors in humans requires very high vector doses in order to achieve a therapeutic efficiency. For example, doses of approximately 10^{12} vector particles per kilogram were required in the initial Hemophilia B trials using AAV2 vectors (5). Even if lower doses were used in the latter trials using other AAV serotypes, the amount of particles delivered as a single injection in patients still remained impressively high. Experiments performed in animals for vaccination with AAVs also used very high doses to achieve an efficient immune response. Therefore, as for gene therapy, application of high vector doses may constitute a safety concern by increasing the risk of inducing detrimental immune responses and of off-target transduction. Hence, future efforts to reduce vector doses should focus on decreasing the vector load by improving transduction efficiencies and increasing the immunogenicity of the vector particles.

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