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# APPROACHES TO BLOCKING THE IMMUNE RESPONSE TO GENE TRANSFER WITH VIRAL VECTORS

Hosted by
Katherine High, Roland W. Herzog and Hildegund C. Ertl

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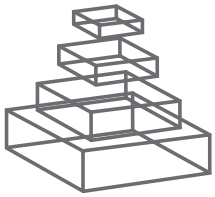

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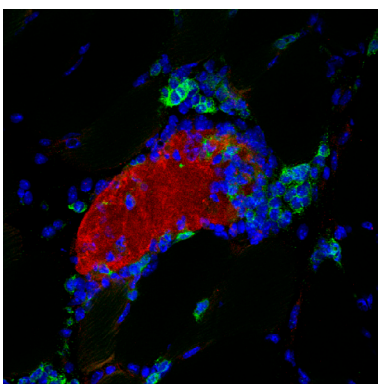
# APPROACHES TO BLOCKING THE IMMUNE RESPONSE TO GENE TRANSFER WITH VIRAL VECTORS

Hosted By:
**Katherine High**, Children's Hospital of Philadelphia, USA
**Roland W. Herzog**, University of Florida, USA
**Hildegund C. Ertl**, Wistar Institute, USA



Viral vectors are superior tools for gene therapy and as a genetic vaccine platform because viruses have evolved to efficiently infect and transfer their genomes to cells. Several impressive successes in viral vector-based gene therapies have been reported in humans, including restoration of vision in patients with Leber's congenital amaurosis by retinal gene transfer and cures for severe immune deficiencies by gene transfer to hematopoietic stem cells. However, the mammalian immune system has evolved in parallel to fend off invading pathogens such as viruses. Innate and antigen-specific adaptive immune responses against viral vectors and therapeutic transgene products pose serious hurdles for successful gene therapy. Pre-existing immunity in humans, resulting from prior exposure to the parent virus that forms the basis for the gene transfer vehicle may be derived from, often prevents efficient gene transfer. This problem also reduces our ability to use certain vectors for genetic vaccination or in anti-cancer therapy. For these reasons, the gene transfer community has been extensively studying the mechanisms of immune responses against viral vectors and has started to develop strategies and protocols to block or circumvent such responses. Choice, design and engineering of a vector as well as the route of administration/target tissue can be optimized/ altered to minimize immune responses or evade pre-existing immunity. Immune suppression and modulation strategies are being developed in order to minimize inflammation, prevent antibody or T cell responses against vectors, and to promote tolerance to therapeutic gene products. Combinations of these approaches will likely facilitate clinical applications of gene therapy for many target diseases and also aid in vaccine development.

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# Gene therapy research at the Frontiers of viral immunology

**Brad E. Hoffman[1]*, Hildegund C. J. Ertl[2], Cox Terhorst[3], Katherine A. High[4,5] and Roland W. Herzog[1]**

[1] Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida College of Medicine, Gainesville, FL, USA
[2] Immunology Program, Wistar Institute, Philadelphia, PA, USA
[3] Division of Immunology, Beth Israel Deaconess Medical Center, Boston, MA, USA
[4] Department of Pediatrics, The Children's Hospital of Philadelphia, University of Pennsylvania Medical Center, Philadelphia, PA, USA
[5] Howard Hughes Medical Institute, Philadelphia, PA, USA
*Correspondence: bhoffman@ufl.edu

Although originally conceived in the mid 1970s as an alternative to transfection (Goff and Berg, 1976), the use of viral vectors as a tool for clinical gene therapy did not emerge until the 1980s. As Li and Ertl (2011) recapitulate in their thought provoking perspective, viruses meet all the requirements needed for gene therapy. Evolving since life's beginning, viruses have established the ability to efficiently infect and transfer their genomes in a wide variety of mammalian cell types. Likewise, and generally considered beneficial for survival, mammals have equally evolved highly complex mechanisms to protect themselves against invading pathogens such as viral gene transfer vectors. However, it is this protective immune response that often presents major obstacles for successful long-term therapy. Fortunately, the gene transfer community has been extensively studying the mechanisms of immune responses against viral vectors and has started to develop strategies and protocols to block or circumvent such responses. In this *Research Topic*, the editors present a collection of mini-Reviews, in-depth Reviews, Perspective, and Primary research articles that highlight both the well established and emerging strategies currently being used in blocking the immune response to gene transfer with viral vectors.

Viral vectors such as adenovirus and adenovirus-associated virus (AAV) are superior tools for gene therapy due to their high efficiency of transduction into a variety of cells *in vivo*. Administration of viral vectors often provokes the initiation of innate and antigen-specific adaptive immune responses against the virus and/or the therapeutic transgene products. Activation of these pathways elicits a flurry of antiviral and pro-inflammatory signals that can recruit effector lymphocytes, inhibit viral transduction, and encourage elimination of transduced cells over a period of time. Additionally, the *de novo* expression of a wild-type protein may trigger an adaptive immune response in the form of neutralizing antibodies. Likewise, pre-existing immunity (immunological memory) to the gene transfer vector resulting from prior exposure to the virus often prevents efficient gene transfer. All of these scenarios pose serious hurdles for successful gene therapy.

The first review article of this special topic provides a comprehensive overview of the innate immune responses to AAV. The authors highlight and discuss recent discoveries regarding strategies to abrogate potentially detrimental signaling pathways (Rogers et al., 2011). Historically, the innate immune response to single-stranded AAVs has been considered weak and transient when compared to the potent and prolonged response elicited by Ad vectors, thus prompting many investigators to focus more on the adaptive immune response to AAV. In more recent years our understanding of the early innate mechanisms of immune responses to viral vectors has greatly improved. The authors present an up-to-date analysis of the mechanisms surrounding the innate immune response to single-stranded and self-complimentary AAVs, including the role of the viral capsid, the effects on target tissue, and the therapeutic potential of blocking innate responses.

An equally impressive review detailing the current understanding relative to Ad vector mediated induction of the innate and adaptive immune responses is presented by Aldhamen et al. (2011). Ad vectors possess several advantages, the most important of which is that they can be easily, and routinely produced to high titers, however they also rapidly activate innate immune responses as well as potent cellular and humoral adaptive immune responses. This review examines the impact these responses have on the safety and efficacy of Ad vector-based therapies (Aldhamen et al., 2011). They also highlight strategies proposed to either mitigate or harness Ad-induced innate and adaptive immune responses for the improved and broadened development of advanced, Ad-based therapies.

Adenovirus-associated virus vectors have shown considerable promise as a gene delivery tool for clinical gene therapy applications. Unfortunately, the presence of pre-formed neutralizing antibodies directed against the AAV capsid in a large proportion of the human population due to widespread prior exposure to the wild-type virus has prevented AAV from reaching its full potential as a gene therapy vector. Bartel et al. (2011) provide an intriguing review that examines the use of capsid engineering as a means to evade pre-existing immunity. The authors discuss the consequences of humoral anti-AAV immune responses and potential strategies to prevent them. Additionally, Arnett et al. (2011) discusses the influence that cross reactivity of pre-existing antibodies has on gene therapy, including neonatal administration of viral vectors as a means to circumvent such antibody responses.

As a target tissue for gene therapy, muscle is appealing because it is abundant, easily accessible, and procedures involving gene transfer to muscle are relatively safe and non-invasive (Hoffman et al., 2007). The mini-review by Wang et al. focuses on recent reports of immunity to AAV capsid proteins and transgene products in the context of gene delivery to muscles for treating both muscular dystrophies and other non-muscle diseases. They provide strategies of immune modulation and tolerance induction in order to prevent unwanted immune responses to the vector and/or the therapeutic gene product (Wang et al., 2011). Additionally, the research article presented by the Boyer group, evaluates a treatment protocol designed to inhibit the deleterious immune activation during muscle gene transfer. Their strategy

is based on the administration of CTLA-4/Ig in order to block the co-stimulatory signals required early during immune priming combined with gene transfer of PD-1 ligands to inhibit T cell functions at the tissue sites (Adriouch et al., 2011).

It has become evident that the manipulation of various co-stimulatory pathways to regulate host immune responses is of therapeutic interest. Addressing this, Huang and Yang, offer a broad review of relevant T cell co-stimulatory pathways in the activation of both T and B-cells, and provide strategies for targeting these co-stimulatory pathways in gene therapy applications. Ultimately, they suggest that studies should focus on targeting multiple pathways including both the positive and negative co-stimulatory pathways (Huang and Yang, 2011). Using a multiple pathway approach in an animal model of hemophilia, the Herzog lab presents new findings that suggest that transient immune modulation using a cocktail of rapamycin, IL-10, and specific peptides could prevent or possibly reverse gene therapy-induced inhibitor formation (Nayak et al., 2011).

The success of *in vivo* gene therapy not only depends on the ability to control the immune response toward the input vector, but also to the therapeutic transgene. Using both vector-based and pharmacological approaches, various groups have explored various approaches to control the immune-mediated clearance of transgene-expressing cells after viral delivery. One approach using micro-RNA transgene regulation to generate a tolerogenic response is reviewed by Goudy et al. They further speculate on possible mechanisms used by the liver to induce the transgene-specific regulatory T cells (Goudy et al., 2011). Another method to modulate tolerance induction is via gene transfer to B-cells and such an approach is presented by Su et al. Here, the authors demonstrate that host IL-10 is critical for the tolerogenicity of B-cell based peptide–IgG gene therapy (Su et al., 2011).

Overall, this Research Topic: "Approaches to Blocking the Immune Response to Gene Transfer with Viral Vectors" provides a well-developed overview of the current therapeutic potentials of viral gene therapy. This collection of articles not only provides a dynamic review and analyses of the complex immune responses present in current gene therapy applications, but they also provide insight for the future direction of the field.

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# Can viruses be modified to achieve sustained gene transfer

***Hua Li and Hildegund C. J. Ertl****

*Immunology Program, Wistar Institute, Philadelphia, PA, USA*

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*George Q. Perrin, University of Florida, USA*
*Arun Srivastava, University of Florida, USA*

****Correspondence:***
*Hildegund C. J. Ertl, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA.*
*e-mail: ertl@wistar.upenn.edu*

It is very easy to replace a faulty gene in an immunocompromised mouse. First, one takes a well-characterized virus, such as an adenovirus or an adeno-associated virus, and incorporates the correct version of the faulty gene together with some regulatory sequences into the genome. Then, one transduces the recombinant genome into helper cells, which will add the viral capsid. At last, one injects the resulting viral vector into the sick mouse, and the mouse is cured. It is not that easy in an immunocompetent mouse, let alone in a human, as over the eons the immune system evolved to eliminate viruses regardless if they penetrate as dangerous pathogens or are injected by a well-meaning gene therapist. Here we offer our perspective on the potential of how viral vectors achieve sustained gene transfer in the face of a hostile immune system.

**Keywords: gene therapy**

Gene therapy, which aims to temporarily or permanently replace a faulty or missing protein at therapeutic levels, requires efficient transfer of genes into a host cell. The gene transfer vehicle has to withstand enzymes in blood, find the right cells, enter them and then their nucleus. Viruses meet these criteria to the dot; their capsids protect the genome, ensure that they enter cells and then facilitate transfer of the gene into the nucleus.

Gene therapists have been attempting to take advantage of viruses' talents for gene transfer focusing on viruses that commonly infect humans. During the milleniums, well before primates climbed down from trees evolution has selected for cellular organisms that can fight off viral infection first through primitive innate immune responses and then about 410 million years ago through an adaptive immune system and its high specificity and ability to remember. Living beings have thus evolved for billions of years to combat viruses. The evolution of gene therapy pales in comparison; the first humans were treated a mere 21 years ago.

Gene therapy can roughly be divided into two fields, transient and permanent gene therapy. The latter can be divided into *ex* and *in vivo* therapy. Transient or *ex vivo* gene transfers have their own sets of problems, but the virtually insurmountable challenges caused by anti-viral immune responses are mainly faced upon direct injection of a viral vector for *in vivo* correction of a gene.

The two viral vectors most commonly used are based on adeno- (Ad) and adeno-associated viruses (AAV).

Humans encounter different serotypes of Ad viruses early in life and develop neutralizing antibodies. Prevalence rates of such antibodies vary depending on the Ad serotype and the geographic region (Chen et al., 2010). Neutralizing antibodies prevent the virus from entering its target and thus reduce transduction rates. This can be overcome, e.g., by using alternative Ad serotypes. $CD8^+$ T cells pose the bigger problem. Humans have robust numbers of Ad virus-specific $CD8^+$ T cells (Chen et al., 2010), which are activated and are chock-full of granules containing lytic enzymes (Hutnick et al., 2010). Even immunosuppression, short of complete T cell ablation, would have no effect on the ~1 billion Ad virus-specific $CD8^+$ T cells of the average adult, which can commence lysis immediately.

In AAVs vectored for use in gene therapy, the ORFs are removed and replaced with the gene of interest flanked by the ITRs. Conversion of the ssDNA genome of AAV vectors into double-stranded (ds)DNA is rate limiting in transduction and delays onset of gene expression (Ferrari et al., 1996). DsAAV vectors have been developed and shown to achieve per vector genome copy higher levels of transgene product with accelerated onset of expression (McCarty et al., 2001; Nathwani et al., 2006).

Prevalence rates of neutralizing antibodies to the different AAV serotypes vary (Calcedo et al., 2009). Neutralizing antibodies, even if present at only low titers, readily blocks AAV-mediated gene transfer. Humans have circulating $CD4^+$ and $CD8^+$ T cells to AAV capsid at low frequencies of approximately 0.1% of a given subset. They less activated than those to Ad viruses and belong predominantly to the effector memory or central memory subset (Li et al., 2011).

Adeno-associated viruses-mediated gene transfer readily results in sustained transgene expression in experimental animals (Song et al., 1998; Mount et al., 2002; Gao et al., 2006) presumably reflecting that AAV vectors are not particularly immunogenic. Based on successes in animals, AAV vectors were tested in clinical gene transfer trials. If given at moderate doses to an immunoprivileged site, they resulted in some correction of disease (Maguire et al., 2009). But if given systemically at the high doses needed to encode beneficial levels of a transgene product, such as factor (F) IX in hemophilia B, ssAAV vectors induced a capsid-specific $CD8^+$ T cell response accompanied by loss of the therapeutic protein (Manno et al., 2006; Mingozzi et al., 2007). The potential success of AAV-mediated gene transfer in humans may turn out to be a numbers game. Transgene product expression by AAV vectors with a dsDNA genome is more efficient and in a recent trial a dsAAV8 vector achieved near therapeutic levels of factor FIX in hemophilia patients without evidence of T cell activation if given at a dose of $2 \times 10^{10}$ vg/kg (Nathwani, 2010). In a preceding trial

with ssAAV2 vectors for FIX, doses of $8 \times 10^{10}$ vg/kg failed to elicit a T cell response but they also did not achieve detectable levels of FIX. At the next tested dose of $4 \times 10^{11}$ vg/kg, T cell expansion was observed although levels of FIX still remained below detection. FIX levels together with increases in circulating AAV capsid-specific T cells were not observed till the final dose of $2 \times 10^{12}$ vg/kg (Manno et al., 2006; Mingozzi et al., 2007). The T cell response to the capsid proteins of AAV vectors is dictated by the input dose as without *de novo* synthesis of capsid proteins, T cells rely on presentation of degrading capsids, which are limiting both in terms of quantity and duration. Assuming that AAV vector doses below $\sim 10^{11}$ vg/kg can dodge recognition by T cells, gene therapists should concentrate on the use of maximally efficient AAV vectors. Nevertheless, this assumption, which is based on low numbers of AAV vector treated patients, has to be viewed with caution. Different serotypes of AAV vectors or even ss versus dsAAV vectors may differ in the degradation rate of their capsids. Processing of capsid for presentation of peptides with MHC class I molecules is mediated by proteases, which in turn are upregulated by proinflammatory cytokines. Capsids that are processed more rapidly may result in a bolus of epitopes, which may favor T cell recognition unlike capsids that are degraded slowly. Frequencies of circulating AAV capsid-specific $CD8^+$ T cells in humans vary and humans with higher frequencies might be more prone to mount effective recall responses to AAV gene transfer than humans with low frequencies. Recommending firm doses for AAV-mediated gene transfer will thus not be feasible till larger cohorts have been tested and one can at this stage only advise a very conservative dose escalation.

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# Immune recognition of gene transfer vectors: focus on adenovirus as a paradigm

***Yasser Ali Aldhamen[1], Sergey S. Seregin[1] and Andrea Amalfitano[1,2]****

[1] *Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA*
[2] *Department of Pediatrics, College of Osteopathic Medicine, Michigan State University, East Lansing, MI, USA*

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Dirk Homann, University of Colorado at Denver, USA*
*Phyllis Flomenberg, Thomas Jefferson University, USA*

***Correspondence:**
*Andrea Amalfitano, Michigan State University, 4194 Biomedical and Physical Sciences Building, East Lansing, MI 48823, USA.*
*e-mail: amalfit1@msu.edu*

Recombinant Adenovirus (Ad) based vectors have been utilized extensively as a gene transfer platform in multiple pre-clinical and clinical applications. These applications are numerous, and inclusive of both gene therapy and vaccine based approaches to human or animal diseases. The widespread utilization of these vectors in both animal models, as well as numerous human clinical trials (Ad-based vectors surpass all other gene transfer vectors relative to numbers of patients treated, as well as number of clinical trials overall), has shed light on how this virus vector interacts with both the innate and adaptive immune systems. The ability to generate and administer large amounts of this vector likely contributes not only to their ability to allow for highly efficient gene transfer, but also their elicitation of host immune responses to the vector and/or the transgene the vector expresses *in vivo*. These facts, coupled with utilization of several models that allow for full detection of these responses has predicted several observations made in human trials, an important point as lack of similar capabilities by other vector systems may prevent detection of such responses until only after human trials are initiated. Finally, induction of innate or adaptive immune responses by Ad vectors may be detrimental in one setting (i.e., gene therapy) and be entirely beneficial in another (i.e., prophylactic or therapeutic vaccine based applications). Herein, we review the current understanding of innate and adaptive immune responses to Ad vectors, as well some recent advances that attempt to capitalize on this understanding so as to further broaden the safe and efficient use of Ad-based gene transfer therapies in general.

**Keywords: adenovirus, innate immunity, adaptive immunity, cellular responses, humoral responses, vaccines**

## INTRODUCTION

Viruses are obligate intracellular, metabolically inert particles composed of DNA or RNA and a protein coat. They are very efficient in transferring (transducing) their simple "genomes" into vulnerable cells of multicellular organisms.

Replication-deficient adenovirus based vectors (Ads) have been the focus of considerable interest in the last few years for their potential applications in both gene therapy and vaccine applications (Amalfitano and Parks, 2002; St George, 2003; Amalfitano, 2004; Waehler et al., 2007; Lasaro and Ertl, 2009; Russell, 2009; Barouch, 2010). Adenoviruses are a family of non-enveloped viruses containing an icosahedral protein capsid with a 30- to 40-kb linear double-stranded DNA (dsDNA) genome. In general, of the immunologically distinct human Ad serotypes, none are associated with any neoplastic disease, with most causing relatively mild, self-limiting respiratory illnesses in immunocompetent individuals (Lichtenstein and Wold, 2004; Russell, 2009). At least 51 serotypes of human Ad have been identified, and Ad serotypes 5 (Ad5) and Ad2, both belonging to subclass C, are the most extensively studied and characterized both relative to general Ad biology, as well in regard to utilization as a gene transfer vector.

Ad vectors continue to be the most widely utilized gene transfer vector in human clinical trials worldwide. More to the point, as of 2010, over 387 human clinical trials have administered Ad-based vectors by any number of routes to both normal human volunteers, as well as patients affected by a number of diseases potentially treatable by an Ad-based gene transfer approach[1] (Seregin and Amalfitano, 2009; Liu, 2010). In China, Ad vectors are routinely administered for the treatment of some forms of cancer (Huang et al., 2009). These facts may be surprising to some, as it is in sharp contrast to the erroneous views widely held by many basic researchers within, as well as outside the field of gene therapy, that Ad vectors are not recommended for human usage. Much of this confusion stems from an incident in 1999 (the "ornithine transcarbamylase, OTC clinical trial"), in which the tragic death of a clinical trial subject occurred after intravascular administration of a large dose of an Ad-based vector (Raper et al., 2002, 2003). The OTC trial was further compromised by problems in the design and conduct of the trial, as acknowledged and fully detailed by the trial's Principal Investigator (Wilson, 2009). To be clear, there have been subsequent human trials to the OTC trial, that have also intravascularly administered (in a similar fashion to the OTC trial) equivalent, or even higher doses of Ad-based vectors to large numbers of trial subjects, and not had the unfortunate outcome reported in the 1999 OTC trial (Atencio et al., 2006). The reasons

[1] http://www.wiley.com/legacy/wileychi/genmed/clinical/

for this dichotomy in responses are numerous, and likely may never be resolved (Wilson, 2009).

Why do Ad vectors continue to be so widely utilized? Ad vectors posses several important advantages, the most important of which is that they can be easily, and routinely produced to high titers in a good manufacturing practice (GMP) compliant fashion (up to $1 \times 10^{13}$ vp/ml). Non-dependence upon transfection based packaging systems for vector production is a major reason for this efficiency, a feature that has likely limited the clinical use of other vector systems that have been available for as long a period of time as Ad vector systems. Additionally, Ad vectors allow for efficient transduction of various proliferating and quiescent cell types, they can allow for transfer of large segments of foreign DNA (up to 35 kb in some systems), and most importantly, Ad vectors do not integrate, and therefore are much less likely to cause insertional mutagenesis or germ line transmission associated problems, in contrast to integrating virus based vectors, such as retrovirus and lentivirus based gene transfer systems (Hacein-Bey-Abina et al., 2003; Modlich et al., 2005; Howe et al., 2008).

As a result of the ability to both easily concentrate and deliver large amounts of Ad vectors, coupled with high level transgene expression derived from the vector once successful transduction *in vivo* has occurred, it has become clear that the use of Ad vectors also rapidly activates innate immune responses as well induces potent cellular and humoral adaptive immune responses, against both the vector and transgene product being expressed. These events likely occur subsequent to gene transduction with use of any gene transfer vector, virus or non-virus based. However, an ability to only deliver very low particle titers of vector, or low overall transduction efficiencies, may alter or prevent detection of innate or adaptive immune responses subsequent to use of other (non-Ad based) gene transfer vectors.

As viruses have evolved to more efficiently transduce host cells, the mammalian immune system has co-evolved cellular and humoral immune responses to prevent or limit the growth of an invading virus or pathogen. The immune response to viruses is generally composed of two branches: a rapid and non-specific response mediated by the innate arm of the immune system, as well as a relatively slower, more highly specific adaptive immune response, the latter being endowed with memory of past infections to respond more efficiently upon repeat exposure to an infecting organism such as a virus. The innate immune response promotes initiation of the adaptive immune response, and can also orchestrate its overall progression.

While innate immune responses are primarily driven by virion components (capsid proteins, DNA, or RNA genomes) present upon initial administration of the Ad virus into a living animal, adaptive immune responses are mainly associated with the leaky expression of Ad derived genes in early generations of Ad vectors (so called E1 deleted Ads), or more importantly driven by whether or not the transgene being expressed by the Ad vector is perceived by the host as immunologically foreign (Tripathy et al., 1994, 1996; Ding et al., 2001; Kiang et al., 2006b). Regardless of the cause, activation of the innate and adaptive immune systems by Ad vectors can lead to tissue or organ inflammation, enhanced immune-mediated clearance of vector transduced cells, and reduced transgene expression (Amalfitano, 2004). The presence of memory T- and B-cells responses in individuals previously exposed to wild-type Ads further limits the potential for benefit when utilizing Ad-based vectors (Barouch, 2010).

Ad vectors with additional deletions in their genome (accommodated by use of newer generation, trans-complementing packaging cell lines) in the E2A, E2B, and E4 Ad genes have been generated (Engelhardt et al., 1994; Amalfitano et al., 1998; Raper et al., 1998; Amalfitano and Parks, 2002). These advanced generation Ad vectors produce fewer Ad derived gene products as compared to first generation Ads, and can minimize the induction of vector-specific adaptive immune responses (Engelhardt et al., 1994; Ding et al., 2001). These benefits are furthered by the use of helper-dependent (HD)-fully deleted Ad vectors, that have their entire genome deleted (thereby can accommodate up to a 35-kb transgene payload) and are propagated with high efficiency via use of a highly engineered helper virus (Parks et al., 1996; Brunetti-Pierri and Ng, 2009). Overall, newer generations of Ad vectors elicit lower immunogenicity (diminished adaptive immune responses to Ad antigens), and allow for longer transgene expression (Parks et al., 1996; Morral et al., 1998; Amalfitano and Parks, 2002; Everett et al., 2003).

Despite the improved features of multiply deleted or HD-Ad vectors, these vectors continue to elicit innate immune profiles in a pattern similar to that induced by wild-type or first generation Ad vectors (Brunetti-Pierri et al., 2004; McCaffrey et al., 2008; Seregin and Amalfitano, 2009). Furthermore, some advanced generation Ad vectors may still be subject to immunological neutralization or cell mediate clearance by the presence of anti-Ad neutralizing antibodies (NAbs) and/or Ad specific memory T cells present in individuals previously exposed to wild-type Ads (Sumida et al., 2004; Hutnick et al., 2010). Exploring and understanding the mechanism by which Ad vectors interact with and activate the innate and adaptive immune systems will not only allow for the safer use of these vectors, but may also allow for pre-emptive and specific modulation of these responses in efforts to allow for better utilization of these important gene transfer platforms. Lessons learned from the use of Ad vectors are also directly applicable to most, if not all gene transfer vectors (virus or non-virus based), as the mammalian innate and adaptive immune systems have evolved to detect and rapidly neutralize all manner of invading particle, especially those containing a DNA or RNA genome. We will review here much of the current understanding relative to Ad vector mediated induction of the innate and adaptive immune responses, and the impact these responses have on the safety and efficacy of Ad vector based therapies. We will also highlight some strategies proposed to either mitigate or harness Ad-induced innate and adaptive immune responses for the improved and broadened development of advanced, Ad-based therapies.

## INNATE IMMUNE RESPONSES TO VIRAL INFECTION

The innate immune system is conserved across species and represents the first line of general defense against pathogenic infections, inclusive of viral infections specifically (Hoffmann et al., 1999). These, "pathogen associated molecular patterns" (PAMPs), are detected by the host's deployment of a wide array of extracellular, cell surface or intracellular molecules, proteins, and receptors generally known as "pattern recognition receptors" (PRRs; Girardin

et al., 2002; Kawai and Akira, 2010). The innate immune system is also composed of a network of different cell types expressing or reacting to PRR activation, including: monocytes/macrophages, dendritic cells (DCs), natural killer (NK) cells, NK T cells, neutrophils, gamma delta T cells, and mast cells. Each cell of the innate immune system expresses various types of PRRs. For example, a widely studied family of PRRs are the toll-like receptors (TLRs; Kawai and Akira, 2010). Emerging data also indicates an important role for other families of intracellular PRRs, including the nucleotide-binding oligomerization domain/leucine-rich repeat receptors (NOD–LRR) and the retinoic acid-inducible gene (RIG)-1-like receptors (RLRs; Takeuchi and Akira, 2010). Once activated by a specific ligand, these various PRRs trigger a series of signaling cascades that result in down-stream activation and transcription of immune response genes via the NFκB, mitogen-activated protein kinases (MAPKs), and/or interferon regulatory factors (IRFs) 3 and/or 7 signaling pathways, as but some examples. Activation of these pathways eventuates in the production and/or rapid release of pro-inflammatory cytokines and chemokines, initiation of type I interferon responses that limit the replication of invading pathogens, as well as the promotion and shaping of pathogen-specific B- and T cell adaptive immune responses (Medzhitov, 2007; O'Neill and Bowie, 2010).

## INNATE IMMUNE RESPONSES TO Ad VECTORS

Similar to any invading pathogen, gene transfer vectors in general, and Ad vectors in particular are recognized by both immune and non-immune cells, recognitions that activate robust innate immune responses *in vivo* (Schnell et al., 2001; Zhang et al., 2001; Hartman et al., 2007, 2008; Rhee et al., 2011). These responses are dose-dependent and can limit the efficacy of gene transfer mediated by these vectors due to the development of inflammation and the rapid activation of the humoral and/or cytolytic arms of the innate and adaptive immune system (Everett et al., 2003).

One must take note that the mammalian innate system has evolved to rapidly detect very low numbers of an invading pathogen such as a virus, and respond accordingly. Therefore, administration of most any gene transfer vector will result in activation of the innate immune system, and likely in an exaggerated manner since large numbers of gene transfer vectors (virus or non-virus based) need to be delivered before evidence of relevant levels of transgene expression can be confirmed. This caveat is in contrast to the generally low numbers of particles being present during the initial stages of a wild-type virus infection. To account for this, the immunological response is much like a rheostat, a rheostat that has a very large and multi-faceted "dynamic range." For example, detection of low levels of pathogen numbers, or replication, by the innate immune system may result in undetectable, or low level cytokine or chemokine elevations resulting in undetectable or milder symptoms, such as arthralgia, malaise, or fever (the latter being much more difficult to ascertain in some animal models). Greater provocation of the innate immune system after exposure to greater numbers of virus particles induces greater levels of pro-inflammatory compounds and cellular activations. These responses can become exaggerated, and manifest as a cytokine "storm" that can cause serious medical complications, including the systemic inflammatory response syndrome, and even death as in OTC clinical trial (Raper et al., 2003; Matsuda and Hattori, 2006). Furthermore, these responses by the innate immune system are also likely occurring at the local level, and/or when lower levels of vector are administered, though these activations may not be easily detected.

Upon initial introduction into a host, The innate immune response can be initiated following the binding or coating of the Ad vector capsid with several humoral (extracellular) factors including: surfactant-A (SP-A), lactoferrin, pre-existing immunoglobulin, and protein members of the complement pathways, both classical (C1q, C4) and alternative (Factor B, Factor D; Jiang et al., 2004; Shayakhmetov et al., 2005; Shifrin et al., 2005; Huarte et al., 2006; Kiang et al., 2006; Johansson et al., 2007; Zhu et al., 2007; Appledorn et al., 2008). These early interactions attempt to usher the virus away from vulnerable tissues or organs, and rather deliver the virus particles to cells of the reticulo-endothelial system (Jiang et al., 2004; Blom and Ram, 2008; Seregin et al., 2010a,b). It should be clear to the reader that these mechanisms are likely at play for any virus vector (or for that matter, complexes of DNA or RNA coupled with complex liposomes; i.e., containing targeting motifs), and may obviate attempts to retarget capsid modified vectors to various tissues beyond the reticulo-endothelial system (RES).

## ADENOVIRUS VECTOR INDUCTION OF PRO-INFLAMMATORY CYTOKINE AND CHEMOKINE RESPONSES

Despite the initial interactions with the several components of the innate immune system, Ad vector particles can and do infect target cells (primarily accomplished by administration of excessive doses of the vector), this many times accomplished by administering large doses of the vector that effectively overruns the capacity of the RES system to sequester virus particles (Bristol et al., 2000; Morral et al., 2002; Ziegler et al., 2002). However, this does not come without a serious cost. The administration of Ad vectors also results in the immediate production (1–6 h post injection) of various pro-inflammatory cytokines and chemokines, as well as type I interferons in mice, non-human, and human primates (Schnell et al., 2001; Basner-Tschakarjan et al., 2006; Hartman et al., 2007, 2008; Appledorn et al., 2008; Appledorn et al., 2010). Specifically, high dose, intravascular administrations of Ad vectors have been found to induce high levels of the cytokines tumor necrosis factor α (TNFα), IL-6, IL-12, interferon γ (IFNγ), IL-1α, and IL-1β and the chemokines RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1(monocytes chemoattractant protein 1), KC, MIP-1α (macrophage inhibitory protein-1 alpha), MIP-1β, and IFNγ inducible protein 10 (IP-10; Hartman et al., 2007; Appledorn et al., 2008c, 2010; Di Paolo et al., 2009), (**Table 1**).

The origins of these pro-inflammatory mediators *in vivo* is not fully known but is likely from multiple sources inclusive of Kupffer cells, macrophages, endothelial cells as well as Ad transduced tissues, and organs themselves (Shifrin et al., 2005; Appledorn et al., 2008c). Some studies suggest that conventional DCs (cDCs) and macrophages are the main source of acute inflammatory cytokines in response to systemic Ad vectors administration (Zhang et al., 2001). Depletion of Kupffer cells in mice, by intravenous injection of gadolinium chloride (GdCl3), resulted in inhibition of Ad-mediated TNFα production. However, robust production of

**Table 1 | Innate recognition of adenovirus vectors.**

| Innate immune receptors | Response/implication | Cytokine responses | Reference |
|---|---|---|---|
| Complement | Ad vector activates the classical (C1q, C4, C3) and alternative (Factor B, Factor D, C3) complement pathways | Production of IL-6, IL-12, G-CSF, MIP-1β, RANTES, and MCP-1 | Shayakhmetov et al. (2005), Appledorn et al. (2008b), Seregin et al. (2010a,b), Kiang et al. (2006a) |
| **TLRs** | | | |
| TLR2 | Activation of NFκB MAPK signaling | Production of MCP-1, RANTES | Appledorn et al. (2008c) |
| TLR3 | Activation of NFκB MAPK signaling via TRIF adaptor | | Appledorn et al. (2008c, 2009) |
| TLR4 | Activation of NFκB MAPK signaling via MyD88 and TRIF adaptor | | Appledorn et al. (2008c, 2009) |
| TLR9 | Activation of NFκB and ERK1/2 MAPK signaling via MyD88 adaptor | Production of type I IFN, IL-6, MCP-1, IL-12p40, G-CSF, and RANTES | Hartman et al. (2007), Appledorn et al. (2008c), Basner-Tschakarjan et al. (2006) |
| **NON-TLRs** | | | |
| CAR | Activation of the PI3K/JAML signaling pathways | | Verdino et al. (2010) |
| RIG-I | Activation of IRF3 and IRF7 signaling | Production of type I interferons | Otake et al. (1998) |
| v5 Integrins | Activation of the PI3K signaling pathway and enhancement of DCs maturation | TNFα | Li et al. (1998), Philpott et al. (2004) |
| **NOD-LIKE RECEPTORS (NLRs)** | | | |
| AIM2 | Activation of caspase-1 and inflammasome | IL-1β processing and release | Hornung et al. (2009), Burckstummer et al. (2009) |
| NALP3/ASC inflamasome | Induces maturation of pro-interleukin-1β and activation of caspase-1 | IL-1β processing and release | Barlan et al. (2011), Lotze and Tracey (2005) |

*Summary of the innate immune pathways that are activated in response to adenovirus vector administration. TLRs, toll-like receptors; CAR, coxsackievirus and adenovirus receptor; AIM2, absent in melanoma 2; NALP3, NACHT-, LRP-, and PYD-containing protein-3; ASC, apoptosis-associated speck-like protein containing a CARD.*

IL-6 has been observed in Kupffer cell depleted mice with no change in NFκB activity, suggesting that there might be additional cell types that contribute to Ad vector-mediate pro-inflammatory cytokine production (Lieber et al., 1997).

Furthermore, Ad vectors can either activate of directly transduce various immune cells types in the liver and spleen including: DCs (Lore et al., 2007), both plasmacytoid DCs (pDCs; Basner-Tschakarjan et al., 2006) and (cDCs; Lindsay et al., 2010), macrophages (Aldhamen et al., 2011), and to a lesser extent (NK) cells (Schroers et al., 2004; Aldhamen et al., 2011). The induction of type I interferon is critical for innate immune defense against Ad vectors *in vivo* (Zhu et al., 2008); the maturation of antigen presenting cells, both DCs and macrophages (Hensley et al., 2005); and the regulation of the induction of pro-inflammatory cytokines (Huarte et al., 2006; Zhu et al., 2007).

In addition to DCs and macrophages, we and others have shown that NK cells are another major group of innate immune effector cells that responds to the presence of Ad vectors. As early as 6 h post injection, NK cells accumulate in the liver and spleen producing high levels of IFNγ, which contributes significantly to the innate immune elimination of Ad vectors and the induction of T helper cell type 1 (TH1) adaptive immune responses (Peng et al., 2001; Ruzek et al., 2002; Zhu et al., 2008, 2010; Appledorn et al., 2010, 2011; Aldhamen et al., 2011). Furthermore, NK cell activation by Ad vectors contributes to liver injury, as NK cell depletion using anti-NK1.1 or anti-asialo GM1 (AsGM1) antibodies reduced Ad vector induced elevations of transaminases, as well as hepatocyte cell death (Liu et al., 2000).

## MOLECULAR BASIS FOR CELLULAR RECOGNITION OF ADENOVIRUS VECTOR INFECTION

Ad5 vectors interact with both the coxsackie-adenovirus receptor (CAR; via the Ad fiber knob domain) and with cellular integrins (via the Ad penton base RGD motifs) to initiate host cell penetration (Wickham et al., 1993; Bergelson et al., 1997). The penetration process itself will also simultaneously trigger cellular pro-inflammatory innate immune responses. Inductions of IP-10 by Ads have been detected after infecting kidney derived epithelial cells with WT capsid vectors, as well as CAR binding ablated fiber knob mutants (Tibbles et al., 2002). However, recent data has shown that binding of CAR by Ads also promotes the clustering of junctional adhesion molecule-like protein (JAML) and activation of the P13K signaling pathway, suggesting another role for CAR binding during the initiation of innate immune responses to Ads (Verdino et al., 2010). Various reports have also shown a significant role for $\alpha_v$-integrins for Ad vector induced innate immune responses (Huang et al., 1996; Li et al., 1998; Nemerow and Stewart, 1999). Specifically, Ad penton base interactions with $\alpha_v$-integrins have also been shown to activate the PI3K signaling pathway and induce DCs maturation via TNFα autocrine signaling (Li et al., 1998; Philpott et al., 2004). Inhibiting PI3K signaling also blocked Ad-induced DC

maturation and reduced Ad-induced TNFα production (Philpott et al., 2004).

After internalization and upon endosomal escape, Ad vectors have been shown to activate MAPK and NFκB signaling pathways via both TLR dependent and TLR non-dependent mechanisms (Zhu et al., 2007; Appledorn et al., 2008c). We and others have shown that, Ad vectors activate TLR signaling and induce various cytokines and chemokines responses in a MyD88 and TLR9 dependent manner (Basner-Tschakarjan et al., 2006; Hartman et al., 2007; Appledorn et al., 2008c). However, the induction of some chemokines, for example KC and MCP-1, was not dependent on TLR9, but still required a functional MyD88 adaptor protein, suggesting either the involvement of other TLRs, or other MyD88 dependent signaling systems, for the complete induction of the innate immune response to Ad vectors (Cerullo et al., 2007). Studies in our laboratory have shown that the induction of several cytokines and chemokines, the expression of innate immune response genes, and the generation of antibodies to both Ad vectors (NAbs) as well as Ad expressed transgene products were also TLR2, TLR3, and TLR4 dependent, and required both functional MyD88 and TRIF (TIR-domain-containing adapter-inducing interferon-beta) adaptors proteins (Appledorn et al., 2008c, 2009). In addition, our studies also demonstrated a suppressive role for TLR4 signaling in some Ad-induced innate immune responses; suggesting a complex role for TLRs in Ad vectors-mediated immune responses (Appledorn et al., 2009).

Besides TLRs, significant evidence has been accumulated in recent years implicating a TLR9-independent mechanism for sensing Ad5 (dsDNA) genomes (Nociari et al., 2007; Zhu et al., 2007; Shayakhmetov et al., 2010). Takaoka et al. (2007) showed that a dsDNA sensor called DNA-dependent activator of interferon regulatory factors (DAI) activates type I interferon in response to DNA viruses in L-929 cells, but subsequent studies suggested the presence of other cytoplasmic DNA sensor(s) (Ishii and Akira, 2006; Ishii et al., 2008). Absent in melanoma 2 (AIM2) has been shown to respond to cytoplasmic dsDNA and activate the inflammasome, driving the activation of caspase-1 and IL-1β processing and release (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). AIM2 was suggested to be important for the induction of pro-inflammatory immune responses against several viruses that produce cytosolic DNA during their lifecycle, such as vaccinia virus, herpes simplex virus-1 (HSV-1), and adenovirus (Kanneganti, 2010; Rathinam et al., 2010). However, it is our understanding that there is as yet no direct evidence linking the activation of the inflammasome by Ads to AIM2.

The activation of the NALP3 inflammasome by Ad derived dsDNA leads to caspase-dependent activation of IL-1β and induction of pro-inflammatory cytokine and chemokine responses including elevations of IL-6, MIP-1β, IP-10, and MCP-1 (Muruve et al., 2008). In addition, Ad-mediated disruption of lysosomal membranes, and the release of cathepsin B into the cytoplasm, are required for Ad-induced NLRP3 inflammasome activation (Barlan et al., 2011). Furthermore, Ad5 activation of NLRP3 also induced necrotic cell death, resulting in the release of the pro-inflammatory molecule High-mobility group box 1 protein (HMGB1), a recently identified damage (or danger)-associated molecular pattern (DAMP) that mediates the response to infection, injury, and inflammation (Lotze and Tracey, 2005; Barlan et al., 2011). It is important to note that, only partial reductions of pro-inflammatory cytokines and chemokines have been observed in Ad treated mice deficient of TLR9, NALP3, or the ASC inflammasome; suggesting the involvement of other innate immune PRRs, or a synergy between the inflammasome and other innate immune receptors during the innate immune recognition of Ad vector derived DNA (Cerullo et al., 2007; Muruve et al., 2008; Hornung et al., 2009). In addition, it has been demonstrated that adenovirus virus-associated RNA (VA) is recognized by retinoic acid-inducible gene I (RIG-I), a cytosolic PRR, and activates RIG-I down-stream signaling, leading to the induction of type I interferons (IFNs; Minamitani et al., 2011).

Together, these findings confirm that Ad vectors activate a number of complex innate immune response networks (**Table 1**). These newly identified networks may also provide targets for the development of new approaches to further improve the safety and efficacy of Ad based, as well as other important gene transfer platforms.

## MODULATION OF INNATE IMMUNE RESPONSES TO Ad VECTORS

Various strategies have been attempted in efforts to manipulate Ad vector-inductions of the innate immune system to develop safe and efficacious Ad-based gene transfer therapies. For gene transfer based applications applied to genetic diseases, multiple approaches have been utilized to minimize innate immune responses to Ad vectors including the use of immunosuppressive agents and depletion of innate immune cells (i.e., DCs and macrophages) thought to be primarily responsible for cytokine and chemokine production following Ad vector administrations. For example, the modulation of Ad vector induced inflammation by prophylactic use of anti-inflammatory corticosteroids (such as Dexamethasone, DEX) was associated with a significantly reduced ability of Ad vectors to induce several acute inflammatory cytokine and chemokine responses including: TNFα, IL-6, IL-12, MCP-1, MIP-1α, G-CSF, in concert with sustained, as well in some cases, improved transgene expression subsequent to gene transduction (Otake et al., 1998; Seregin et al., 2009). These improvements were in some instances coupled with reductions in pro-inflammatory leukocyte infiltrations into the liver, as well reduced induction of pro-inflammatory gene expression in Ad transduced liver hepatocytes and spleen derived cells (Seregin et al., 2009). In addition, the use of more targeted strategies in mice, such as: TLR9 blockade with ODN-2088, administration of anti-TNFα monoclonal antibodies, and administration of Erk or p38 MAPK inhibitors (U0126 and SB203580, respectively) prior to Ad vector administration each significantly reduced Ad-triggered innate immune responses (Tibbles et al., 2002; Wilderman et al., 2006; Cerullo et al., 2007; Appledorn et al., 2008c).

Ad vectors have also been engineered to enhance innate immune responses subsequent to administration. In tumor immunotherapy based approaches, Ad vectors expressing pro-inflammatory mediators, such as IFNγ, IL-2, IL-12, IL-18, and IL-23, have been shown to possess potent anti-tumor activities in several pre-clinical and clinical investigations utilizing the vectors as tumor-lytics (Siddiqui et al., 2007; Urosevic et al., 2007; Reay et al., 2009; Choi et al., 2011). Administration of Ad vectors

over-expressing MyD88 has been shown to improve the induction of antigen specific adaptive immune responses, and enhance tumor cell lysis in a mouse model *in vivo* (Hartman et al., 2010). Studies in our laboratory have shown that Ad vectors expressing a recombinant Eimeria tenella derived TLR agonist (*rEA*) induced potent innate immune responses that correlated with an improved induction of cellular immune responses to several target antigens (Appledorn et al., 2010). Analogous to use of MyD88 (Hartman et al., 2010), we have also recently shown that Ad vectors expressing EAT-2 (Ewing's sarcoma-associated transcript-2), a signaling lymphocytic activation molecules (SLAM) derived adaptor protein, enhanced innate immune cell (NK-, DCs, and macrophages) activation and induced beneficial cytokine and chemokine responses. These responses also correlated with improved inductions of antigen specific adaptive immune responses (including induction of cytotoxic T cell responses) by Ads expressing EAT-2 *in vivo* (Aldhamen et al., 2011).

## ADAPTIVE IMMUNE RESPONSES AGAINST ADENOVIRUS VECTORS

### DEVELOPMENT OF NEUTRALIZING ANTIBODIES AND CD8$^+$ T CELL RESPONSES TO THE Ad VECTOR

When attempting to summarize adaptive immune responses to Ad-based vectors, one must consider two primary issues, adaptive immune responses to Ad derived proteins (either Ad genes still expressed from early generation vectors, or the Ad capsid proteins themselves) as well the adaptive immune responses to the transgene expressed by a respective Ad vector.

Both gene therapy and vaccine applications utilizing Ad-based vector platforms may suffer from diminished efficacy (i.e., diminished antigen specific immune responses or blunted transient expression of therapeutic transgene) when pre-existing Ad5 immunity is present. A majority (over 60%) of the worldwide human population have had previous exposure to wild-type Ads in childhood or adulthood, these exposures result in the development of various forms of pre-existing immunity to the most common Ad serotypes (Abbink et al., 2007; Lasaro and Ertl, 2009; Seregin and Amalfitano, 2009). NABs to Ad vectors are primarily directed against the surface loops of the viral hexon protein, however, antibodies to the penton base or the fiber can also neutralize Ads (Sumida et al., 2005). Ad NABs affect the efficacy of Ad vector gene transfer by blocking cell transduction (Smith et al., 2008; Parker et al., 2009; Pichla-Gollon et al., 2009; Seregin and Amalfitano, 2009).

When utilizing early generation Ad5 based vectors that are deleted for only the E1 genes, or alternative serotype Ad vectors that are also only E1 deleted, this results in reduced transgene product expression and thus, reduced induction of transgene-specific CD8$^+$ T cell immune responses in vaccine applications (Sumida et al., 2004; Thorner et al., 2006; McCoy et al., 2007; Liu et al., 2008; Gabitzsch et al., 2009b; Osada et al., 2009; Haut et al., 2011). Since the presence of high levels of NABs to the most commonly utilized Ad vector, Ad5, are highly prevalent in certain populations, including sub-Saharan Africa (Abbink et al., 2007; Barouch et al., 2011), recent efforts have focused on developing Ad vectors that have either had immunogenic portions of the Ad5 capsid replaced with homologous regions from alternative serotype Ads, or on Ad vectors entirely derived from rarer human, or chimp derived Ad serotypes (Abbink et al., 2007; Barouch, 2008; Seregin and Amalfitano, 2009; Geisbert et al., 2011). While the use of these alternative serotypes may allow for partial overcoming of transductional inefficiencies due to the presence of pre-existing Ad5 NABs, their use will also have several limitations, including altered biodistribution and biosafety profiles, as well they are also subject to neutralization upon their readministration (Barouch et al., 2004; Thorner et al., 2006; Appledorn et al., 2008a; Liu et al., 2009).

Additionally, several studies emphasize a significant role for Ad specific CD8$^+$ T cells in pre-existing Ad immunity. Ad vector specific (mostly against hexon capsid protein) CD8$^+$ T cells can be readily detected in PBMCs in a high percentage of healthy adults (Molinier-Frenkel et al., 2000, 2002; Tang et al., 2006). This latter point must also be considered when alternative, rarer Ad serotypes are utilized in attempts to circumvent pre-existing common Ad serotype specific antibodies, since pre-existing T cell responses to one Ad serotype can still be harnessed when a host is exposed to an alternative Ad serotype (Heemskerk et al., 2003; Leen et al., 2004; Hutnick et al., 2010). An important and recent study demonstrated that CD8$^+$ T cells against the E2b encoded polymerase protein can be found at frequencies as high as found for CD8$^+$ T cells against the major immunodominant Ad protein hexon in Ad immune individuals (Joshi et al., 2009). This feature may be responsible for the recent finding that administration of Ad5 vectors devoid of expression of the Ad polymerase (E2b) protein can induce beneficial adaptive immune responses to expressed antigens, even in the Ad5 immune host (Gabitzsch et al., 2009a,b, 2010; Weaver et al., 2009; Clarke et al., 2010).

### ADAPTIVE IMMUNE RESPONSES TO Ad VECTOR EXPRESSED TRANSGENES

A continued fallacy regarding the use of Ad-based gene transfer is the notion that Ads are incapable of allowing for long term transgene expression in the immune competent host. However, Ad vectors have been repeatedly shown to allow for long term transgene expression, especially in animals that do not perceive the transgene being delivered by the Ad vector as immunologically foreign. We and others have reviewed this topic extensively in the past, and refer the reader to those publications for full validation and understanding of these views (Amalfitano, 2004; Seregin and Amalfitano, 2009, 2010).

Conversely, Ad-based vectors have a potent ability to induce potent humoral, but more importantly, cellular immune responses to expressed foreign antigens, and have therefore recently received much attention for use in a number of vaccine based applications (Lasaro and Ertl, 2009; Barouch, 2010). Specifically, E1 deleted Ad5 vectors expressing the HIV-1 *gag*, *pol*, and *nef* genes have been utilized in human trial subjects (Buchbinder et al., 2008; Priddy et al., 2008). The results from the early-phase clinical trials demonstrated that the Ad5 vector-based vaccines elicited some of the most potent, HIV specific cellular immune responses in humans to date, however, the presence of pre-existing Ad5-specific NABs partially suppressed these responses (Buchbinder et al., 2008; Priddy et al., 2008).

Utilization of advanced generation Ad vectors have also recently been shown to allow for improved efficacy in several vaccine

based applications (Gabitzsch et al., 2009b, 2010, 2011; Weaver et al., 2009). Specifically, [E1-, E2b-]Ad5 vectors were able to induce heightened gene specific T cell responses in mice and primates (Gabitzsch et al., 2009a,b, 2010). In contrast to E1 deleted Ad vectors, these types of Ad vaccines can show strong efficacy despite the existence of of pre-existing Ad immunity possibly by their avoidance of $CD8^+$ T cell responses directed to the Ad polymerase gene (Gabitzsch et al., 2009a,b, 2010, 2011; Osada et al., 2009). [E1-, E2b-]Ad vectors, expressing tumor derived antigens were also able to induce beneficial, cytolytic T cell responses that promoted tumor regression in murine models (Gabitzsch et al., 2011). Based upon these improvements, a phase I/II clinical trial is currently underway, utilizing a CEA (Carcinoembryonic Antigen) expressing [E1-, E2b-]Ad5 vector in an attempt to safely induce beneficial, CEA specific adaptive immune responses in patients (both Ad5 naive and Ad5 immune) bearing CEA expressing tumors[2].

## FURTHER STRATEGIES FOR EVADING HOST IMMUNE RESPONSES AFTER Ad VECTOR MEDIATED GENE TRANSFER

Several elegant approaches have been developed in various attempts to diminish Ad vector and transgene-triggered innate and/or adaptive immune responses, thereby, maximizing the efficiency and persistence of Ad-mediated gene transfer for a variety of applications. This chapter will briefly summarize the latest advances, and refer readers to several recent reviews summarizing earlier papers on these topics (Seregin and Amalfitano, 2010).

The approaches attempting to maximize the duration of transgene expression from an Ad vector and minimize potential side effects can be categorized as follows: (Waehler et al., 2007) preemptive transient immune modulation of the host; consisting of the use of immunosuppressive drugs or specific compounds to block important immune pathways, which are known to be induced by Ad vectors and (St George, 2003) selective modification of the Ad vector itself. The latter approaches includes several innovative strategies, with the most prominent being covalent modifications of the entire Ad vector capsid moiety; Ad capsid-display of specific inhibitors or ligands; the use of tissue specific promoters to drive transgene expression in selected tissues to minimize adaptive immune responses, and as alluded to previously, the use of genome modified Ads, chimeric Ads, and alternative Ad serotypes (Seregin and Amalfitano, 2010).

Transient, non-specific immunosuppression of the host with (Dexamethasone, FK506, cyclosporine A), or selective immunosuppression of the host (TLR9 or TNFα blockers, CTLA4-Ig, anti-CD40 antibodies), and/or attempts at induction of a generalized tolerance state (IL-10, TGFβ) have all been strategies that have been described in animal models to successfully improve outcomes of Ad-based gene transfer. Moreover, some of these immunosuppression approaches have been tested in clinical trials, as reviewed (Seregin and Amalfitano, 2010). In regards to the non-specific modification of the Ad vector capsid itself, Ad vectors have also been complexed with several polymers in a manner to shield the capsid from either innate or adaptive humoral immune components, such as NABs. These moieties included the use of polyethylene glycol (PEG; O'Riordan et al., 1999), polylactic glycolic acid (PLGA; Matthews et al., 1999) or other lipids (Lee et al., 2000), each of which have been reported to improve efficacy and/or safety of Ad-mediated gene transfer, when the vector is produced in a complex with these moieties.

The non-enveloped Ad virion is composed of a large capsid of about 90 nm in diameter, containing nine proteins (Rux and Burnett, 2004; Parks, 2005; Vellinga et al., 2005). Several novel and specific modifications to the Ad capsid have also been engineered in attempts to improve the efficacy of the basic Ad vector platform. The fiber, penton, protein IX, and hexon proteins have all been exploited for genetic insertion of foreign peptides, either as "in-frame" insertions within the proteins, or as "in-frame" C-terminal fusions.

In a vaccine targeted application, antigenic epitopes, derived from the hemagglutinin (HA) protein of the influenza A virus, were incorporated into, and displayed from the Ad capsid as hexon, penton base, fiber knob, or protein IX fusions (Krause et al., 2006). The fiber-displaying Ads induced the highest levels of HA-specific immunity, as determined by measuring production of HA-specific IgM and IgG humoral responses, as well as HA-specific IL-4 or IFNγ producing $CD4^+$ T cellular immune responses (Krause et al., 2006). In another example, the immunodominant portions of the *Bacillus anthracis* protective antigen (PA) were genetically inserted and displayed from the HVR5 site of the Ad5 hexon (McConnell et al., 2006). Intramuscular injections of the novel vector into BALB/c mice resulted in generation of PA-specific IgG1 and IgG2a antibodies, possibly indicating that TH1 and TH2 immunity to the antigen was generated, surpassing the efficacy of synthetic peptide based vaccination strategies (McConnell et al., 2006). The HVR5 site of hexon has also been exploited for display of the *Pseudomonas aeruginosa* B cell epitope-encoding peptide. Footpad vaccinations with the novel vaccines induced antibody responses to the *P. aeruginosa* antigen (Worgall et al., 2005). Both IFNγ-positive $CD4^+$ and $CD8^+$ *P. aeruginosa* specific T cell responses were also generated. Most convincingly, mice vaccinated with the *P. aeruginosa* B cell epitope displaying Ad were protected against subsequent lethal pulmonary challenge with several *P. aeruginosa* strains (Worgall et al., 2005).

In contrast, Ad capsid-display of immuno-evasive proteins can also dramatically improve the efficacy and/or safety of Ad-based gene transfer (Seregin et al., 2010a, 2011). Specifically, the human decay-accelerating factor (DAF) natural complement inhibitor was shown to retain anti-complement activity when displayed from the surface of the Ad capsid in a retro-oriented fashion (Seregin et al., 2010). Subsequent studies have shown that mice injected with the "DAF-displaying" Ad5 vector, demonstrated significant reductions in pro-inflammatory cytokine release, avoidance of thrombocytopenia, reduced endothelial cell activation, minimized activation of pro-inflammatory genes expression, and reduced plasma ALT levels in mice as compared to unmodified Ad5 vectors. Moreover, these results correlated positively with a significantly decreased activation of DCs, NK cells, $CD3^+CD8^+$ T cells, and $CD3^+CD8^-$ T cells (Seregin et al., 2010a, 2011). Importantly, this modulation of the complement dependent arm of the

[2]http://clinicaltrials.gov/ct2/show/NCT01147965?term = adenovirus + CEA& rank = 1

innate immune response resulted in significantly reduced induction of Ad neutralizing antibody responses, as well as in blunted T cell responses to the transgene (i.e., HIV-gag or GFP) by the DAF-displaying Ad (Seregin et al., 2011).

In addition, genetic modifications, or wholesale "swapping" of the Ad capsid hexon and fiber proteins from one serotype to another have been undertaken in efforts to avoid pre-existing Ad serotype specific neutralizing antibody responses, as previously described (Gall et al., 1998; Roy et al., 1998; Molinier-Frenkel et al., 2002; Ritter et al., 2002; Wu et al., 2002).

## CONCLUSION AND FUTURE PERSPECTIVES

After full analysis of the several points covered in this review, the reader should understand that the interactions of Ad-based vectors with the host innate and adaptive immune systems are multifaceted and complex. These complexities should make it clear that one cannot make simple assertions regarding the potential for use of Ad vectors in a specific gene therapy or vaccine based application, especially, if only limited information is provided, or general assumptions are being promulgated. It is also clear that despite this level of complexity and potential for several limitations, Ad vectors continue to be the platform of choice for an ever increasing number of clinical trials worldwide.

Utilization of Ad vectors (or any gene transfer vector) for implementing gene therapy for genetic disorders is very challenging. The induction of potent innate immune responses by the vector may limit its utility for this purpose in some instances. However, this same limitation may be of benefit when considering utilization of the vector in various vaccine based applications. It should also be clear that there have been a number of vector modifications attempted to improve the safety or efficacy of Ad-based gene transfer, some of these modified Ads may well be better utilized for some clinical applications but not for others. Despite this context-specific utility, it is obvious that the Ad-based gene transfer platform appears to be highly "plastic" and capable of tolerating a number of elegant molecular manipulations. Importantly, solely with use of first generation (E1 deleted) Ad vectors, the number of Ad vector utilizing clinical trials has doubled within the last 5 years. Bolstered by a clearer understanding as to the benefits, and more importantly, identified limitations of Ad-based vectors, we predict a continued expansion for clinical use of Ad vectors in general, an expansion that will be bolstered by the current and expanded future use of modified Ad vectors, as well inclusion of pharmacological and/or other supportive interventions to further enhance both the safety and efficacy of Ad-mediated gene transfer into humans.

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# Innate immune responses to AAV vectors

**Geoffrey L. Rogers[1], Ashley T. Martino[1], George V. Aslanidi[1], Giridhara R. Jayandharan[2], Arun Srivastava[1,3,4,5,6]* and Roland W. Herzog[1,3,4,5,6]***

[1] Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida College of Medicine, Gainesville, FL, USA
[2] Department of Haematology, Centre for Stem Cell Research, Christian Medical College, Vellore, Tamil Nadu, India
[3] Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL, USA
[4] Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, FL, USA
[5] Genetics Institute, University of Florida College of Medicine, Gainesville, FL, USA
[6] Shands Cancer Center, University of Florida College of Medicine, Gainesville, FL, USA

**Edited by:**
*Katherine High, Children's Hospital of Philadelphia, USA*

**Reviewed by:**
*Dirk Dittmer, University of North Carolina at Chapel Hill, USA*
*Melanie M. Brinkmann, Helmholtz Centre for Infection Research, Germany*

***Correspondence:**
*Arun Srivastava, University of Florida, 2033 Mowry Road, Room 492-A, Gainesville, FL 32610, USA.*
*e-mail: aruns@peds.ufl.edu;*
*Roland W. Herzog, Cancer and Genetics Research Complex, University of Florida, 2033 Mowry Road, Room 203, Gainesville, FL 32610, USA.*
*e-mail: rherzog@ufl.edu*

Gene replacement therapy by *in vivo* delivery of adeno-associated virus (AAV) is attractive as a potential treatment for a variety of genetic disorders. However, while AAV has been used successfully in many models, other experiments in clinical trials and in animal models have been hampered by undesired responses from the immune system. Recent studies of AAV immunology have focused on the elimination of transgene-expressing cells by the adaptive immune system, yet the innate immune system also has a critical role, both in the initial response to the vector and in prompting a deleterious adaptive immune response. Responses to AAV vectors are primarily mediated by the TLR9–MyD88 pathway, which induces the production of pro-inflammatory cytokines by activating the NF-κB pathways and inducing type I IFN production; self-complementary AAV vectors enhance these inflammatory processes. Additionally, the alternative NF-κB pathway influences transgene expression in cells transduced by AAV. This review highlights these recent discoveries regarding innate immune responses to AAV and discusses strategies to ablate these potentially detrimental signaling pathways.

**Keywords: AAV vectors, gene therapy, innate immunity**

## INTRODUCTION

Adeno-associated virus (AAV) has emerged as a promising vector for viral gene therapy over the past 20 years (Mueller and Flotte, 2008; Mays and Wilson, 2011; Mingozzi and High, 2011). AAV is a parvovirus, which is a family of small, non-enveloped viruses containing a single-stranded linear DNA genome of about 5 kb; the wild-type virus is replication-deficient, requiring a helper virus in order to reproduce (Srivastava et al., 1983). In humans, AAV has not been found to be pathogenic. This fact, along with the tendency for the genomes of recombinant AAV (rAAV) vectors to remain as episomal concatemers rather than integrating into the host genome (reducing the risk for insertional mutagenesis), makes AAV a relatively safe gene therapy vector for testing in the clinic (Nakai et al., 2001). Indeed, AAV has become a preferred choice by many investigators for *in vivo* viral gene transfer, and due to its wide tissue tropism, it has been used in over 20 clinical trials to treat a wide variety of monogenetic diseases, including but not limited to: hemophilia B, α1 antitrypsin deficiency, cystic fibrosis, Parkinson's disease, and Leber's congenital amaurosis (LCA; Zhao et al., 2006; Nathwani et al., 2011).

It is widely accepted that the trial for LCA represents the first example of successful AAV gene therapy in humans without immune consequences. LCA is a genetic disease characterized by severe vision deficits due to a mutation in *RPE65*. Patients who received one subretinal injection of rAAV encoding RPE65 tolerated the transgene well and showed improved visual capability both in psychophysical (e.g., visual acuity) and functional (e.g., ability to navigate an obstacle course) measures (Maguire et al., 2009). So far, the improvements have persisted for over 3 years (Mingozzi and High, 2011). However, other clinical trials using AAV have not been as successful due to interference from the immune system (Manno et al., 2006). The success of the LCA trials is likely due to unique features of the target tissue that make it ideally suited for gene transfer. The retina is easy to access without potential exposure of vector to other tissues, and the relatively small number of target cells allow a small volume and low vector titer to result in a high multiplicity of infection (Mccarty, 2008). Additionally, the eye is generally regarded as an immunoprivileged site, greatly reducing the likelihood of developing a deleterious immune response (Hauswirth et al., 2008).

Conversely, other target tissues do not possess the near complete immunological ignorance displayed by the retina. The potential effects of an immune response on gene transfer are illustrated by the 2006 trial using AAV serotype 2 (AAV2) to transfer human blood coagulation factor IX (hF.IX) to the livers of hemophilia B patients deficient in factor IX. In this trial, one patient showed elevated circulating F.IX at 2 weeks, followed by a decline of transgene with a concomitant rise in liver enzymes, indicative of the destruction of hepatocytes; this damage was most likely mediated by a $CD8^+$ T cell response against the AAV capsid (Manno et al., 2006; Mingozzi et al., 2007). Subsequently, it was shown that input capsid-derived peptides are presented by MHC class I

molecules on the surface of hepatocytes following transduction with AAV2 vectors (Pien et al., 2009). Therefore, transduced cells may become targets for AAV capsid-specific CTLs. Another subject with a higher neutralizing antibody (NAB) titer against the capsid did not show appreciable levels of circulating F.IX, likely because the antibodies prevented transduction of hepatocytes (Mingozzi and High, 2007). These results illustrate a major problem with using AAV for *in vivo* gene therapy – pre-existing immunity. Since AAV is a naturally occurring infection in the human population, it is not surprising that reports have indicated that $CD8^+$ memory T cells as well as NAB to AAV are common (Mingozzi and High, 2007; Calcedo et al., 2009; Boutin et al., 2010).

Studies in animal models have also revealed concerns beyond pre-existing immune responses to AAV. Without a memory response against the capsid developed due to natural infection, it is easier to successfully transduce wild-type mice with hF.IX via hepatic gene transfer; the resulting induction of tolerance to the transgene is thought to be mediated by hF.IX-specific regulatory T cells ($T_{regs}$; Dobrzynski et al., 2006; Cao et al., 2007). However, even in animal models, sustained transgene expression is not guaranteed. Hemophilic mice with missense mutations in transgenically expressed hF.IX genes are more tolerant to hF.IX gene transfer than total deletion mutants. The target tissue for transgene expression can also affect the outcome of gene transfer. In the same hemophilic mouse strains, hF.IX was less tolerated when expressed in skeletal muscle than when expressed in hepatocytes (Cao et al., 2009). Furthermore, tolerance can be affected by the serotype of AAV that is used; increased transduction efficiency in the liver is more likely to lead to tolerance to the transgene. In this regard, AAV8 is more tolerogenic than AAV2 (Cooper et al., 2009). Transduction efficiency can be also be increased by mutating surface exposed tyrosine residues on the capsid, which is thought to reduce proteasomal degradation, increasing trafficking to the nucleus (Zhong et al., 2008; Markusic et al., 2010). Though a variety of mechanisms are involved in these studies, they, along with other studies in animals, are united by a common theme: in current murine models, functional $CD8^+$ T cell infiltrates in AAV transduced tissues are primary directed against the transgene product rather than the capsid, while an antibody response is often observed to both potential immunogens (Siders et al., 2009).

With these concerns in mind, many investigators have focused more on the adaptive immune response to AAV2. Additionally, a previous study comparing adenoviral vectors and AAV2 found that the innate immune response to AAV was weak and transient relative to the potent and prolonged response to adenovirus, suggesting that innate immunity to AAV2 may be insignificant (Zaiss et al., 2002). It is commonly accepted that innate responses provide activation signals critical for subsequent adaptive immunity. Even though the adaptive immune system has the effector functions that impact viral gene transfer, signals provided by the innate immune system can recruit and activate antigen presenting cells, T cells, and B cells (Hensley and Amalfitano, 2007). In the absence of proper activation signals, lymphocytes may be unresponsive to the presence of antigen. In this article, we will review the mechanisms that the innate immune system uses to respond to viruses, and then specifically consider how responses to rAAV vectors are mediated and how they affect successful transgene expression.

## OVERVIEW OF INNATE IMMUNE RESPONSES TO VIRUSES

As with other pathogens, in order to respond to viruses, the innate immune system needs to identify the particle as foreign and potentially dangerous. This occurs by recognizing structural motifs unique to non-self organisms, commonly referred to as pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs). The innate immune system distinguishes the unique characteristics of viruses via PRRs that can recognize both viral nucleic acids and membrane glycoproteins (Akira et al., 2006).

The most studied family of PRRs are the toll-like receptors (TLRs), of which 13 have been described so far (Huang and Yang, 2009). These transmembrane proteins are characterized by an extracellular domain that binds to the receptor's cognate PAMP and an intracellular Toll/IL-1R homology (TIR) domain. TLR2 (glycoproteins and lipoproteins), TLR3 (dsRNA), TLR4 (glycoproteins and bacterial LPS), TLR7 (ssRNA), TLR8 (ssRNA), and TLR9 (unmethylated CpG DNA) have all been implicated in initiating inflammatory responses to viruses (Kawai and Akira, 2011). The glycoprotein-recognizing receptors are generally found on the cell surface, while the nucleic acid specific TLRs are most commonly located in endosomal compartments. TLRs are mostly found in immune cells – including DCs, macrophages, B cells, and some T cells – but also in some non-immune cells such as fibroblasts and epithelial cells (Akira et al., 2006).

Upon receptor engagement, most TLRs recruit MyD88, which causes phosphorylation of IRAK4 and IRAK1. Subsequent interaction of the IRAKs with TRAF6 and NEMO leads to ubiquitination of the latter molecules. Once ubiquitinated, TRAF6 and NEMO recruit TAK1, which activates the MAP kinase and classical NF-κB pathways (via ubiquitin-mediated degradation of IκB, freeing the RelA-p50 heterodimer to enter the nucleus). This results in the production of pro-inflammatory cytokines such as TNF-α and IL-6. TLR3, on the other hand, uses TRIF as an adaptor molecule instead of MyD88, but this pathway also leads to TRAF6-mediated activation of TAK1 and the same downstream inflammatory pathways. TLR2 and TLR4 can also induce an inflammatory response by signaling through TRIF (Kawai and Akira, 2007).

TLR7 and TLR9 signaling can induce the production of type I interferons (IFNs) in plasmacytoid dendritic cells (pDCs), which are a subset of DCs specifically designed to sense and respond to viral infections by producing large amounts of IFN. This signaling pathway is also dependent on MyD88; however, in order to stimulate IFN production, TLR9 must interact with AP-3 and traffic to the $LAMP2^+$ lysosome-related organelle (LRO), where it can engage TRAF3 and signal IRF7 (Gilliet et al., 2008; Sasai et al., 2010). It is important to note that this pathway need not be activated in the same cell as the classical NF-κB pathway. IFNs are a class of soluble cytokines uniquely suited to combat intracellular infections by stimulating the production of over 100 interferon response genes (ISGs). The combined actions of these genes induce an antiviral state that renders the cell resistant to viral infection. Although the individual effects of most of these genes are still unknown, the family includes proteins such as Mx, which sequesters viral ribonucleoproteins, PKR, which leads to phosphorylation of eIF2α, inhibiting translation, and OAS, which activates RNAse L to degrade cellular and viral RNA

(Garcia-Sastre and Biron, 2006). Furthermore, type I IFNs play a role in augmenting the innate immune response and stimulating the adaptive immune response to a viral infection by activating NK cells, inducing short-term proliferation of memory $CD8^+$ T cells, aiding expansion and functionality of naïve T cell populations, and acting on $CD4^+$ T cells and B cells to induce antibody isotype switching (Garcia-Sastre and Biron, 2006; Zhu et al., 2007, 2008).

In addition to the transmembrane TLRs, soluble PRRs can be found in the cytoplasm. As with the TLRs, these receptors mostly respond to viral nucleic acids. RIG-I and MDA5 detect dsRNA formed during the life cycle of most RNA viruses, while DAI has been implicated in cytoplasmic responses to DNA (Akira et al., 2006). There is also evidence that NLR proteins in association with mitochondrial antiviral signaling protein (MAVS; also known as IPS-1) are capable of regulating type I IFN and NF-κB production in response to viral single-stranded or double-stranded RNA (Ting et al., 2010). Finally, the NLRP3 inflammasome, a cytoplasmic complex that consists of NLRP3, ASC, and procaspase-1, can cleave pro-IL-1β and pro-IL-18 into their active and secreted forms in response to viral DNA (Muruve et al., 2008).

In summary, the innate immune system has a wide variety of membrane-bound and cytoplasmic mechanisms to detect viral components and initiate inflammatory responses that are primarily mediated by NF-κB and type I IFNs. Only some of these pathways and mechanisms are involved in innate immunity to AAV, which elicits a limited response. Our current knowledge of the interactions between the innate immune system and AAV vectors is summarized in the following.

## INNATE RESPONSES TO AAV

As previously noted, the innate immune responses to single-stranded AAV vectors (ssAAV) are typically low, particularly when compared with adenoviral vectors (Zaiss et al., 2002). In some situations, these vectors appear to lack the inflammatory signals that allow transduced cells such as hepatocytes to be targeted by a CTL response (Somanathan et al., 2010). Nevertheless, other studies have indicated that innate immune signaling through TLR9 can have a crucial effect on the development of a $CD8^+$ T cell response against a transgene product (Zhu et al., 2009). The authors showed that AAV can induce the production of type I IFNs in pDCs [but not cDCs, Kupffer cells (KCs), or macrophages] *in vitro*, and that this induction is dependent on the TLR9–MyD88 pathway. In order to be exposed to this endosomal PRR, AAV must enter the endosomal pathway. There are two potential mechanisms by which AAV particles may enter APCs and traffic to endosomes. Binding to a specific receptor and co-receptor cause the particle to be endocytosed into a clathrin-coated pit (**Figure 1A**). Receptor specificity varies among the AAV serotypes. Examples of primary receptors utilized by some serotypes include heparin sulfate proteoglycan (HSPG) or sialic acid. AAV2 can utilize several different co-receptors, including αVβ5 integrin and platelet-derived growth factor receptor (PDGFR; Ding et al., 2005; Muzyczka, 2010). Alternatively, the virus may enter APCs via a clathrin-independent mechanism, such as pinocytosis (**Figure 1A**; Harbison et al., 2008). Once in the endosome, degradation of the viral capsid can expose the genome to TLR9, which then signals through MyD88 to activate NF-κB and ISGs (**Figures 1B–F**; Zhu et al., 2009).

Zhu et al. (2009) also demonstrated that this pathway is critical for the development of an anti-transgene CTL response and NAB to both the transgene product and the AAV capsid. This study represented a significant breakthrough in attempts to elucidate the source of innate immune responses to AAV and their effects on the formation of an adaptive immune response that can lead to transgene rejection. A recent study by Martino et al. (2011) explored the innate immune responses to ssAAV and self-complementary (scAAV vectors) during hepatic gene transfer. Such scAAV vectors could potentially display altered responses due to their unique genome organization (dsDNA rather than single-stranded) and transduction kinetics (Mccarty, 2008).

### TLR9 FACILITATES ENHANCED RESPONSES TO scAAV VECTORS DURING HEPATIC GENE TRANSFER

Overall, the study by Martino et al. (2011) found that the innate responses to scAAV vectors were increased relative to ssAAV (**Figure 2**). Consistent with previous findings, the response to single-stranded vectors was rapid yet transient. Within 2 h after administration, a small increase in type 1 IFNs, TLR9, MyD88, and TNF-α RNA levels could be observed, which faded within 6 h. Conversely, the scAAV vectors induced much higher expression of these genes, plus increases in IL-6 (which now could be observed

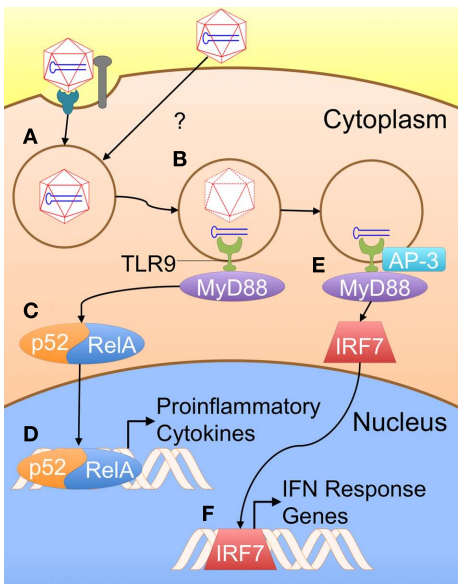


**FIGURE 1 | A proposed model for innate immune recognition of AAV vectors (based on TLR9 dependence of the response and general knowledge of TLR9 signaling). (A)** AAV particles are taken up by APCs; the exact mechanism of uptake in these cell types is not presently known. Typically, AAV is taken up via receptor/co-receptor interactions that lead to internalization in a clathrin-coated pit. However, viral particles might also enter APCs through other pathways such as pinocytosis. **(B)** Some capsid breaks down in the endosome, exposing the genome to TLR9. **(C)** MyD88 initiates a signaling cascade, resulting in activation of NF-κB1 (p52–RelA heterodimer). **(D)** NF-κB enters the nucleus, initiating transcription of pro-inflammatory cytokines. **(E)** Alternatively, TLR9 may be rerouted to a $LAMP2^+$ compartment by AP-3, allowing it to activate IRF7. **(F)** IRF7 translocates into the nucleus, inducing transcription of interferon response genes, including IFNα/β.

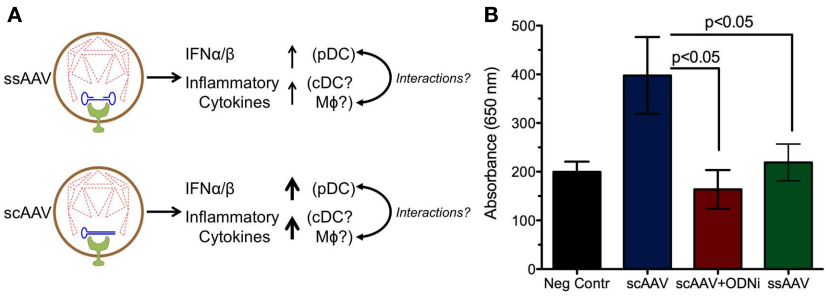


**FIGURE 2 | Increased innate responses are induced by scAAV relative to ssAAV. (A)** Degradation of the capsid within the endosome can expose the vector genome to TLR9, which subsequently induces production of inflammatory cytokines and type I IFNs. The stronger response to self-complementary genomes may be due to reduced capsid stability of scAAV, leading to more genome exposure to TLR9. Alternatively, the structure of the double-stranded DNA may lead to increased TLR9 signaling and heightened inflammation relative to single-stranded DNA. **(B)** Experiments in a TLR9 reporter cell line demonstrate that sensing of scAAV results in stronger TLR9 signaling compared to ssAAV; this response can be blocked by TLR9 antagonists. Secreted embryonic alkaline phosphatase (SEAP) levels were measured by absorbance at 650 nm following TLR9 activation in a 293 reporter cell line (Invivogen, San Diego, CA, USA) after infection at an MOI of $10^4$ with ssAAV, scAAV, or scAAV with a TLR9 inhibitory oligonucleotide (ODNi).

systemically by 2 h), CCL5, MIP-1, and TLR2. Cytokine responses varied with vector dose for scAAV but not ssAAV. Other pro-inflammatory genes, such IL-1α/1β, TLR1, and TLR3-8, were not upregulated following injection with either single-stranded or self-complementary AAV. Unlike adenoviral vectors, neither type of AAV vector could activate the inflammasome (Martino et al., 2011).

In accordance with the differential induction of chemokines by ssAAV and scAAV, the vectors provoked dissimilar levels of innate immune cell infiltrates 2 h after vector administration. The ssAAV vectors caused a slight increase in neutrophil and macrophage infiltration, whereas scAAV injection led to more substantial infiltration by neutrophils, macrophages, and NK cells.

The production of pro-inflammatory cytokines in response to scAAV is almost entirely dependent on TLR9. TLR9$^{-/-}$ mice injected with scAAV displayed no increases in RNA transcripts for any of the markers previously upregulated, except for a marginal increase in TLR2. The same effect can be achieved using an inhibitory oligodeoxynucleotide (ODN); these molecules binds strongly to TLR9 without inducing signaling, allowing them to serve as antagonists and block normal TLR9 signals (Stunz et al., 2002). Co-administration of inhibitory ODN with scAAV broadly blocked the cytokine response in a similar manner to the TLR9 knockout mice. It was also able to effectively blunt infiltration by macrophages, NK cells, and neutrophils, demonstrating that the recruitment of those cells depends on chemokines induced by TLR9 signaling. The rapid activation of pro-inflammatory cytokines suggests that the classical NF-κB pathway initiates their production, which is supported by other studies (Jayandharan et al., 2011).

At the cellular level, innate immune responses to scAAV in the liver were found to be partially dependent on KCs, concurring with previous data from ssAAV (Zaiss et al., 2002). Inactivation of KCs via gadolinium chloride ($GdCl_3$) injection prior to scAAV administration significantly altered the profile of the immune response. Transcripts for IFNα/β, IP-10, and IL-6 were not increased by scAAV injection following $GdCl_3$ treatment. More minor changes in TLR9, MyD88, MCP-1, and MIP-1 were observed, while transcripts for TNF-α and TLR2 were not significantly affected by KC inactivation. This treatment was also able to diminish recruitment of neutrophils and NK cells, but only slightly reduced macrophage accumulation. The underlying mechanism of these results is not yet clear. However, since changing the scAAV capsid to serotype 8, which more effectively transduces hepatocytes, did not increase the innate immune response, the hepatocytes themselves may not respond to the vector genomes; it is therefore more likely that the response is due to an uptake of AAV particles by APCs. Additionally, inactivation of KCs in the presence of scAAV vector prevented the type I IFN response (Martino et al., 2011).

Not surprisingly, the disparity found in innate immune responses between ssAAV and scAAV correlates with differences observed in adaptive responses. At equal doses, scAAV vectors induced higher CD8$^+$ T cell and antibody responses against the vector capsid compared to ssAAV. Prevention of TLR9 signaling either via injection into TLR9$^{-/-}$ mice or co-administration of an inhibitory ODN caused a significant reduction in CTL responses and delayed the formation of anti-AAV antibodies. This suggests that the increased adaptive response to scAAV, like the innate response, depends on TLR9 signaling. However, following liver-directed delivery, the adaptive response to the F.IX transgene was unchanged between ssAAV and scAAV, most likely due to the temporal separation between the innate signals and transgene expression. The pro-inflammatory cytokine response induced by scAAV occurs primarily during the first 9 h after vector administration, while the transgene is only expressed after viral uncoating and transcriptional activation. Thus, the immune system would not encounter hF.IX in the presence of inflammatory signals that could induce an effector response (Martino et al., 2011). In contrast to hepatic gene transfer, there is some evidence that, in skeletal muscle, scAAV vectors can direct a stronger response to the transgene product (H. C. Ertl, personal communication). Still, despite the lack of anti-transgene response after scAAV liver gene transfer, inhibition of TLR9 signaling increases systemic F.IX expression. This would imply that factors other than an anti-transgene

adaptive response, such as NK cells or type I IFN activity, could affect the overall levels of transgene expression in animal models for AAV, as is known to be the case for adenovirus (Zhu et al., 2008).

Interestingly, the heightened responses observed for scAAV vectors appear to be entirely dependent on their unique genome conformation. Single-stranded AAV8 and ssAAV–tyrosine mutant (TM; an altered AAV2 vector with three surface tyrosines changes to phenylalanine) displayed a comparable cytokine response to ssAAV2 vectors, while scAAV8–GFP was similar to scAAV2–hF.IX. Thus, the capsid serotype and transgene do not appear to affect the TLR9-mediated innate responses to scAAV during hepatic gene transfer (Martino et al., 2011).

The highly transient nature of the innate response further explains why hepatic gene transfer with AAV vectors has been successful for tolerance induction to transgene products, which is further facilitated by the anti-inflammatory microenvironment of the liver (Breous et al., 2009; Loduca et al., 2009; Martino et al., 2009; Somanathan et al., 2010; Hoffman et al., 2011).

### ROLE OF THE AAV CAPSID IN IMMUNE RESPONSES

Although capsid alterations do not modify the TLR9 dependent innate response, variable immune responses to different AAV capsids has been demonstrated. Some reports have suggested that certain serotypes, specifically AAV1, are capable of transducing DCs (Lu and Song, 2009). After transduction and maturation, DCs may initiate an immune response to the foreign transgene product; thus, the report by Lu and Song found that transgene delivered to NOD mice in an AAV1 capsid was more immunogenic and less tolerated than when it was delivered with AAV8, which does not effectively transduce DCs.

Other AAV capsid types have also been shown to have variable immunogenicity. AAVrh32.33, an evolutionarily divergent serotype isolated from rhesus macaques, yields reduced transgene expression and stronger anti-vector and anti-transgene $CD8^+$ T cell responses than AAV8 when injected into skeletal muscle (Mays et al., 2009). This enhanced response was shown to depend on $CD4^+$ helper T cells, plus CD40L and CD28, both of which are co-stimulatory molecules involved in immune activation. Interestingly, anti-CD40 antibody, which is usually able to restore immune responsiveness in $CD40L^{-/-}$ mice, was ineffective in this case.

Interactions between the viral capsid and complement have also been shown to play a role in the innate immune response to AAV (Zaiss et al., 2008). Immunoprecipitation studies showed that iC3b can bind to the AAV capsid, and that, *in vitro*, complement binding to AAV2 can increase capsid uptake and pro-inflammatory cytokine production by macrophages. This effect was also observed *in vivo*. Mice deficient in complement receptor 1/2 or C3 were less able to mount a humoral immune response to AAV than wild-type mice. However, AAV was not found to activate the alternative complement pathway. The capsid appears to bind factor H, which can then inhibit factor I-mediated degradation of C3b to iC3b, providing the virus some protection against complement.

The observation that scAAV vectors can slightly activate TLR2 transcription raises the question of whether this PRR may play a minor role in responses to AAV (Martino et al., 2011). While TLR2 is more commonly associated with sensing surface glycoproteins of enveloped viruses, leading to the production of type I IFNs in inflammatory monocytes, it has also been demonstrated to be involved in sensing adenoviral particles, which, like AAV, are non-enveloped (Appledorn et al., 2008; Barbalat et al., 2009; Quigley et al., 2009). Further, it has been reported that empty AAV capsids can induce innate immune responses (Hoesel et al., 2010). Though that mechanism is unclear, it is possible that the capsid-based inflammatory response could involve TLR2; further study will be required to elucidate this molecule's role in innate immune responses to AAV.

### EFFECTS OF TARGET TISSUE ON INNATE IMMUNE RESPONSIVENESS

As has been mentioned previously, the target tissue transduced by AAV can significantly impact the resulting immune response. Preclinical and clinical studies of gene therapy for hemophilia have focused on two target tissues: the liver and skeletal muscle (Matrai et al., 2010). Generally, the tolerogenic nature of the liver renders hepatic gene transfer more conducive to long-term transgene expression than gene transfer to skeletal muscle, as demonstrated by an 8-year study in hemophilic dogs and a detailed analysis in hemophilia B mice (Cao et al., 2009; Niemeyer et al., 2009). The divergent outcomes between muscle and liver gene transfer do not appear to be related to differences in innate responses due to TLR3 signaling (Cao and Herzog, 2008). Despite this, skeletal muscle gene transfer remains a useful tool for investigators. The more pronounced immune responses generated in muscle make it easier to elucidate the mechanisms of immune responsiveness to AAV. Muscular injection of AAV–CMV–OVA vectors demonstrated that a strong, transgene specific immune response could cause a loss of transgene expression (Wang et al., 2005). Other studies in muscle have demonstrated that the nature of the transgene can affect the development of an immune response. Membrane-bound β-gal delivered via AAV was found to be more immunogenic than cytoplasmic transgene, presumably because it was more accessible for uptake by DCs and presentation to T cells on MHC class I and II (Sarukhan et al., 2001). Hopefully, expanding studies of scAAV to muscle can further our understanding of the immune responses to these vectors and help determine organ-specific effects on innate immunity.

## THE ALTERNATIVE NF-κB PATHWAY AND AAV

So far, this article has focused on traditional innate responses to AAV initiated through the classical NF-κB pathway. These responses use NF-κB1 (p105), which is constitutively cleaved into p50 and complexes with RelA; this heterodimer can be disinhibited through ubiquitin-mediated degradation of IκB by IKKβ (Kawai and Akira, 2007). In contrast, the alternative pathway uses NF-κB2 (p100). NIK activates IKKα, which induces proteolytic cleavage of p100 into p52, allowing p52–RelB dimers to translocate to the nucleus and initiate transcription (Senftleben et al., 2001; Lawrence and Bebien, 2007). While the classical pathway is strongly involved in the production of pro-inflammatory cytokines during the innate immune response, the alternative pathway is considered important for adaptive immunity (Lawrence and Bebien, 2007).

However, a recent study has also described a role for the alternative pathway in AAV transduction (Jayandharan et al., 2011). The authors report that, in addition to classical NF-κB activation by 2 h (**Figure 3A**), infection with AAV is capable of inducing alternative pathway activation after 9 h, as demonstrated by increased levels of nuclear p52 both *in vitro* and *in vivo*. Expression of an EGFP transgene was also increased by 20–25 fold following alternative pathway activation. VP16, an activator of both pathways, increased fluorescence in HeLa cells, while Bay11, which can inhibit both the classical and alternative pathways, ablated transgene expression. PDTC (an inhibitor of the classical pathway), on the other hand, did not affect transgene expression. This result concurs with previous studies demonstrating that the AAV genome can be bound and transcriptionally inhibited by cellular proteins, one of which was later shown to be an NF-κB-repressing factor (Qing et al., 1997; Jayandharan et al., 2011).

Adeno-associated virus was also able to induce alternative pathway signaling *in vivo* (Jayandharan et al., 2011). Liver homogenates from 9 h after injection showed increased nuclear p52, suggesting NF-κB2 signaling; p52 elevation could be ablated if Bay11 was administered before the vector (**Figure 3D**). That alternative pathway signaling could be detected from whole liver suggests the transduced hepatocytes, rather than tissue-resident APCs, are responsible for this expression. Indeed, DCs exposed *in vitro* to AAV did not active the classical or alternative NF-κB pathways and were not effectively transduced to produce EGFP. Addition

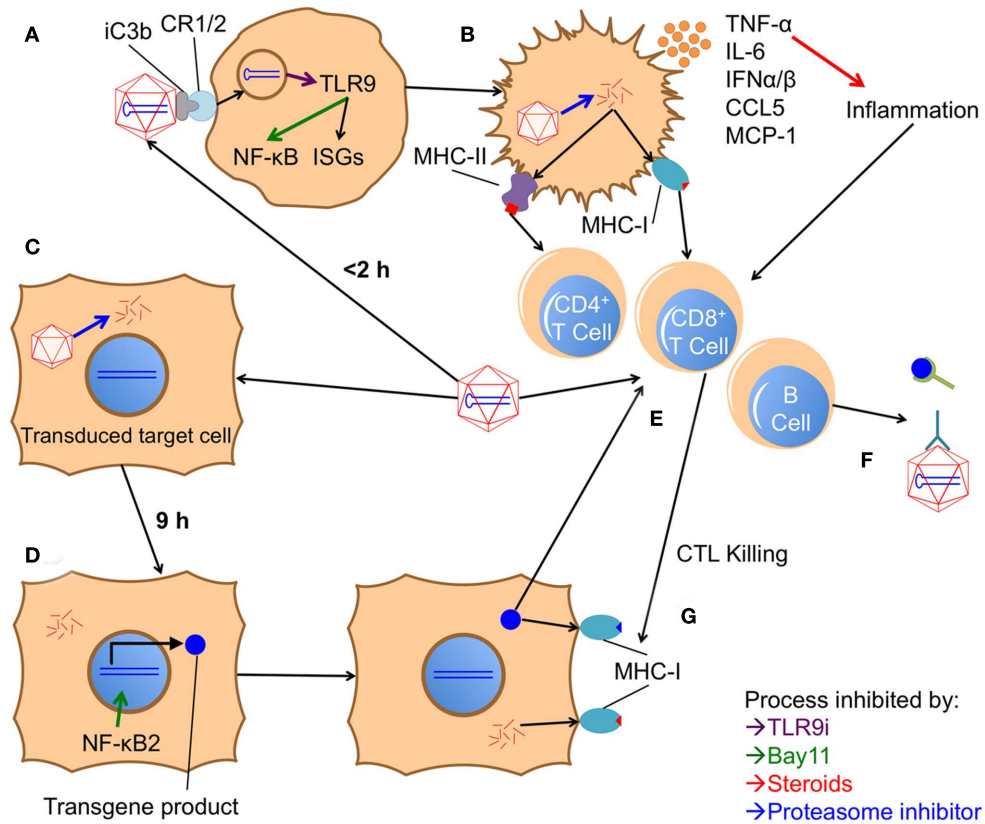


**FIGURE 3 | Model of proposed *in vivo* innate and adaptive immune responses to AAV vectors. (A)** Within 2 h after vector injection, AAV is phagocytosed by an APC; it is unclear whether initial contact is through a cDC, pDC, KC, MΦ, or other cell type. Phagocytosis may be aided by opsonization through iC3b and complement receptor 1/2 (CR1/2). Once in the endosome, the virus's genome is detected by TLR9, which subsequently activates the classical NF-κB pathway and interferon response genes (ISGs). TLR9i can inhibit both these responses, while Bay11 can block the NF-κB pathway. **(B)** Initiation of these pathways causes the APC to mature, up-regulating costimulatory molecules and producing pro-inflammatory cytokines like TNF-α, IL-6, CCL5, MCP-1, and type I IFNs. Steroids can inhibit the inflammatory activity of these cytokines. Mature APCs also present capsid fragments on MHC class I and II, unless protease inhibitors are utilized to hinder degradation of the capsid. **(C)** Concurrently, AAV productively transduces target cells. **(D)** Within 9 h, the alternative NF-κB pathway is triggered in these cells, enhancing expression of the transgene product. Bay11 can also blockade this NF-κB pathway. **(E)** Over the course of several days, the inflammatory signals from internalized AAV particles and the local environment induce maturation of APCs. Activation of the alternative NF-κB pathway in these APCs may enhance transgene expression and cross-presentation, augmenting their ability to prime an adaptive response (involving B cells, $CD4^+$ T cells, and $CD8^+$ T cells) to both the AAV capsid and the transgene product. **(F)** B cells subsequently produce antibodies against the capsid, inhibiting further transduction, and against the transgene product, inhibiting its efficacy. **(G)** Capsid or transgene specific $CD8^+$ T cells recognize peptide fragments of either protein in class I MHC on transduced cells. Recognition prompts the CTLs to attack and kill the target cell. Death of a significant number of transduced cells can lead to a reduction or complete elimination of transgene expression. Capsid-specific CTL killing can be reduced by inhibiting capsid degradation in target cells with proteasome inhibitors.

of VP16 or pro-inflammatory cytokines, which did activate both pathways, caused the DCs to become permissive to AAV transduction. Addition of cytokines and Bay11 resulted in reduced NF-κB activation and transduction relative to non-inhibited cells.

Thus, the alternative pathway seems to support AAV transduction in multiple cell types. While increased transduction efficiency is desired in hepatocytes, expression in APCs is more likely to lead to an enhanced immune response against the transgene. Activation of the alternative pathway can also enhance the ability of DCs to cross-present exogenous antigen to $CD8^+$ T cells, so even in the absence of APC transduction, NF-κB2 activation could induce a stronger immune response (Lind et al., 2008). The authors therefore investigated the effects of transient NF-κB suppression via Bay11 on long-term transgene expression (Jayandharan et al., 2011). Administration of Bay11 prior to gene transfer abrogated the rapid production of pro-inflammatory cytokines mediated by the classical pathway and reduced the anti-capsid antibody response. Additionally, 2 weeks post injection, both Bay11 treated and untreated mice showed comparable EGFP expression, suggesting that temporary inhibition of the alternative pathway does not interfere with sustained transgene expression. The mechanism by which AAV infection activates the alternative pathway is unclear and requires further study.

However, preliminary studies to test the mechanism of NF-κB-mediated increases in transgene expression from AAV vectors suggest that activation of the classical NF-κB p65 component in the cytosol may require the vector capsid, while the activation and function of alternative NF-κB p52 is subsequently amplified by its binding to AAV2 inverted terminal repeats (ITRs; Jayandharan et al., 2011; Jayandharan, unpublished results). Transfection in HeLa cells with plasmids containing increasing numbers of functional ITRs + D-sequence in the presence of the NF-κB activator VP-16 showed an increase in EGFP expression in an ITR + D-sequence-dependent fashion, whereas Bay11 ablated this effect. This suggested that putative NF-κB-responsive transcription factor binding sites exist in the AAV–ITRs. Further *in silico* analysis with a human transcription factor database demonstrated the presence of several binding sites for NF-κB binding co-factors, such as p300, TFIIB, and SplI (Jayandharan et al., 2011). One of these is the p300/CREB transcription factor that has been recently shown to be associated with the AAV genome (Dean et al., 2009).

## THERAPEUTIC POTENTIAL OF BLOCKING INNATE RESPONSES TO AAV

After examining what is known about the innate immune responses to AAV, it is prudent to discuss how these findings could be translated into improved therapies in the clinic. Given the brief nature of these responses, it seems that transient immunosuppression will down-regulate immune responses that are potentially deleterious to successful gene therapy. The recent reports reviewed here have, through preclinical studies, highlighted the potential for this sort of protocol to succeed (**Figure 3**). Inhibition of TLR9 signaling through an ODN antagonist and suppression of both NF-κB pathways were each able to reduce, but not completely eliminate, immune responses to the vector (Jayandharan et al., 2011; Martino et al., 2011). Bay11 alone was probably only partially successful because, as an NF-κB inhibitor, it does not block production of type I IFNs, which are critical mediators of responses to viruses (**Figures 3A,D**). Obstructing TLR9 activation (**Figure 3A**), on the other hand, seemed mostly successful, but an anti-capsid antibody response began to develop at 4 weeks post injection. Although either alone was not completely effective, perhaps a combination of these two therapies would more successfully abolish the immune response to AAV.

While primary adaptive immune responses rely heavily on activation by inflammatory signals such as those derived from TLR signaling, a recent report has indicated that secondary expansion of memory $CD8^+$ T cells occurs independently of MyD88 signaling (Rahman et al., 2011). Hence, therapeutics directed against specific innate pathways such as TLR–MyD88 may not be capable of preventing the loss of transduced cells due to capsid-specific memory $CD8^+$ T cells.

Other groups are also exploring different transient treatments to co-administer with vectors to increase the success of the therapy. Treatment with glucocorticoids prior to systemic delivery of adenoviral vectors could ablate immunotoxicity without negatively impacting transduction efficiency (**Figure 3B**; Seregin et al., 2009). This strategy may also be applicable to AAV vectors. Adenoviral research has also indicated that complement inhibition may reduce the immune response to viral vectors. Fusion of decay-activating factor (DAF), a complement inhibitor, to the capsid of adenovirus 5 reduced the antibody and $CD8^+$ T cell response to Ad-encoded HIV-Gag; antibody responses to the adenovirus capsid were also diminished by this strategy (Seregin et al., 2011). Thus, complement inhibition may be another strategy to increase the efficacy of AAV-mediated gene transfer.

Instead of artificially blocking the immune response with drugs, the other way to reduce inflammation would be to reduce vector load. To that end, as mentioned previously, tyrosine mutant AAV vectors have been developed that enhance transduction efficiency by resisting proteasomal degradation; this allows fewer vector particles to achieve the same expression levels as significantly higher titers of wild-type AAV (Li et al., 2010; Markusic et al., 2010). A similar effect could also potentially be achieved using proteasome inhibitors at the time of vector administration to enhance transduction efficiency (**Figures 3B,C**).

These two treatment modalities do not have to be mutually exclusive. It is possible that a combination of methods that enhance vector efficiency and suppress the immune response could lead to safer and more effective AAV gene therapy in humans.

## CONCLUSION

It is clear that, despite its low immunogenicity, AAV is detected and can cause innate immune responses. These responses can interfere with transgene expression, negatively impacting the outcome of gene therapy. Among the variety of mechanisms to respond to viruses, sensing of vector genomes by TLR9 appears to be the key mediator of these responses. Through MyD88, TLR9 induces the production of pro-inflammatory cytokines and chemokines (via NF-κB) and type I IFNs (**Figure 1**). These recruit additional pro-inflammatory cells, interfere with transduction, and lead to the development of an adaptive immune response (**Figures 3E–G**). Preventing signaling through TLR9 completely blocks the innate response and reduces adaptive

responses, suggesting that it is the most important innate response element to AAV. However, there is also evidence that the capsid can be involved in these responses, by dictating which cell types are transduced, by binding complement, and perhaps by some signaling through TLR2. Additionally, the alternative NF-κB pathway has a role in immune responses and transgene expression.

Though we have a much greater understanding of AAV immunology due to recent findings, a number of questions remain unanswered. For instance, it is unclear why the response to AAV disappears so rapidly compared to other viral vectors. Is the initial inflammatory signal too weak to continue to self-propagate, is the duration of active signaling following pathogen recognition reduced compared to other vectors, or could the vector trigger a pathway that down-regulates the response? Also, the mechanisms behind the enhanced responses observed to scAAV vectors need further investigation. Although there is evidence that self-complementary TLR9 agonists can signal more strongly than single-stranded agonists, other factors maybe involved (Struthers et al., 2010). The capsid of self-complementary vectors may be less stable, causing more DNA to be released in the endosome, or perhaps the self-complementary genome is not completely encapsulated (**Figure 2A**). The vector cassette does not affect the response, and it is therefore unlikely that specific sequences in the DNA are responsible.

Furthermore, the cell–cell interactions during *in vivo* responses to AAV are still uncertain. *In vitro* data indicate that pDCs are responsible for type I IFN production, yet *in vivo* results from hepatic gene transfer demonstrate that KCs are required (Zhu et al., 2009; Martino et al., 2011). These cell types, and perhaps others, likely cooperate within in the host to mount an immune response. Blocking innate responses noticeably reduces the adaptive response to capsid, yet it is uncertain whether this effect extends to the transgene product. Nonetheless, in TLR9$^{-/-}$ mice, AAV2–influenza hemagglutinin (HA) gene transfer to skeletal muscle exhibits diminished responses against HA (Zhu et al., 2009). On the other hand, in the liver, increasing signaling through TLR9 with scAAV vectors did not increase the adaptive response to hF.IX (Martino et al., 2011). Whether adaptive immune responses to the transgene product can be effectively prevented by blocking innate immunity can be addressed in future studies.

Clearly, a number of additional studies will be required in order to better understand the innate immunology of AAV. Hopefully, in answering these questions, we can define interventions that will allow us to improve AAV gene therapy and make it a practical treatment for a variety of genetic diseases.

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# Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity

***Melissa Bartel*[1,2,3]*, David Schaffer*[1,2,3]**†* *and Hildegard Büning*[4,5]**†***

[1] *Department of Chemical and Biomolecular Engineering, University of California at Berkeley, Berkeley, CA, USA*
[2] *Department of Bioengineering, University of California at Berkeley, Berkeley, CA, USA*
[3] *The Helen Wills Neuroscience Institute, University of California at Berkeley, Berkeley, CA, USA*
[4] *Department I of Internal Medicine, University Hospital Cologne, Cologne, Germany*
[5] *Center for Molecular Medicine Cologne (ZMMK), University of Cologne, Cologne, Germany*

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Joseph Zabner, University of Iowa, USA*
*Sergei Zolotukhin, University of Florida, USA*

****Correspondence:***
*David Schaffer, Departments of Chemical and Biomolecular Engineering, Bioengineering, The Helen Wills Neuroscience Institute, University of California at Berkeley, 176 Stanley Hall, Berkeley, CA 94720-3220, USA.*
*e-mail: schaffer@berkeley.edu;*
*Hildegard Büning, Department I of Internal Medicine, Center for Molecular Medicine Cologne (ZMMK), ZMMK Research Building, University of Cologne, Robert-Koch-Str. 21, 50931 Cologne, Germany.*
*e-mail: hildegard.buening@uk-koeln.de*

*†Joint senior authorship.*

Vectors based on adeno-associated viruses (AAV) have shown considerable promise in both preclinical models and increasingly in clinical trials. However, one formidable challenge is pre-existing immunity due to widespread exposure to numerous AAV variants and serotypes within the human population, which affect efficacy of clinical trials due to the accompanying high levels of anti-capsid neutralizing antibodies. Transient immunosuppression has promise in mitigating cellular and humoral responses induced by vector application in naïve hosts, but cannot overcome the problem that pre-existing neutralizing antibodies pose toward the goal of safe and efficient gene delivery. Shielding of AAV from antibodies, however, may be possible by covalent attachment of polymers to the viral capsid or by encapsulation of vectors inside biomaterials. In addition, there has been considerable progress in using rational mutagenesis, combinatorial libraries, and directed evolution approaches to engineer capsid variants that are not recognized by anti-AAV antibodies generally present in the human population. While additional progress must be made, such strategies, alone or in combination with immunosuppression to avoid *de novo* induction of antibodies, have strong potential to significantly enhance the clinical efficacy of AAV vectors.

**Keywords: adeno-associated virus, viral vector, immune response, neutralizing antibodies, bioconjugation, mutagenesis, directed evolution**

## INTRODUCTION

### AAV BIOLOGY

Adeno-associated virus (AAV) is a non-pathogenic parvovirus composed of a 4.7-kb single-stranded DNA genome packaged into a non-enveloped, icosahedral capsid (Knipe and Howley, 2006). The viral genome contains three open reading frames (ORF) between two inverted terminal repeats (ITR), which function as the origin of replication and as the packaging signal (Knipe and Howley, 2006). The *rep* ORF encodes four non-structural proteins that function in viral replication, transcriptional regulation, site-specific integration, and virion assembly (Knipe and Howley, 2006). The *cap* ORF encodes three structural proteins that assemble to form a 60-mer viral capsid (Knipe and Howley, 2006). An alternative ORF located in the same region of the genome produces the assembly activating protein (AAP; Sonntag et al., 2010), a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly reaction (Sonntag et al., 2010).

The capsid proteins determine the virus' ability to interact with and infect cells, from their initial binding to various cell surface receptors, to their trafficking inside the cell, to gaining access to the nucleus. Specifically, AAV is internalized rapidly via receptor-mediated endocytosis from clathrin-coated pits (Bartlett et al., 2000). Following cellular internalization, the virion escapes from early endosomes and traffics to the perinuclear area. Evidence exists for AAV uncoating prior to viral DNA entry into the nucleus (Lux et al., 2005), as well as viral trafficking into the nucleus prior to uncoating (Bartlett et al., 2000; Sonntag et al., 2006). In either case, once AAV localizes to the nucleus, second-strand synthesis – i.e., conversion of its single-stranded genome into double-stranded, transcriptionally active DNA – must occur for viral gene expression (Ferrari et al., 1996). In the absence of helper virus co-infection, AAV enters a latent life-cycle with viral genomes integrated in human chromosome 19 or non-integrated as extrachromosomal episomes.

Recombinant versions of AAV can be created in which a gene of interest is inserted between the ITRs, and the ORFs for structural and non-structural proteins are supplied separately (Flotte, 2004). This system allows the gene of interest to be packaged inside the viral capsid and delivered to the cell. Dividing as well as non-dividing cells are transduced, and gene expression – which occurs in the absence of helper virus function – is stable for years

in post-mitotic tissue. There are several naturally occurring variants and serotypes of AAV, each of which differs in amino acid sequence, in particular in the hypervariable regions of the capsid proteins, and thus in their gene delivery properties (Wu et al., 2006; Schaffer et al., 2008). Importantly, no AAV has been associated with any human disease, making it a desirable gene delivery vector for clinical applications (Knipe and Howley, 2006).

## CLINICAL TRIALS INVOLVING AAV

Adeno-associated virus has been employed with promising results in a number of clinical trials. During a Phase I dose-escalation trial for gene therapy of Leber's congenital amaurosis (LCA), for example, all 12 patients who received a subretinal injection of AAV2 encoding a protein required for the isomerohydrolase activity of retinal pigment epithelium showed sustained improvement in both subjective and objective measurements of vision (Bainbridge et al., 2008; Maguire et al., 2008, 2009). Furthermore, there were no significant adverse events during either the pre-trial efficacy studies or the Phase I trials (Bainbridge et al., 2008; Maguire et al., 2008, 2009). As another example, in a Phase I study for gene therapy of Canavan disease, 10 patients received intracranial infusions of AAV2 encoding human aspartoacylase (McPhee et al., 2006). Of importance with respect to vector re-application, 7 out of 10 patients in this trial had low or undetectable levels of neutralizing antibodies to AAV2 following administration of the gene therapy vector (McPhee et al., 2006).

In contrast to these clinical studies, which targeted immune privileged sites, anti-AAV host responses most likely mediated by cytotoxic T-cells limited the therapeutic efficacy of rAAV vectors following intrahepatic and intramuscular administration. Briefly, a Phase I/II dose-escalation study in which patients received a hepatic artery infusion of AAV2 encoding human Factor IX initially achieved therapeutic levels of Factor IX expression. However, the therapeutic levels were only present for 2 months (Manno et al., 2006), and follow-up experiments concluded that T-cell-mediated immunity to the AAV capsid antigens induced the destruction of AAV2-transduced hepatocytes (Manno et al., 2006). Furthermore, all subjects had a several log increase in neutralizing antibody titer following vector administration (Manno et al., 2006). Similarly, in a gene therapy study on lipoprotein lipase (LPL) deficiency, all eight patients achieved decreased median triglyceride levels and increased local LPL protein levels and activity 12 weeks post-administration of the AAV1 vector encoding $LPL^{S447X}$ (Stroes et al., 2008). But, also in this trial, triglyceride levels returned to baseline levels 18–31 months post-administration (Stroes et al., 2008).

## IMMUNE RESPONSES TOWARD AAV

Viruses are, in general, recognized by the innate immune system and induce the production of inflammatory cytokines, chemokines, and/or interferons that foster adaptive immune responses. The latter mainly comprises induction of antibodies produced by B lymphocytes following presentation of viral antigens by antigen-presenting cells, as well as direct cell killing mediated by cytotoxic T-cells (Knipe and Howley, 2006).

Knowledge on the mechanisms that lead to recognition and elimination of AAV in particular is still limited. Only recently, Zhu et al. (2009) provided evidence that AAV's genome is recognized by the innate immune system through the Toll-like receptor 9-MyD88 pathway. In this study, plasmacytoid dendritic cells (pDCs) were identified as sentinel cells, and activation of the Toll-like receptor 9-MyD88 pathway was shown to elicit humoral and cytotoxic T-lymphocyte immune responses in mice.

Humans become naturally infected by numerous AAV serotypes, and in particular AAV2, during childhood, as indicated by sero-conversion. As a result of this exposure, memory B and eventually memory T-cells are induced. The latter are assumed to be the cause for the lack of long-term gene expression and loss of therapeutic efficacy in the clinical trials on hemophilia and on LPL mentioned above. Currently, it is not clear how this response is launched, and consequently strategies for its avoidance are lacking. One hypothesis is that memory T-cells were re-activated upon vector administration through presentation of capsid fragments following intracellular degradation of incoming capsids. Alternatively, contamination of the gene therapy vector preparations with *cap* DNA impurities could result in persistent expression of capsid antigens and thus the destruction of transduced cells, though a detailed analysis was unable to find *cap* DNA sequences in the vector preparation used for the hemophilia trial (Hauck et al., 2008). Should this prove to be a problem, microRNA approaches or an oversized *cap* ORF could be employed to avoid *de novo* capsid production and consequently MHC loading following AAV vector application (Halbert et al., 2011; Lu et al., 2011). Employing these strategies may help to reduce the cytotoxic immune response to successfully transduced cells. Furthermore, immunosuppression is exploited as a strategy to avoid induction of T as well as of B cell responses (Jiang et al., 2006). An as of yet even greater challenge is the high prevalence of pre-existing neutralizing antibodies that significantly hamper the efficacy of *de novo* cell transduction.

Natural exposure to AAV serotypes 1, 2, 5, 6, 8, and 9 results mainly in production of IgG1, but low amounts of IgG2, IgG3, and IgG4 antibodies are also produced (Boutin et al., 2010). With respect to the total anti-AAV IgG prevalence for each serotype, a recent, comprehensive analysis revealed that AAV2 (72%) and AAV1 (67%) antibodies were the most common, but AAV9 (47%), AAV6 (46%), AAV5 (40%), and AAV8 (38%) antibodies were also present in a large portion of the population studied (Boutin et al., 2010). Furthermore, these anti-AAV antibodies may cross-react with other related AAV serotypes. Differences in antibody prevalence in the human population are likely due to human genetic variation and/or frequency of exposure to the various serotypes.

Animal studies on the mechanisms of anti-AAV antibody induction using class I-deficient (unable to mount a cellular immune response) and class II-deficient (unable to mount a humoral response) mice found that the latter could readily express high levels of transgenes upon a second administration of the vector (Manning et al., 1998). In addition, using several types of immunodeficient mice (RAG1 knock-out, CD40 ligand knock-out, and nude), Chirmule et al. (2000) showed that the AAV neutralizing antibody response is T-cell dependent. Viral capsid antigens are presented to B cells in the lymph nodes, resulting in CD4+ T-cell activation (Bessis et al., 2004). The B cells then

differentiate into plasma cells, which produce antibodies against the viral capsid proteins (Bessis et al., 2004). The route of administration has been shown to also have an impact on the induction of immune responses. Rhesus macaques receiving wild-type AAV via intramuscular or intravenous injection developed a humoral immune response to the AAV capsid, even without helper virus co-infection, while animals that received vectors via the intranasal route did not develop an immune response unless co-infected with a helper virus (Hernandez et al., 1999). Analogous results on the role of route of administration have been found in mice. Ge et al. (2001) found that intramuscular vector administration was more effective at inducing a humoral immune response than intraportal vein delivery. Nevertheless, independent of the initial administration route, re-administration of AAV vectors via the tail vein did not lead to transgene expression (Ge et al., 2001).

Recently, an AAV variant derived from rhesus macaques (AAVrh32.33) was found to elicit an immune response in mice similar to that against other AAV vectors seen in primate species (Mays and Wilson, 2009). Specifically, this AAV generates a strong CD8+ T-cell response to the AAV capsid and to the delivered transgene in mice, and its use may thus provide a more accurate murine model of AAV immune activation, and help to better explain the loss of transgene expression in human gene therapy clinical trials. In parallel to developing a deeper mechanistic understanding of anti-vector immune responses, for AAV to effectively function in clinical gene therapy, strategies must be developed to create vectors that evade the body's immune response.

## STRATEGIES TO AVOID NEUTRALIZATION BY HUMORAL ANTI-AAV IMMUNE RESPONSES

### TRANSIENT IMMUNOSUPPRESSION

In the naïve host, humoral immune responses are elicited upon AAV vector application. In order to circumvent induction of this response, transient immunosuppression has been exploited. This strategy has been tested in preclinical animal models, using antibodies or small molecule inhibitors against T-cell functionality. Manning et al. (1998) reported that successful vector re-administration was achieved in 60% of mice treated with anti-CD40 antibodies during the first vector administration. However, the mice developed neutralizing antibodies to AAV following the second administration of the vectors, which was conducted without immunosuppression (Manning et al., 1998). Another study by Halbert et al. (1998) attempted transient immunosuppression using MR1 (a monoclonal anti-CD40 ligand antibody) and CTLA4Ig (a CTLA4-immunoglobulin fusion protein) alone or in combination. Mice that received only MR1 or CTLA4Ig were capable of expressing the delivered transgene, but at a lower level than the combination treatment in which the animals also developed low to undetectable levels of neutralizing antibodies to AAV. Also in these animals, neutralizing antibodies were elicited upon re-administration (Halbert et al., 1998).

In response to the above mentioned clinical trial that reported destruction of AAV2-FIX transduced hepatocytes (Manno et al., 2006), Jiang et al. (2006) tested transient immunosuppression as a means to prevent AAV capsid-directed T-cell response against liver cells expressing the FIX transgene. Immunosuppression of rhesus macaques was achieved using a combination of mycophenolate mofetil and tacrolimus (FK506). Neither transaminitis nor expansion of memory T-cells was observed in any of the animals, and consequently only the impact of immunosuppression on humoral, but not on anti-capsid T-cell responses, could be investigated. Of the three animals receiving immunosuppression during vector administration, one developed a strong anti-AAV antibody response. Furthermore, withdrawal of the immunosuppression therapy after 6 weeks lead to a 2-log increase in neutralizing antibody titer, revealing that at least in rhesus macaques long time immunosuppression is required (Jiang et al., 2006).

In summary, immune suppression is an effective strategy to mitigate the body's immune response long enough to allow the capsid proteins to clear from the cell surface and prevent the formation of neutralizing antibodies in order to facilitate re-administration of the vector. However, transient immunosuppression is not a solution to pre-existing neutralizing antibodies to the AAV capsid. Modifications, either chemical modifications that protect surface exposed parts of the protein capsid or genetic modifications that result in changes to the protein capsid, must be made to the vector to evade these neutralizing antibodies.

### CHEMICAL MODIFICATION OF AAV CAPSID TO AVOID ANTIBODY NEUTRALIZATION

One approach to reduce antibody neutralization of vector particles is to graft chemical moieties onto the virion surface to shield neutralizing epitopes, as recently reviewed (Jang et al., 2011). For example, crosslinking synthetic polymers onto the vector can reduce neutralization by anti-virus antibodies as well as enable evasion of innate immune responses, thereby enhancing gene transfer in the presence of existing antibodies and facilitating repeated administration by reducing subsequent adaptive immune responses to the vector (Kreppel and Kochanek, 2008). Polymeric materials that have been explored in conjunction with adenoviral and adeno-associated viral vectors have included polyethylene glycol (PEG; O'Riordan et al., 1999; Croyle et al., 2000, 2005; Cheng et al., 2003; De Geest et al., 2005; Eto et al., 2005; Lee et al., 2005; Mok et al., 2005; Oh et al., 2006; Hofherr et al., 2007), poly-*N*-(2-hydroxypropyl) methacrylamide (poly-HPMA; Fisher et al., 2001; Green et al., 2008), polysaccharides (Espenlaub et al., 2008), and others (Kasman et al., 2009; Kim et al., 2010). They are typically covalently conjugated through the reaction of active groups on the polymer termini to nucleophilic amino acid side chains on the viral surface, such as lysines and cysteines.

Polyethylene glycol is a non-toxic material known for its capacity to resist protein binding – likely through steric hindrance and blocking of protein surface charges – and it has been extensively conjugated to proteins to extend their circulatory half-life and reduce immune responses (Delgado et al., 1992; Edwards et al., 2003; Haag and Kratz, 2006; Romberg et al., 2008). Likewise, PEGylation has been utilized to protect viral vectors from neutralizing antibodies (O'Riordan et al., 1999; Croyle et al., 2001; Cheng et al., 2003; De Geest et al., 2005; Eto et al., 2005; Lee et al., 2005; Mok et al., 2005; Hofherr et al., 2007), enable vector retargeting (Oh et al., 2006), and enhance vector stability (Croyle et al., 2000). Several studies have PEGylated AAV, using several different crosslinking chemistries. In one report, high molecular weight PEG conjugated to AAV using a terminal *N*-hydroxysuccinimidyl

ester to attach the polymer to viral surface lysines modestly protected AAV from neutralizing serum in culture (2.3-fold) at intermediate levels of PEG/virus. However, above a key stoichiometric ratio of PEG/AAV, viral infectivity was lost for both high and low molecular weight PEG, presumably due to a loss of key lysine residues and/or steric hindrance of viral surface regions critical for viral infectivity. This loss of infectivity was accompanied by alterations of AAV particles that could be visualized by electron microscopy (i.e., virion shape and size; Lee et al., 2005). Another study, which used different PEGylation chemistries, found more positive results. Specifically, AAV reacted with PEG using succinimidyl succinate chemistry (SSPEG) was partially protected from antibodies. In contrast, AAV coated with PEG via tresyl chloride reactive groups (TMPEG) was more effectively protected from neutralizing antibodies both *in vitro* and *in vivo* (Le et al., 2005). Over time, hydrolysis of the SSPEG linkages may progressively unmask antigen binding sites, rendering this conjugation less effective.

Other human viruses also face challenges with pre-existing immunity, and chemical conjugation has, for example, been more extensively explored to shield adenoviral vectors from serum, including both PEG (Croyle et al., 2005; De Geest et al., 2005; Mok et al., 2005; Kasman et al., 2009) and other polymers such as poly-HPMA (Fisher et al., 2001; Green et al., 2008). In one study, adenoviral vectors were coated with a random copolymer containing *N*-(2-hydroxypropyl)methacrylamide and methacryloyl-Gly-Gly-4-nitrophenoxy (pHPMA–ONp), via reaction with adenoviral surface amino groups. A binding assay involving ELISA measurement of free antibodies found that even at high excess, coated virus did not deplete anti-viral antibodies from solution, indicating the polymer shielded the adenovirus from the antibodies. Furthermore, since polymer coatings can block regions responsible for natural viral tropism, this modification afforded the further opportunity to retarget the virus via addition of fibroblast growth factor (FGF)-2 or vascular endothelial growth factor (VEGF) to the polymer. Adenoviral vectors coated with the polymer plus FGF-2 were 10-fold more resistant to antibody neutralization *in vitro* (Fisher et al., 2001). In addition, the vectors exhibited selective delivery to cell lines expressing receptors for FGF-2 or VEGF, depending on the ligand attached to the particle surface.

In addition to direct polymer grafting, an alternate strategy for protecting vectors from serum is to encapsulate them inside polymeric gels or particles designed to progressively degrade and release the virus (Jang et al., 2011). In early work, Beer et al. (1998) encapsulated adenovirus in poly-lactic glycolic acid using a double emulsion technique. They found that the encapsulated virus retained 27–50 and 62–65% infectivity in culture at 1:100 and 1:500 dilutions of rat serum, respectively, while non-modified vectors retained less than 1% of infectivity at both dilutions. In addition, they assessed the development of neutralizing antibodies in naïve mice upon administration of protected vector and found that animals receiving encapsulated adenovirus did not develop anti-adenovirus antibodies until after the third dose, and the resulting titers were 45-fold lower than mice receiving unmodified adenovirus (Beer et al., 1998). In another report, adenovirus was encapsulated in microspheres generated from alginate, a linear copolymer of anionic saccharides isolated from brown algae. Upon intranasal or intraperitoneal administration of virus-loaded microspheres, high level LacZ expression was observed in numerous organs in mice (including spleen, liver, lung, kidney, and lymph node) and was not significantly inhibited by the presence of neutralizing anti-adenovirus antibodies. By contrast, administration of un-encapsulated control vector was significantly reduced in animals harboring neutralizing antibodies (Sailaja et al., 2002).

In summary, direct chemical conjugation of protective polymers to AAV shows promise, though with somewhat mixed results, and future work may investigate the application of alternate polymers and controlled release strategies that have shown promise with adenoviral and other vectors.

## GENETIC MODIFICATION OF AAV CAPSID PROTEINS TO AVOID ANTIBODY NEUTRALIZATION

As suggested by the term "serotype," AAV variants with unique serological characteristics have evolved in nature (Gao et al., 2004). Variations in the amino acid composition of the capsid proteins, in particular at protruding sites, can lead to low serum cross-reactivity between serotypes (Lochrie et al., 2006). For example, mice immunized with AAV2 show only a slight reduction in transduction efficacy if subsequently injected with AAV1 (which differs from AAV2 at 16.3% of the capsid amino acids; Gao et al., 2004), while anti-AAV2 antibodies impair re-application of AAV2 (Xiao et al., 1999). In principle, such findings raise the idea that one could harness natural AAV diversity to overcome problems of pre-existing immunity. That is, identification of one or more serotypes to which specific patients have not previously been exposed could allow recombinant vector administration or even re-administration. This idea suffers from several problems, however. First, such personalized therapy poses practical and regulatory concerns. In addition, capsid variability among serotypes affects not only antigenicity but also tropism, so that different serotypes could not be readily alternated. Furthermore, the patterns of pre-existing AAV immunity within the human population are complex (Boutin et al., 2010). As already mentioned, sera are frequently found to neutralize more than one serotype, and for example in >93% of sera positive for AAV2, neutralizing antibodies toward AAV1 are additionally present. Interestingly, antibodies against these two serotypes were also detectable in sera positive for AAV5, 6, 8, or 9 (Boutin et al., 2010). However, the observation that different serotypes exhibit different extents of cross-reactivity raises the idea that a given capsid can be engineered or mutated to retain the natural tropism of, yet evade pre-existing antibodies directed against, its parental serotype. Both rational design and directed evolution approaches have been pursued toward this goal.

### *Peptide scanning for immunogenic epitopes*

The rational design of AAV variants that evade pre-existing humoral immunity necessitates basic knowledge of immunogenic epitopes. In an early study, Moskalenko et al. (2000) utilized peptide scanning to map neutralizing epitopes for antibodies present in human serum samples and found a total of six linear epitopes that are targets of neutralizing antibodies (**Table 1**). Wobus et al. (2000) mapped additional linear and conformational immunogenic epitopes neutralized by mouse monoclonal antibodies. Linear epitopes in the VP1 unique region, in VP1/VP2, and at the

**Table 1 | Immunogenic sites of the AAV2 capsid mapped by peptide scan.**

| Amino acid position | Localization | Sera | Reference |
|---|---|---|---|
| $E_{17}$GIRQWWKLKPG | VP1 | Polyclonal human | Moskalenko et al. (2000) |
| $K_{123}$RVLEPLGL | VP1 | A1 | Wobus et al. (2000) |
| $N_{113}$LGRAVFQAKKR | VP1 | Polyclonal human | Moskalenko et al. (2000) |
| $L_{171}$NFGQTGDADSV | VP1/VP2 | A69 | Wobus et al. (2000) |
| $K_{321}$EVT## | VP3 region | Polyclonal human | Moskalenko et al. (2000) |
| $T_{337}$STV | VP3 region | Polyclonal human | Moskalenko et al. (2000) |
| $V_{369}$FMVPQYGYL (main contribution), $H_{381}$YFGYSTPWG (minor contribution), $R_{566}$TTNPVAT$_{573}$EQ (minor contribution) | VP3 region | A20 | Wobus et al. (2000) |
| $Q_{473}$SRNWLPGPCYR | VP3 region | Polyclonal human | Moskalenko et al. (2000) |
| $S_{474}$RNWLPGPCY# | VP3 region | D3 | Wobus et al. (2000) |
| $S_{493}$ADNNNSEYSWT (main contribution), $L_{601}$PGMVWQDRD (minor contribution) | VP3 region | C37-B | Wobus et al. (2000) |
| $I_{726}$GTRYLTR | VP3 region, C′-terminus | B1 | Wobus et al. (2000) |

*#Additional sequences likely contribute to the immunogenic epitope (Wobus et al., 2000).*

*##Described as part of a conformational epitope (Moskalenko et al., 2000).*

C′-terminus of the VP3 region are recognized by antibodies A1, A69, and B1, respectively (**Table 1**), while conformational epitopes were bound by C24-B, C37-B, D3, and A20 (**Table 2**). In order to map the latter, Wobus and colleagues used peptide insertion mutants of AAV2 (Girod et al., 1999) that displayed an integrin binding ligand, L14, at defined and surface exposed positions of the capsid (Wobus et al., 2000).

The same mutants were subsequently screened for their immune escape phenotype by Huttner et al. (2003) using a panel of human polyclonal sera. Forty-two percentage of the sera showed a significant reduction (∼31%) in antibody binding affinity when the capsid position 534 or 573 were subjected to peptide insertion, while 21% of the sera additionally showed a reduced binding affinity for the other six mutants (**Table 2**). These findings for polyclonal human sera agreed with the results of Wobus et al. (2000) obtained with mouse monoclonal antibodies and identified positions 534 and 573 as major antigenic determinants in humans. Peptide insertion not only impacts antibody binding affinity, but also the transduction ability. Further investigations by Huttner et al. (2003) revealed that the peptide insertion mutant I-587, which displayed the L14 peptide at position 587 as ligand for targeting B16F10 cells, transduced this cell line despite the presence of neutralizing antibodies. Exchanging the peptide for a 7-mer peptide targeting Mec1 cells (Perabo et al., 2003) yielded similar results, demonstrating that insertion of peptides at 587 can modulate both cell tropism and antibody neutralization (Huttner et al., 2003).

### *Rational design of AAV variants via peptide insertion or site-directed mutagenesis*

Lochrie et al. (2006) utilized extensive site-directed mutagenesis of the AAV2 capsid to develop variants with immune escaping properties. *In silico* structural analysis of potential docking for a murine IgG2a antibody with the AAV2 surface yielded a number of sterically accessible, candidate positions that were then subjected to point mutagenesis. The resulting collection of mutants were assessed for antibody binding using A20 (mouse anti-AAV2 capsid antibody), human sera (three different donors), and IVIG (pooled human IgG isolated from thousands of blood donors). As may be anticipated for polyclonal antibody mixtures, in contrast to work with A20 that identified a set of variants with mutations at a specific epitope, analysis with human sera (single donors) and IVIG did not map a single epitope. At any rate, point mutations at distinct sites spread across the capsid reduced immune neutralization.

### *Directed evolution of AAV variants via random mutagenesis*

In contrast to rational design based approaches, directed evolution strategies can be exploited in the absence of knowledge on the capsid biology or on immunogenic sites. This strategy is based on the generation and high-throughput selection of diverse genetic libraries to create variants with enhanced biological functions (Neylon, 2004). Maheshri et al. (2006) and Perabo et al. (2006) applied this technology to AAV to overcome the obstacle of pre-existing immunity. Both approaches used error-prone mutagenesis to randomize the AAV2 capsid. Random point mutations to the AAV *cap* ORF are introduced using a "sloppy" polymerase chain reaction (PCR) to amplify and mutate the *cap* DNA sequence at a defined rate. The PCR conditions can be adjusted to tune the average number of mutations in each *cap* gene sequence (**Figure 1A**; Pritchard et al., 2005). Viral capsid mutants are then packaged by standard production protocols and screened for infectivity despite the presence of neutralizing antibodies. Progeny production is induced by helper virus co-infection and followed by a new round of selection.

Perabo et al. (2006) selected in presence of human sera on a highly permissive cell line, HeLa. In contrast to selections in the presence of non-neutralizing sera, selections in the presence of neutralizing antibodies yielded viral mutants in which 73% of point mutations clustered in the same, surface exposed region. The most frequent selected clones carried mutations at capsid positions 459 and 551. Introduction of selected amino acid substitution at position 459 and/or 551 of capsids of recombinant AAV vectors conferred the vectors with an improved ability to evade

**Table 2 | Immunogenic sites at the AAV2 capsid[#].**

| Amino acid position | Localization | Sera | Detection method | Reference |
|---|---|---|---|---|
| E12 | VP1 unique region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| S42 | VP1 unique region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| A117 | VP1 unique region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| A152 | VP1/VP2 | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| D180 | VP1/VP2 | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| K258 | VP3 region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| S261-S262 | VP3 region | A20, C37-B, D3 (*), polyclonal human | Peptide insertion | Wobus et al. (2000), Huttner et al. (2003) |
| Q263 | VP3 region, edge of plateau | A20 | Rational design | Lochrie et al. (2006) |
| S264 | VP3 region, edge of plateau | A20, IVIG | Rational design | Lochrie et al. (2006) |
| G265 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| D269 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| N381-N382 | VP3 region | A20, D3 (*), polyclonal human | Peptide insertion | Wobus et al. (2000), Huttner et al. (2003) |
| S384 | VP3 region, edge of plateau | A20 | Rational design | Lochrie et al. (2006) |
| Q385 | VP3 region, edge of plateau | A20 | Rational design | Lochrie et al. (2006) |
| V418 | VP3 region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| R447-T448 | VP3 region | C37-B, polyclonal human | Peptide insertion | Huttner et al. (2003) |
| R459 | VP3 region, threefold symmetry axis | polyclonal human | *In vitro* evolution | Perabo et al. (2006) |
| R471 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| T491 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| A493 | VP3 region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| N497 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| S498 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| W502 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| K527 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| E531 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| K532 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| F534-F535 | VP3 region | A20, C24-B, C37-B, D3 (*), polycloncal human | Peptide insertion | Wobus et al. (2000), Huttner et al. (2003) |
| K544 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| E548 | VP3 region, spike region | A20, polyclonal human | Rational design | Lochrie et al. (2006) |
| T550 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| N551 | VP3 region, threefold symmetry axis | Polyclonal human | *In vitro* evolution | Perabo et al. (2006) |
| T567 | VP3 region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| T573-E574 | VP3 region | A20, C24-B, C37-B, D3 (*), polyclonal human | Peptide insertion | Wobus et al. (2000), Huttner et al. (2003) |
| E574 | VP3 region | IVIG | Rational design | Lochrie et al. (2006) |
| G586 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| N587 | VP3 region, threefold symmetry axis, heparin binding motif | Polycloncal rabbit, polyclonal human | *In vitro* insertion, rational design | Maheshri et al. (2006), Lochrie et al. (2006) |
| N587-R588 | VP3 region | C24-B, C37-B, polyclonal human | Peptide insertion | Wobus et al. (2000), Huttner et al. (2003) |
| N705 | VP3 region | Polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |
| K706 | VP3 region | Polyclonal human | Rational design | Lochrie et al. (2006) |
| V708 | VP3 region, edge of plateau | A20, polyclonal human, IVIG | Rational design | Lochrie et al. (2006) |

*(Continued)*

**Table 2 | Continued**

| Amino acid position | Localization | Sera | Detection method | Reference |
|---|---|---|---|---|
| T713 | VP3 region | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |
| T716 | VP3 region, surface of the twofold dimple | Polyclonal rabbit | *In vitro* evolution | Maheshri et al. (2006) |

*[#]Viral vectors, i.e., intact capsid assayed.*

*(*)Reduced binding affinity maybe due to a conformational change in the epitope caused by the peptide insertion (Wobus et al., 2000).*

*A20 = mouse monoclonal antibody (Wistuba et al., 1995), conformational epitope.*

*C37-B = mouse monoclonal antibody (Wobus et al., 2000), conformational epitope.*

*Human sera = serum obtained from single donors.*

*IVIG = purified human IgG prepared from thousands of blood donors.*

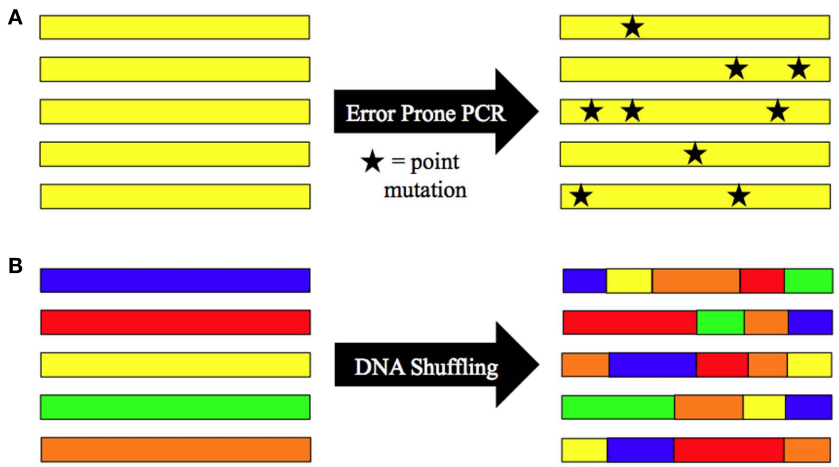


**FIGURE 1 | Schematic of library AAV capsid protein engineering strategies for evasion of antibody neutralization.** Methods for generation of highly diverse viral libraries include **(A)** random point mutagenesis (error-prone PCR) and **(B)** *in vitro* recombination (DNA shuffling). Directed evolution strategies use these approaches as part of an iterative strategy to increase AAV's ability to avoid antibody neutralization.

neutralization. The highest immune evasion was observed for the sera used to select the mutants, but with each of the seven different sera assayed, a notable higher serum concentration was required to halve the transduction efficacy of the mutants compared to AAV2 (up to 5.3-fold).

The viral library used by Perabo et al. (2006) possess on average 0.9 mutations per clone making it unlikely that multiple combinations of mutations are found on a single clone. The latter, however, increases the likelihood of selecting potent immune escaping variants. An efficient strategy to achieve this goal was exploited by Maheshri et al. (2006), who combined error-prone PCR mutagenesis and DNA shuffling, in their approach to establish directed evolution strategies for AAV. Random DNA recombination (**Figure 1B**) can be achieved using several different methods, including the staggered extension process (StEP) recombination (Zhao et al., 1998; used by Maheshri et al., 2006) and DNase I digestion followed by fragment reassembly with DNA polymerase (Stemmer, 1994; used by Grimm et al., 2008; Koerber et al., 2008). The StEP consists of repeated cycles of denaturation, annealing, and short polymerase-catalyzed extension steps. The abbreviated extension step results in short fragments of DNA that can then anneal to new templates and further extend, creating genes that contain sequence information from multiple templates. DNA shuffling uses DNase I digestion to create small fragments of DNA similar to the fragments created in StEP. These fragments are then reassembled into new genes through repeated annealing cycles in the presence of DNA polymerase. Both methods can be used to create point mutations (when all *cap* gene templates are from the same serotype) or to generate chimeric capsids (when *cap* gene templates are from different serotypes).

The viral library produced by Maheshri et al. (2006) was selected for mutants that productively infect HEK293 in the presence of a strongly neutralizing rabbit anti-AAV2 serum. Interestingly, each mutant selected in this screen contained a threonine to alanine substitution at position 716, near the C-terminus of the common VP3 region. In order to further optimize the selection procedure, the viral library was subjected to a further round of mutagenesis. While transduction of AAV2 in the presence of rabbit sera was neutralized at a 1:1,500 dilution, the most promising candidate of the second screen was only mildly neutralized at a

1:2.35 serum dilution. More importantly, this mutant mediated transgene expression *in vivo* following pre-incubation with anti-AAV serum at levels 2–3 orders of magnitude higher than serum concentrations that neutralized AAV2 (Maheshri et al., 2006).

To date, AAV libraries based on error-prone PCR mutagenesis possess a diversity of $10^7$–$10^8$ clones (Maersch et al., 2009). However, in order to screen every possible amino acid substitution for their immune escape properties, a diversity of $3.2 \times 10^{17}$ would be required (Maersch et al., 2009). A possibility to overcome this technical limitation and identify the most effective amino acid substitution for a given position, and hence improve the antibody evasion capacity of immune escape mutants was proposed by Maersch et al. (2009). In this procedure, only regions implicated as immunogenic sites are randomized, and these mutants are subjected to high-throughput selections for viral infectivity in the presence of neutralizing sera. In their proof-of-principle study, Märsch and colleagues focused on five immunogenic sites: positions 449, 558, 459, 551, and 493 (Maheshri et al., 2006; Perabo et al., 2006; Maersch et al., 2009), which were subjected to saturation mutagenesis. Due to this restriction, the library purportedly contained all possible amino acid substitutions for these positions (required diversity $20^5$, obtained diversity $6 \times 10^6$). From the mutants selected in the presence of human sera on HEK293 cells, six mutants were analyzed in comparison to AAV1, AAV2, and AAV2 R459K/N551D, a double mutant selected in the previous study of Perabo et al. (2006). Two of these mutants were significantly less neutralized by human sera from single donors and by IVIG compared to AAV2 and AAV2 R459K/N551D. In agreement with the initial hypothesis that the level of immune evasion can be improved if an optimal amino acid substitution is introduced to disrupt an immunogenic epitope, both mutants contained novel substitutions at positions 459, 493, and 551. In addition, the relatively minor sequence changes – compared for example to the 120 amino acid differences between AAV2 and AAV1 – likely did not change the mutant's tropism relative to AAV2.

#### *Directed evolution of AAV variants via serotype shuffling*

Grimm et al. (2008) implemented an *in vitro* evolution strategy that utilized a library of chimeric capsids. DNA shuffling through fragment reassembly was exploited to randomly combine *cap* sequences of eight AAV serotypes [AAV2, 4, 5, 8, and 9, and caprine (CAAV), avain (AAAV), and bovine AAV (BAAV)]. The library was firstly selected for the ability to transduce human hepatoma cell lines, followed by further selection in the presence of IVIG. The result was a single mutant, AAV-DJ, a chimera of AAV2, 8, and 9 in which the majority of non-AAV2 amino acid sequences were found in immunogenic epitopes of the capsid. For in depth analysis of the immune profile of AAV-DJ, Grimm et al. (2008) passively immunized mice with IVIG followed by vector injection. At lower IVIG levels, AAV-DJ possessed an *in vivo* immune evasion capacity comparable to AAV8 and AAV9 as indicated by the comparable level of transgene expression, though both serotypes clearly outperformed AAV-DJ at a higher IVIG concentration. Interestingly, although re-administration of AAV-DJ was impaired, and although mice pre-immunized with AAV8 or 9 could not be transduced by AAV-DJ, AAV2, 8, and 9 were able to mediate transgene expression in AAV-DJ pre-immunized mice.

In another study involving DNA shuffling of AAV, Koerber et al. (2008) performed a detailed analysis of seven chimeric AAV vectors, which had been selected in the absence of antibodies in a single selection round for viability. The library used for their selection contained DNA shuffling based virus chimeras of AAV1, 2, 4, 5, 6, 8, and 9, i.e., they included additional non-human primate serotypes but not more distantly related serotypes. Sequencing of candidate chimeras revealed a greater than 90% similarity to AAV2 for three of the mutants, and to AAV1 and 6 for the other four mutants. In addition, all clones contained a high proportion of sequences originating from non-AAV2 and non-AAV1/6 serotypes, respectively, in the surface exposed regions of the capsid. Of note, four of these seven mutants showed, in the absence of a selection pressure, a naturally greater resistance to neutralization by IVIG than the parental serotypes. The highest immune evasion capability was detected for a mutant with a greater than 90% similarity to AAV1/6. This mutant, cB4, was 400-fold more resistant to neutralization by IVIG than AAV2 and is as of yet one of the most potent immune escaping AAV vectors. This work by Koerber et al. (2008) and Grimm et al. (2008) clearly reveal the great potential of chimeric virions. The immune evasion phenotype is, however, due to a new combination of *cap* sequences of different serotypes and thus requires the selection of chimeric mutants which in addition to immune evasion possess a desired tropism in order to fully exploit this strategy to improve AAV's *in vivo* application.

## CONCLUSION

Adeno-associated virus possesses several characteristics that have contributed to its growing popularity as a gene delivery vector for clinical gene therapy applications. Unfortunately, pre-existing neutralizing antibodies against the AAV capsid and humoral immune responses following vector administration have prevented AAV from reaching its full potential as a gene therapy vector. Chemical modifications that protect exposed immunogenic epitopes of the capsid and genetic modifications that mutate these epitopes have demonstrated that AAV vectors can be altered to decrease antibody neutralization, while still effectively delivering genetic material, both *in vitro* and *in vivo*. The modification methods described above can be used to isolate new AAV variants with more improved immune evasion characteristics and thus possibly stronger clinical potential.

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# Immunity and AAV-mediated gene therapy for muscular dystrophies in large animal models and human trials

***Zejing Wang[1,2]*, Stephen J. Tapscott[2,3,4], Jeffrey S. Chamberlain[3,4,5] and Rainer Storb[1,2]***

[1] *Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA, USA*
[2] *Department of Medicine, University of Washington, Seattle, WA, USA*
[3] *Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA, USA*
[4] *Department of Neurology, University of Washington, Seattle, WA, USA*
[5] *Department of Biochemistry, University of Washington, Seattle, WA, USA*

***Edited by:***
*Hildegund C. J. Ertl, Wistar Institute, USA*

***Reviewed by:***
*Roland W. Herzog, University of Florida, USA*
*Valder Arruda, University of Pennsylvania and The Children's Hospital of Philadelphia, USA*

****Correspondence:***
*Zejing Wang, Transplantation Biology Program, Division of Clinical Research, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, D1-100, Seattle, WA 98109-1024, USA.*
*e-mail: zwang@fhcrc.org*

Adeno-associated viral (AAV) vector-mediated gene replacement for the treatment of muscular dystrophy represents a promising therapeutic strategy in modern medicine. One major obstacle in using AAV vectors for *in vivo* gene delivery is the development of host immune responses to the viral capsid protein and transgene products as evidenced in animal models and human trials for a range of genetic diseases. Here, we review immunity against AAV vector and transgene in the context of gene delivery specific to muscles for treating muscular dystrophies and non-muscle diseases in large animal models and human trials, factors that influence the intensity of the immune responses, and immune modulatory strategies to prevent unwanted immune responses and induce tolerance to the vector and therapeutic gene for a successful gene therapy.

**Keywords: AAV, immunity, muscular dystrophy, immune modulation, review**

## INTRODUCTION

Muscular dystrophies are a group of heterogeneous diseases that primarily affect striated muscles throughout the body. Many of these myopathies are caused by mutations in genes that encode structural proteins which link the cytoskeleton of muscle fibers to the extracellular matrix. The absence of functional proteins results in destabilization of the muscle membrane, increased muscle fragility and degeneration, and progressive muscle wasting, all of which compromise the patients' mobility and, in the severe disease forms (Emery, 2002) such as Duchenne muscular dystrophy (DMD), lead to death.

Over the past decades, viral vector-mediated gene replacement has evolved as an attractive treatment strategy. Studies focusing on adenoviral (Ad), retroviral, and adeno-associated viral (AAV) vectors have shown that delivery via these vectors of full-length or truncated but functional genes improves the disease phenotype in animal models (Gregorevic and Chamberlain, 2003; Dudley et al., 2004; Gregorevic et al., 2004; Li et al., 2005; Liu et al., 2005), which has led to initiation of early phase clinical trials (Mendell et al., 2009, 2010a). However, a major obstacle identified from preclinical and clinical studies is the host immune response to the viral capsid protein and the therapeutic transgene product, which may limit the efficacy or efficiency of this approach.

This review will focus on recent reports of immunity to AAV capsid proteins and transgene products in large animal models and human trials of muscular dystrophy (summarized in the **Table 1**), as well as strategies that are needed for a successful *in vivo* gene transfer to muscle.

## IMMUNITY TO AAV AND TRANSGENE WHEN DELIVERED TO MUSCLE

### INTRAMUSCULAR INJECTION OF AAV

Administration of AAV vectors to skeletal muscle via direct intramuscular injection has been the most common route utilized for gene delivery while developing treatments for the muscular dystrophies. While fairly promising safety profiles were reported in mouse models, cytotoxic T cell responses to AAV vector and transgene product in muscle of large animal models have been reported recently. In immunocompetent dogs, we reported that local intramuscular injection of AAV2 or AAV6 vectors in wild type or DMD dogs induced robust T cell-mediated immune responses against AAV capsid proteins that peaked 4 weeks after vector injection and eliminated much of the transgene expression by week 10 (Wang et al., 2007a). The immune responses were triggered regardless of the nature of the transgene delivered or the vector purification methods. Yuasa et al. (2007) reported that injection of AAV2 evoked strong inflammatory responses to transgene products in dog muscles. Robust cellular infiltration was also reported by Yue et al. (2008) when AAV9 vectors were injected into muscles of normal adult dogs, but not in muscles of neonatal dogs.

The binding ability of AAV capsids to heparan sulfate proteoglycans (HSPG) was suggested to facilitate the uptake of certain AAV serotypes, such as AAV2, by dendritic cells (DC), which leads to antigen processing, MHC class I antigen presentation, and a consequent cell-mediated cytotoxic T cell responses (Vandenberghe et al., 2006). However, this notion has not been confirmed in large animal or humans. Studies by Takeda's group (Ohshima et al., 2009) demonstrated that cellular immune responses were

**Table 1 | Summary of studies using AAV gene transfer to muscle.**

| Disease model | Species in study | Transgene product | Serotype (route of injection) | Drugs (regimen) | Immune responses | Transgene expression |
|---|---|---|---|---|---|---|
| DMD (Wang et al., 2007a, 2010) | WT dogs | cFIX, β-gal | AAV1, 2, or AAV6 (IM) | None | + | <8 weeks |
| DMD (Wang et al., 2007b) | WT dogs | cFIX | AAV6 | CSP/MMF (8 weeks) | − | Sustained |
| | DMD | hu-μ-dys | AAV6 | CSP/MMF (8 weeks) | + | <12 weeks |
| | | k9-μ-dys | AAV6 | ATG/CSP/MMF (16 weeks) | Limited | >22 weeks |
| DMD (Yuasa et al., 2007) | WT dogs | β-gal | AAV2 (IM) | None | + | <2 weeks |
| | | β-gal | AAV2 (IM) | CSP (2 weeks) | + | <2 weeks |
| | | β-gal | AAV2 (IM) | CSP/MMF (2 weeks) | Reduced + | >4 weeks |
| DMD (Yue et al., 2008) | WT dogs (adult) | hu-PLAP | AAV9 (IM) | None | + | Limited at 8 weeks |
| | WT dogs (neonatal) | hu-PLAP | AAV9 (IM, i.v.) | | Limited | >24 weeks |
| DMD (Ohshima et al., 2009) | WT dogs | β-gal | AAV2 or AAV8 (IM) | None | + | <4 weeks |
| | WT dogs | β-gal | AAV8 (i.v.) | | Reduced + | <8 weeks |
| LGMD (Mendell et al., 2009) | Human trial | α-sarcoglycan | AAV1 (IM) | Prednisolone (days 0–2) | Limited | 6 weeks – 3 mos |
| LGMD (Mendell et al., 2010b) | Human trial | α-sarcoglycan | AAV1 (IM) | Prednisolone before injection | + in 1 out of 3 pts | 6 mos for 2 out of 3 pts |
| LGMD (Herson et al., 2011) | Human trial | α-sarcoglycan | AAV1 (IM) | None | Moderate + | Limited expression at week 4 |
| DMD (Mendell et al., 2010a) | Human | mini-dys | IM, but not specified on serotype | Glucocorticoid prior to and throughout the study course; prednisolone before injection | + in 4 out of 6 pts | Not detectable |
| DMD (Gregorevic et al., 2009) | WT dogs | hu-PLAP | AAV6 (i.v.) | CSP/MMF (6 weeks) | + | Limited at week 6 |
| | | hu-PLAP | AAV6 (i.v.) | | Limited | Much enhanced at week 6 |
| DMD (Kornegay et al., 2010) | DMD(neonatal) | hu-mini-dys | AAV9 (i.v.) | None | + | 16 weeks for 2 out of 4 dogs |
| DMD (Toromanoff et al., 2008) | NHP | rtTA, cmEpo | AAV1 (IM, i.v.) | AAV-LEA29Y (Belatacept, i.v.) | − | Sustaining |
| | | rtTA, cmEpo | AAV1 (IM, i.v.) | AAV-LEA29Y (Belatacept, IM) | + | |
| DMD (Rodino-Klapac et al., 2010) | NHP | hu-u-dys | AAV8 (IM, i.v.) | None | − | 5 mos |
| HB (Herzog et al., 1999) | Dogs | cFIX | AAV2 (IM) | None | + | >17 mos |
| HB (Herzog et al., 2001) | Dogs | cFIX | AAV2 (IM, i.v.) | Cyclophosphamide (6 weeks) | ± | Sustained |
| HB (Haurigot et al., 2010) | Dogs | cFIX | AAV2 or AAV6 (i.v.) | Cyclophosphamide (6 weeks) | ± | 6 mos |
| HB (Wang et al., 2005) | Dogs | cFIX | AAV8 (i.v.) | None | + | 1−2 years |

*(Continued)*

**Table 1 | Continued**

| Disease model | Species in study | Transgene product | Serotype (route of injection) | Drugs (regimen) | Immune responses | Transgene expression |
|---|---|---|---|---|---|---|
| HB (Manno et al., 2003) | Human trials | hu-cFIX | AAV2 (IM) | None | − | 10 mos |
| LPL (Mingozzi et al., 2009) | Human | LPL | AAV1 (IM) | None | + | 6 mos |
| AAT (Brantly et al., 2009) | Human | AAT | AAV1 (IM) | None | + | ∼12 mos |
| AAT (Flotte et al., 2011) | Human | AAT | AAV1 (IM) | None | + | >3 mos |

*ATG, anti-thymocyte globulin; AAT, alpha-1 antitrypsin; β-gal, β-galactosidase; cFIX, canine factor IX; cmEpo, cynomolgus macaque erythropoietin; CSP, cyclosporine; DMD, Duchenne muscular dystrophy; HB, hemophilia B; hu, human; IM, intramuscular; i.v., Intravascular; k9, canine; LGMD, limb-girdle muscular dystrophy; LPL, lipoprotein lipase; MMF, mycophenolate mofetil; NHP, non-human primate; pts, patients; PLAP, placenta alkaline phosphates; rtTA, reverse tetracycline transactivator; WT, wild type, +, positive for either humoral or cellular or both immune responses.; ±, limited humoral or cellular responses.*

elicited in dog muscles transduced with either AAV2 or AAV8, a non-HSPG binding serotype. The immune responses can be induced by both the vector capsid protein and the transgene product. Nevertheless, the authors documented less inflammation by histological analysis following intramuscular injection of AAV8-β-galactosidase compared to AAV2-β-galactosidase into dog muscle, and AAV8-β-galactosidase expression did not last for more than 4 weeks (Ohshima et al., 2009). AAV1, another serotype that does not bind HSPG, also induced significant cellular immune responses when injected into dog muscles (Wang et al., 2010).

In humans, a phase I clinical trial of limb-girdle muscular dystrophy (LMGD-2D; Mendell et al., 2009) reported both humoral (in all three patients seen as early as 2 weeks) and cellular immunity (in one patient detected from weeks 2 to 12) to AAV1 capsids following intramuscular injection of $3.25 \times 10^{11}$ vg/site, though the immune response did not diminish transgene expression. However, the data are difficult to interpret because of the use of (1) a 3-day course of methylprednisolone (from day of injection to day 2) in all patients; and (2) the extensor digitorum brevis muscle which is generally spared from the dystrophic process, hence dose not display pre-existing and ongoing inflammation, which is characteristic of dystrophic muscle. The use of a muscle specific promoter may also contribute to the lessened immune responses. In a follow-up paper by Mendell et al. (2010b), two out of three patients had sustained transgene expression at 6 months, and humoral and T cell responses to AAV1 were detected in the patient failed to show expression. Herson et al. (2011) reported modest cellular infiltration at week 4 following injection of $4.5 \times 10^{10}$ vg/site of AAV1, but not after a lower dose of $3 \times 10^{9}$ vg/site, into muscle of LGMD patients with transgene expression being detected in limited muscle fibers (4.7–10.5%). A recent phase I trial on DMD from Mendell et al. (2010a) also raised the potential of cellular immune responses to either self or non-self dystrophin epitopes. In the study, AAV vectors carrying a truncated but functional dystrophin gene were delivered intramuscularly into DMD patients. None of the patients displayed mini-dystrophin gene expressing myofibers at the two muscle biopsy time points, days 42 and 90. Four out of six patients had detectable dystrophin-specific T cells, including $CD4^+$ and $CD8^+$ T cells, in peripheral blood, with two patients having dystrophin-specific T cells before vector treatment. In contrast, immune responses to AAV capsid were not reported. The authors suggested that T cells targeting self or non-self dystrophin epitopes may have eliminated mini-dystrophin expressing muscle fibers in the study.

## INTRAVASCULAR ADMINISTRATION OF AAV TO MUSCLES

Administration of AAV vectors to skeletal muscles via an intravascular route is another strategy. Reports of immunity to AAV vectors delivered intravascularly have not been consistent. Studies by Gregorevic et al. (2009) documented marked inflammatory responses in dog muscle following limb artery infusion; however, the use of transient immunosuppression enhanced transgene expression. Yue et al. (2008) did not observe similar immune responses following intra-jugular injection of AAV9 into neonatal dogs, and the authors attributed this to the immature immune system in neonatal dogs. Following intravenous injection into neonatal dogs, AAV9 carrying a human mini-dystrophin, which is a potential antigen, induced inflammatory responses detected by magnetic resonance imaging at 8 weeks and by histological analysis of muscle samples at 16 weeks (Kornegay et al., 2010). Pelvic limb muscle atrophy and contractures detected at 16 weeks may be a result of the early marked inflammation responses (Kornegay et al., 2010).

In non-human primates (NHP), both humoral and cellular immunity were observed when AAV1 and AAV8 vectors were delivered intramuscularly, but not with regional i.v. injection. The immunity was not observed when animals were under immunosuppression with either mycophenolate mofetil plus prednisone for 3 weeks, or LEA29Y, a mutant of CTLA4-Ig which blocks costimulatory signaling through CD28 pathway that is required for optimal T cell activation (Toromanoff et al., 2008, 2010). Here, LEA29Y was expressed from AAV vector delivered intravenously to ensure therapeutic level. When LEA29Y was delivered intramuscularly, it did not eliminate immune responses to transgene product. On the other hand, no cellular immunity was observed to either AAV capsid or transgene following either intramuscular or i.v. delivery to NHP muscle by Chicoine's group (Rodino-Klapac et al., 2010).

### ADMINISTRATION OF AAV TO MUSCLES OF NON-MUSCULAR DYSTROPHIES

Muscle has also been a target for AAV-mediated gene transfer as a potential means to deliver secreted gene products to the blood for treating other genetic diseases due to easy access and muscle tropism of some AAV serotypes. Muscles in these disease models are normal comparing to the diseased muscle in muscular dystrophies. Immune responses to AAV viral capsid and/or transgene products have been reported in some studies but not in the others. In muscle-directed, AAV2-mediated canine factor IX (cFIX) transfer for treating hemophilia B in dogs, while no cellular responses were reported to either AAV2 capsid or cFIX, inhibitory anti-cFIX antibodies were reported to develop in a dose–dependent manner (Herzog et al., 1999, 2002). Transient immunosuppression with cyclophosphamide prevented anti-cFIX antibody formation and resulted in sustained transgene expression via either an intramuscular or intravascular delivery route (Herzog et al., 2001; Arruda et al., 2005, 2010). In another study by Haurigot et al. (2010), 6 weeks of immunosuppression with cyclophosphamide did not prevent the development of anti-AAV antibody and T cell infiltrates in muscle, and the T cells were transgene specific.

In human hemophilia B patients, AAV–FIX transfer to skeletal muscle did not result in therapeutic levels of FIX; however, no immune responses were observed (Manno et al., 2003). T cell responses to AAV1 capsid proteins were observed in a clinical trial for lipoprotein lipase (LPL) deficiency (Mingozzi et al., 2009). Specifically, AAV1 capsid-specific CD8 + T cells were detected at 4 weeks in half of the patients after intramuscular injection with kinetics that is dose–dependent, while no T- or B-cell responses to the LPL transgene product were seen. In a clinical trial for alpha-1 antitrypsin (AAT) deficiency (Brantly et al., 2009), humoral (by day 14) and T cell (by day 14, responses to AAV1 capsid were observed in all subjects though these immune responses did not diminish the low level of transgene expression that was observed. A recent phase II clinical trial by Flotte et al. (2011) further confirmed moderate to marked CD8 + T cell responses against AAV1 capsid protein following delivery of AAV1–AAT to muscles of patients. AAT expression was sustained but declined after day 30, and serum therapeutic AAT level was not achieved in any of the patients receiving either $6 \times 10^{11}$, $1.9 \times 10^{12}$, or $6 \times 10^{12}$ vg/kg.

## IMMUNE MODULATION

The ultimate goal of AAV-mediated gene therapy is to achieve persistent transgene expression at therapeutic levels, however, at present this goal is compromised by development of *de novo* or amplification of pre-existing humoral or cellular host immune responses to both AAV capsid and transgene products. Preventive strategies, such as use of alternative serotypes, changes in vector dosing, route of administration, and fetal or neonatal administration may not be feasible or effective in many situations. Immune modulation designed to prevent activation and proliferation of antigen-specific T- and B-cells is more potent and may be required in addition to non-immunosuppressive preventive strategies.

Studies using AAV-based therapy coupled with immunosuppression in muscular dystrophy are limited but encouraging. In a dog model of DMD (Wang et al., 2007b), it was demonstrated that a combination of cyclosporine (CSP), a calcineurin inhibitor that impairs T cell activation; and mycophenolate mofetil (MMF), an anti-metabolite that prevents proliferation of both T- and B-cells, was able to prevent immune responses to AAV in muscles of normal dogs; in dystrophic muscle which contains pre-existing and ongoing inflammation, a 5-day course of anti-thymocyte globulin (ATG) was needed in addition to a 16-week course of CSP/MMF to suppress immune responses to both AAV capsid and the canine (k9) micro (μ)-dys transgene. More importantly, robust and prolonged expression of k9-μ-dys persisted for at least 22 weeks after withdrawal of all immunosuppression. Immunosuppression by CSP and MMF also prevented cellular infiltration and led to enhanced transgene expression following intravascular delivery of AAV to muscle in dogs (Gregorevic et al., 2009). In human patients, a short course of methylprednisolone was used with LMGD and DMD (Mendell et al., 2009, 2010a), which lessened cellular responses, however, there was no transgene expression.

The generation and maintenance of immunological memory is critical for host protection when re-encountering a pathogen. However, memory cells can be a barrier in viral-mediated gene therapy for patients with muscular dystrophies who may require multiple treatments with vectors throughout life. Despite high transduction efficiency of AAV-mediated gene transfer, re-administration of AAV vectors has resulted in little or no further transduction events in tissues, such as skeletal muscle and lung. This was correlated with the appearance of viral or transgene specific neutralizing antibodies (Halbert et al., 1997). Pre-existing neutralizing antibodies or memory T cells specific to viral capsid protein or transgene product were also suggested to prevent sustained transgene expression in recent human trials (Kessler et al., 1996; Xiao et al., 1996; Manno et al., 2006; Mingozzi et al., 2007; Mendell et al., 2010a). However, a study on AAV delivery into lung in mice (Halbert et al., 1998) demonstrated that transient immunosuppression through blocking costimulatory signaling pathways with a combination of anti-CD40 ligand antibody and CTLA4-Ig at the time of primary AAV exposure allowed successful repeat administration of AAV to lung. More recently, Arruda and Colleagues reported that the presence of neutralizing antibodies to AAV2 did not prevent Intravascular AAV6 delivery to muscles of hemophilia B dogs given immunosuppression with cyclophosphamide 1 day prior to vector injection and weekly thereafter for 6 weeks (Herzog et al., 2001). Studies by the Wilson's group also showed sustained expression of factor IX carried by AAV8 in AAV2-pretreated hemophilia dogs (Wang et al., 2005).

## CONCLUSION

Adeno-associated viral-mediated gene therapy for muscular dystrophies is a promising yet fairly complex therapeutic approach under development for approximately 20 years. The advances in the last two decades in the field are remarkable and have energized the planning and implementation of phase I clinical trials. Identification of immunity to AAV vectors emerged as a major challenge from these studies and emphasizes the importance of appropriate animal models to address safety and efficacy of the approach and predict clinical outcomes. Combining immunosuppressive therapy with other preventive strategies may be necessary to induce persistent antigen-specific tolerance in the gene therapy setting.

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# Immune responses to rAAV6: the influence of canine parvovirus vaccination and neonatal administration of viral vector

***Andrea L. H. Arnett[1,2], Dilip Garikipati[2†], Zejing Wang[3], Stephen Tapscott[3] and Jeffrey S. Chamberlain[2,4,5]****

[1] *Medical Scientist Training Program, University of Washington School of Medicine, Seattle, WA, USA*
[2] *Department of Neurology, University of Washington School of Medicine, Seattle, WA, USA*
[3] *Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA*
[4] *Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA*
[5] *Department of Biochemistry, University of Washington School of Medicine, Seattle, WA, USA*

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Dongsheng Duan, University of Missouri, USA*
*Tim Nichols, University of North Carolina at Chapel Hill, USA*

***Correspondence:**
*Jeffrey S. Chamberlain, Department of Neurology, University of Washington School of Medicine, 1959 NE Pacific Street, Box 357720, Seattle, WA 98195-7720, USA.*
*e-mail: jsc5@uw.edu*

**†Present address:**
*Dilip Garikipati, Department of Animal Sciences, Washington State University, Pullman, WA, USA.*

Recombinant adeno-associated viral (rAAV) vectors promote long-term gene transfer in many animal species. Significant effort has focused on the evaluation of rAAV delivery and the immune response in both murine and canine models of neuromuscular disease. However, canines provided for research purposes are routinely vaccinated against canine parvovirus (CPV). rAAV and CPV possess significant homology and are both parvoviruses. Thus, any immune response generated to CPV vaccination has the potential to cross-react with rAAV vectors. In this study, we investigated the immune response to rAAV6 delivery in a cohort of CPV-vaccinated canines and evaluated multiple vaccination regimens in a mouse model of CPV-vaccination. We show that CPV-vaccination stimulates production of neutralizing antibodies with minimal cross-reactivity to rAAV6. In addition, no significant differences were observed in the magnitude of the rAAV6-directed immune response between CPV-vaccinated animals and controls. Moreover, CPV-vaccination did not inhibit rAAV6-mediated transduction. We also evaluated the immune response to early rAAV6-vaccination in neonatal mice. The influence of maternal hormones and cytokines leads to a relatively permissive state in the neonate. We hypothesized that immaturity of the immune system would permit induction of tolerance to rAAV6 when delivered during the neonatal period. Mice were vaccinated with rAAV6 at 1 or 5 days of age, and subsequently challenged with rAAV6 exposure during adulthood via two sequential IM injections, 1 month apart. All vaccinated animals generated a significant neutralizing antibody response to rAAV6-vaccination that was enhanced following IM injection in adulthood. Taken together, these data demonstrate that the immune response raised against rAAV6 is distinct from that which is elicited by the standard parvoviral vaccines and is sufficient to prevent stable tolerization in neonatal mice.

**Keywords: AAV, muscle, immunity, tolerance, parvovirus, vaccination, canine, immune response**

## INTRODUCTION

Adeno-associated virus (AAV) is a non-enveloped, single-stranded DNA virus that is a member of the Parvovirus family. The AAV genome is approximately 5 kb in size and is packaged within an icosahedral capsid that facilitates viral entry into susceptible cells (Schultz and Chamberlain, 2008). AAV-mediated gene transfer has been successfully demonstrated in numerous large and small animal models of human disease (Arnett et al., 2009; Wang et al., 2009), and recombinant AAV (rAAV) vectors are thus considered a prime candidate for use in the development of gene replacement strategies. rAAV vectors are limited by their small carrying capacity, but possess several attractive features that are advantageous for use as therapeutic reagents, including a broad range of tissue tropism and lack of pathogenicity (Schultz and Chamberlain, 2008). Over 12 serotypes and numerous variants of AAV have been identified. Each serotype has demonstrated a unique profile of tissue tropism that can be utilized to develop targeted therapies with enhanced tissue specificity (Zincarelli et al., 2008; Vandenberghe et al., 2009). For example, rAAV2 exhibits a high tropism for liver and has been used to treat hemophilia B via expression of Factor IX (Manno et al., 2006). rAAV6 has been shown to achieve a high level of transduction in both lung (Halbert et al., 2001, 2007) and striated muscle (Blankinship et al., 2004; Gregorevic et al., 2004, 2006), and is thus being studied to develop treatments for diseases such as cystic fibrosis (Flotte et al., 2007; Halbert et al., 2007), α1-antitrypsin deficiency (Halbert et al., 2010), and the muscular dystrophies (Arnett et al., 2009; Wang et al., 2009).

Stable transgene expression in transduced cells can be harnessed to treat diseases resulting from genetic deficiencies. However, a significant obstacle to the use of viral vectors is the development of host immune responses to both the transgene and the vector (Zaiss and Muruve, 2005, 2008;

Nayak and Herzog, 2010). The rAAV genome is one of the simplest of viral gene therapy vectors, containing only the transgene expression cassette flanked by non-coding viral inverted terminal repeats that facilitate packaging and capsid assembly (Samulski et al., 1982). No viral genes are encoded within the engineered genome, which significantly reduces the risk of viral protein synthesis within the host and limits the potential immunogenicity of rAAV vectors. Initial studies regarding rAAV delivery have demonstrated the relative lack of a cell-mediated immune response to rAAV infection in naïve animals (Athanasopoulos et al., 2004; Warrington and Herzog, 2006). In contrast to adenovirus, rAAV does not efficiently trigger a strong, acute inflammatory response, resulting in inefficient activation of dendritic cells and other antigen presenting cells that influence a cytotoxic immune response (Zaiss and Muruve, 2005). These and other factors are thought to contribute to sustained transgene expression in targeted tissues. However, despite promising results in animal studies, clinical trials of rAAV gene delivery in humans have failed to demonstrate the same level of success. Results from multiple studies indicate that capsid-specific humoral and cell-mediated immunity limit tissue transduction and lead to gradual clearance of transduced cells (Manno et al., 2006; Mingozzi and High, 2007; Mingozzi et al., 2009). Thus, renewed effort is being made to understand and modulate factors governing the immune response to rAAV across multiple routes of delivery.

Evaluation of therapeutic constructs and delivery strategies in large animal models is a necessary step in the assessment of potential gene replacement therapies destined for clinical trials. In this regard, dogs can suitably model the physics and challenges of vector delivery to large volumes of tissue and model potential adverse reactions of an evolved mammalian immune system (Wang et al., 2009). The immune response to rAAV in canines has been investigated in several studies (Mount et al., 2002; Wang et al., 2007a, 2010; Yuasa et al., 2007; Ohshima et al., 2009; Halbert et al., 2010; Haurigot et al., 2010). We and others have previously evaluated rAAV delivery to striated muscle (Yuasa et al., 2007; Gregorevic et al., 2009; Ohshima et al., 2009) and have observed significant humoral and cell-mediated immunity utilizing a variety of rAAV serotypes (Wang et al., 2007a, 2010; Yuasa et al., 2007; Ohshima et al., 2009). However, canines used for research purposes are routinely vaccinated against several potential pathogens, one of which is canine parvovirus (CPV). CPV infection is associated with high mortality in young puppies and is very contagious (Patel and Heldens, 2009). High risk of CPV infection necessitates early vaccination in the majority of kennels. Like AAV, CPV is a member of the Parvovirus family and shares significant sequence identity with AAV and other family members. As follows, any immune response generated against CPV vaccination has the potential to cross-react with other members of the Parvovirus family, including AAV. Thus, it is important to consider the influence of CPV vaccine-related immunity on the rAAV-directed immune response in canines, as well as the influence of early exposure to virus and vaccine constituents in the maturing mammalian immune system. In this study, we investigate the immune response to rAAV6 delivery in CPV-vaccinated canines and evaluate multiple vaccination regimens in a mouse model of CPV-vaccination. In addition, we explore the influence of early rAAV6-vaccination on the immune response to repeat rAAV6-infection in adulthood.

## RESULTS

### CPV VACCINATION AND ANTI-AAV ACTIVITY IN DOGS

Canine parvovirus-vaccination has the potential to stimulate production of antibodies that cross-react with rAAV, and may contribute to the rAAV-directed immune response that has been previously observed in canines (Wang et al., 2007a, 2010; Yuasa et al., 2007; Ohshima et al., 2009). To address this concern, we tested serum from a cohort of wild-type (*wt*) beagles that had been vaccinated following a standard vaccination regimen that included three separate intramuscular CPV-vaccinations between the ages of 3 and 7 weeks. Serum was collected at 8 weeks of age and evaluated for rAAV6 cross-reactive neutralizing antibodies (**Figure 1**). Assay results demonstrate that the neutralization efficiency of serum from CPV-vaccinated animals is minimal. At the strongest serum dilution tested (1:50), less than 10% inhibition was observed. Following the initial serum collection, the dogs were treated with rAAV6 via intravascular injection ($5 \times 10^{12}$ vector genomes/kg), and serum was again sampled for analysis 5 weeks later. As expected, exposure to rAAV6 capsids elicited a strong neutralizing antibody response (**Figure 1**), but prior CPV-vaccination did not prevent transduction of skeletal muscle (published in Gregorevic et al., 2009; Wang et al., 2010).

We also performed a series of Westerns to further analyze whether or not CPV vaccination generated antibodies that cross react with AAV capsids. Serum samples were collected from two groups of dogs (**Figure 2**). For Group 1, sera were collected from five CPV-vaccinated dogs between the ages of 8–12 months old as post-CPV but pre-rAAV samples, and then collected once more at 4 weeks after intramuscular (IM) injection of rAAV6 vectors. For Group 2, sera were collected from 5-week-old pups ($n = 3$) prior to CPV vaccination, followed by collection at 3 months after CPV-vaccination, and then 4 weeks after IM injection of rAAV6. **Figure 2** shows representative data generated from the two groups, and essentially identical results were seen with all dogs. Pre-CPV serum isolated from the three dogs in Group 2 showed no obvious reactivity to rAAV2, rAAV6, or parvoviral capsids by

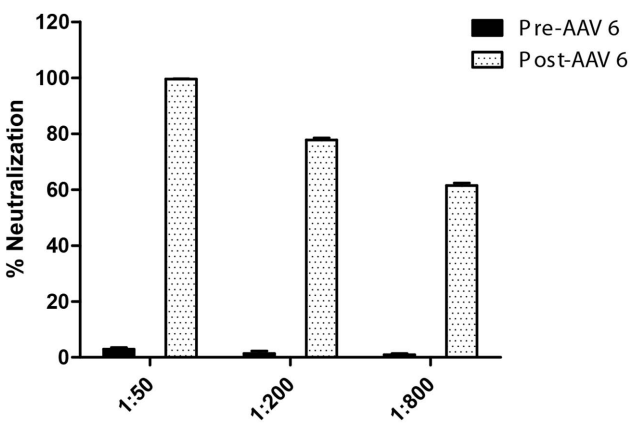


**FIGURE 1 | rAAV6 neutralization activity in CPV-vaccinated canines, pre- and post-rAAV6 injection.** Beagle pups ($n = 3$) were vaccinated against CPV prior to 7 weeks of age. Serum neutralization activity was minimal prior to rAAV6 injection.

western analysis. While parvovirus capsids were detected when post-parvo but pre-rAAV serum was used, the serum was not able to detect rAAV6 or rAAV2 above background (BSA was used as negative control). As expected, serum collected after rAAV6 treatment showed strong reactivity to both rAAV2 and 6, whereas the parvoviral signal remained similar in strength to what was observed prior rAAV injection. Together, these data suggested that CPV vaccination did not generate antibodies with detectable cross-reactivity to rAAV.

### CPV-VACCINATION AND rAAV6 TRANSDUCTION IN MICE

CPV-vaccines have been developed and marketed by multiple agencies. Thus, both vaccination reagents and the timing of administration can vary from kennel to kennel. It is important to recognize that variation in vaccination regimens could influence the nature of the immune response, and it may not be appropriate to generalize results obtained from a single vaccination regimen in a relatively small cohort of animals. The above results from vaccinated beagles provide some insight regarding the immune response to CPV-vaccination and cross-reactivity to rAAV6. However, the study is limited by issues inherent to large animal models. Both ethical considerations and high cost necessitate the use of a small number of animals, and thus it is impractical to effectively assess multiple vaccination regimens in a canine model. In consideration of these factors, we continued the remainder of our vaccination studies in the mouse, which is more amenable to larger sample sizes. In addition, their smaller mass facilitates administration of a higher dose (per kilogram) of rAAV6 that can achieve body-wide transduction of skeletal muscle (Blankinship et al., 2004) and is more relevant to therapeutic dosing levels. While significant differences exist between canine and murine immune systems, the mouse has been utilized extensively as a model system for the study of vaccination responses, autoimmunity, and other aspects of mammalian immunology. Thus, commonalities between the immune systems of both animal models predict that the results regarding CPV-vaccination and generation of any potential rAAV6-directed immune response in the mouse may also be applicable to the canine model.

Mice were vaccinated following two different regimens that represent those commonly employed in kennels (**Figure 3A**). Regimen 1 utilized reagents that were equivalent to the vaccines administered to the above cohort of beagles. It consisted of a dose of Galaxy® PV, a modified live CPV vaccine, coupled with Intra trac® 3, an upper respiratory vaccine that is directed against adenovirus type-2, parainfluenza, and *Bordetella bronchiseptica*. Regimen 2 consisted of a combination of two commercial vaccines, Vanguard® Plus and Duramune® Max, both of which contain attenuated CPV, parainfluenza, adenovirus type-2, canine coronavirus, and canine distemper. The two vaccines are given together for the initial dose, followed by a single, additional dose of Vanguard® Plus 1 week later. Eight weeks post-vaccination, serum was collected from both cohorts and analyzed for cross-reactivity with rAAV6 capsids. Vaccinated animals exhibited a slightly elevated neutralizing antibody response compared to unvaccinated mice (**Figure 3B**). However, this difference was statistically significant only at the highest serum concentration.

These three cohorts of mice were then injected with rAAV6 carrying the human placental alkaline phosphatase (hPlAP) reporter gene. Vector was administered via IV injection ($2 \times 10^{12}$ vg) and IM injection ($1 \times 10^{10}$ vg). Dual injection methods were employed to ensure maximum presentation of rAAV6 capsids to monitoring immune cells in both interstitial and intravascular compartments. One month post-rAAV injection, serum samples, and striated muscle tissues were collected for analysis (**Figure 4**). Transgene

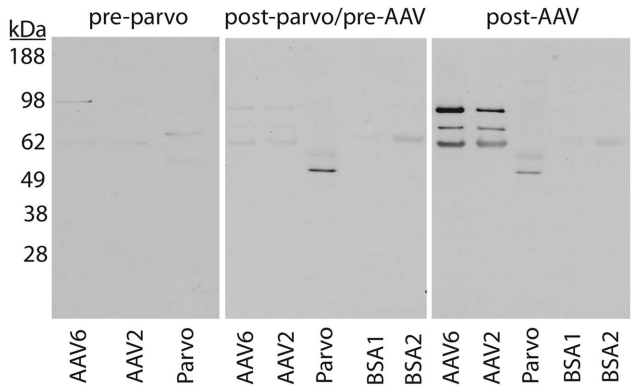


**FIGURE 2 | Western analysis of CPV vaccination and serum anti-AAV activity in canines.** Serum isolated from CPV-vaccinated animals does not exhibit enhanced anti-rAAV6 activity in comparison to unvaccinated controls. Serum from dogs before CPV vaccination (pre-parvo), after CPV vaccination but before rAAV6 administration (post-parvo/pre-AAV), or after rAAV6 administration (post-AAV) were used as probes. kDa, kilodalton molecular marker; BSA, bovine serum albumin, used as negative control.

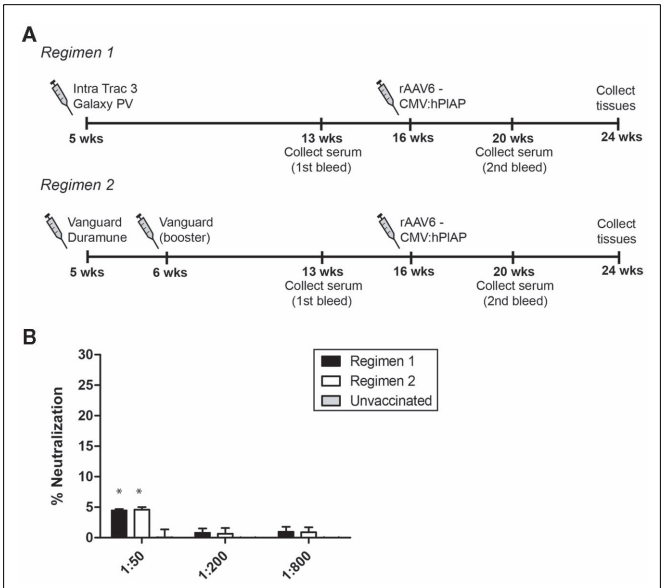


**FIGURE 3 | AAV6 neutralization activity and CPV vaccination in mice.** **(A)** Schematic representing the timeline of CPV vaccination and rAAV6 injection. Serum was collected 4 weeks prior to rAAV6 injection and neutralization activity was quantified **(B)**. Both vaccination schedules elicited a slight neutralizing antibody response that was detectable at 1:50 serum dilution (* indicates statistical significance compared to unvaccinated control, $p < 0.05$).

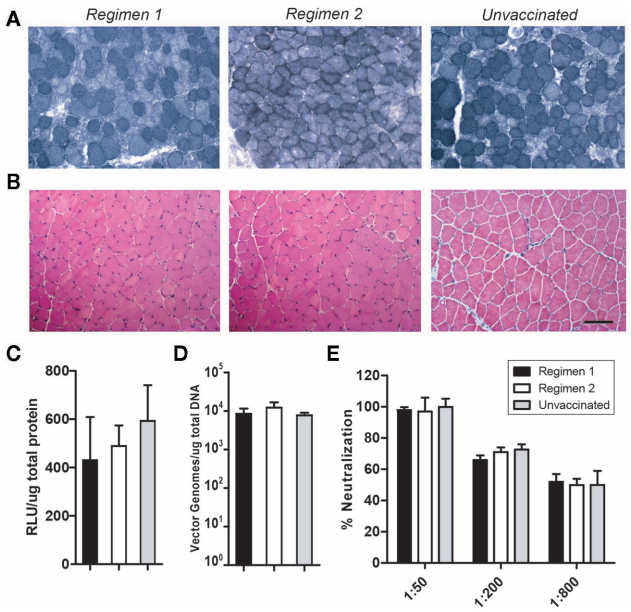


**FIGURE 4 | rAAV6 transduction is not impaired in CPV-vaccinated mice.** Vaccinated animals ($n = 6$) received IM and IV injections of rAAV-hPlAP. Serum and tissues were collected 1 month post-injection. Histochemical staining of muscle cross-sections demonstrates robust hPlAP expression **(A)** and no significant inflammatory infiltrate **(B)**. Quantification of hPlAP expression **(C)** and vector genome number **(D)** in muscle lysates revealed no significant difference between vaccinated and unvaccinated animals. CPV-vaccination did not significantly influence the neutralizing antibody response to rAAV6 injection **(E)**. Scale bar 50 μm.

expression and vector genomes were quantified across different groups, but no significant difference in hPlAP enzymatic activity or genome copy number was found between either of the two vaccination regimens or the unvaccinated control mice (**Figures 4C,D**). In addition, no significant cellular inflammatory response was observed in transduced muscle isolated from vaccinated or unvaccinated mice (**Figure 4B**), suggesting that CPV-vaccination does not contribute to chronic inflammatory infiltration in rAAV-transduced muscle. As expected, neutralizing antibodies were generated following rAAV6 injection, but the response in CPV-vaccinated mice was not significantly different compared to control animals.

### rAAV6 VACCINATION IN NEONATAL MICE

The immune system in young mammals is immature, and neonatal exposure to antigen does not always elicit the same type of immune response as in the adult (reviewed in Jaspan et al., 2006). A diminished cytotoxic T cell response and the presence of circulating maternal antibodies can result in impaired induction of memory cells and persistent infection in newborns (Adkins et al., 2001). In addition, a tolerogenic response to antigens can be initiated in neonates (Morein et al., 2007; Verhasselt, 2010a), which could theoretically be harnessed to facilitate repeat administration of therapeutic vectors. Thus, it is possible that very early vaccination with rAAV6 could modulate the immune response to rAAV infection in a permissive manner. To investigate this prospect, we vaccinated mice with rAAV6 at either 1 or 5 days of age. Vector was delivered intraperitoneally at a dose of $1 \times 10^{11}$ vg per mouse (**Figure 5A**). Serum was collected 4 weeks post-vaccination and rAAV6 neutralization activity was quantified prior to IM injection of rAAV6-hPlAP ($1 \times 10^{10}$ vg) in the left tibialis anterior (TA). Both cohorts of mice generated a significant neutralizing antibody response to neonatal rAAV6 vaccination (**Figure 5B**). However, neutralization activity in the Day 1 vaccination cohort was significantly lower compared to the Day 5 cohort. In addition, animals in the Day 1 cohort demonstrated a greater degree of individual variability in neutralization activity, which may be related to differences in immune system maturity and development between newborn and 5-day old mice. Interestingly, transduction of skeletal muscle was not significantly impacted by early vaccination in either cohort, despite the presence of circulating anti-rAAV6 antibodies (**Figure 5C**). These results suggest that the rAAV6-directed immune response initiated in vaccinated, neonatal mice may represent either a weak, ineffective primary immune response against re-infection or may instead be indicative of a tolerant response with the potential to remain permissive to viral infection upon rAAV6 re-exposure during adulthood.

### PERMISSIVE IMMUNITY DOES NOT PERSIST IN rAAV6-VACCINATED MICE

It has been previously shown that IM injection of rAAV stimulates a robust humoral immune response that is sufficient to prevent skeletal muscle transduction upon subsequent exposure to the same vector serotype (Burger et al., 2004; Riviere et al., 2006; Sabatino et al., 2007). As shown above, rAAV6-vaccinated mice responded positively to the first IM injection of rAAV6, but it was important to determine whether this permissive state would persist through repeated exposure to rAAV6 during adulthood. To evaluate whether mice had developed a functional tolerance for rAAV6, both Day 1 and Day 5 cohorts were given an additional IM injection of $1 \times 10^{10}$ vg into the contralateral TA muscle. Serum was collected immediately prior to injection, and neutralization activity was quantified (**Figure 6A**). Both cohorts showed a strong neutralizing antibody response that was significantly enhanced when compared to the response obtained from the first bleed (**Figure 5B**). No significant difference in neutralization activity was observed between vaccinated animals and un-vaccinated controls. In addition, neutralization activity remained high even at more dilute serum concentrations, suggesting that the 4 week IM injection enhanced the rAAV6-directed immune response. Consistent with these observations, transduction in the contralateral TA was dramatically reduced and was limited to a small number of sparsely distributed muscle fibers (**Figure 6B**). These data indicate that rAAV6-vaccinated mice did not remain permissive to repeat infection and suggest that the success of the initial IM injection may be related to a weak primary immune response to rAAV6-vaccination, rather than induction of tolerance.

## DISCUSSION

The immune response to rAAV vectors has emerged as a prominent issue in the development of gene replacement strategies. Initial studies suggested a relative lack of immunogenicity of rAAV vectors, but the success achieved in animal models has not

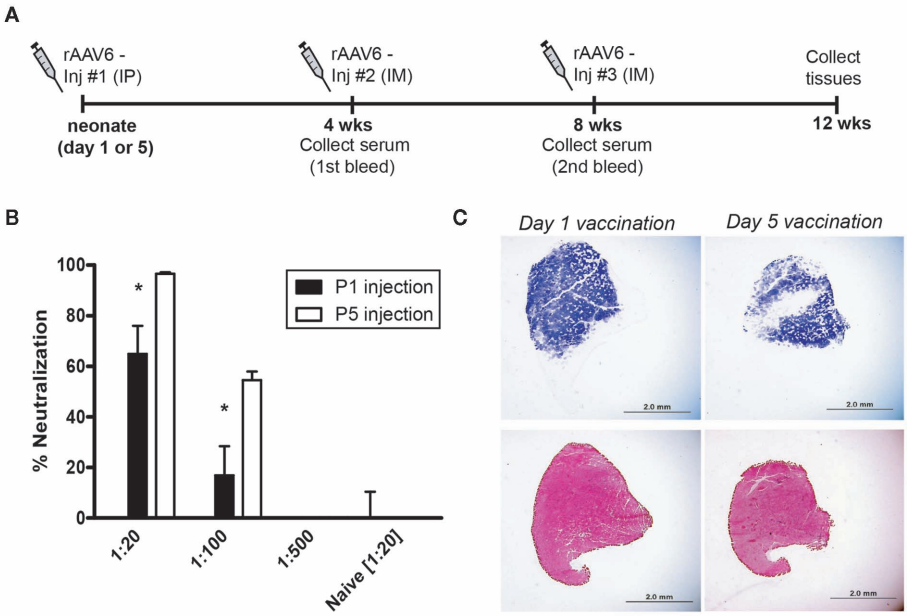


**FIGURE 5 | rAAV6 vaccination and neutralizing antibody response in neonatal mice. (A)** rAAV6 injection and serum collection timeline. Mice were vaccinated (IP) at P1 or P5, and received two sequential injections (IM) of rAAV6 at 4 and 8 weeks. **(B)** Quantification of neutralizing antibody response from the first bleed, 4 weeks post-vaccination. Animals vaccinated on Day 1 generated a weaker response than those injected Day 5 (* indicates significant difference compared to P5 cohort, $p < 0.05$). **(C)** Cross-sections of tibialis anterior injected with rAAV6-hPlAP (Inj #2) at 4 weeks post-vaccination. Neutralizing antibody response did not prevent rAAV6 transduction in either cohort. hPLAP staining (top) and hematoxylin and eosin staining (bottom). Scale bar 2 mm.

been mirrored in human trials. Significant immune responses to the vector have been observed following intravascular delivery of rAAV to liver and direct injection into skeletal muscle. Exposure to wild-type AAV is common within the human population, and the frequency of sero-positivity for AAV-directed neutralizing antibodies approaches nearly 30% for AAV2 (Mingozzi and High, 2007). Pre-existing neutralizing antibodies to AAV have been shown to significantly inhibit transduction of hepatocytes in a clinical trial evaluating delivery of rAAV2 to hemophilia patients (Manno et al., 2006). A similar humoral response has been observed in cystic fibrosis gene therapy trials involving repeat administration of rAAV2 (Flotte et al., 2007). Disappointingly, the majority of clinical trials have resulted in the gradual loss of transgene expression. rAAV2-mediated delivery of human Factor IX (hFIX) to either skeletal muscle (Kay et al., 2000) or liver (Manno et al., 2006) resulted in therapeutic levels of hFIX in a subset of patients, but hFIX expression was eventually eliminated or reduced to non-therapeutic levels in all participants. In another clinical trial, rAAV1 was used to deliver lipoprotein lipase (LPL) via IM injection to patients with LPL-deficiency (Mingozzi et al., 2009). Once again, transgene expression rose to effective levels for a relatively short period before gradually dropping below therapeutic threshold. In a clinical trial of rAAV1-mediated delivery of α-sarcoglycan, two out of three patients responded well to the treatment, whereas the third patient failed to demonstrate successful gene transfer (Mendell et al., 2010). Extensive analysis of data generated from these clinical trials has indicated that both humoral and cell-mediated anti-capsid immune responses likely play a significant role in the elimination of transduced tissues (Manno et al., 2006; Mingozzi et al., 2009; Mendell et al., 2010).

A significant immune response has been observed in several studies of rAAV delivery in canines. Aspects of the canine immune response have mirrored findings in human clinical trials, including detection of a capsid-directed T cell response and gradual loss of transgene expression in non-immunosuppressed animals. Ohshima et al. (2009) observed a strong inflammatory response and T cell infiltration following delivery of rAAV2 or rAAV8 to skeletal muscle, though the magnitude of the inflammatory response was reduced in rAAV8-injected muscles. In addition, we have previously shown that injection of rAAV6 or rAAV1 in immunocompetent animals results in a similar sequence of events. An anti-capsid immune response led to local inflammation and clearance of the majority, but not all, transduced cells over a period of several weeks (Wang et al., 2007a, 2010). Loss of transgene expression was prevented by a short course of immunosuppression (Wang et al., 2007b). An rAAV-directed immune response has also limited transgene expression in a canine model of α1-antitrypsin deficiency (Halbert et al., 2010). These studies raise concerns regarding the immunogenicity of rAAV vectors and emphasize the need for careful evaluation of the potential immune response to rAAV delivery, especially in disorders that may require very high vector doses for systemic delivery, such as the muscular dystrophies.

Further study of the canine immune response to rAAV may provide insight toward addressing immunogenicity in clinical trials, but it is important to rule out the influence of

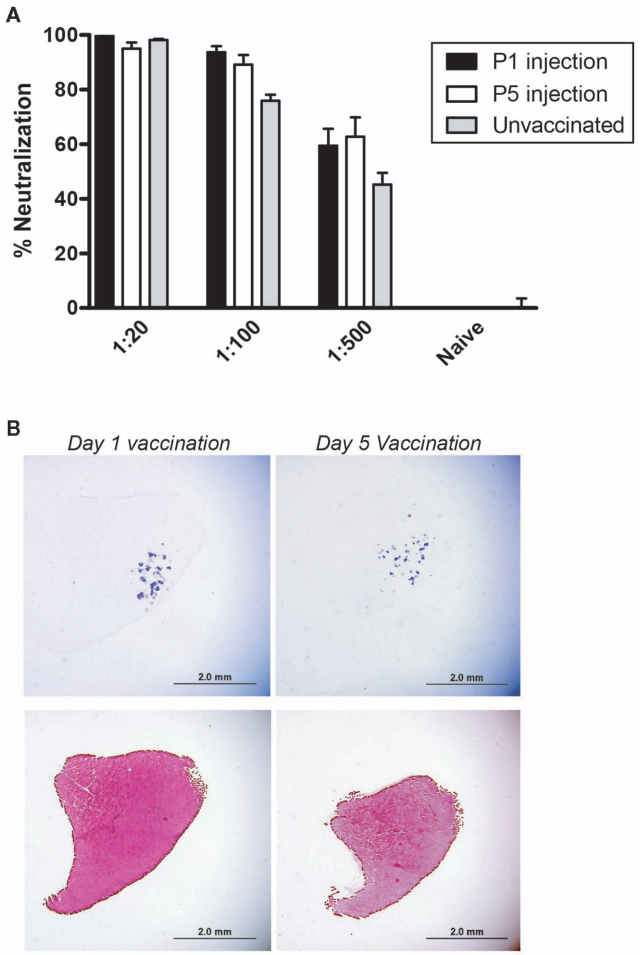


**FIGURE 6 | Permissive immunity does not persist in rAAV6-vaccinated mice.** Animals received a third injection of rAAV6-hPlAP at 8 weeks post-vaccination. Serum collected immediately prior to injection (Inj #3) demonstrated high neutralization activity **(A)**. rAAV6 transduction was dramatically reduced **(B)**. hPLAP staining (top) and hematoxylin and eosin staining (bottom). Scale bar 2 mm.

CPV-vaccination regarding the generation of an rAAV-directed immune response. Antibody cross-reactivity has been demonstrated with closely related variants of AAV and between other related members of the Parvovirus family (Patel and Heldens, 2009). In addition, it has been shown that T cell receptors are reactive to epitopes that are conserved between different serotypes (Mingozzi and High, 2007; Mingozzi et al., 2009; Wang et al., 2010), suggesting that a cell-mediated response may be more broadly reactive across a related group of viral vectors. Here, we observed a low level of neutralization activity in both mouse and canine models of CPV-vaccination at serum dilutions of 1:50 (**Figures 1** and **3**). This activity was not measurable at more dilute serum concentrations, and Western analysis of serum from parvo-vaccinated and unvaccinated canines did not exhibit enhanced binding to rAAV particles (**Figure 2**). These results may indicate the presence of anti-CPV antibodies that possess low, cross-reactive affinity for rAAV6. Cross-reactive antibodies with weak affinity for rAAV6 would require a higher concentration of neutralizing antibodies to achieve significant neutralization. At more dilute serum concentrations, inefficient binding of cross-reactive antibodies would significantly limit neutralization activity. Further characterization of the antibody response would be necessary to quantify affinity strength and identify common epitope targets before cross-reactivity can be confirmed. However, it is important to emphasize that CPV-vaccination did not correlate with any significant inhibition of rAAV6 transduction (**Figure 4**), suggesting that a low level of neutralization activity may not play a role in generating a functionally significant rAAV6-directed immune response. In addition, we did not detect any significant inflammatory infiltration, nor did we observe any changes in muscle architecture that would be indicative of a cell-mediated, cytotoxic response (**Figures 4A,B**).

The CPV vaccination regimen utilized in these studies was chosen to reflect the vaccination regimens routinely employed in large kennels within the United States (Bioresources, 2006). A variety of CPV vaccines are available from different manufacturers, and availability in different regions is dependent on local laws, international licensing, and market preferences (Patel and Heldens, 2009). The regimens evaluated in this study are representative of the majority of vaccine components that are used by major manufacturers, and thus, our results are likely applicable to a wide selection of vaccines. However, it is possible that subtle differences in vaccines and vaccination schedules could influence the immune response. Additional studies evaluating vaccines from a larger number of manufacturers would be necessary to rule out this possibility.

In addition to CPV-vaccination, we explored the response to rAAV6 vaccination in neonatal mice. The immune system in newborn mammals is under-developed, and significant differences exist in the pattern of T and B cell activation between newborns and adults. Newborns exhibit a generalized deficiency in adaptive cellular responses, with a bias toward prolonged Th2-type immunity (Adkins et al., 2001). This observation has been attributed to the influence of maternal cytokines and hormones that promote maternal tolerance to fetal antigens. These cytokines persist in the fetal circulation and influence the neonatal immune response for a significant period after birth, leading to a relatively tolerogenic, and vulnerable state in the neonate (Morein et al., 2002). In this regard, the newborn period represents a time when the immune system is learning to balance induction of tolerance to self antigens with appropriate reactive immunity to foreign pathogens. Thus, viral vectors delivered during this developmental window have the potential to initiate a tolerant immune response.

The ability to achieve successful transduction following repeat administration of viral vectors would be advantageous, both in the clinic and in the realm of basic research. Unfortunately, repetitive administration of identical serotypes has been ineffective in the majority of cases due to the development of a significant humoral immune response to rAAV vectors (Burger et al., 2004; Riviere et al., 2006; Sabatino et al., 2007; Petry et al., 2008). It has been speculated that early vaccination with rAAV could stimulate a tolerant response to vector in the immature immune system and thus permit re-administration in adulthood. In this regard, previous studies have investigated the response to *in utero* adenoviral (Lipshutz et al., 2000; Bouchard et al., 2003) or AAV (Jerebtsova

et al., 2002; Bouchard et al., 2003; Sabatino et al., 2007) vaccination in mice. These studies were limited to IM injection of rAAV serotypes 1, 2, and 5, and did not evaluate rAAV6. Considering that serotype-specific transduction profiles, dosage, and the route of administration can significantly influence the immune response to viral vectors (Mingozzi and High, 2007; Petry et al., 2008; Zaiss and Muruve, 2008), it is important to empirically determine the immune response rAAV6 vaccination.

Unfortunately, we were unable to induce persistent tolerance to rAAV6 utilizing neonatal vaccination. The neutralizing antibody response to the initial IP vaccination did not inhibit transduction during the first IM injection at 4 weeks of age. However, animals did not remain permissive to rAAV6 transduction. On the contrary, neutralization activity was significantly enhanced following the first IM injection, resulting in near-complete inhibition of transduction during the second IM injection. These results suggest that the initial vaccination triggered a weak primary immune response, which led to an enhanced secondary reaction to the first IM injection. However, without a more detailed characterization of the antibody response, we cannot rule out the existence of partial tolerance to specific viral epitopes. These findings are consistent with those previously observed following *in utero* delivery of rAAV1 and rAAV2 (Jerebtsova et al., 2002; Sabatino et al., 2007) and indicate that neonatal IP injection of rAAV6 does not facilitate repetitive administration of vector beyond a single, repeat injection.

In summary, we have evaluated the effects of CPV and rAAV vaccination on rAAV6-mediated transduction. The neutralizing antibody response to CPV-vaccinated animals is minimal and does not appear to significantly enhance either the humoral or cellular response to rAAV6 transduction. These data suggest that CPV-immunity is not a significant component of the rAAV6-directed immune response in canines, and support the use of canines as a valid model for further characterization of the immune response to rAAV6. In contrast, vaccination with rAAV6 in neonatal mice leads to a significant immune response that prevents repetitive administration of rAAV6. However, additional methods of tolerance induction may warrant further consideration, including oral delivery (Verhasselt, 2010b) and thymic expression of viral proteins (Chu et al., 2010).

## MATERIALS AND METHODS

### AAV PRODUCTION AND CHARACTERIZATION

rAAV6 vector was generated as previously described (Grimm et al., 2003). Cells were co-transfected with an rAAV6 packaging plasmid pDGM6 and plasmid containing the expression cassette flanked by viral ITRs. Cellular pellets and supernatants were collected and processed through a 110S microfluidizer (Microfluidics, Newton, MA, USA), followed by clarification of the homogenate by filtration through a 0.22-μm filter. Additionally, an Amersham AKTA10 HPLC machine (Amersham, Piscataway, NJ, USA) was used for affinity purification on a HiTrap heparin column (Amersham). The column was then washed and vector was eluted and dialyzed against physiological Ringer's solution. Vector was titered using HT-1080 cells as transduction targets, and Southern analysis was utilized to determine the number of genome-containing particles in the vector preparation.

### ANIMAL EXPERIMENTS

Animal studies were performed in accordance with the guidelines set forth by the institutional Review office of University of Washington. C57BL/6 mice were bred in our animal facility. Mice were given vaccines or AAV injections according to the indicated schedule (see Results). Vaccinations were administered using either Intra Trac® 3 and Galaxy PV® (both supplied by Intervet/Schering-Plough Animal Health, Millsboro, DE, USA), Vanguard® Plus (Pfizer Animal Health, Exton, PA, USA) and Duramune® Max (Boehringer-Ingelheim Vetmedica, Inc., St. Joseph, MO, USA), or intraperitoneal administration of rAAV6-CMV-cre. rAAV6-CMV-hPlAP was delivered via either retro-orbital injection or IM injection (into the left TA muscle), as indicated. Blood samples were collected via retro-orbital route, under isofluorane-induced anesthesia. At the indicated timepoints, mice were euthanized according to approved protocol and tissue samples were collected for analysis.

### WESTERN ANALYSIS

$3 \times 10^9$ vector genome/well of AAV6 and AAV2 and 10 μl/well of parvovirus vaccine at 1:10 dilution were loaded onto a 4–12% NuPAGE Bis–Tris gel (Bio-Rad, USA). The gel was transferred onto nitrocellulose membrane (Bio-Rad, USA). Membranes were blocked with 5% non-fat milk, 0.1% Tween–PBS (w/v) overnight at 4°C and then incubated with serum at 1:200 dilution. Horseradish peroxidase (HRP)-labeled rabbit anti-dog Ig was used as secondary antibody at 1:25,000 (Jackson ImmunoResearch, USA). Immunoreactive proteins were visualized using the ECL system (Amersham, USA).

### CELL CULTURE

The 293 human embryonic kidney cells and HT-1080 human fibrosarcoma cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum supplemented with penicillin and streptomycin. Cells were cultured at 37°C in an atmosphere of 5% $CO_2$.

### VIRUS NEUTRALIZATION ASSAY

Serum was prepared by centrifugation at 3000 rpm for 5 min, followed by heat inactivation at 56°C for 30 min. Virus neutralization assays were done as previously described (Halbert et al., 2000; Calcedo et al., 2009). Briefly, rAAV6-CMV-GFP or rAAV6-CMV-hPlAP was diluted to $1 \times 10^9$ genome-containing particles per ml. Serum was added to 100 μl of diluted virus to achieve the desired final serum dilution (1:20, 1:50, 1:200, 1:500, or 1:800). The virus and serum were incubated for 1 h at 37°C, and 80 μl were added to HT-1080 cells plated at $2 \times 10^4$ cells per well (12 well plates) the previous day. Two days following infection, plates transduced with rAAV6-CMV-GFP were counted with fluorescence activated cell sorting (FACS). Cells transduced with rAAV6-CMV-hPlAP were stained for hPlAP expression and the number of positive cells per field was quantified.

### HISTOLOGICAL ANALYSIS

Muscle tissue was frozen in liquid nitrogen-cooled isopentane embedded in Tissue-Tek OCT medium (Sakura Finetek USA, Torrance, CA, USA) and sectioned transversely in cryostat at

10 μm. For hPLAP staining, sections were fixed with ice cold 4% paraformaldehyde, washed three times in cold phosphate-buffered saline, placed in 65°C phosphate-buffered saline for 90 min, rinsed in room temperature phosphate-buffered saline, and washed in alkaline phosphatase buffer (0.1 mol/l Tris–HCl pH 9.5, 0.1 mol/l NaCl, 0.01 mol/l $MgCl_2$) for 10 min. Excess liquid was removed from the sections, and Sigma *FAST* BCIP/NBT substrate solution (Sigma, St Louis, MO, USA) was applied to each section for 30 min at room temperature, in the dark. Slides were rinsed three times in room temperature phosphate-buffered saline, dehydrated in 70% EtOH for 5 min, 2× (95% EtOH for 2 min), 2× (100% EtOH for 2 min), 2× (xylene for 3 min), and coverslipped with Permount mounting media (Fisher Scientific, Fair Lawn, NJ, USA). Images were captured using QIcam or Olympus digital cameras and processed using QCapture Pro (QImaging, BC, Canada). For hematoxylin and eosin m thickness were briefly fixed in methanol and staining, cryosections of 10 stained with Gill's hematoxylin and eosin–phyloxine. The sections were washed, dehydrated, and cleared in xylene before mounting with Permount.

### LUMINOMETRY ASSAY

After sacrifice of CPV-vaccinated mice, the gastrocnemius muscle was rapidly excised and flash frozen in liquid nitrogen. Frozen muscles were then powdered using a mortar and pestle and the protein was extracted with a protease-inhibiting buffer containing 137 mM NaCl, 20 mM Tris–HCl, pH 7.6, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.2% Tween 20 (Amersham), and 1× Complete protease inhibitor (Roche, Indianapolis, IN, USA). Protein was quantified via spectrophotometric absorption using Bradford reagent (Peirce, Rockford, IL, USA). The extract was then analyzed for hPlAP expression using a commercial luminometry kit (Applied Biosystems, Carlsbad, CA, USA)

### VECTOR GENOME QUANTIFICATION

Muscles were snap frozen in liquid nitrogen and then pulverized with a mortal and pestle. Pulverized muscle tissue was resuspended in tissue lysis buffer [0.5% NaDOC, 50 mM Tris, 150 mM NaCl, 1% Triton, 0.8% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)]. DNA was isolated from cell and tissue lysates using a DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA) according the manufacturer's guidelines. Genome quantification was performed utilizing a SV40 polyA-specific probe and quantitative-PCR, as previously described (Gregorevic et al., 2004).

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# Improved immunological tolerance following combination therapy with CTLA-4/Ig and AAV-mediated PD-L1/2 muscle gene transfer

***Sahil Adriouch[1,2]*, Emilie Franck[1,2], Laurent Drouot[1,2], Carole Bonneau[1], Nelly Jolinon[3], Anna Salvetti[3] and Olivier Boyer[1,2,4]****

[1] *INSERM U905, Faculty of Medicine and Pharmacy, Rouen, France*
[2] *IFRMP 23, Institute for Biomedical Research, University of Rouen, Rouen, France*
[3] *INSERM U758 and École Normale Supérieure de Lyon, Lyon, France*
[4] *Department of Immunology, Rouen University Hospital, Rouen, France*

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Ashley Thomas Martino, St. John's University, USA*
*Ou Cao, University of Florida, USA*

***Correspondence:***
*Sahil Adriouch and Olivier Boyer, INSERM U905, Faculty of Medicine and Pharmacy, 22 boulevard Gambetta, F-76000 Rouen, France.*
*e-mail: sahil.adriouch@univ-rouen.fr; olivier.boyer@chu-rouen.fr*

Initially thought as being non-immunogenic, recombinant AAVs have emerged as efficient vector candidates for treating monogenic diseases. It is now clear however that they induce potent immune responses against transgene products which can lead to destruction of transduced cells. Therefore, developing strategies to circumvent these immune responses and facilitate long-term expression of transgenic therapeutic proteins is a main challenge in gene therapy. We evaluated herein a strategy to inhibit the undesirable immune activation that follows muscle gene transfer by administration of CTLA-4/Ig to block the costimulatory signals required early during immune priming and by using gene transfer of PD-1 ligands to inhibit T cell functions at the tissue sites. We provide the proof of principle that this combination immunoregulatory therapy targeting two non-redundant checkpoints of the immune response, i.e., priming and effector functions, can improve persistence of transduced cells in experimental settings where cytotoxic T cells escape initial blockade. Therefore, CTLA-4/Ig plus PD-L1/2 combination therapy represents a candidate approach to circumvent the bottleneck of immune responses directed toward transgene products.

**Keywords: gene therapy, AAV vectors, costimulation, CTLA-4, PD-1, PD-L1, PD-L2, tolerance**

## INTRODUCTION

Since the original reports describing the use of adeno-associated virus (AAV) vectors for transfer of β-galactosidase gene to muscle (Kessler et al., 1996; Xiao et al., 1996; Fisher et al., 1997), recombinant AAVs (rAAV) have emerged as very efficient and potentially non-immunogenic vector candidates for delivering therapeutic genes to a variety of tissues and treating monogenic diseases. As they poorly activate innate immunity and weakly transduce dendritic cells, rAAV appear as far less immunogenic than adenoviral vectors (Zhang et al., 2000; Zaiss et al., 2002; McCaffrey et al., 2008). Nevertheless, it has rapidly become clear that rAAV vectors carrying various transgenes can, under different conditions, induce potent immune responses that could ultimately lead to destruction of transduced cells *in vivo* and consecutive disappearance of transgene expression (Manning et al., 1997, 1998; Halbert et al., 1998; Brockstedt et al., 1999). Not surprisingly therefore, their use has even been proposed in genetic vaccination protocols aimed at eliciting cellular and humoral immune responses against different microorganisms (Kuck et al., 2006; Du et al., 2008). In primates and humans, rAAV administration has also been documented to elicit significant cytotoxic $CD8^+$ T cell responses directed against the viral as well as the transgenic "exogenous" proteins, resulting in the destruction of transduced cells and complete loss of transgene expression (Manno et al., 2006; Mingozzi et al., 2007; Gao et al., 2009). Additionally, on the side of the humoral immunity, production of neutralizing antibodies targeting capsid proteins may also prevent vector readministration and accelerate the loss of the therapeutic protein through the formation of immune complexes. Such immune complexes may further sensitize the cellular immune response by enhancing cross-presentation of the transgenic protein by the antigen-presenting cells (APC). Therefore, developing strategies to circumvent immune responses and facilitate long-term expression of transgenic therapeutic proteins has been identified as one of today's main challenges for the translation of rAAV vectors into the clinic (Mingozzi and High, 2011a,b; Nayak and Herzog, 2011).

Depending on the experimental situation, rAAV-mediated gene transfer can either lead to durable transgene expression or, conversely, to the rapid formation of neutralizing antibodies and/or destruction of transduced cells by cytotoxic cells. Several factors influencing the immune response against transgenic proteins encoded by the rAAV vectors have now been identified including host species, route of administration, vector dose, immunogenicity of the transgenic protein, inflammatory status of the host and capsid serotype (Mays and Wilson, 2011). These factors are thought to influence immunogenicity by triggering innate immunity, cytokine production, APC maturation, antigen presentation and, ultimately, priming of naïve T lymphocytes to functional effectors. Therefore, the idea to dampen immune activation by

interfering with these very mechanisms has logically emerged with the aim to induce a short-term immunosuppression, avoid the early immune priming that follows vector administration and promote long-term tolerance (Zaiss and Muruve, 2008).

Here, we evaluated two different strategies to inhibit the undesirable immune activation that follows muscle gene transfer by acting at two different checkpoints of the immune response, i.e., on T cell priming or on the functions of activated T cells that may escape such priming blockade. We used the administration of CTLA-4/Ig to inhibit the substantial immune priming that immediately follow vector injection. Indeed, CTLA-4/Ig represent a potent immunosuppressive fusion protein that reversibly prevents T cell activation (Wallace et al., 1995) and is now used in the clinic to treat inflammatory diseases such as rheumatoid arthritis (Bluestone et al., 2006). Its immunomodulatory action depends on its competitive inhibitory effect on the CD28/B7 pathway thereby preventing the pivotal CD28-dependent costimulation required to fully activate T lymphocytes (Salomon and Bluestone, 2001). As a second strategy, we turned to immunomodulatory molecules that could protect transduced muscle fibers from immune attacks by activated T cells. For that, we aimed at stimulating the inhibitory PD-1 molecule expressed on T cells upon activation, by the gene transfer of its ligands PD-L1 or PD-L2 to muscle cells (Freeman et al., 2000; Latchman et al., 2001; Ishida et al., 2002). We show herein that acting on these two non-redundant mechanisms of tolerance provides synergistic effects that prolong transgene expression in muscle even in the presence of circulating cytotoxic T cells directed against the transgene product.

## MATERIALS AND METHODS

### ANIMAL EXPERIMENTS

Female C57BL/6 (B6) mice were obtained from Centre d'Elevage Janvier (Le Genest Saint Isle, France). Mice were all between 7 and 10 weeks of age at beginning of experiments. For transduction with rAAV vectors, mice back legs were shaved under general anesthesia and titrated $1 \times 10^{11}$ vector genomes (vg) rAAV2/1-Ova or rAAV2/8-Ova were injected (50 µl in PBS) in the gastrocnemius muscles. Where indicated, $10^{11}$ vg rAAV2/1-PD-L1 or rAAV2/1-PD-L2 were mixed with $10^{11}$ vg rAAV-Ova and co-injected using the same procedure. For costimulation blockade experiments, mice were injected i.p. with 200 µg CTLA-4/Ig (Chimerigen laboratories, MF-110A4) diluted in 200 µl of PBS.

In some experiments, lymphocytes from tolerized mice were transferred to conditioned recipients to further evaluate the presence of anti-Ova lymphocytes and to study their functionality *in vivo*. For that, $50 \times 10^6$ splenocytes harvested from individual mice that have be treated 80 days before with AAV-Ova, with or without immunomodulatory regimens, were injected i.v. into 5 Gy-irradiated syngenic C57BL/6 recipient. One day after, each individual mouse was shaved and injected subcutaneously with $1 \times 10^6$ syngenic EG7 tumor cells expressing the Ova antigen and known to be sensitive *in vivo* to $CD8^+$ T cell cytotoxicity in primed animals (Moore et al., 1988). Tumor sizes were measured with a digital caliper three times a week during 26 days. Tumor volume was calculated as length × width × [(length + width)/2].

All animal experiments were approved by the local institutional ethic committee for animal experimentation (authorization #0211-22 "Comité Régional d'Éthique en Expérimentation Animale de Normandie").

### PLASMIDIC CONSTRUCTS AND PREPARATION OF RECOMBINANT AAV VECTORS

The rAAV-Ova vector, a kind gift of Roland W. Herzog, was described previously (Wang et al., 2005). The cDNA encoding mouse PD-L1 (CD274) or PD-L2 (CD273) were cloned using standard molecular biology procedures and introduced in the SSV9–CAG plasmidic backbone after digestion with *Eco*RI. The resulting expression cassette, flanked by AAV serotype 2 inverted terminal repeats (ITRs), contains the CAG promoter combining the cytomegalovirus early enhancer and the chicken β-actin promoter, a chicken β-actin intron, and a rabbit β-globin polyadenylation signal. rAAV2/1 and rAAV2/8 vectors were generated using a standard helper-virus free three-plasmid transient transfection method and pseudotyped with either AAV1 or AAV8 capsid proteins. Vectors were purified by two cesium chloride gradient centrifugations and dialyzed against PBS as described (Salvetti et al., 1998). Genome titers of vector preparations were assayed by Dot-blot hybridization using a probe to detect the CAG or CMV promoter.

### sOVA AND ANTI-OVA IgG ELISA

Quantification of soluble Ova (sOVA) concentration in serum was performed by Ova-specific ELISA. Microtiter plates were coated with polyclonal rabbit anti-Ova antibodies (1:3000 dilution, Ray-Biotech) and bound sOVA was detected using biotinylated rabbit polyclonal anti-Ova antibodies (1:5000 dilution, Abcam) and streptavidin–peroxidase (1:15000, Roche).

Detection of serum anti-Ova IgG antibodies was performed by ELISA using Ova-coated microtiter plates. Anti-Ova IgG antibodies were detected using biotinylated polyclonal goat anti-mouse IgG and revealed using the mouse ExtrAvidin kit (Sigma-Aldrich). IgG titers were defined as the dilution yielding the half maximum optical density obtained with control serums and was calculated using sigmoid curve fitting using GraphPad prism software.

### QUANTIFICATION OF Ova cDNA AND mRNA IN TRANSDUCED MUSCLES

To analyze the quantities of Ova DNA and Ova mRNA present in transduced muscles of treated mice at indicated time points, a real-time PCR assay was developed. Muscles collected from each mouse were kept at −80°C before DNA and RNA extraction performed using the phenol/chloroform method and the RNeasy Fibrous Tissue Mini Kit (Qiagen) following the manufacturer's instructions.

For quantification of Ova DNA, the primers used were Ova-F (5′-AAG CAG GCA GAG AGG TGG TA-3′), Ova-R (5′-GAA TGG ATG GTC AGC CCT AA-3′), CD8a-F (5′-GGT GCA TTC TCA CTC TGA GTT CC-3′), and CD8a-R (5′-GCA GAC AGA GCT GAT TTC CTA TGT G-3′). For all reaction mixtures, 10 µl of FastStart Universal SYBR green master mix (Roche) was used in a final volume of 20 µl. Ova primers were used at 500 nM and CD8a primers at 400 nM. Approximately 10 ng of DNA was added in a 5-µl volume and always set up in duplicate. Each qPCR was performed under the following conditions: 10 min hot-start denaturation at 95°C and 40 amplification cycles (10 s at 95°C,

30 s at 60°C). The melting temperatures of the final double-strand DNA products were determined by gradual heating from 60 to 95°C over 20 min. All qPCRs were performed with a StepOnePlus real-time PCR system (Applied Biosystems) and corresponding software. Absolute amounts of Ova and CD8a amplicons, in arbitrary units, were determined using serial dilutions of pAAV–CMV–OVA plasmid or pTOPO-CD8a plasmid as a standard. The data were expressed as Ova/CD8a ratios, fixed at 1 for PBS-injected control mice.

For quantification of Ova mRNA, 100 ng of total RNA were reverse transcribed using iScript DNA Synthesis Kit (Biorad). Then, 2 μl of cDNA were subjected to real-time PCR amplification using Ova primers, β-actin-F (5′-AAG ATC TGG CAC CAC ACC TTC T-3′) and β-actin-R (5′-TTT TCA CGG TTG GCC TTA GG-3′) primers. For all reaction mixtures, 10 μl of FastStart Universal SYBR green master mix (Roche) was used in a final volume of 20 μl, Ova primers were used at 500 nM and β-actin primers at 400 nM. The same qPCR program as above-described conditions were used. The absolute amount of Ova mRNA for each sample was then normalized against the β-actin mRNA amount (arbitrary units) and determined using serial dilutions of pAAV–CMV–OVA plasmid and β-actin purified PCR product.

### FLOW CYTOMETRY AND ELISPOT ASSAYS

Fluorescently labeled anti-CD4 (RM4-5), -CD8 (53-6.7), -CD44 (IM7), -CD62L (MEL-14), -PD-1 (J43), -PD-L1 (B7-H1), -PD-L2 (B7-DC) monoclonal antibodies (mAb), and unlabeled anti-CD16/CD32 antibodies were all purchased from eBioscience. PE-conjugated H-2K$^b$/Ova$_{257–264}$ pentamers were used to detect the CD8$^+$ cells that specifically recognize the immunodominant Ova-derived SIINFEKL peptide using the manufacturers' protocol (Proimmune). Flow cytometry measurements of single cell suspensions derived from lymph nodes, spleen, or blood samples were performed using standard procedures and acquired on a FACSCanto (BD Biosciences) instrument. Flow cytometry analyses were performed using the FlowJo software (Tree Star).

Enzyme-linked immunospot (ELISpot) assays were performed following the instructions of the manufacturer (Diaclone). Briefly, PVDF membrane plates were coated with capture antibody against mouse IFNγ and blocked with a 2% skimmed milk solution prepared in PBS. $2 \times 10^5$ Splenocytes per well were cultured *in vitro* for 36 h in the presence of 10 μg/ml SIINFEKL peptide. Plates were revealed after incubation with anti-mouse IFNγ detection antibody coupled to biotin followed with a streptavidin–alkaline phosphatase conjugate and exposure to a ready-to-use solution of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP) for chromogenic development. Plates were analyzed with an ELISpot plate reader and a dedicated ImmunoSpots software (C.T.L.).

### DATA REPRESENTATION AND STATISTICAL ANALYSIS

Results were expressed as mean ± SEM. Significance was assessed by non-parametric one-way ANOVA (Kruskal–Wallis tests) using the GraphPad Prism software. Results were considered statistically significant when the *p* value was inferior to 0.05 (*), to 0.01(**) or to 0.001 (***).

## RESULTS

### CHARACTERIZATION OF THE ANTI-Ova CELLULAR AND HUMORAL IMMUNE RESPONSES AFTER rAAV2/1-Ova OR rAAV2/8-Ova-MEDIATED MUSCLE GENE TRANSFER

We used rAAV2/1-Ova and rAAV2/8-Ova administration to model a gene therapy setting where the transgene encodes for a highly immunogenic secreted protein. We first evaluated the capacity of these vectors to transduce muscle cells upon direct i.m. injection. Injection of $10^{11}$ viral genome (vg) rAAV2/1-Ova or rAAV2/8-Ova similarly resulted in efficient transduction, as attested by the detection of Ova DNA and mRNA in the injected muscles quantified by qPCR and qRT-PCR respectively (**Figure 1A**). No significant difference was found when comparing the transduction efficiency of rAAV2/1-Ova and rAAV2/8-Ova in these conditions.

Transgene expression in muscle was found to be high early after transduction but rapidly declined over time, suggesting the occurrence of an immune response against transduced cells (**Figure 1A**). To investigate this point, we quantified the cellular and humoral immune responses directed against the xenogenic Ova transgene product by flow cytometry and ELISA. Staining with MHC-I pentamers presenting the immunodominant Ova-derived SIINFEKL peptide, designated hereafter as H-2K$^b$/Ova pentamers, allowed to monitor the expansion of CD8$^+$ lymphocytes in spleen and draining lymph nodes of rAAV-Ova challenged animals. This analysis revealed that both rAAV2/1-Ova and rAAV2/8-Ova injected in the gastrocnemius muscles induced a robust expansion of anti-Ova CD8$^+$ lymphocytes (**Figures 1B,C**). Interestingly, the kinetics and intensity of T cell priming was found to be different when comparing the cells harvested from the draining lymph nodes and from the spleen. In the lymph nodes draining the injected muscles, anti-Ova CD8$^+$ T cells expanded early after AAV challenge to reach around 2% of the CD8$^+$ T cell compartment at day 7 post-injection (**Figure 1C**, left panel). In contrast, when analyzing splenocytes, anti-Ova CD8$^+$ T cells were found to reach up to 10% of the total CD8$^+$ subset but only at day 14 after i.m. AAV injection (**Figure 1C**, right panel). These results are consistent with a model in which the local cellular immune response measured in the draining lymph nodes would be induced by an rAAV leakage from the injected muscle and the consequent transduction of non-muscle cells such as dendritic cells, as shown for AAV5 (Xin et al., 2006) or AAV1 (Lu and Song, 2009) for instance. The systemic response detected in the spleen would rather reflect systemic immunization directed against the circulating sOva protein after it has been produced by transduced muscle cells. This interpretation is supported by the kinetics of sOva apparition and antibodies production (**Figure 1D**). Indeed, in the serum of AAV-treated mice, sOva reached a maximum at day 7 post-injection and rapidly declined thereafter (**Figure 1D**, green curves). Consistently, anti-Ova IgG appeared between day 7 and 12 after AAV i.m. injection, correlating with the disappearance of sOva in the serum after day 7 (**Figure 1D**, blue curves). The latter probably reflects the *in vivo* clearance of sOva following the formation of antigen–antibody immune complexes, although we cannot completely rule out that anti-Ova IgG antibodies may mask Ova epitopes in the immunoassay. Together, these data reveal that i.m. injection of

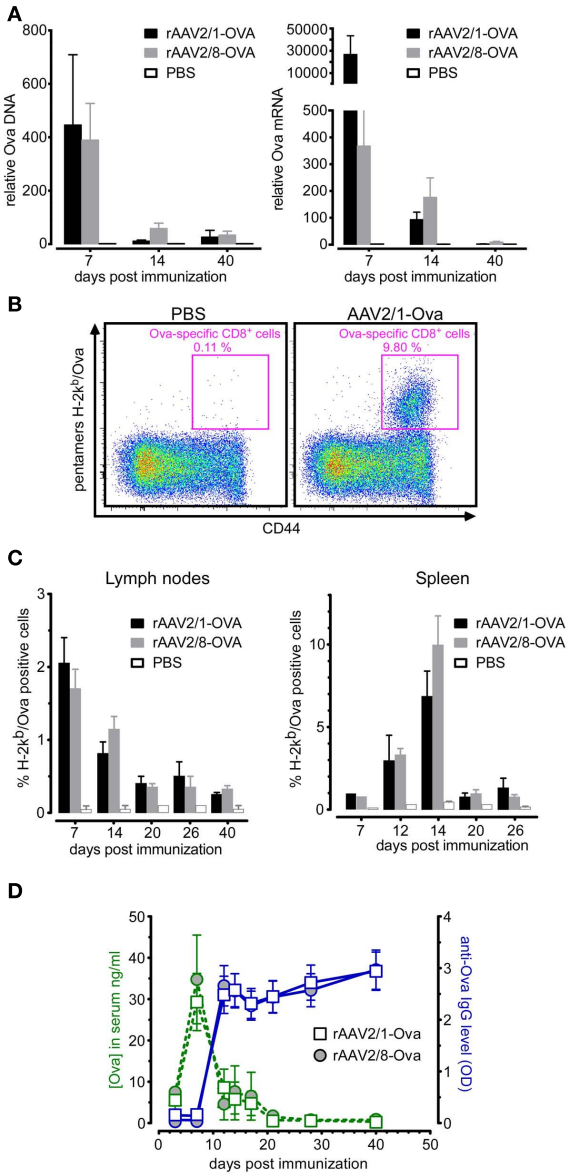


**FIGURE 1 | Characterization of anti-Ova immune responses following muscle gene transfer using rAAV2/1-Ova or rAAV2/8-Ova.** Group of four to seven mice were injected with titrated $1 \times 10^{11}$ viral genomes (vg) of either rAAV2/1-Ova or rAAV2/8-Ova in the gastrocnemius muscles at day 0 and then culled at different time points. **(A)** Gastrocnemius muscles were analyzed for the presence of Ova DNA and mRNA by qPCR and qRT-PCR, respectively, at 7, 14, and 40 days post-gene transfer. **(B)** Representative flow cytometry profile and **(C)** bar graphs showing the percentage of Ova-specific $CD8^+$ T cells positively stained with H-$2K^b$/Ova pentamers in draining lymph nodes and spleen at different time points after gene transfer. **(D)** Sera were harvested from individual mice at different time points and analyzed by ELISA for the detection of sOva (green lines) and anti-Ova IgG (blue lines).

rAAV encoding sOva is strongly immunogenic and provides a mouse model that is suited to stringently evaluate the efficacy of tolerance induction protocols.

## CTLA-4/Ig BUT NOT PD-L1 GENE TRANSFER PREVENTS THE PRIMING OF THE IMMUNE RESPONSES FOLLOWING rAAV2/1-Ova ADMINISTRATION

We investigated the possibility to block immune responses directed against the transgene product by two distinct immunomodulatory strategies, one blocking lymphocyte priming (i.e., CTLA-4/Ig) and the other inhibiting the function of lymphocytes targeting transduced muscle fibers (i.e., activation of the PD-1 pathway by muscle gene transfer of PD-1 ligands). Indeed, we have previously shown in transgenic mice that express Ova as a neo-autoantigen in muscle that tolerant transgenic anti-Ova $CD8^+$ T cells up-regulated PD-1, suggesting that PD-1 plays a role in the induction of tolerance to skeletal muscle-expressed Ag (Calbo et al., 2008). As expected, the anti-Ova $CD8^+$ T cell response that followed an i.m. injection of rAAV2/1-Ova (**Figures 1B,C**) was accompanied by an up-regulation of PD-1 on $CD8^+$ T cells that exhibit an activated $CD44^{hi}$ phenotype (**Figure 2A**). As activated T cells express PD-1 and PD-1 ligands inhibit their activation and function, we generated rAAV2/1-PD-L1 and rAAV2/1-PD-L2 vectors and first verified that they were able to efficiently transduce HEK cells *in vitro* (**Figure 2B**). The expected consequence of injecting these vectors i.m. is that they should not prevent T cell priming but rather inhibit the cytotoxic activity of T cells directed against muscle-expressed transgenic proteins.

To investigate the *in vivo* immunomodulatory potential of CTLA-4/Ig and rAAV2/1-PD-L1, we either administered 200 μg of CTLA-4/Ig i.p. or co-injected rAAV2/1-PD-L1 i.m. at the same time as rAAV2/1-Ova, and monitored the cellular and humoral immune response 14 day after. Results showed that even a single dose of CTLA-4/Ig was efficient to completely prevent the priming of anti-Ova $CD8^+$ T cells (**Figure 2C**, left panel) and the apparition of anti-Ova IgG in the serum of treated animals (**Figure 2C**, middle panel). Accordingly, this immunosuppressive treatment allowed a sustained sOva production that could be assayed in the serum at day 14 whereas it was undetectable in controls (**Figure 2C** right panel). We concluded from these data that transient immunosuppression by a single dose of CTLA-4/Ig is very efficient to prevent the priming of the cellular and humoral response at early time points after AAV transduction.

In contrast, despite a slight but not statistically significant tendency to reduce immune responses, rAAV2/1-PD-L1 co-injected with rAAV2/1-Ova failed to provide the same effect on immune priming (**Figure 2C**, left and middle panels). Also, treatment with rAAV2/1-PD-L1 did not allow the persistence of sOva in the serum in agreement with the significant amount of anti-Ova IgG detected in the serum of these animals (**Figure 2C**). This expected result confirmed that local transduction of muscle cells with rAAV2/1-PD-L1 can not interfere with the systemic immune priming against sOva since activation of the PD-1/PD-L1 pathway was anticipated to regulate T cells functions rather than their priming.

We further analyzed the presence of Ova DNA and mRNA in muscles at day 40 in the different groups of treated mice by qPCR and qRT-PCR, respectively. This further confirmed that suppression of immune priming by CTLA-4/Ig promotes the persistence of transduced cells *in vivo* as measured by the detection of significantly higher levels of Ova DNA and mRNA in the muscles of treated animals (**Figure 2D**). By contrast, injection

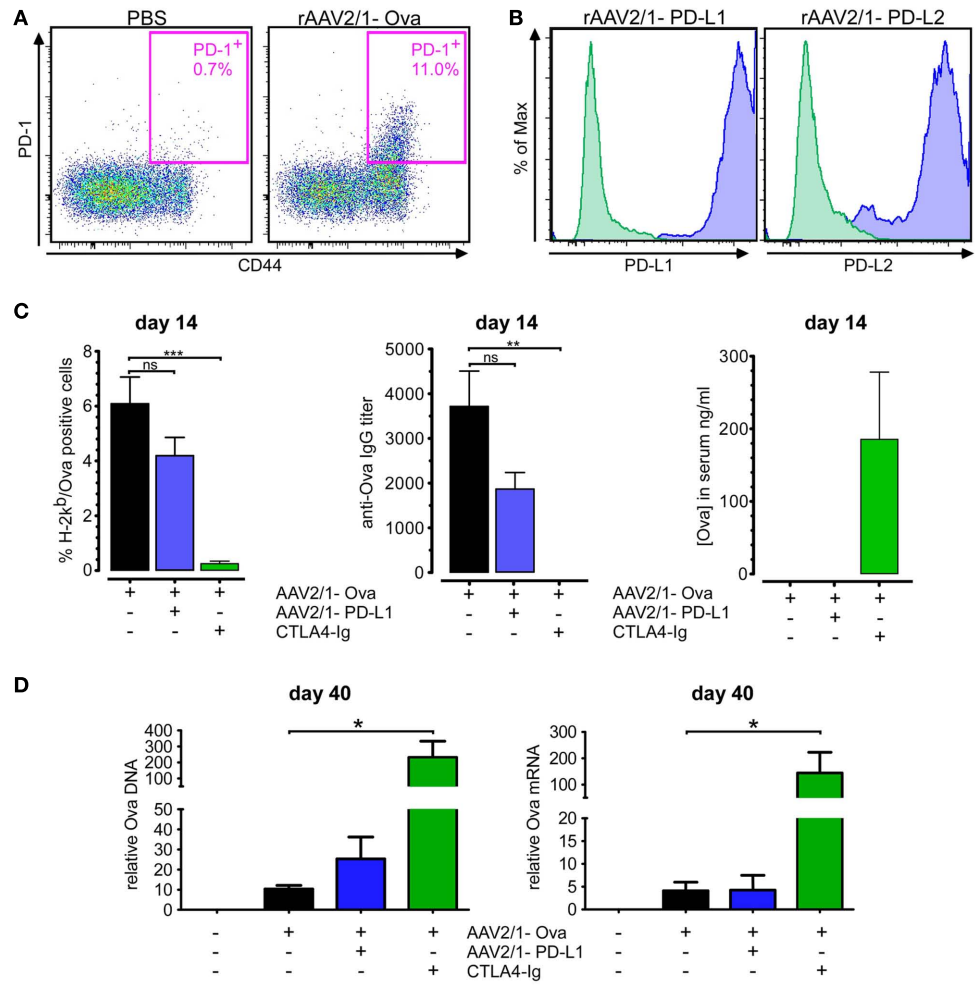


**FIGURE 2 | A single injection of CTLA-4/Ig dramatically inhibits immune responses and improves transgene persistence at early time points. (A)** Mice were injected as in **Figure 1** with $1 \times 10^{11}$ vg rAAV2.1-Ova in the gastrocnemius muscles at day 0. Splenic $CD8^+$ T cells were analyzed at day 14 by flow cytometry for expression of CD44 and PD-1. **(B)** rAAV2/1-PD-L1 and rAAV2/1-PD-L2 vectors were designed, produced, and tested for their capacity to transduce HEK-293 cells *in vitro*. For this, cells were analyzed by flow cytometry 3–5 days after their transduction with 1/10th dilution of the concentrated virus stocks. Controls correspond to unmanipulated HEK-293 parental cells stained with the same antibodies (green histograms). **(C)** The immunosuppressive potential of rAAV2/1-PD-L1 and CTLA-4/Ig were evaluated *in vivo*. Mice were injected with $10^{11}$ vg rAAV2/1-Ova in the gastrocnemius muscles and received or not a co-injection of $10^{11}$ vg rAAV2.1-PD-L1, or 200 μg of CTLA-4/Ig injected contemporaneously by the i.p. route. Blood samples were then collected 14 days later to analyze the percentage of anti-Ova $CD8^+$ T cells, the level of anti-Ova IgG and the presence of sOva in the serum. **(D)** Gastrocnemius muscles were then collected at day 40, and Ova DNA and mRNA were quantified by qPCR and qRT-PCR.

of rAAV2/1-PD-L1 did not significantly improve transgene persistence in these settings, in line with the prominent systemic immune response detected at day 14 (**Figure 2D**).

## COMBINED TREATMENT WITH CTLA-4/Ig AND rAAV2/1-PD-L1 SYNERGISTICALLY ACTS TO IMPROVE TRANSGENE PERSISTENCE AND EXPRESSION

Whereas a single dose of CTLA-4/Ig was efficient to suppress the systemic immune response at day 14 (**Figures 3A,B** left panels), this was not the case when further monitoring the immune response at day 40. Indeed, anti-Ova $CD8^+$ T cell expansion as well as anti-Ova IgG immune responses were readily detectable at day 40 in mice that had received rAAV2/1-Ova and CTLA-4/Ig at day 0 (**Figures 3A,B**). Therefore, early suppression of immune priming by a single dose of CTLA-4/Ig is not sufficient to permanently wipe out the immune responses against transgene product in these experimental conditions. Instead, continuous production of sOva by transduced muscle cells sensitizes the immune response at later time points when CTLA-4/Ig has been cleared from the circulation of treated mice.

We next tested whether rAAV2/1-PDL-L1 or rAAV2/1-PDL-L2 could synergize with CTLA-4/Ig to improve transgene tolerance at later time points when the initial CTLA-4/Ig monotherapy was evidently not efficient alone to completely block immune sensitization against sOva. This combined strategy may indeed target two non-redundant mechanisms of immunomodulation acting

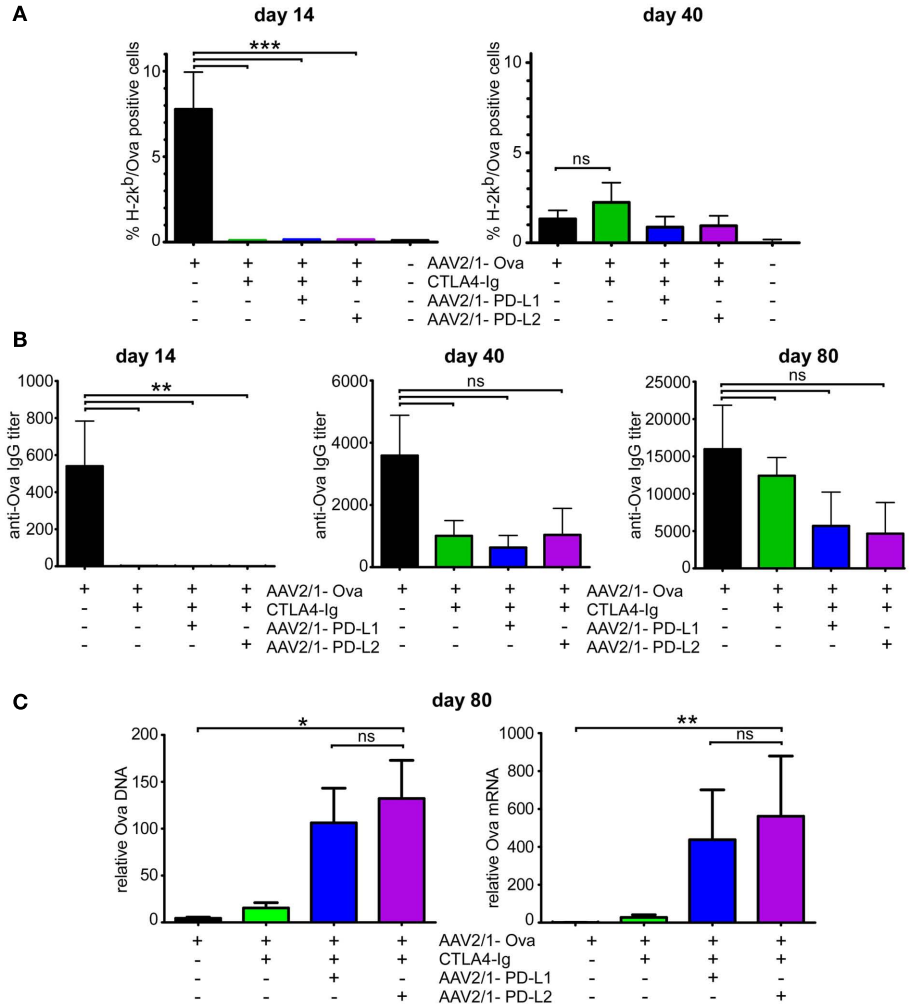


**FIGURE 3 | Combination therapies using CTLA-4/Ig and rAAV-PD-L1 or rAAV-PD-L2 significantly improve transgene persistence and transcription.** Mice were injected with $1 \times 10^{11}$ vg rAAV2/1-Ova in the gastrocnemius muscles at day 0 and received at the same time an immunomodulatory regimen consisting of either 200 μg of CTLA-4/Ig alone or the same amount of CTLA-4/Ig together with $1 \times 10^{11}$ vg rAAV2/1-PD-L1 or rAAV2/1-PD-L2. **(A)** Blood samples were collected at day 14 and 40 to evaluate the percentage of $CD8^+$ T cells specifically recognizing the immunodominant epitope derived from Ova by flow cytometric analyses. **(B)** Sera were collected at days 14, 40, and 80 to assay the level of anti-Ova IgG antibodies by ELISA. **(C)** Injected gastrocnemius muscles were collected at day 80 and analyzed for the presence of Ova DNA and mRNA by qPCR and qRT-PCR respectively.

either on the APC side for CTLA-4/Ig or on the target tissue side for rAAV2/1-PD-L1 and rAAV2/1-PDL-L2. To test this possibility, groups of mice were transduced i.m. with rAAV2/1-Ova and also received at the same time either rAAV2/1-PD-L1 or rAAV2/1-PD-L2 i.m. and CTLA-4/Ig i.p. combination therapies. We then evaluated the cellular and humoral immune responses at different time points and finally evaluated the persistence of transduced muscle cells by quantification of Ova DNA and mRNA at day 80 (**Figure 3**).

Remarkably, whereas CTLA-4/Ig alone was inefficient, rAAV2/1-PD-L1 co-administered with CTLA-4/Ig at day 0 significantly improved transgene persistence, as measured by quantification of Ova DNA by qPCR (**Figure 3C**, left panel). Accordingly, mRNA analysis by qRT-PCR at day 80 revealed sustained transcription of the transgene in muscle (**Figure 3C**, right panel). Of note, rAAV2/1-PD-L2 appeared equally as effective as rAAV2/1-PD-L1 to provide this effect when combined with CTLA-4/Ig. Importantly, this protective effect occurred in a setting where neither CTLA-4/Ig alone nor the tested combination therapies could block immune response at days 40 and 80 (**Figures 3A,B**). To further attest the presence of functional anti-Ova cytotoxic T cells at day 80 (a time point when pentamer staining is not sensitive enough to detect anti-Ova T cells, not shown), we analyzed by ELISpot the capacity of $CD8^+$ T cells to secrete IFNγ when stimulated with the Ova-derived immunodominant SIINFEKL peptide. This assay revealed the presence of low numbers of functional anti-Ova $CD8^+$ T cells in the three groups of animals that had received CTLA-4/Ig with or without rAAV-PD-L1 or rAAV-PD-L2,

whereas the assay was negative for mice that did not receive rAAV-Ova and strongly positive for those that received rAAV-Ova in the absence of any immunoregulatory adjuvant therapy (**Figure 4A**). To definitively demonstrate that these IFNγ-secreting T cells were functional *in vivo*, we performed adoptive transfer experiments to non-lethally irradiated syngenic recipients that were inoculated with Ova-bearing tumor cells. Whereas tumors rapidly developed in recipients that had received T cells from control mice that only received PBS, recipient mice receiving lymphocytes from rAAV2/1-Ova injected donors rapidly developed an anti-tumor immune response that resulted in complete tumor rejection (**Figure 4B**). In agreement with the lower numbers of anti-Ova $CD8^+$ T cells detected by ELISpot, recipient mice that had received immune cells from donors treated with CTLA-4/Ig or the combined CTLA-4/Ig plus rAAV-PD-L1 or rAAV-PD-L2 therapy reacted with a slight delay but were also was also capable to efficiently control tumor growth (**Figure 4B**). Together, these results indicate that the cytotoxic activity of transgene product specific T cells that have escaped costimulation blockade can be functionally blocked by AAV-mediated gene transfer of PD-1 ligands in muscle tissues while they remain capable to reject tumor cells.

## DISCUSSION

Gene therapy for muscular dystrophies and other monogenic diseases aims at achieving long-term expression of a functional form of an otherwise deficient gene in target tissues. In this context, the product of the therapeutic gene is structurally different from its abnormal or absent counterpart, and consequently viewed as non-self by the immune system. Additional signals from the viral vector may further strengthen adverse innate and adaptive immune reactions against the transgene product, while the vector itself can be targeted by antiviral pre-existing or acquired immunity. Although the presence of natural epitopes in the non-mutated regions of the protein or the occasional occurrence of mutation reversion in a minute population of patients' cells (Klein et al., 1992) may yield some level of immunological tolerance, it remains that acquired immunity to the transgene product still represents one of the major obstacles to the success of gene therapy. Here, we provide the proof of principle that a combination immunoregulatory therapy targeting two non-redundant checkpoints of the immune response, i.e., priming and effector functions, can promote persistence of transduced target cells and transgene transcription thereof even when some cytotoxic T cells have escaped initial control.

A first strategy for the blockade of adaptive immunity is obviously to target costimulation pathways and therefore provide an early control on lymphocyte priming. Whereas several costimulatory pathways have now been identified, CD28-mediated T cell costimulation by APC expressing CD80/CD86 molecules from the B7 family is clearly prominent. Indeed, CD28 signaling strongly enhances T cell proliferation and survival, cytokine production and prevents induction of anergy after TCR-mediated activation (Boise et al., 1995). Consequently, anti-CD80 (B7.1) or anti-CD86 (B7.2) antibodies inhibit T cell priming and genetically engineered CD28-deficient T cells are strongly impaired in their capacity to expand (Green et al., 1994; Salomon and Bluestone, 2001). T cells activation also promotes up-regulation of the negative regulator CTLA-4, another ligand of CD80 and CD86 molecules, which transmits inhibitory signals that regulate activated T cell and provides a key homeostatic mechanism of the immune response (Fife and Bluestone, 2008). One of the early striking evidence of the key role of this molecule is that CTLA-4-deficient animal rapidly die

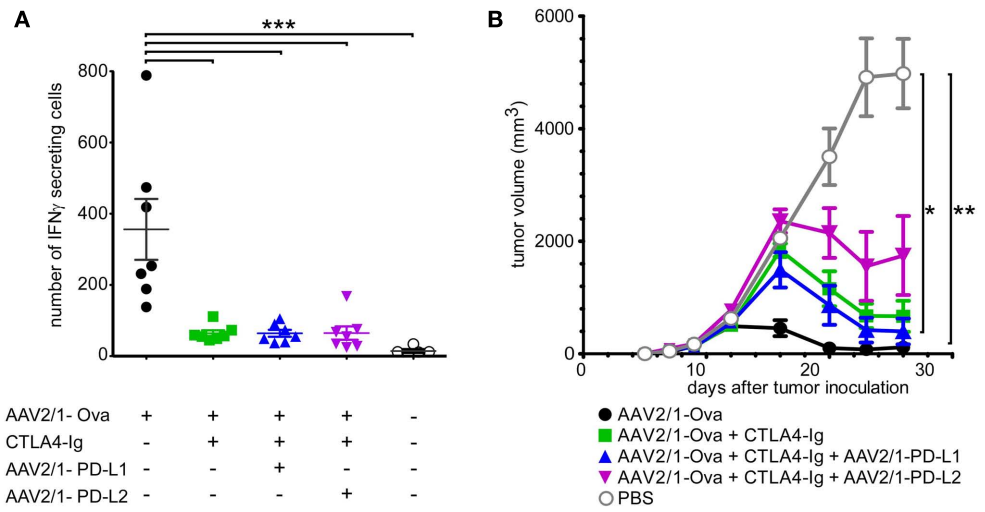


**FIGURE 4 | Detection of anti-Ova T cells in animals treated with combination therapies.** Seven mice per group were injected with $1 \times 10^{11}$ vg rAAV2/1-Ova in the gastrocnemius muscles at day 0 and received at the same time combination therapies as in **Figure 3**. Splenocytes were then harvested 80 days after and analyzed for the presence of lymphocytes capable to respond to Ova antigen. **(A)** Splenocytes were analyzed by ELISpot as described in the Section "Materials and Methods" for their capacity to secrete IFNγ after *in vitro* restimulation with the Ova-derived immunodominant SIINFEKL peptide. **(B)** $5 \times 10^7$ Splenocytes from the same mice were also adoptively transferred to 5 Gy-irradiated syngenic mice inoculated 1 day after with Ova-bearing EG7 tumor cells. The capacity of transferred lymphocytes to reject the tumors was monitored three times per week by measuring the tumor size with a digital caliper.

from massive lymphoproliferative syndrome and dysregulation of immunity (Waterhouse et al., 1995).

One convenient way to pharmacologically block the CD28 costimulation pathway is to use the soluble CD80/CD86 ligand CTLA-4/Ig. This fusion protein prevents CD28 interaction with CD80 and CD86 molecules on naïve and activated T cells and therefore provides a strong and early inhibition on T cell priming (June et al., 1990; Lenschow et al., 1992; Larsen et al., 2005). CTLA-4/Ig may also induce a negative signalization on the APC side through the induction of indoleamine2,3-dioxygenase (IDO) which catalyses the production of inhibitory kynurenine from tryptophan (Grohmann et al., 2002). CTLA-4/Ig may also possibly provide some level of APC depletion *in vivo*, although this aspect has been poorly documented and should critically depend on the form of CTLA-4/Ig used. Importantly, CTLA-4/Ig is now clinically available for the treatment of diseases such as rheumatoid arthritis (Dumont, 2004) and it is thus reasonable to propose its evaluation in the field of gene therapy. Indeed, there has been experimental evidence that CTLA-4/Ig in combination with a monoclonal antibody to CD40L (MR1) in the context of gene therapy can block immune responses and allow vector readministration in the mouse (Halbert et al., 1998; Lorain et al., 2008). It is difficult to weight the relative roles of CTLA-4/Ig and MR1 in these experiments but a plausible hypothesis is that CTLA-4/Ig provides the strongest immunoregulatory contribution since MR1 alone has been reported to be less efficient in similar experimental conditions (Manning et al., 1998). The results reported herein are consistent with these earlier reports and provide definitive evidence that CTLA-4/Ig alone efficiently, but only transiently, blocks anti-transgene T cell priming.

One concern about using CTLA-4/Ig in gene therapy is that discontinuation of treatment exposes patients to immunization against the transgene product. The present results illustrate this point since initial CTLA-4/Ig efficiently blocks the anti-Ova cellular and humoral responses while sustained sOva production finally elicits this undesired response when CTLA-4/Ig is probably cleared from the circulation. Our experimental conditions have been purposely designed to model this very situation where the immune response escapes the initial immunoregulatory regimen in order to evaluate if a second strategy could provide additional protection of transduced cells, i.e., PD-L1/2 gene therapy.

The PD-1/PD-L1 pathway has recently been identified as a negative regulator of immunity (Sharpe et al., 2007). The PD-1 immunoreceptor is also a member of the CD28/CTLA-4 family which, upon interaction with either one of its two ligands PD-L1 or PD-L2, down-modulates TCR signaling, reduces cytokine production and affects T cell survival by recruiting the SHP-2 tyrosine phosphatase, thereby dampening the PI3K and Akt pathway (Francisco et al., 2010). In BALB/c mice, PD-1 deficiency leads to the spontaneous development of a lethal autoimmune cardiomyopathy (Nishimura and Honjo, 2001). This disease is mediated by autoantibodies directed against the cardiac muscle autoantigen troponin I (Okazaki et al., 2003). Hence, PD-1 controls the physiological tolerance to muscle autoantigens. Also, PD-L1 was recently shown to regulate $CD8^+$ T cell-mediated muscle injury in a mouse model of myocarditis (Grabie et al., 2007). Several PD-1/PD-L1 dependant mechanisms may act synergistically to induce an effective effector T cell blockade and contribute to tolerance induction. As mentioned earlier, PD-1 engagement on the surface of T cell is known to significantly inhibit TCR signaling thereby preventing T cell activation and cytokine production. In addition to this mechanism, PD-L1 was recently shown to be a potent inducer of adaptive Tregs. Indeed, PD-L1 expressed on $CD8^+$ dendritic cell subset plays an important role on the conversion of naive lymphocytes toward $FoxP3^+$ regulatory T cells (Wang et al., 2008). Thus, engagement of PD-1 on T cells could induce cell-intrinsic tolerance (by blocking TCR signaling) as well as a dominant form of immunological tolerance by promoting the emergence of $FoxP3^+$ adaptive Tregs. Of note, manipulation of the PD-1/PD-L1 axis is also used by some tumors cells to tolerize surrounding lymphocytes. Indeed, expression of PD-L1 on the surface of malignant cells has been suggested to represent a subversive strategy used by tumors to escape to immuno-surveillance (Iwai et al., 2002). Thus, we believe that up-regulation of the expression of PD-L1 on the surface of muscle cells by the means of gene transfer should be able to induce tolerization of the immune system by preventing cytotoxicity of PD-1-expressing activated lymphocytes. However, PD-L1 gene transfer alone was not sufficient in our model to prevent the immune response directed against transduced muscle fibers (**Figure 2**), suggesting that muscle expression of PD-L1, i.e., at distance from lymphoid compartments, is probably not capable of inducing sufficient adaptive $FoxP3^+$ Tregs. Another explanation would be that PD-L1 expressed in muscle is not effective to block fully activated T cells that have received normal costimulatory signals (i.e., in the absence of CTLA-4/Ig co-administration). This interpretation would be in line with the finding that PD-1 inhibitory pathway could be overcome by CD28 costimulatory ligation in the presence of IL-2 (Freeman et al., 2000; Latchman et al., 2001; Carter et al., 2002; Ishida et al., 2002). Hence, the use of CTLA-4/Ig to block early CD28 costimulatory signaling may create favorable conditions where escaping T cells could become sensitive to PD-L1/2. This strengthens the notion that CTLA-4 and PD-1 signaling represent two distinct pathways acting synergistically to maintain tolerance (Fife and Bluestone, 2008). As shown herein, neither CTLA-4/Ig alone nor combination therapies could block immune response at days 40 and 80 (**Figures 3** and **4**). Our results are thus compatible with the notion that the use of rAAV2/1-PD-L1 or rAAV2/1-PD-L2 in combination with CTLA-4/Ig, does not completely inhibit the immune priming against sOva that most probably occurs at distance from the site of transduction (e.g., spleen and/or lymph nodes) but instead provides an additional protection by disarming lymphocytes within the target tissue.

It has been argued that the priming of T cells directed to the transgene product may be somehow defective after rAAV-mediated gene transfer (Lin et al., 2007a,b). However, in our experimental settings, transduced muscle fibers progressively disappear in the absence of immunoregulatory treatment (**Figure 2D**). Further, we show that even after CTLA-4/Ig alone or in combination therapies, an anti-Ova immune response is evidenced in ELISpot assays and in an *in vivo* model of tumor rejection upon adoptive transfer of T cells in recipient animals (**Figure 4**). Hence, forced up-regulation of PD-1 ligands in muscle cells yielded a level of protection of transduced cells from the cytotoxic assault

of circulating anti-Ova lymphocytes and provided in combination therapy a means to prolong transgene persistence and transcription. Therefore, CTLA-4/Ig plus PD-L1/2 combination therapy represents a candidate approach to circumvent the bottleneck of immune responses directed toward the transgene product and may deserve further investigation for non-secreted transgenic protein models and in larger animals.

## CONTRIBUTION

Sahil Adriouch designed research, performed experiments, analyzed data, and co-wrote the manuscript; Anna Salvetti and Olivier Boyer designed research and co-wrote the manuscript; and Emilie Franck, Laurent Drouot, Carole Bonneau, and Nelly Jolinon performed experiments.

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# Prevention and reversal of antibody responses against factor IX in gene therapy for hemophilia B

**Sushrusha Nayak[1], Debalina Sarkar[1], George Q. Perrin[1], Babak Moghimi[1], Brad E. Hoffman[1], Shangzhen Zhou[2], Barry J. Byrne[1] and Roland W. Herzog[1]***

[1] Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL, USA
[2] Division of Hematology, Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

**Edited by:**
*Katherine High, Children's Hospital of Philadelphia, USA*

**Reviewed by:**
*Dirk Dittmer, University of North Carolina at Chapel Hill, USA*
*Sing Sing Way, University of Minnesota, USA*

***Correspondence:**
*Roland W. Herzog, Cancer and Genetics Research Center, University of Florida, Room 203, 2033 Mowry Road, Gainesville, FL 32610, USA.*
*e-mail: rherzog@ufl.edu*

Intramuscular (IM) administration of an adeno-associated viral (AAV) vector represents a simple and safe method of gene transfer for treatment of the X-linked bleeding disorder hemophilia B (factor IX, F.IX, deficiency). However, the approach is hampered by an increased risk of immune responses against F.IX. Previously, we demonstrated that the drug cocktail of immune suppressants rapamycin, IL-10, and a specific peptide (encoding a dominant $CD4^+$ T cell epitope) caused an induction of regulatory T cells (Treg) with a concomitant apoptosis of antigen-specific effector T cells (Nayak et al., 2009). This protocol was effective in preventing inhibitory antibody formation against human F.IX (hF.IX) in muscle gene transfer to C3H/HeJ hemophilia B mice (with targeted F9 gene deletion). Here, we show that this protocol can also be used to reverse inhibitor formation. IM injection of AAV1–hF.IX vector resulted in inhibitors of on average 8–10 BU within 1 month. Subsequent treatment with the tolerogenic cocktail accomplished a rapid reduction of hF.IX-specific antibodies to <2 BU, which lasted for >4.5 months. Systemic hF.IX expression increased from undetectable to >200 ng/ml, and coagulation times improved. In addition, we developed an alternative prophylactic protocol against inhibitor formation that did not require knowledge of T cell epitopes, consisting of daily oral administration of rapamycin for 1-month combined with frequent, low-dose intravenous injection of hF.IX protein. Experiments in T cell receptor transgenic mice showed that the route and dosing schedule of drug administration substantially affected Treg induction. When combined with intravenous antigen administration, oral delivery of rapamycin had to be performed daily in order to induce Treg, which were suppressive and phenotypically comparable to natural Treg.

**Keywords: adeno-associated virus, factor IX, hemophilia, tolerance, antibody, gene therapy, muscle, rapamycin**

## INTRODUCTION

Gene therapy offers many advantages for treatment of the X-linked bleeding disorder hemophilia B, which is caused by mutations in coagulation factor IX (F.IX). In particular, adeno-associated viral (AAV) *in vivo* gene transfer to skeletal muscle or liver has been shown to direct long-term expression of functional F.IX in animal models, thereby reducing the incidence of spontaneous bleeding (Mingozzi and High, 2011b). A series of pioneering clinical trials on muscle- and liver-directed AAV gene transfer recently culminated in successful treatment patients with severe hemophilia B (Kay et al., 2000; Manno et al., 2003, 2006; Ponder, 2011). However, despite the low immunogenicity profile of AAV vectors, several concerns about immune responses to gene transfer remain. For example, prior natural infection with the parent or related virus may cause pre-existing immunity in humans, including neutralizing antibodies to viral particles (preventing gene transfer). Memory $CD8^+$ T cell responses to capsid are also known to occur and may target transduced cells (Manno et al., 2006; Mingozzi et al., 2007b; Li et al., 2011; Mingozzi and High, 2011a). Immune responses to the transgene product are an additional concern. Large F9 gene deletions or other "null" mutations substantially increase the risk of immune responses, likely due to a lack of central tolerance (Cao et al., 2009a). A particular concern for gene therapy for inherited protein deficiencies such as hemophilia is the potential for antibody formation, which could then also negate conventional protein replacement therapy.

Muscle-directed gene transfer is an attractive treatment modality because of the safety and simplicity of vector administration, as demonstrated in clinical trials, but is hampered by an increased risk of immune responses against the transgene product (Herzog et al., 2001; Manno et al., 2003). For example, animals with null mutations are at high risk of formation of inhibitory antibodies (inhibitors) against F.IX (Herzog et al., 2001; Cao et al., 2009a). Previously, we demonstrated that the drug cocktail of immune suppressants rapamycin, IL-10, and a specific peptide (encoding a dominant $CD4^+$ T cell epitope) caused an induction of $CD4^+CD25^+FoxP3^+$ Treg with a concomitant antigen-specific apoptosis of effector T cells (Teff; Nayak et al., 2009). This protocol was effective in preventing inhibitor formation against human F.IX (hF.IX) upon subsequent IM administration of an AAV–hF.IX vector in hemophilia B mice with targeted F.IX gene deletion. Here, we used this model to test whether an inhibitor response to

gene therapy can be reversed and whether a similar protocol can be developed that prevents inhibitor formation in a prophylactic manner without prior knowledge of T cell epitopes. Such epitopes may differ from one patient to another and may be difficult to map in a person who does not already have an immune response.

## MATERIALS AND METHODS

### ANIMALS

All mice were 6- to 12-week-old males at the onset of the experiments and housed in specific pathogen free conditions. Hemophilia B mice (F9 gene deletion) on C3H/HeJ background were as previously described (Cao et al., 2006, 2009a; Verma et al., 2010). DO11.10-tg Rag-$2^{-/-}$ BALB/c mice were obtained from Taconic (Germantown, NY, USA). This strain is transgenic for ovalbumin-specific DO11.10 $CD4^+$ TCR and knock out for Rag-2 (DO11.10-tg Rag-$2^{-/-}$; Cao et al., 2007a). Drug cocktails were administered via different routes in 200 μl of sterile PBS, three-times per week at the following doses: ovalbumin (ova) peptide (amino acid residues 323–339) or hF.IX-specific peptide (2A-54) were given at 100 μg/dose, rapamycin at 4 mg/kg/dose, and IL-10 at 50 ng/kg/dose (Nayak et al., 2009). In an alternative regimen, rapamycin was given by oral gavage at the same dose (but in a volume of only 100 μl) daily for 1 month. During that time, recombinant human F.IX (Benefix, Wyeth, Madison, NJ, USA) was given intravenous (IV) into the tail vein at 0.1 IU/mouse twice per week. Viral vector was administered at $1 \times 10^{11}$ vector genomes (vg)/mouse by intramuscular (IM) injection into quadriceps and tibialis anterior muscles. Blood samples from hemophilia B mice were collected by tail-bleed into 3.8% sodium citrate buffer.

### REAGENTS AND VIRAL VECTOR

Ova and hF.IX peptides were synthesized by AnaSpec (San Jose, CA, USA). Murine IL-10 (Sigma Aldrich, St. Louis, MO, USA) and rapamycin (LC Laboratories, Woburn, MA, USA) stock solution were made in 0.02% carboxymethylcellulose and 0.25% Tween80. The previously published AAV-CMV-hF.IX vector contains the hF.IX cDNA (plus a 1.4-kb portion of the F9 gene) expressed from the cytomegalovirus immediate-early enhancer/promoter (Arruda et al., 2004). Vector genomes were packaged into serotype 1 capsid following triple transfection of HEK-293 cells, purified by PEG-precipitation and gradient centrifugation, and titers determined by quantitative slot–blot hybridization as published previously (Liu et al., 2003; Ayuso et al., 2010).

### ASSAYS ON PLASMA SAMPLES

Levels of hF.IX were determined by ELISA as previously described (Cao et al., 2009a). Activated partial thromboplastin time (aPTT) was measured using a fibrometer (Fibrosystem; BBL, Cockeysville, MD, USA) and previously published protocols (Cao et al., 2009a). Bethesda assay to determine inhibitor titers was also performed as previously described. By definition, 1 Bethesda Unit (BU) represents a residual hF.IX activity of 50%.

### FLOW CYTOMETRY

Splenocytes were isolated using standard protocols, and cell viability was measured using a hemocytometer and trypan blue staining. Antibody stains for surface and intracellular molecules were applied according to manufacturers' protocols. Antibodies against mouse antigens CD4-FITC, CD25-PE, FoxP3-Alexa Fluor 647 were from eBioscience (San Diego, CA, USA). Isotype controls included Rat IgG2a-FITC, Rat IgG2b-PE, Rat IgG2a-Alexa Fluor 647, and other controls were unstained splenocytes and anti-Rat Ig Compensation Particles (BD Biosciences/Pharmingen). Data acquisition was carried out using the BD LSR II. Data analysis was done with FCS 3.0 software. Antibodies against mouse antigens CD4-PB, CD25-PE-Cy7, FoxP3-Alexa Fluor 647, GITR-PE, CD127-Alexa Flour 780 were from eBioscience (San Diego, CA, USA), and antibodies against mouse antigens CD25-PE, CTLA-4-PE were from BD Biosciences (San Jose, CA, USA).

### *IN VITRO* SUPPRESSION ASSAY

Antigen presenting cells (APCs) were isolated from naïve D011.10-tg mice using MACS CD90.2 MicroBeads (Miltenyi Biotec, Auburn, CA, USA). $CD4^+CD25^+$ Treg were isolated from DO11.10-tg RAG-$2^{-/-}$ mice using mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotech) and the $CD4^+CD25^-$ Teff were isolated from DO11.10-tg RAG-$2^{-/-}$ mice using mouse $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). $CD4^+CD25^-$ cells were labeled with 0.5 μM CFSE (carboxyfluorescein diacetate, succinimidyl ester, Invitrogen, Carlsbad, CA, USA) in PBS containing 2% FBS at a cell concentration of $3 \times 10^6$ cell/ml at room temperature for 5 min in the dark. These cells were then washed three times in PBS containing 2% FBS and re-suspended in DMEM containing 10% FBS and penicillin/streptomycin. $CD4^+CD25^+$ Treg were cultured with $2 \times 10^5$ CFSE-labeled Teff at different ratios as indicated and stimulated with 1 or 0.1 μg/ml ova peptide (AnaSpec), along with $2 \times 10^4$ APCs in DMEM supplemented with 10% FBS and penicillin/streptomycin in Thermo Scientific Nunclon D round bottom 96-well plates (Pittsburgh, PA, USA) and incubated at 37°C in 5% $CO_2$ for 4 days.

### STATISTICAL ANALYSIS

Results are reported as mean ± SD or ±SEM. Significant differences between an experimental group and control groups were determined with unpaired Student's *t*-test. *P* values of $<0.05$ were considered significant. For comparison between multiple experimental groups, one-way ANOVA with variance was applied using Prism software (Irvine, CA, USA).

## RESULTS

### TRANSIENT IMMUNE MODULATION WITH RAPAMYCIN/IL-10/SPECIFIC PEPTIDE CAN REVERSE GENE THERAPY-INDUCED INHIBITOR FORMATION

Hemophilia B mice with an F9 gene deletion on a C3H/HeJ genetic background have more robust immune responses to hF.IX than other strain backgrounds such as C57BL/6 or BALB/c (Mingozzi et al., 2003; Wang et al., 2005; Cao et al., 2006, 2009a; Verma et al., 2010). As expected, IM injection of the AAV1–hF.IX vector in these mice resulted in a high-titer inhibitory antibody response of on average 8–10 BU (up to 14 μg IgG1/ml plasma) within 1 month (**Figures 1B–E**). Coagulation times were not corrected (average aPTTs of ∼80 s; an aPTT of 60 s corresponds to a coagulation activity of approximately 1%), and transduced mice lacked systemic hFIX expression (**Figures 1F–I**). Previously, we found that inhibitor formation in these mice, induced by muscle-directed hF.IX gene transfer, could be prevented by repeated with

intraperitoneal (IP) administration of a cocktail comprised of rapamycin/IL-10/hFIX peptide; while cocktails lacking either IL-10 or the peptide were less effective (Nayak et al., 2009). Therefore, we decided to test whether the most optimal regimen could not only prevent but also reverse inhibitor formation in muscle gene transfer.

Therefore, 1.5 months after vector administration, animals were randomly divided into the two groups ($n = 6$/group) graphed in the left and right columns of **Figure 1**. Animals of one group were not further manipulated (control group, **Figures 1B,D,F,H**), while the animals of the second group (**Figures 1C,E,G,I**) were treated with IP injections of rapamycin/IL-10/hFIX peptide, three-times per week for 4 weeks (**Figure 1A**). These treatments resulted in a substantial reduction of inhibitor titers to <2 BU by 1 month after the transient immune modulatory regimen had been stopped (**Figure 1C**). The difference between inhibitor titers at 1 month (i.e., prior to immune modulation) and all times points after immune modulation was highly significant ($P < 0.001$). Antibody titers to hF.IX were low to undetectable for the duration of the experiment (7 months after vector administration or 4.5 months after immune modulation; **Figures 1C,E**). The reduction in inhibitor/antibody titers was accompanied by an increase in systemic hF.IX antigen from 0 ng/ml before the tolerance regimen to an average level of ∼250 ng/ml by 5–7 months (**Figures 1C,D**). Clotting times gradually declined to an average of 58–60 s (**Figure 1E**), representing a coagulation activity of approximately 1% of normal.

In contrast, transduced control mice that were not treated with the tolerance protocol maintained inhibitors for at least 5 months (**Figure 1B**). As seen in previously (Nayak et al., 2009), there was a gradual spontaneous decline in hF.IX-specific IgG titers over time and some improvement in the aPTT (**Figures 1D,H**). Nonetheless, no systemic hF.IX was detected in these mice for the duration of the experiment (i.e., 7 months after gene transfer, **Figure 1F**).

## PREVENTION OF INHIBITOR FORMATION USING THE HF.IX PROTEIN AS THE TOLERIZING ANTIGEN

A problem with the prophylactic use of this protocol against inhibitor formation is that the specific T cell epitopes may not be known in a patient. However, when we used hF.IX protein (1 IU) instead of the peptide in an otherwise identical protocol, we failed to prevent inhibitor formation after subsequent administration of the AAV1–CMV–hF.IX vector (data not shown). Therefore, the protocol needed to be modified to use the entire hF.IX protein as the tolerizing antigen.

Keeping translatability in mind, subsequent experiments were adjusted for the fact that rapamycin is typically given orally in humans (IP administration is not a suitable route for humans) and that the cytokine IL-10 is not readily available for clinical application. Thus, a protocol that could realistically be used in humans would likely be based on oral administration of rapamycin and IV delivery of hF.IX protein in the absence of IL-10.

In a first attempt with such a modified protocol, rapamycin was given orally three-times per week for 4 weeks to hemophilia B mice (same C3H/HeJ strain described above). In addition, hF.IX was given IV at a reduced dose of 0.1 IU/mouse, twice per week. However, this protocol was only partially effective in preventing

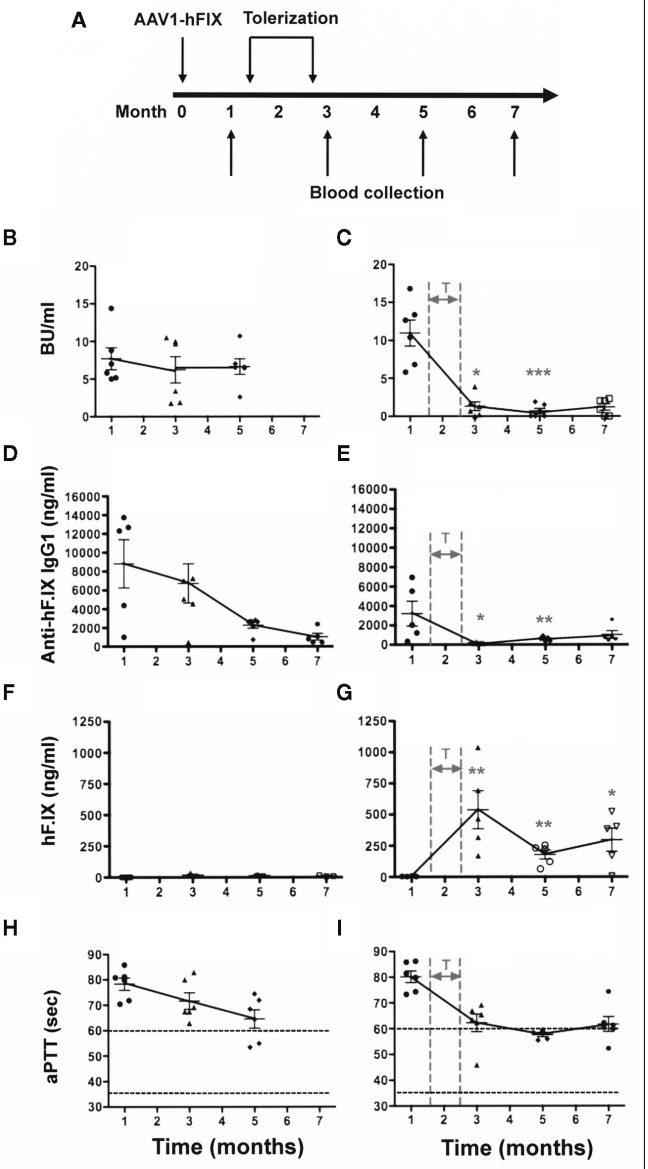


**FIGURE 1 | Elimination of a pre-existing/ongoing anti-hF.IX immune response.** C3H/HeJ F9$^{-/-}$ mice ($n = 12$) were injected IM with AAV1-CMV-hF.IX ($1 \times 10^{11}$ vg/mouse) to generate an immune response. After demonstrating the existence of an immune response, half of the animals were given rapamycin/IL-10/specific peptide for 4 weeks [right panels: **(C,E,G,I)**]. Controls [left panels: **(B,D,F,H)**] only received gene transfer. **(A)** Experimental timeline. **(B,C)** Inhibitory antibody titers (in BU/ml). **(D,E)** Anti-hF.IX IgG1 antibody (ng/ml). **(H,G)** hF.IX plasma levels (ng/ml). **(H,I)** Coagulation times (aPTT in s). Data are shown as a function of time after vector administration in control mice (treated with vector only, left panels) or mice that received the immune modulation/tolerance regimen (starting 1.5 months after vector administration, indicated as "T" in right panels). Results are shown as averages at each time point (±SEM) and for individual animals. Statistically significant differences between the immune modulated and control groups ($n = 6$ per group) at a specific time point are indicated as gray asterisk in the panels of the immune modulated group with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

the immune response to hF.IX in gene therapy (**Figures 2B,F**). Two of five animals still developed IgG1 and >5 BU (**Figure 2F**). Next, we decided to test whether more frequent oral administration rapamycin could improve the protocol. Hence, rapamycin was given orally on a daily schedule for 1 month to hemophilia B mice, during which time a low-dose of hF.IX protein (0.1 IU) was also given IV twice per week (**Figure 2A**). AAV1-CMV-hF.IX was again injected IM 1 week before the end of the tolerance protocol. This regimen was highly effective and prevented antibody/inhibitor formation, resulting in systemic hF.IX (ranging from 50 to 300 ng/ml) and more robust correction of the aPTT to on average ∼50 s (**Figures 2B–I**). Three of the initial five treated animals were still alive >8 months after gene transfer (>1 year old), at which time they still maintained their hF.IX protein levels with no detectable antibody (data not shown).

### EFFECT OF THE ROUTE OF DRUG ADMINISTRATION ON THE EFFECTIVENESS OF THE PROTOCOL

Experimental results presented in **Figure 2** suggested that the route of rapamycin administration and the dosing schedule had an impact on the effectiveness of the tolerance regimen. These questions are addressed in the following. In order to directly measure the ability to promote a shift from Teff to Treg as a function of the route of drug administration, we used T cell receptor (TCR) transgenic mice lacking endogenous Treg (DO11.10-tg Rag-$2^{-/-}$ BALB/c mice with a $CD4^+$ TCR specific for ovalbumin). In these mice, IP, subcutaneous (SQ), intravenous (IV), and oral routes of drug administration were compared using our original cocktail. Rapamycin was either given as a cocktail with IL-10 and ova peptide, as published, three-times per week for 3 weeks via IP, SQ, or the IV route (Nayak et al., 2009). Alternatively, the IL-10/ova mix was given IP as published, while rapamycin was fed on the same day by oral gavage (within 30 min of administration of the other drugs).

All routes resulted in a substantial reduction in the frequency of ova-specific $CD4^+$ T cells (**Figure 3A**). However, oral delivery of rapamycin was significantly less effective than the other routes (**Figure 3A**). Induction of ova-specific $CD4^+CD25^+FoxP3^+$ Treg with a frequency of up to 20% of $CD4^+$ T cells was achieved with all these routes (**Figure 3B**). Oral administration of rapamycin was again less effective in this regard, albeit not significantly different, from the other routes (**Figure 3B**).

Data presented in **Figure 2** showed that IV administration of antigen combined with daily gavage of rapamycin was superior in tolerance induction compared to performing gavage of rapamycin less frequently. To study this further, rapamycin was given orally to DO11.10-tg Rag-$2^{-/-}$ BALB/c mice either three-times per week or daily for 3 weeks. During this time, ova peptide was given IV three-times per week. For comparison, a rapamycin/ova peptide cocktail was injected IP three-times per week. As expected, reduction of Teff cells and Treg induction was not as effective for the IP method using this cocktail, which lacks IL-10 (**Figures 4A,B**). Both oral rapamycin regimens were less effective compared to the IP route (**Figures 4A,B**). Nonetheless, amongst the two oral rapamycin protocols, daily administration was significantly better for Treg induction (**Figure 4B**). At a frequency of three-times per week, nearly no Treg induction was observed. Reduction in total $CD4^+$ T cell frequency was similar for both frequencies of oral delivery (**Figure 4A**).

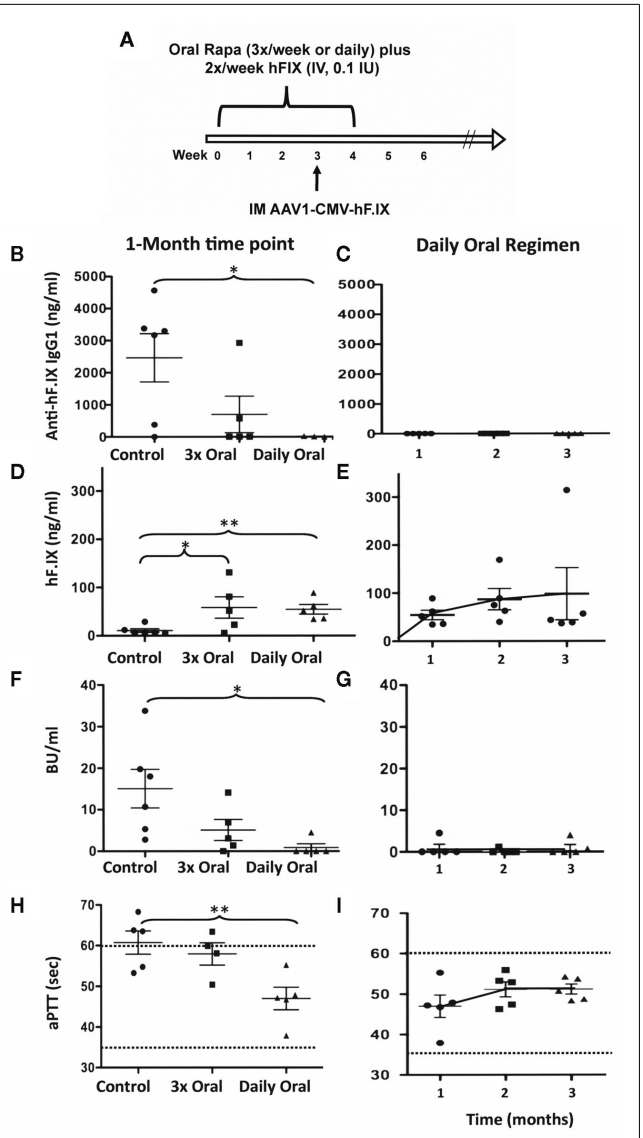


**FIGURE 2 | Optimization of a protocol that prevents inhibitor formation by administration of hF.IX protein and rapamycin.** **(A)** Experimental timeline of the optimal protocol. For immune modulation, C3H/HeJ $F9^{-/-}$ mice received hF.IX IV injections (0.1 IU/dose) twice per week for 4 weeks. During that time, rapamycin was administered orally once per day. One week before the end of this 1-month regimen, AAV1-CMV-hF.IX ($1 \times 10^{11}$ vg/mouse) was given IM. Left panels: Comparison of IgG1 **(B)** and inhibitor **(F)** formation against hF.IX, of systemic hF.IX expression **(D)**, and of coagulation times [aPTT **(H)**] at 1 month after vector administration in mice that had received vector only; or 2 IV doses of hF.IX (0.1 IU) plus 3 oral doses of rapamycin per week (with the first and last 30 min prior to hF.IX administration); or the optimal protocol (2 IV doses of F.IX plus daily rapamycin). Right panels: IgG1 **(C)** and inhibitor **(G)** formation against hF.IX, systemic hF.IX expression **(E)**, and coagulation times [aPTT **(I)**] as a function of time after vector administration in mice that had received the optimal protocol (2 IV doses of F.IX plus daily rapamycin).

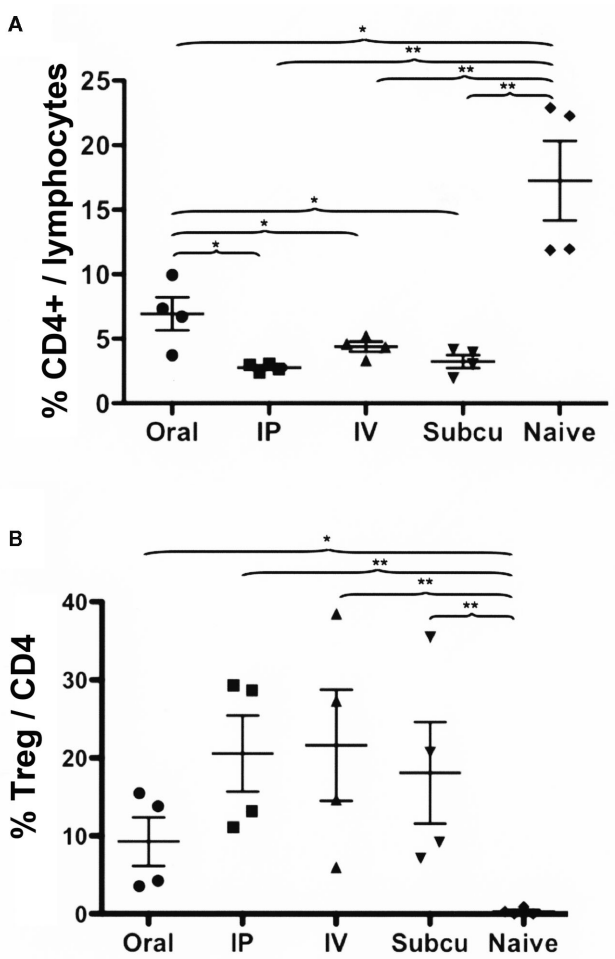


**FIGURE 3 | Comparison of Rapa/IL-10/specific peptide tolerization protocol using different routes of drug administration.** DO.11.10-tg Rag$^{-/-}$ mice ($n = 4$/group) were treated for 3 weeks with Rapa/IL-10/ova-specific peptide, after which the spleens were harvested, and flow cytometry was used to determine: **(A)** percentage of CD4$^+$ T cells of total gated splenocytes and **(B)** percentage of CD25$^+$FoxP3$^+$ cells of CD4$^+$ T cells. Statistically significant differences between two groups are indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

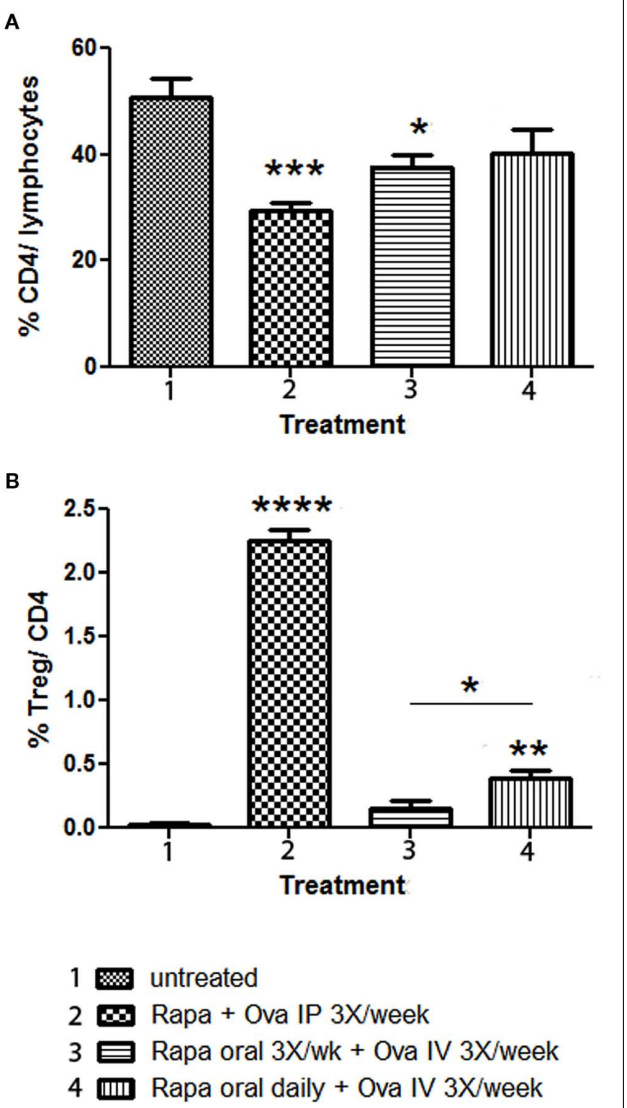


**FIGURE 4 | Comparison of Rapa/specific peptide tolerization protocol using different routes and schedules of drug administration.** DO.11.10-tg Rag$^{-/-}$ mice ($n = 4$ per group) were treated for 3 weeks with Rapa/ova-specific peptide, after which the spleens were harvested, and flow cytometry was used to determine: **(A)** percentage of CD4$^+$ T cells of total gated splenocytes, and **(B)** percentage of CD25$^+$FoxP3$^+$ cells of CD4$^+$ T cells. Statistically significant differences between each group and untreated (naïve) control mice are indicated as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. In **(B)**, a statistically significant difference between mice that received rapamycin orally three-times per week or daily is also indicated.

### ANTIGEN/RAPAMYCIN-INDUCED TREG SHOW AN IMMUNE SUPPRESSIVE PHENOTYPE

Further characterization of oral rapamycin/IV antigen induced Treg was carried out by extending treatment to 4 weeks. This resulted in higher Treg frequencies in the IP and oral protocols when performed in parallel for comparison (**Figure 5E**). The IP regimen was again superior in deletion of the ova-specific CD4$^+$ T cells and in Treg induction (**Figures 5D,E**). Immunophenotyping, as shown in the form of examples in **Figures 5A–C**, suggested that FoxP3 expression was comparable for "natural," IP-, and oral-induced Treg (**Figure 5F**; "natural" Treg refers to CD4$^+$CD25$^+$FoxP3$^+$ cells isolated from spleens of naïve immune competent DO11.10-tg Rag-2$^{+/+}$ BALB/c mice as opposed to Treg induced by exogenous antigen administration). Orally induced Treg were most consistently CD25$^{hi}$. Similarly, all three types of Treg stained positive for surface expression of GITR (**Figure 5G**). Interestingly, oral-induced Treg showed a higher frequency of CTLA-4 expression compared to natural or IP-induced Treg (**Figure 5H**). Finally, ~97% of natural Treg and ~93% of IP-induced Treg were CD127$^{-/low}$, respectively, while ~99% of oral-induced Treg were CD127$^{-/low}$ (data not shown).

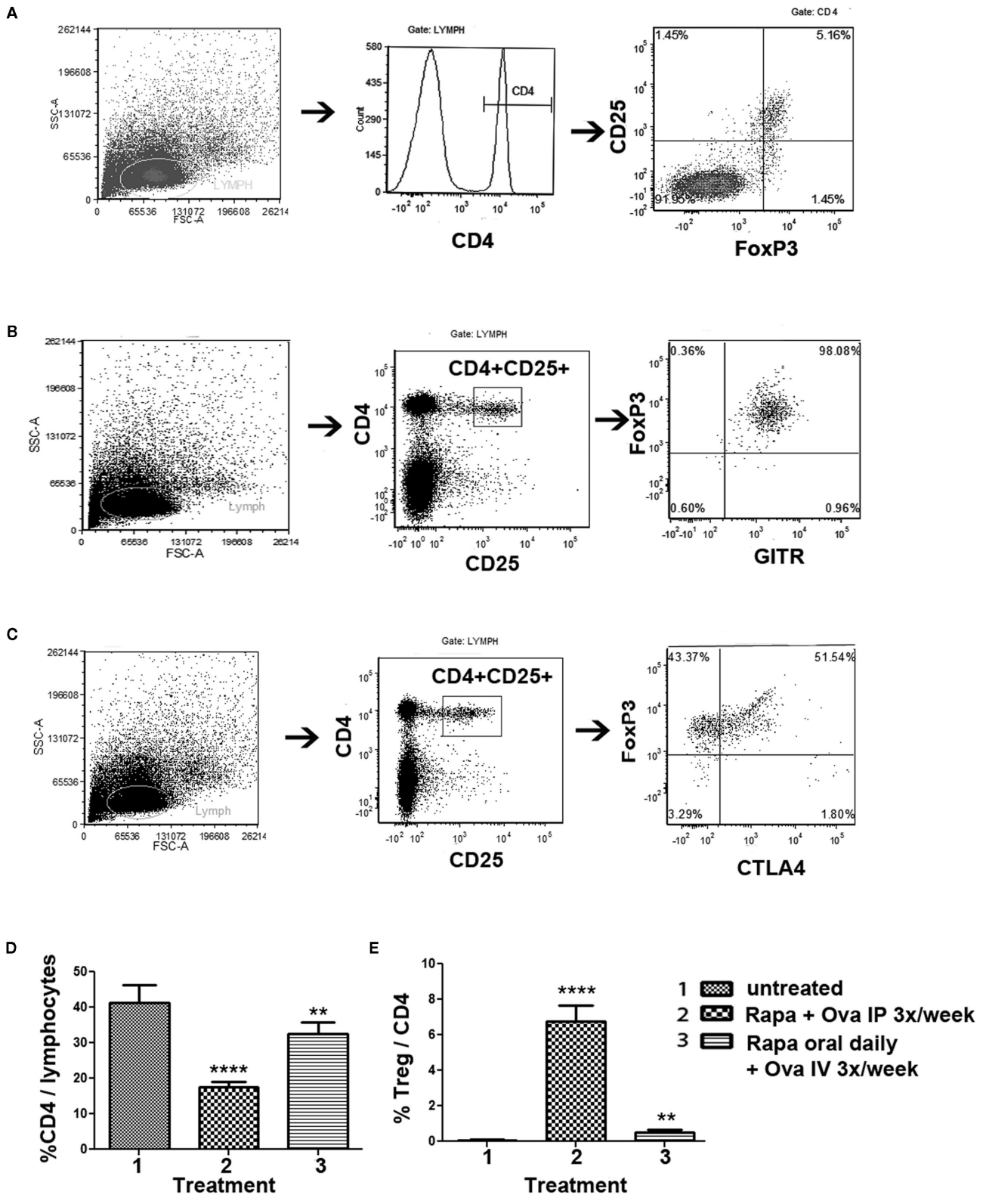


**FIGURE 5 | Continued**

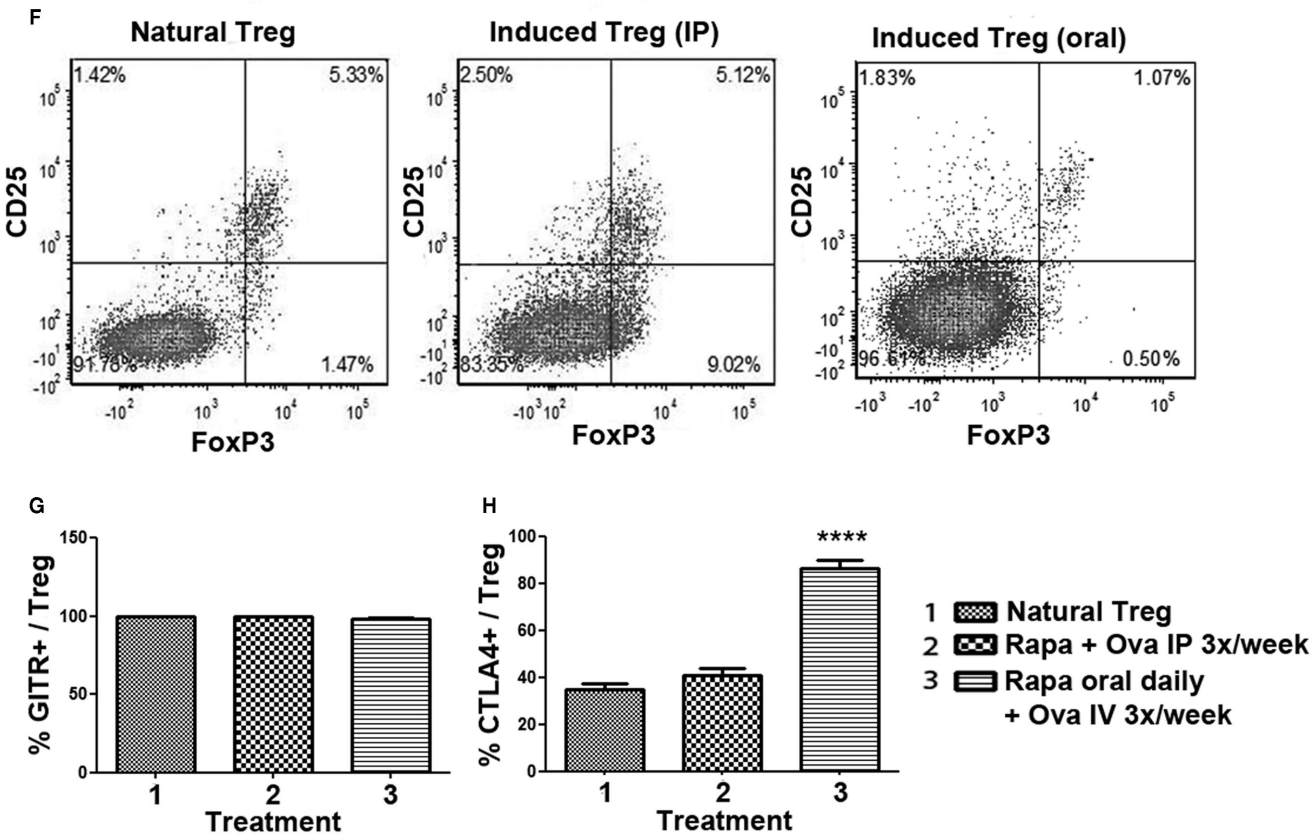


**FIGURE 5 | Phenotypic analysis of Treg induced by the rapa/specific peptide tolerization protocol using different routes and schedules of drug administration.** DO11.10-tg Rag$^{-/-}$ BALB/c mice ($n = 5$ per group) were treated for 4 weeks with rapamycin/ova-specific peptide, after which splenocytes were analyzed by antibody stains and flow cytometry. **(A–C)** Examples for gating scheme and flow cytometric analysis of FoxP3 **(A)**, GITR **(B)**, and CTLA-4 **(C)** expression by induced CD4$^+$CD25$^+$ Treg. **(D)** Percentage of CD4$^+$ T cells of total gated splenocytes, and **(E)** Percentage of CD25$^+$FoxP3$^+$ cells of CD4$^+$ T cells as a function of treatment. Statistically significant differences between each group and untreated (naïve) control mice are indicated as $^{**}P < 0.01$, and $^{****}P < 0.0001$. **(F)** Representative examples of CD25 and FoxP3 staining (gated on CD4$^+$ splenocytes) for "natural" Treg (isolated from immune competent DO11.10-tg Rag$^{+/+}$ mice), and induced Treg from DO11.10-tg Rag$^{-/-}$ mice treated with the IP or the oral protocol. **(G)** Percent GITR$^+$ cells, and **(H)** percent CTLA-4$^+$ cells amongst CD4$^+$CD25$^+$FoxP3$^+$ Treg. All bar graphs are average ±SD.

In order to demonstrate that orally induced Treg were functional suppressors, proliferation of ova-specific CD4$^+$ Teff was monitored by CFSE-dilution assay upon co-culture with increasing numbers of Treg isolated from spleens of DO11.10-tg Rag-2$^{-/-}$ BALB/c mice that had been treated for 4 weeks with the oral rapamycin/IV ova combination (**Figure 6**). Treg induced by daily oral rapamycin/IV ova suppressed Teff proliferation in response to stimulation with ova peptide in a Treg dose-dependent manner, resulting in more complete suppression at lower antigen doses (**Figures 6A,B**). Suppression was at least as effective for orally induced as for IP-induced Treg (compare **Figures 6A,C**).

## DISCUSSION

Gene therapy with viral vectors holds a great deal of promise for the treatment of inherited protein deficiencies such as hemophilia. As with any protein or gene replacement therapy, there are inherent risks of immune responses to the therapeutic gene product. In the treatment of hemophilia, antibody ("inhibitor") formation against the functional coagulation factor is of particular concern as a major complication of therapy. Our lab and others have used preclinical models to assess immune suppressive regimens for their ability to prevent this immune response when a drug or combination of drugs is given in conjunction with gene therapy (Sack and Herzog, 2009; Miao, 2010; Nayak and Herzog, 2010). However, the ability to reverse an immune response caused by gene transfer has not been studied thoroughly. Preliminary data suggested that monoclonal antibodies (anti-CD20/cyclosporine A combination or anti-CD3) might be useful (Nathwani et al., 2006; Peng et al., 2009; Sack and Herzog, 2009). Here, we clearly demonstrate that transient immune modulation using rapamycin can reverse antibody ("inhibitor") formation.

### EFFECTS OF ANTIGEN/RAPAMYCIN COMBINATION

Previously, we found that MHC II antigen presentation to CD4$^+$ T cells in the presence of the specific mTOR inhibitor rapamycin resulted in activation-induced cell death, primarily of Teff. In addition, CD4$^+$CD25$^+$FoxP3$^+$ Tregs are induced in secondary lymphoid organs and expanded (Nayak et al., 2009). These Tregs are able to utilize alternative signaling pathways such as STAT5 to proliferate in response to cytokine growth factors, thereby bypassing

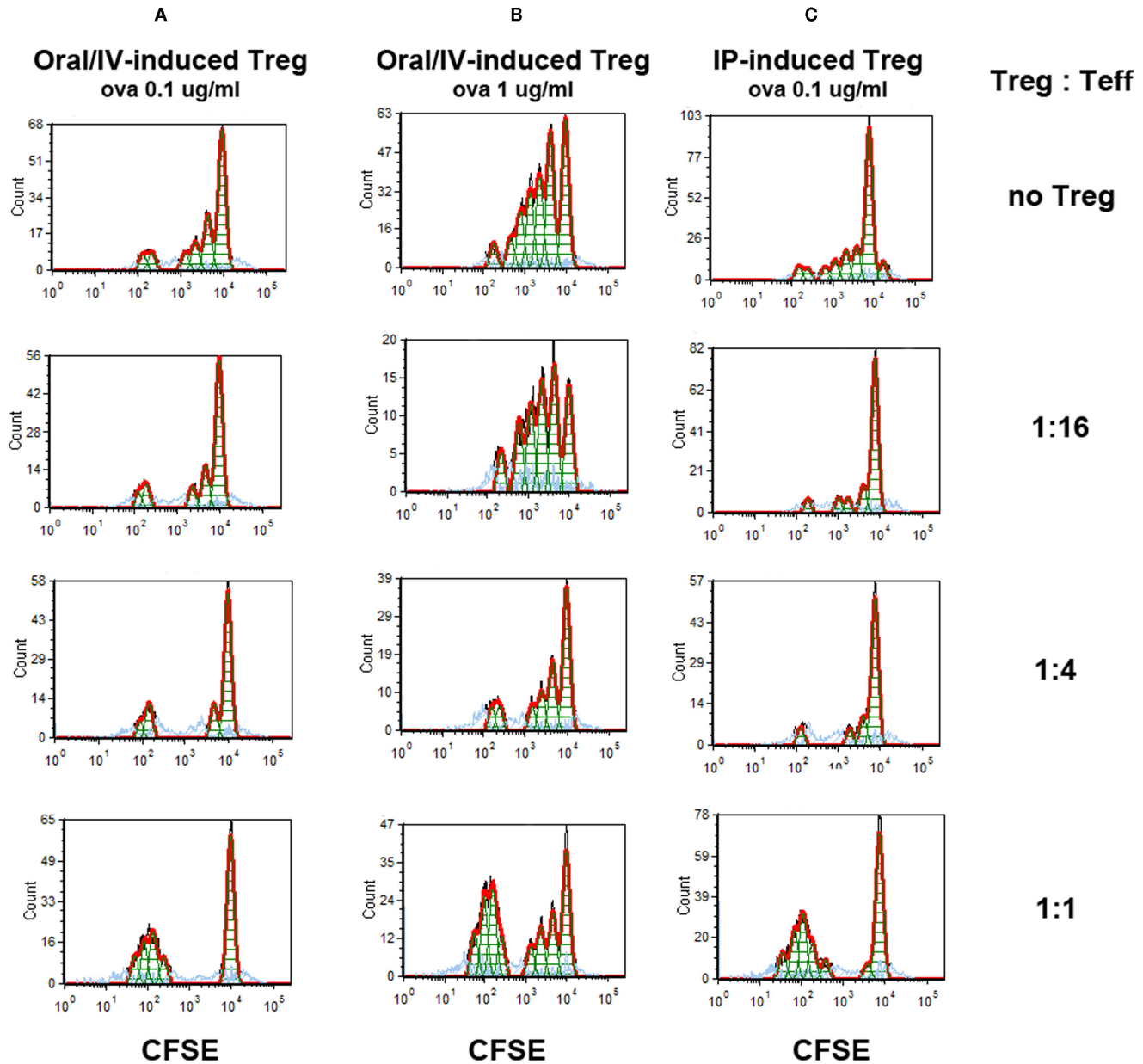


**FIGURE 6 | Suppression of antigen-specific Teff proliferation by induced Treg.** Treg induced by 4 weeks of oral rapamycin/IV ova peptide-treated **(A,B)** or rapamycin/ova peptide IP-treated **(C)** DO11.10-tg Rag$^{-/-}$ BALB/c mice were co-cultured in the indicated ratios with Teff cells and APCs. Teff were CFSE-labeled ova-specific CD4$^{+}$ T cell isolated from DO11.10-tg Rag$^{-/-}$ BALB/c mice. Conditioned media contained ova peptide at 0.1 μg/ml **(A,C)** or 1 μg/ml **(B)**. CFSE-dilution was assayed by flow cytometry after 4 days of *in vitro* stimulation.

the need for the mTOR pathway (Zeiser et al., 2008; Moghimi et al., 2011). The shift from an effector to a Treg response is further enhanced by the immunosuppressive cytokine IL-10, and the induced suppressive response can be adoptively transferred using CD4$^{+}$CD25$^{+}$ splenocytes (Battaglia et al., 2006a,b; Nayak et al., 2009; Moghimi et al., 2011).

While we previously showed prophylaxis against inhibitor formation in muscle-directed AAV–hF.IX gene transfer in hemophilia B C3H/HeJ mice, this new data demonstrates the enhanced reversal of a pre-existing response by our regimen. In this strain of hemophilia B mice, IM administration of AAV–hF.IX vector induces not only an inhibitor but also a CD8$^{+}$ T cell response to hF.IX (Cao et al., 2009a). However, these infiltrating CD8$^{+}$ T cells are not fully functional and fail to eliminate hFIX-expressing muscle fibers (Wang et al., 2005; Lin et al., 2007; Velazquez et al., 2009). Therefore, production of hF.IX protein persists in the muscle, which is critical for the restoration of systemic expression after antibody elimination. Consequently, we achieved systemic hF.IX expression

and partial correction of the coagulation defect. Although the data demonstrate elimination of inhibitors (as measured by Bethesda assay) and restoration of coagulation following the 1-month immune modulatory protocol, circulating antigen levels (on average ∼5% of normal) were higher than the level of coagulation activity (1–2% of normal). Low-titer antibodies against hF.IX were detectable by ELISA in rapamycin/IL-10/peptide-treated mice at late time points and may have limited coagulation activity.

#### USE OF PEPTIDE VS. PROTEIN ANTIGEN FOR TOLERANCE INDUCTION

We have now shown in two different models (F.VIII replacement therapy in hemophilia A mice and F.IX muscle gene transfer in hemophilia B mice) that IV delivery of protein combined with daily feeding of rapamycin prevents inhibitor formation (Nayak et al., 2009; Moghimi et al., 2011). Use of the protein antigen for tolerance induction has the advantage that knowledge of specific epitopes is not required. Reduced protein doses appear better suited for tolerance induction with rapamycin (Moghimi et al., 2011). However, in the context of an ongoing immune response, the protein antigen may further stimulate B cell responses. In some cases (such as enzyme replacement therapy for hemophilia B or Pompe disease), anaphylactic reactions against the protein are a concern (Dimichele, 2007; Nayak et al., 2009; Sun et al., 2010; Verma et al., 2010). In this case, T cell epitope mapping followed by co-administration of rapamycin and specific peptides may represent a more effective means for targeting antigen-specific effector $CD4^+$ T cells and expansion of Treg. This would promote hyporesponsiveness to the transgene product/therapeutic protein and reversal of the T help-dependent antibody response. Treg appear critical for tolerance to coagulation factors and other transgene products (Cao et al., 2007a,b, 2009b; Mingozzi et al., 2007a; Miao et al., 2009; Miao, 2010; Hoffman et al., 2011).

#### IMPACT OF THE ROUTE OF DRUG ADMINISTRATION ON TOLERANCE INDUCTION

The dose of rapamycin in our experiments was chosen based on prior published animal studies (Teachey et al., 2006; Nayak et al., 2009; Moghimi et al., 2011). This dose has been determined to maintain a trough level of hF.IX in the blood (10–15 ng/ml) that is known to cause a tolerogenic effect (Teachey et al., 2009). Using direct injections such as IP, IV, or subcutaneous (rather than delivery via oral gavage), it is likely that maintenance of such a trough level requires less frequent administration. In addition, co-administration of a mixture of the antigen and rapamycin may have induced a more effective antigen presentation to T cells with a blocked mTOR pathway. Interestingly, there are now also IV preparations of rapamycin available for patients and could be considered for use in this protocol.

Nonetheless, our data demonstrate that hemophilic mice can be effectively tolerized to F.VIII (Moghimi et al., 2011) or F.IX (this study) using a combination of oral delivery of rapamycin and IV delivery of low-dose protein. Daily rapamycin was required for Treg induction. Orally induced $CD4^+CD25^+$ Treg expressed transcription factor FoxP3 and the co-stimulatory molecule GITR at levels comparable to IP-induced or natural Treg, while the inhibitory signaling molecule CTLA-4 was even more frequently expressed, and CD127 expression was low. Therefore, orally induced Treg had all the hallmark features of suppressor cells. A suppressive phenotype was confirmed by inhibition of Teff proliferation. Although not tested here, we previously found no evidence of antigen/rapamycin-induced splenic Treg to express IL-10, but provided evidence for TGF-β expression (Nayak et al., 2009; Moghimi et al., 2011). Experiments in the DO11.10-tg model as well as our published adoptive transfer studies (Nayak et al., 2009; Moghimi et al., 2011) demonstrate induction of antigen-specific Treg. However, we had also found that daily rapamycin administration for 1 month caused a general increase in frequency of circulating Treg (to 27–38% of $CD4^+$ cells) in immune competent BALB/c mice, which within 6 weeks returned to the baseline level of approximately 10% (Moghimi, unpublished observations). We had also previously found general transient decreases in B and T cell frequencies by repeated rapamycin administration (Nayak et al., 2009). Therefore, the intravenously introduced antigen is likely presented in a generally immune suppressive context, while at the same time inducing antigen-specific deletion of Teff and induction of Treg. The combination of these effects likely facilitates tolerance induction. However, results from oral rapamycin scheduling strongly suggest that Treg induction is required for tolerance.

#### ADVANTAGES AND POTENTIAL COMPLICATIONS OF RAPAMYCIN TREATMENT

There is ample clinical experience with rapamycin, including use in pediatric patients. Rapamycin is administered in combination with other drugs in solid organ transplantation and has recently been successfully tested for the treatment of autoimmune lymphoproliferative syndrome (ALPS; Teachey et al., 2009). It is also a promising drug for the treatment of autoimmune diabetes and can be combined with cytokines such as IL-2 or IL-10 for optimal results (Wells et al., 1999; Zheng et al., 2003; Battaglia et al., 2006a; Koulmanda et al., 2007; Nayak et al., 2009). Conceptually, the mode of action through which Teff are deleted and Treg are induced and expanded make rapamycin ideal for tolerance induction protocols. Rapamycin also has anti-inflammatory properties such as interfering with DC mobilization and function and increasing levels of hemeoxygenase-1 (HO), a stress-inducible, anti-inflammatory enzyme (Hackstein et al., 2003; Visner et al., 2003; Dimitrov et al., 2010). Furthermore, mTOR inhibition can reduce IFN I expression in response to TLR signaling (Manicassamy and Pulendran, 2009). Thus, there are multiple effects of rapamycin that could contribute to tolerance induction and it will be of great interest to study the effects of rapamycin on innate immune responses to AAV vectors, which are largely TLR9-dependent.

On the other hand, prolonged use of rapamycin may actually increase Th1/Th17/inflammatory responses, likely because blockage of mTOR can increase the use of the NF-κB pathway. This, however, may be countered by addition of IL-10 (Weichhart et al., 2008; Huber et al., 2011). In addition, rapamycin counteracts the immunosuppressive effects of glucocorticoids (Weichhart et al., 2011).

In general, immune suppressive regimens pose an increased risk for opportunistic infection and other possible side effects. Although we can achieve coagulation factor-specific tolerance with the 1-month rapamycin-based regimens, there are also

transient effects on total B and T cell frequencies. In our initial rapamycin/IL-10 protocol, we found that B and T cell frequencies returned to normal with 2.5 months after treatment (Nayak et al., 2009). With the oral rapamycin protocol, these cell frequencies return to normal even faster (5 weeks), and subsequently, mice respond properly to viral immunization illustrating the highly transient nature of the immune suppressive protocol (Moghimi et al., 2011).

Neutropenia and dysregulation of lipid metabolism are additional side effects of rapamycin. In addition, rapamycin can also delay wound healing, suggesting that the drug should not be used during or early after surgery or other extensive tissue damage (Stallone et al., 2009). Many other side effects, such as kidney problems or increased cancer risk, are more linked to long-term use of the drug than to a very transient protocol.

In summary, we propose that alternative protocols, based on co-administration of rapamycin with the complete antigen or a known T cell epitope (which can be mapped during the course of an immune response), can be applied to prevent or reverse inhibitor responses in gene therapy for hemophilia. This approach could also be useful in gene therapy for other genetic diseases.

## AUTHOR CONTRIBUTION

Sushrusha Nayak, Debalina Sarkar, George Q. Perrin, Babak Moghimi, and Brad E. Hoffman performed experiments; Shangzhen Zhou provided AAV vector; Barry J. Byrne and Roland W. Herzog supervised the study; Sushrusha Nayak, Debalina Sarkar, George Q. Perrin, and Roland W. Herzog wrote the manuscript. Roland W. Herzog has been receiving royalty payments by Genzyme Corp. for license of AAV-FIX technology.

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# Targeting co-stimulatory pathways in gene therapy

***Xiaopei Huang*[1] *and Yiping Yang*[1,2]***

[1] Department of Medicine, Duke University Medical Center, Durham, NC, USA
[2] Department of Immunology, Duke University Medical Center, Durham, NC, USA

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Hildegund C. Ertl, Wistar Institute, USA*
*Ashley T. Martino, St John's University, USA*

****Correspondence:***
*Yiping Yang, Departments of Medicine and Immunology, Duke University Medical Center, Box 103005, 106 Research Drive, Durham, NC 27710, USA.*
*e-mail: yang0029@mc.duke.edu*

Gene therapy with recombinant viral vectors such as adenovirus and adenovirus-associated virus holds great promise in treating a wide range of diseases because of the high efficiency with which the viruses transfer their genomes into host cells *in vivo*. However, the activation of the host immune responses remains a major hurdle to successful gene therapy. Studies in the past two decades have elucidated the important role co-stimulation plays in the activation of both T and B cells. This review summarizes our current understanding of T cell co-stimulatory pathways, and strategies targeting these co-stimulatory pathways in gene therapy applications as well as potential future directions.

**Keywords: co-stimulation, viral vectors, gene therapy**

## INTRODUCTION

Virus-based vectors such as adenovirus and adenovirus-associated virus (AAV) have been widely used in gene therapy applications due to their high efficiency of transduction into a variety of cells *in vivo*. Indeed, several impressive results using viral vector-based gene therapy have been reported in humans (Ashtari et al., 2011; Fischer et al., 2011). However, one major barrier to successful gene therapy with these viral vectors is the host immune responses to both viral vectors and the transgenes (Huang and Yang, 2009). Our understanding of the mechanisms of immune responses to viral vectors has greatly improved over the past two decades, and as a result, strategies have been developed to manipulate various immune pathways in order to regulate the immune responses to viral vectors. One promising therapeutic strategy is targeting the co-stimulatory pathways to modulate the function of T and B cells.

The original *two-signal hypothesis* (Lafferty et al., 1974, 1980) proposes that full T cell activation requires two signals: antigen-specific T cell recognition (signal 1) provided by the engagement of the TCR–CD3 complex with a processed antigenic peptide bound to the major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell (APC), and an antigen-non-specific co-stimulatory signal (signal 2) provided by the interactions between cell surface molecules on the APC and the T cell. Indeed, early work by Jenkins and Schwartz showed that in the absence of the co-stimulatory signal, T cells are rendered anergic, a state marked by the inability of T cells to respond to subsequent antigenic stimulation, leading to a model that co-stimulation plays a critical role in controlling the fate of T cell responses: activation or anergy (Schwartz et al., 1989; Jenkins et al., 1990). Since then, these seminal studies have stimulated tremendous growth of the co-stimulation field. In addition to signals 1 and 2, we now appreciate that other signals also participate in determining the fate of T cell activation: some of these signals are stimulatory and others are inhibitory (Sharpe, 2009). Furthermore, some other signals are required to promote the differentiation into different T helper cell subsets (Pipkin and Rao, 2009; Simpson et al., 2010).

Because of the importance of co-stimulation in determining the outcome of the immune response, manipulation of various co-stimulatory pathways to regulate host immune responses is of therapeutic interest. In this review, we first summarize the current knowledge of T cell co-stimulatory pathways. We then focus on strategies targeting co-stimulatory pathways and their potential implications in viral vector-mediated gene therapy.

## T CELL CO-STIMULATORY PATHWAYS

Several co-stimulatory pathways have been characterized including both activating and inhibitory pathways (**Figure 1**). The positive activating signals are balanced by the negative inhibitory signals to achieve optimal control of T and B cell activation. The activating pathways such as B7 and CD28, CD40 ligand (CD40L) and CD40, inducible co-stimulatory molecule (ICOS) and ICOS-ligand (ICOS-L), and OX40 (CD134) and its ligand, OX40L, are critical for the activation of T and B cells, whereas inhibitory pathways such as B7 and cytotoxic T lymphocyte antigen-4 (CTLA-4), and programmed cell death-1 (PD-1) and PD ligand (PD-L) downregulate T cell activation. Here we mainly focus on the activating co-stimulatory pathways.

### B7-CD28/CTLA-4 PATHWAY

The CD28 glycoprotein, a member of the immunoglobulin superfamily, is expressed on the T cell membrane as disulfide-linked homodimers. It is expressed on the surface of 90% of human CD4+ T cells and 50% of human CD8+ T cells, and nearly 100% of murine T cells. The ligands for CD28 are B7-1 (CD80) and B7-2 (CD86), also members of the immunoglobulin superfamily, which are expressed by APCs such as DCs, macrophages, and activated B cells. Although B7-1 and B7-2 are co-expressed and share similar overall structure, they differ in their temporal responses to stimuli, with B7-2 induction occurring earlier and usually at

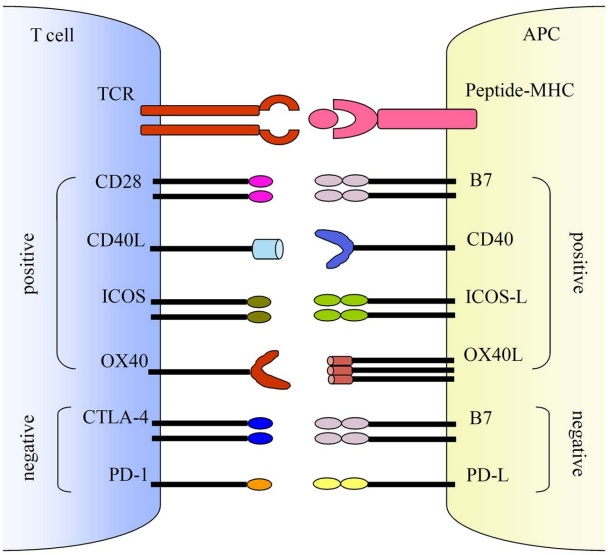


**FIGURE 1 | Co-stimulatory pathways.** Co-stimulations either enhance or down-regulate T cell activation following the initial TCR and peptide-MHC ligation. Positive co-stimulatory pathways include B7–CD28, CD40L–CD40, ICOS–ICOS-L, and OX40–OX40L. Negative co-stimulatory pathways include B7–CTLA-4 and PD-1–PD-L.

much higher level than B7-1 in activated B cells and DCs (Hathcock et al., 1994). The expression of B7-1 and B7-2 is controlled by cytokines and cell–cell interactions. IL-4 is a potent inducer of B7-2 on B cells, whereas IFN-γ and IL-10 differentially regulate B7 expression, depending on cell types (Valle et al., 1991; Stack et al., 1994). Studies have shown that signals transmitted through the MHC class II cytoplasmic domain, which is required for effective antigen presentation, induce B7 expression on B cells (Nabavi et al., 1992).

Mice deficient in CD28 or treated with antagonists of CD28 exhibit substantially reduced proliferative responses (Green et al., 1994). Similarly, mice deficient in both B7-1 and B7-2 have compromised T cell-mediated responses (Borriello et al., 1997; Mcadam et al., 1998), whereas addition of B7 transfectants could augment T cell proliferation and IL-2 production induced with anti-CD3 or PMA in a CD28-dependent manner (Gimmi et al., 1991; Linsley et al., 1991). B7/CD28-mediated signaling enhances the production of IL-2 and IL-2 receptor in a nuclear factor (NF)-κB dependent manner, and upregulates the expression of other cytokines such as IL-1, IL-4, IL-5, TNF, and IFN-γ (reviewed in Lenschow et al., 1996; Kane et al., 2002); accelerates resting T cell entry into and progression through the cell cycle; plays a critical role in the development and differentiation of Th1 and Th2 T cell subsets (Van Der Pouw-Kraan et al., 1992; Seder et al., 1994; King et al., 1995); prevents anergy, and promotes T cell survival by enhancing the expression of the anti-apoptotic proteins such as BCL-xL. Recent studies have reported chromatin structural changes within minutes following T cell activation, and induction of DNA demethylation of *IL*-2 gene as early as 20 min after TCR/CD28 stimulation, suggesting a role for CD28 signaling in these early nuclear events, which are critical to the ensuing processes of cell proliferation and differentiation (Zhao et al., 1998; Acuto and Michel, 2003; Bruniquel and Schwartz, 2003).

Cytotoxic T lymphocyte antigen-4, also known as CD152, is expressed on the T cell membrane as disulfide-linked homodimers. Like CD28 and B7, they are also members of the immunoglobulin superfamily. The ligands for CTLA-4 are also B7-1 and B7-2, but its binding affinity for B7 molecules is about 20-fold greater than that of CD28. Although CTLA-4 and CD28 share sequence homology, CTLA-4 acts antagonistically of CD28, and delivers an inhibitory signal that down-regulates T cell activation. CTLA-4 knockout mice manifest lymphoproliferative disorder which is lethal by 3–4 weeks after birth, supporting the notion that CTLA-4 acts as a negative regulator of T cell activation and is vital for the control of lymphocyte homeostasis (Waterhouse et al., 1995). While CD28 is expressed by both resting and activated T cells, CTLA-4 is only expressed on activated T cells at much lower levels. CTLA-4 induction can be detected 24 h after T cell stimulation, with cell surface expression peaking around 2 days post activation, and returning to background levels by day 4 (Linsley et al., 1992; Walunas et al., 1994). Interestingly, CD28/B7-mediated signaling upregulates the expression of CTLA-4 which effectively competes with CD28 for B7-1/B7-2 at later stages of the immune response to counter T cell activation, suggesting a mechanism for achieving balance between positive and negative stimulation.

## CD40L–CD40 PATHWAY

The CD40 glycoprotein is a member of the tumor necrosis factor-receptor (TNFR) superfamily. It is expressed on APCs such as B cells, DCs, activated macrophages as well as Langerhan cells, endothelial cells, and thymic epithelial cells (Buhlmann and Noelle, 1996). The ligand for CD40 (CD40L), a member of the TNF family, is preferentially expressed on the surface of activated CD4+ T cells in both humans and mice. Early studies have established an important role for CD40L–CD40 interactions in B cell activation and proliferation, as well as immunoglobulin (Ig) class switching (Allen et al., 1993; Disanto et al., 1993). For example, the underlying cause for a severe form of immunodeficiency, X-linked hyper IgM syndrome (HIGM) is a mutation in CD40L (Allen et al., 1993; Disanto et al., 1993). Patients with this disorder have elevated levels of IgM, but little to none IgG or IgA, and absent germinal centers.

In addition to the role in the humoral response, two seminal reports also demonstrate a critical role for the CD40L–CD40 pathway in the activation of T cell responses (Grewal et al., 1996; Yang and Wilson, 1996). In a murine model of experimental allergic encephalomyelitis (EAE), CD4+ T cell priming is defective in CD40L-deficient mice. These mice failed to develop EAE after priming with antigen and produced little to no IL-4 and IFN-γ (Grewal et al., 1996). Similarly, in a murine model of liver-directed gene transfer with adenoviral vectors, CD40L–CD40 signaling on APCs is essential for the development of CD8+ T cell response to adenoviral vector (Yang and Wilson, 1996). This is mediated by upregulating the expression of B7 molecules on APCs, which in turn promote the B7–CD28 pathway, leading to T cell activation (Yang and Wilson, 1996). Indeed, subsequent studies have further delineated that CD40–CD40L interactions between DCs and CD4+ T cells provide DCs with "licensing" in the activation of

CD8+ T cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

The broad spectrum of cells that express CD40 indicates that CD40L–CD40 interactions may exert regulatory effects at multiple levels. CD40L–CD40 interactions also result in the production of IL-12, which promotes the differentiation of Th1 immunity (Trinchieri, 1994). Furthermore, the interactions between CD40L on activated T cells and CD40 on vascular endothelial cells can stimulate the vasculature at sites of inflammation and may play a role in inflammatory responses (Hollenbaugh et al., 1995).

### ICOS PATHWAY

ICOS, a third member of the CD28/CTLA-4 family, is expressed on activated T cells, but very little on resting naïve T cells. ICOS binds specifically to its ligand (ICOS-L), also known as B7-related protein-1 (B7RP-1), which is expressed constitutively on B cells. *In vivo*, the interaction of ICOS with ICOS-L is critical for T cell-dependent B cell responses (Mcadam et al., 2001; Tafuri et al., 2001). In the absence of ICOS, germinal center formation is impaired and immunoglobulin class switching is defective.

In addition to providing help for B cells, ICOS plays an important role in the differentiation of unpolarized CD4+ T cells into Th1, Th2, Th17, and Treg lineages (Simpson et al., 2010). It has been shown that ICOS can promote both Th1 and Th2 responses. However, ICOS and ICOS-L interaction during the early stages of T cell differentiation favors Th2 response (Nurieva et al., 2003). Although ICOS is not required for Th17 differentiation during primary responses, it plays a pivotal role in promoting Th17 compartment by upregulating IL-23R during secondary responses (Bauquet et al., 2009). Recent studies also demonstrate a critical role of ICOS in maintaining the homeostasis of CD4 + Foxp3 + Treg (Burmeister et al., 2008; Ito et al., 2008).

### PD-1 PATHWAY

Programmed cell death-1, an inhibitory receptor in the CD28 family, is expressed on CD4 and CD8 T cells, B cells, NKT cells, and some DC subsets upon activation (Keir et al., 2008). PD-1 has two ligands, PD-L1 and PD-L2. Upon ligation to either PD-L1 or PD-L2, PD-1 attenuates TCR signaling, leading to suppression of T cell responses. Indeed, in a model of chronic LCMV infection, PD-1 is overexpressed on the exhausted T cells and blockade of PD-1 and PD-L1 interaction led to the functional restoration of these exhausted T cells (Barber et al., 2006). Furthermore, the PD-1 pathway plays an important role in regulating immune responses to acute infections (Brown et al., 2010). In addition to providing inhibitory signals to T cells, a recent study has also revealed an essential role for PD-1 signaling in germinal center B cell survival and the formation and affinity of long-lived plasma cells (Good-Jacobson et al., 2010).

## TARGETING THE B7–CD28/CTLA-4 PATHWAY

Early studies in transplant setting have demonstrated that blockade of the CD28/B7 co-stimulation with CTLA-4Ig, a soluble fusion protein consisting of the extracellular domains of CTLA-4 and the constant region of the IgG1 heavy chain, could cause host T cells directed against the grafted tissue anergic, therefore leading to the long-term survival of the graft (Lenschow et al., 1993). CTLA-4Ig acts through competition with CD28 for its ligands, B7-1 and B7-2, disrupting the co-stimulation required for complete T cell activation. In addition, studies using a combination of anti-B7-1 and anti-B7-2 monoclonal antibodies (mAbs) in an allogeneic pancreatic islet transplant setting showed this approach selectively delayed CD4+ T cell infiltration into the graft, leading to inhibition of transplant rejection (Lenschow et al., 1995). Similarly, CTLA-4Ig has been used successfully to suppress T cell responses in animal models of autoimmunity (Dall'era and Davis, 2004). These studies provide evidence that blocking co-stimulatory pathway is a viable strategy in inducing T cell anergy.

A number of studies have also investigated the effect of blocking the B7–CD28 co-stimulatory pathway in viral vector-mediated gene therapy. Administration of CTLA-4Ig blocked the formation of anti-β-galactosidase antibodies following retrovirus-mediated gene transfer to the liver (Puppi et al., 2004). Similarly, treatment with CTLA-4Ig resulted in more stable transgene expression after retrovirus-mediated gene therapy in mucopolysaccharidosis I mice (Ma et al., 2007). Furthermore, potent immunosuppression has been observed with a high-affinity variant of CTLA-4Ig, LEA29Y (belatacept; Larsen et al., 2005). In a mouse model of Duchenne muscular dystrophy (DMD), adenovirus vectors carrying murine CTLA-4Ig (AdmCTLA-4Ig) was coadministered with an adenoviral vector carrying a full-length murine dystrophin cDNA (AdmDys) into skeletal muscle (Jiang et al., 2004a). Stable expression of dystrophin in skeletal muscle up to 8 weeks was achieved, in contrast to the control group without CTLA-4Ig where dystrophin expression was significantly diminished within 8 weeks. CTL response to adenoviral vector was markedly suppressed in the group receiving co-administration of AdmCTLA-4Ig at 2-week time point, and cytokine production such as IFN-γ, IL-4, and IL-6 were much lower than the control group as well. No dystrophin-specific CTL response was observed in either group during the 2-week period. Neutralizing antibody against adenoviral vector was detected in both groups, with highest levels found in the control group, indicating that CTLA-4Ig partially blocked the humoral response against adenoviral vectors.

Similar strategies employing CTLA-4Ig to block the B7–CD28 co-stimulatory pathway have been investigated in a murine model of type II collagen-induced arthritis (Ijima et al., 2001). However, in other model systems, the effect of AdmCTLA-4Ig has been modest (Laumonier et al., 2003), suggesting other strategies in combination with CTLA-4Ig may be needed to achieve successful therapeutic effect.

## TARGETING THE CD40/CD40 LIGAND PATHWAY

The critical role of the CD40L–CD40 pathway in activation of both T and B cells suggests interfering with this pathway may prevent both cellular and humoral immune responses to virus, which is critical for stable transgene expression and the repeated administration of the viral vectors. Indeed, initial studies revealed that a transient blockade of CD40 signaling with an antibody to CD40L infused at the time of vector administration resulted in stable transgene expression and diminished production of neutralizing antibodies in a murine model of adenovirus-mediated gene transfer to the liver and the lungs (Yang et al., 1996). Later, anti-mouse CD40L mAb in combination with CTLA-4Ig has been

shown to further prolong adenovirus-mediated transgene expression after both primary and secondary vector delivery (Kay et al., 1995). Similarly, the combined CD40L blockade and CTLA-4Ig approach also proved effective in a murine model of retrovirus-mediated gene therapy for mucopolysaccharidosis (Ma et al., 2007). More recently, the dual blockade strategy has been shown to act synergistically to prevent antigen-specific immune responses in non-human primates, leading to stable expression of transgene and diminished neutralizing antibody production, and in some cases allowing for repeated administration of adenovirus (Haegel-Kronenberger et al., 2004).

The effectiveness of the combined antibody blockade strategy led to the evaluation for the effect of systemic administration of AdmCTLA-4Ig and AdmCD40Ig (adenoviral vector carrying murine CD40Ig) in murine models of gene delivery to the muscle (Jiang et al., 2004b) and the liver (Schowalter et al., 1997). These studies showed that systemic delivery of both AdmCTLA-4Ig and AdmCD40Ig was required to inhibit the production of anti-adenoviral vector neutralizing antibody, and that AdmCTLA-4Ig alone was insufficient. This may be due to insufficient circulating level of mCTLA-4Ig to prevent anti-adenoviral capsid protein neutralizing antibody production, and that addition of circulating levels of mCD40Ig decreases B cell activation by T cells hence leading to diminished neutralizing antibody production.

Taken together, these data lend support to the effectiveness of the combined CD40 and CD28 blockade strategy in viral vector-mediated gene therapy in animal models. However, the efficacy of this approach in human gene therapy requires further exploration in clinical trials.

## TARGETING THE ICOS/ICOS-L PATHWAY

T cell-dependent B cell responses are critically dependent on the interaction of ICOS with ICOS-L, which promotes germinal center formation and immunoglobulin class switching *in vivo* (Mcadam et al., 2001; Tafuri et al., 2001). It has been shown that anti-ICOS alone or in combination with CD40Ig or anti-CD40L can induce tolerance to islet allografts (Nanji et al., 2006) and inhibit allograft rejection in murine models of transplantation (Guillonneau et al., 2005; Taylor et al., 2005). In a murine model of non-viral mediated gene therapy for hemophilia A, blockade of the ICOS–ICOS-L pathway by anti-ICOS antibody alone was effective in inhibiting the formation of anti-factor VIII antibodies following plasmid DNA-mediated gene transfer (Peng et al., 2008). In this study, anti-ICOS treatment also resulted in elevation of Treg numbers and their suppressive activity (Peng et al., 2008). However, the efficacy of ICOS blockade has not been tested in animal models of viral vector-mediated gene transfer.

## NOVEL STRATEGIES FOR TARGETING THE CO-STIMULATORY PATHWAY

Recent advances in our understanding of the role of the innate immunity in adaptive immune responses suggest potential novel strategies for targeting the co-stimulatory pathways. The innate immune system is the first line of defense against invading pathogens through recognition of pathogen-associated molecular patterns (PAMPs) by a set of receptors called pattern recognition receptors (PRRs; Huang and Yang, 2009). Among the PRRs identified to date, the best studied is the toll-like receptor (TLR) family. Thirteen TLRs have been identified in mammals, with each recognizing a unique set of PAMPs. Most TLRs signal through the myeloid differentiating factor 88 (MyD88)-dependent pathway, initiating the downstream signaling cascade leading to the induction of proinflammatory response characterized by the production of cytokines and chemokines (Akira et al., 2006). In addition, TLR pathways are critical for the ensuing adaptive immune responses by promoting DC maturation and function through upregulation of co-stimulatory molecules (Iwasaki and Medzhitov, 2004; Huang and Yang, 2010).

Recent studies have demonstrated that the innate immune response to AAV is mediated by TLR9 (Zhu et al., 2009; Martino et al., 2011). AAV mainly activates the TLR9–MyD88 pathway in pDCs, leading to the production of type I IFNs (Zhu et al., 2009). *In vivo*, the TLR9–MyD88 pathway is crucial to the activation of CD8 T cell response to both the transgene product and the AAV vector, leading to the loss of transgene expression and the generation of neutralizing antibodies to transgene product and AAV (Zhu et al., 2009). Thus, the TLR9–MyD88-type I IFN pathway plays a crucial role in promoting the adaptive immune responses to AAV.

Stimulation of TLR9 with its ligand, CpG-DNA leads to upregulation of surface expression of co-stimulatory molecules, such as CD40, B7-1/B7-2, and MHC class II on cDCs and macrophages (Hemmi et al., 2003). Therefore, DC maturation and Th1-like cytokine induced by TLR9 stimulation result in efficient and robust activation of Th1 and CD8 cytotoxic T lymphocytes (CTL; Lipford et al., 2000; Horkheimer et al., 2009). Type I IFNs, in addition to their direct anti-viral effects (Vaidya and Cheng, 2003), have also been shown to induce DC maturation by upregulating the expression of co-stimulatory molecules, which in turn promotes T cell responses (Hoebe et al., 2003). DCs matured by type I IFNs promote cross-priming of virus-specific CD8 T cells (Le Bon et al., 2003). Type I IFN can also promote effector function of virus-specific T cells (Cousens et al., 1999), and enhance the survival of activated T cells (Marrack et al., 1999). Furthermore, type I IFN signaling on DCs is important for the production of virus-specific IgM, whereas the generation of protective neutralizing antibodies to adenovirus critically depends on the type I IFN signaling on both CD4 T cells and B cells (Zhu et al., 2007).

A critical role for the TLR9 signaling pathway in the upregulation of co-stimulatory molecules and the induction of adaptive immune response to AAV suggests that strategies targeted to interfere with this signaling pathway may diminish T cell responses to the AAV vector and the transgene product, leading to prolonged transgene expression and reduction in inflammation, improving the safety and efficacy of AAV vectors for gene therapy in humans. Furthermore, blockade of the TLR9 signaling pathway will also likely inhibit antibody responses to the transgene product as well as the AAV vector.

One way to interfere with the TLR9–MyD88-type I IFN signaling pathway is to use antagonists specific to TLR9 such as inhibitory ODN to block the TLR9–MyD88 pathway (Zhu et al., 2009; Martino et al., 2011). Alternatively, blockade of type I IFNs

by administering neutralizing antibodies to IFN-α and IFN-β may also prove effective as type I IFNs are required for adaptive immune responses to AAV (Zhu et al., 2009).

## FUTURE PERSPECTIVES

In this review, we have summarized the role of various T cell co-stimulatory pathways in the activation of both T and B cells and strategies targeting these pathways to improve the outcome of viral vector-mediated gene therapy. The majority of the studies so far are focused on targeting the B7–CD28 and the CD40L–CD40 pathways. It may be beneficial to investigate the effect of blocking other positive co-stimulatory pathways such as ICOS and ICOS-L, and OX40 and OX40L. In addition, strategies for stimulating the negative co-stimulatory pathways such as PD-1 and PD-L may prove effective. Given that the combined blockade of CD40 and CD28 pathways is more effective than the blockade of a single pathway alone, future studies should focus on targeting multiple pathways including both the positive and negative co-stimulatory pathways. In this regard, since the innate immunity is critical in regulating multiple co-stimulatory pathways, strategies targeting innate immune pathways may warrant further investigation. Ultimately, the utility of these strategies in improving the outcome of gene therapy with viral vectors will need to be tested in human clinical trials.

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# B-cell gene therapy for tolerance induction: host but not donor B-cell derived IL-10 is necessary for tolerance

**Yan Su[1,2†], Ai-Hong Zhang[1,2], Nancy Noben-Trauth[3] and David W. Scott[1,2,4†]***

[1] Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, Baltimore, MD, USA
[2] Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA
[3] University of Maryland at Shady Grove Center, Rockville, MD, USA
[4] Department of Surgery, University of Maryland School of Medicine, Baltimore, MD USA

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Brad E. Hoffman, Temple University School of Medicine, USA*
*George Aslanidi, University of Florida, USA*

***Correspondence:**
*David W. Scott, Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA.*
*e-mail: david.scott@usuhs.edu*

[†] ***Present address:***
*Yan Su and David W. Scott, Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA.*

Genetically modified B cells are excellent tolerogenic antigen-presenting cells (APCs) in multiple models of autoimmunity. However, the mechanisms of action are still not completely understood. In our models, we generate antigen-specific tolerogenic B cells by transducing naïve or primed B cells with an antigen–immunoglobulin G (peptide–IgG) construct. In order to be transduced, B cells require activation with mitogens such as LPS. We and others have found that LPS stimulation of B cells upregulates the production of IL-10, a key cytokine for maintaining immune tolerance. In the current study, we defined the role of B-cell produced IL-10 in tolerance induction by using IL-10 deficient B cells as donor APCs. We found that peptide–IgG transduced IL-10 KO B cells have the same effects as wt B cells in tolerance induction in an experimental autoimmune encephalomyelitis model. Moreover, we demonstrated that the tolerogenic effect of peptide–IgG B cells was completely abrogated in anti-IL-10 receptor antibody treated recipients. Taken together, our results suggest that tolerance induced by peptide–IgG B-cell gene therapy requires IL-10 from the host but not donor B cells. These data shed important insights into the mechanisms of tolerance induction mediated by B-cell gene therapy.

**Keywords: gene therapy, B cells, IL-10, IL-10 receptor, tolerance**

## INTRODUCTION

Immune tolerance to self-antigens must be maintained or autoimmunity occurs. Based on the excellent tolerogenicity of IgG (Aldo-Benson and Borel, 1976; Borel et al., 1976) and the capacity of B cells to be tolerogenic antigen-presenting cells (APCs; Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Gilbert and Weigle, 1994), our laboratory has developed a B-cell based gene therapy approach for actively inducing tolerance in animal models of autoimmune diseases and hemophilia (Skupsky et al., 2007). For example, when retrovirally transduced with a fusion construct encoding IgG heavy chain and factor VIII (fVIII) immunodominant domain C2 or A2, B cells are tolerogenic in both naïve and fVIII-primed hemophilic mice (E16, fVIII$^{-/-}$) as measured by specific cellular response and inhibitory antibody titers (Lei and Scott, 2005). In experimental autoimmune encephalomyelitis (EAE), myelin oligodendrocyte glycoprotein (MOG)–IgG, or myelin basic protein (MBP)–IgG transduced B cells can protect mice from EAE (Xu and Scott, 2004; Zhang et al., 2010). In other models of autoimmune diseases including experimental autoimmune uveitis, type I diabetes, and arthritis, animals that received peptide–IgG transduced B cells exhibited reduced specific humoral and cellular responses, delayed disease onset, and amelioration of ongoing disease (Agarwal et al., 2000; Melo et al., 2002; Soukhareva et al., 2006; Satpute et al., 2007). Achievement of antigen-specific tolerance in these models indicates the promise of this method as therapy for patients with autoimmune diseases or hemophiliacs with fVIII inhibitor formation. Similar results were reported by several other groups where B cells were also utilized for tolerance induction in EAE (Chen et al., 2001, 2004) and in a mouse model of asthma (Ahangarani et al., 2009).

The underlying mechanisms for tolerance induction by B-cell delivered gene therapy remains incompletely understood. IL-10 is a key regulator of immune-suppression and for maintenance of peripheral tolerance (Kuhn et al., 1993). Originally, IL-10 was defined as a Th2 cytokine that inhibits Th1 T-cell responses (Fiorentino et al., 1989). Later studies suggest that IL-10 can be produced by multiple types of T cells including CD4$^+$CD25$^+$ regulatory T cells (Tregs), as well as APCs. In the resting state, B cells produce only low levels of IL-10. However, activated B cells dramatically upregulate the production of IL-10 (Fillatreau et al., 2002; Barr et al., 2007; Lampropoulou et al., 2008). Whether IL-10 is involved in the mechanisms of tolerance induction by B-cell based gene therapy remains controversial. Ahangarani et al. (2009) demonstrated that genetically modified B cells can induce tolerance in an allergy model in an IL-10 dependent manner. On the other hand, Frommer et al. (2008) found that production of IL-10 by MOG presenting B cells plays no role in tolerance induction in an EAE model. To optimize our B-cell based gene therapy protocol for future clinical study, we screened multiple mitogens for B-cell activation and, interestingly, found that the manner of B-cell activation was critical for tolerance induction. Thus, LPS-activated B cells are tolerogenic, but unmethylated DNA CpG stimulated

B cells are not (Lei et al., 2005). Further investigation indicated that LPS-treated B cells produce significantly higher amounts of IL-10 than CpG-treated B cells (Skupsky et al., 2007). These findings raise an interesting question regarding the mechanisms of tolerance induction: Do B cells really need IL-10 to induce tolerance?

In the current study, the role of IL-10 was tested by using IL-10 KO mice as B-cell donors or by treating recipient animals with an anti-IL-10 receptor (IL-10R) antibody. We found that IL-10 produced by host but not by donor B cells is critical for tolerance induction. Since we previously showed that $CD4^+CD25^+$ Tregs were required for the induction and maintenance of tolerance (Lei and Scott, 2005; Soukhareva et al., 2006), we propose that these cells may be the source of endogenous IL-10 in the host.

## MATERIALS AND METHODS

### MICE

C57BL/6, Balb/c, membrane ovalbumin expressing (mOVA) transgenic, and H-$2^b$ IL-10 KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). H-$2^d$ IL-10 KO mice were a kind gift from Dr. Wendy Davidson (University of Maryland School of Medicine, Baltimore, MD, USA). All recipient animals were used at 5–8 weeks of age and housed in pathogen-free microisolator cages in the animal facility at the University of Maryland, Baltimore. All animal experiments were approved by IACUC of University of Maryland School of Medicine.

### ANTIBODIES

Anti-IL-10R (1B1.3a, rat IgG1) and control mAb (GL113, anti-*E.coli* β-galactosidase, rat IgG1) hybridomas were originally provided by Dr. Kevin Moore (DNAX, Palo Alto, CA, USA). The anti-IL-10R and control mAbs used *in vivo* were purified from ascites produced in nude mice by ammonium sulfate precipitation and ion exchange chromatography (Harlan Bioproducts).

### VIRUS-PRODUCING CELL LINES

The cDNA of the peptide–$IgG_1$ heavy chain was subcloned into the murine Moloney leukemia retroviral vector (MBAE), as previously described (Zambidis et al., 1997a,b). Briefly, a DNA fragment of $pOVA_{323–339}$ or full-length OVA was inserted into the BSSK-IgG vector and then the IgG fusion construct was subsequently subcloned into the MBAE retroviral vector. Virus-producer cell line was prepared by stable transfection of GP + E86 packaging cells with the engineered construct. Viral producing packaging cells were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and 2-mercaptoethanol.

### B-CELL PURIFICATION AND RETROVIRAL TRANSDUCTION

Splenic B cells from naïve mice were purified to approximately 95% homogeneity with anti-T-cell antibody cocktail (anti-Thy1, anti-CD4, and anti-CD8) followed by complement (Low-Tox-M, Cedarlane Laboratories, Accurate Chemical and Scientific Corporation, Westbury, NY, USA). Purified B cells were prestimulated with 1 μg/ml LPS (E. coli 055:B5, Sigma, St. Louis, MO, USA) overnight and then transduced *in vitro* via co-culture with 1500 rad irradiated virus-producing packaging cells for 24 h in the presence of 6 μg/ml polybrene.

### TOLERANCE INDUCTION TO OVA AND T-CELL PROLIFERATION ASSAY

$1 \times 10^7$ $pOVA_{323–339}$-IgG or OVA–IgG transduced wt or IL-10 KO B cells were transferred into naïve mice intraperitoneally. One week later, recipient mice were immunized with 25 μg OVA protein emulsified in CFA in one hind footpad and the base of tail. Two weeks after immunization, mice were bled and then sacrificed. Draining inguinal and popliteal LNs were removed for T-cell proliferation assay. Single cell solution was prepared at $5 \times 10^6$ cells/ml. One hundred microliter cells were seeded onto 96-well plate in the presence of indicated concentrations of pOVA peptide. 48 h later, cell culture were pulsed with 1 μCi [$^3$H] thymidine (Amersham Life Sciences, Arlington Heights, IL, USA) and incubated for another 16–20 h. Cells were then harvested on glass fiber filters and [$^3$H] thymidine incorporation was counted by using Scintillation Counter. Data were presented as mean Δcpm (count per minute by subtraction of the background) ± SE. Anti-OVA–IgG titers were determined by endpoint ELISA methods.

### IL-10R BLOCKING *IN VIVO*

Anti-IL-10R (1B1.3a, rat IgG1) mAb was used to block IL-10 function *in vivo* and anti-*E.coli* β-galactosidase was used as isotype control. Mice were received B-cell gene therapy (retrovirally transduced wt B cells or mOVA transgenic B cells in different experiments) on day 0 and challenged on day 7. These recipient mice were injected four times on day −3, 0, 7, and 14, respectively. Each time 1 mg/mouse of anti-IL-10R or control mAb was injected intraperitoneally.

### B-CELL GENE THERAPY FOR TOLERANCE INDUCTION IN MURINE EAE

One week before the active induction of EAE, C57BL/6 (B6) were adoptively transferred with $1 \times 10^7$ retrovirally transduced syngeneic tolerogenic B cells expressing $MOG_{35–55}$–Ig (MOG–Ig), intraperitoneally. For EAE induction, 6-week-old female B6 mice were subcutaneously immunized on the flanks with 200 μg of $MOG_{35–55}$ peptide emulsified in CFA containing 4 mg/ml of *Mycobacterium tuberculosis* H37Ra (DIFCO, Detroit, MI). On the day of immunization and 48 h later, the mice also received 200 ng of Pertussis toxin (Sigma-Aldrich) in 0.2 ml PBS intraperitoneally. Clinical signs of EAE were assessed daily with a 0–5 scoring system (Stromnes and Goverman, 2006): 0, normal; 0.5, partially limp tail; 1, paralyzed tail; 2, loss in coordinated movement; 2.5, one hind limb paralyzed; 3, hind limbs paralyzed; 3.5, hind limbs paralyzed and forelimbs weakness; 4, forelimbs paralyzed; 5, moribund.

### STATISTICS

Paired or unequal variances one-tailed Student's *t*-test statistics were applied with differences considered significant at $^*p < 0.05$.

## RESULTS

### IL-10 PRODUCED BY DONOR B CELLS IS NOT REQUIRED FOR TOLERANCE INDUCTION

Our laboratory has successfully applied the B-cell based peptide–IgG gene therapy protocol in multiple animal models for autoimmunity such as type 1 diabetes, EAE, experimental autoimmune uveitis, and also in a mouse model for hemophilia A. In this gene therapy protocol, B cells have to be activated before retroviral

transduction as only dividing cells can be effectively transduced with the peptide–IgG construct. We routinely use LPS to stimulate B cells. Other B-cell mitogens such as anti-IgM and anti-CD40 have similar effects as LPS on B cells in term of tolerance induction. Surprisingly, although it could activate B cells effectively, unmethylated CpG DNA stimulation results in B cells losing their tolerogenic phenotype (Lei et al., 2005). We systematically compared the phenotypic changes of B cells upon LPS and CpG stimulation. Compared to the resting B cells, although both LPS and CpG B-cell blasts have upregulated expression of IL-10 at protein and messenger RNA levels, LPS B cells produce much more IL-10 than CpG B cells (Skupsky et al., 2007). These findings, together with the contradict reports from the literature, led us to ask whether IL-10 is critical for tolerance induction through our B-cell delivered gene therapy protocol. Therefore, in the current study, we first studied the role of B-cell derived IL-10 in our gene therapy system by using H-$2^d$ IL-10 KO mice as B-cell donors. We transduced LPS-activated B cells with a retroviral construct encoding an immunodominant epitope 323–339 fragment of ovalbumin (OVA) and IgG1 heavy chain ($pOVA_{323–339}$–IgG) or a mock control (phospholipid protein, PLP). As expected, mice that received $pOVA_{323–339}$–IgG transduced wt B cells exhibited reduced anti-OVA antibody. Surprisingly, $pOVA_{323–339}$–IgG transduced IL-10 KO B cells also induced a significant reduction of antibody to OVA (**Figure 1**). This suggests that IL-10 KO B cells have normal antigen-presenting function and more importantly, that IL-10 produced by donor B cells is not required for tolerance induction. In addition, both $pOVA_{323–339}$–IgG transduced wt or IL-10 KO B cells significantly reduced the T-cell response to $pOVA_{323-339}$ in recipients. We confirmed these results by using H-$2^b$ IL-10 KO B cells as donors to induce tolerance to OVA protein in C57BL/6 mice (data not shown).

To validate that IL-10 produced by tolerogenic B cells is not required for tolerance induction, we used IL-10 KO B cells as tolerogenic APCs to induce tolerance in the EAE model. We have previously showed that MOG–IgG transduced B cells were highly tolerogenic and adoptive transfer of MOG–IgG B cells protected recipients from EAE induction (Zhang et al., 2010). In the current study, we first transduced IL-10 KO C57BL/6 B cells with MOG–IgG or a mock control (OVA–IgG). Transduced B cells ($1 \times 10^7$) were injected intraperitoneally into naïve C57BL/6 mice 1 week before active induction of EAE with $MOG_{35–55}$. After EAE induction, we monitored the disease development daily. We found that MOG–IgG transduced wt B cells protected mice from EAE in terms of mean disease score and disease incidence during the early phase of the disease development (**Figures 2A,B**). However, we observed an overlap of the average clinical scores in the end of this experiment. Based on our previous findings (Zhang et al., 2010), we believe this is due to the relatively short observation period and does not indicate that the disease protection by wt B cells was transient. Interestingly, MOG–IgG transduced IL-10 KO B cells also protected mice from EAE, suggesting that IL-10 deficiency does not affect tolerance inducing property of B cells (**Figures 2A,B**). Furthermore, in a hemophilia mouse model, we found that fVIII domain C2–IgG transduced IL-10 KO B cells are tolerogenic to C2 peptide *in vivo* (data not shown). Taken together, we confirmed that tolerance induction by our B-cell gene therapy protocol does not depend on the IL-10 produced by donor B cells in multiple animal models from different genetic background.

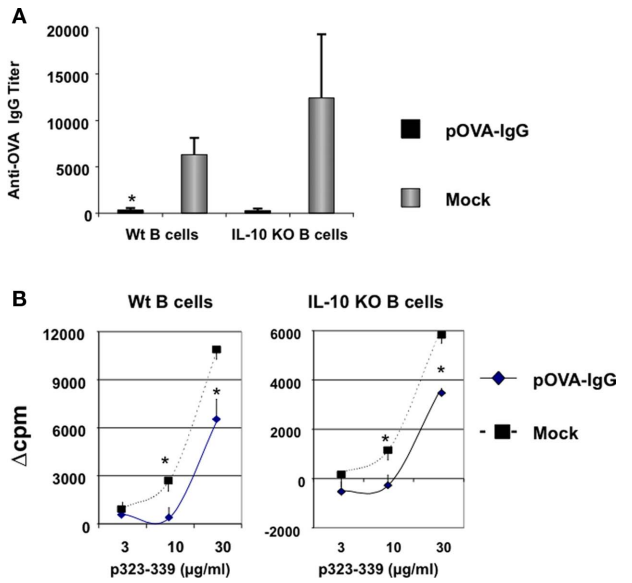


**FIGURE 1 | Donor B cells from IL-10 KO mice are tolerogenic. (A)** LPS-activated wt or IL-10 KO B cells were transduced with $pOVA_{323–339}$–IgG or a mock control vector (PLP–IgG) and $1 \times 10^7$ B cells per mouse were injected i.p. into naïve Balb/c recipients. Seven days later, mice were immunized with 25 μg OVA protein emulsified in CFA via a hind footpad and the base of tail. Two weeks after immunization, sera were collected and assayed for total anti-OVA–IgG by the endpoint ELISA method ($n = 4$). **(B)** Animals were treated as described in **Figure 1A**. Mice were sacrificed 2 weeks after immunization and draining lymph nodes were removed for $CD4^+$ T-cell proliferation assay. Five $\times 10^5$ cells from draining LNs were seeded per well in 96-well plates in the presence of indicated concentrations of p323–339. After 48 h, cultures were pulsed with 1 μCi/well of [$^3$H] thymidine (Amersham Life Sciences, Arlington Heights, IL, USA) and incubated for another 16–20 h. Cells were then harvested on glass fiber filters and T-cell proliferation to p323–339 was determined by [$^3$H] thymidine incorporation. Values represent mean Δcpm (count per minute by subtraction of the background) ± SE for four animals. Data are representative of two independent experiments.

## IL-10 PRODUCED BY THE HOST IS REQUIRED FOR TOLERANCE INDUCED BY PEPTIDE–IGG EXPRESSING B CELLS

In multiple models of infection and autoimmune diseases, mice that are genetically deficient in IL-10 exhibit dramatically exacerbated diseases (Kuhn et al., 1993; Bettelli et al., 1998; Yin et al., 2002). Since IL-10 plays such a critical role in immune regulation and maintenance of tolerance, we next sought to ask whether IL-10 produced *by the host* is required for tolerance induction in our model. To this end, we utilized *in vivo* anti-IL-10R antibody treatment to block IL-10 function in normal mice, rather than using IL-10 KO mice which have an autoimmune syndrome. Following the standard tolerance induction protocol, LPS-activated, OVA–IgG transduced B cells were transferred into naïve mice on day 0. On day −3, 0, 7, and 14, recipients were injected four times with anti-IL-10R or control rat IgG1 mAb. These recipients were immunized with OVA protein in CFA on day 7 and the effect of anti-IL-10R blocking on tolerance induction was evaluated on

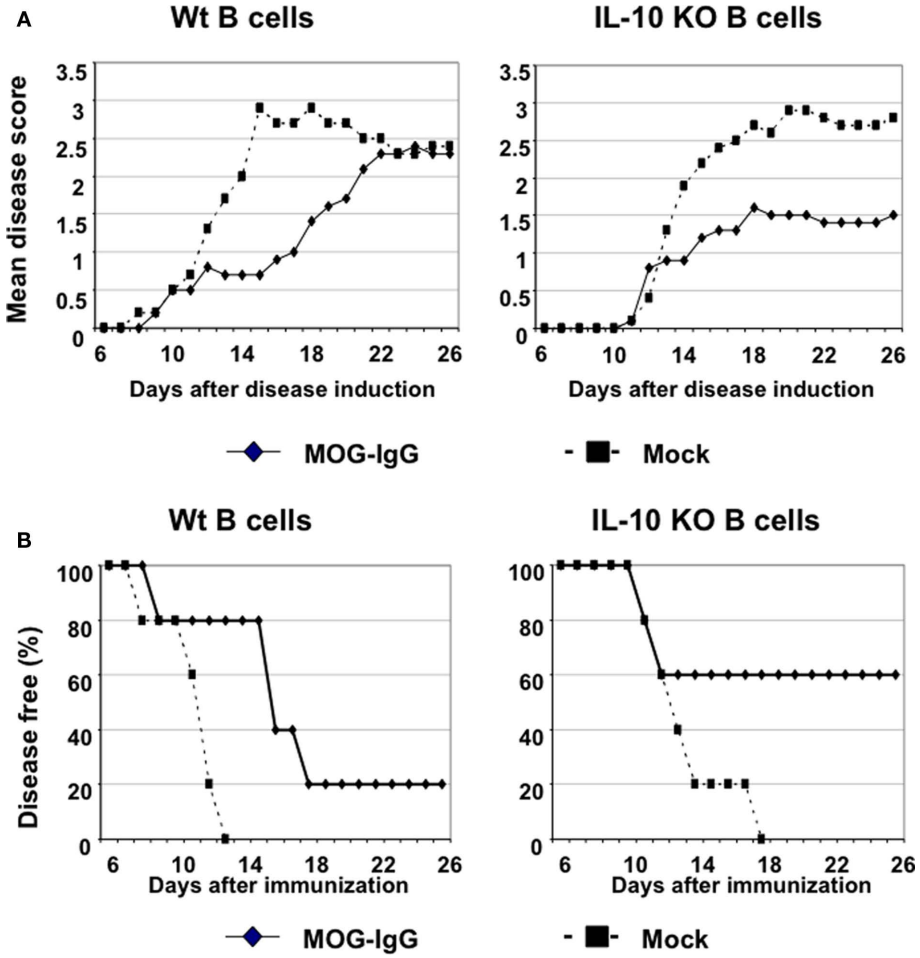


**FIGURE 2 | IL-10 deficient B cells are tolerogenic in an EAE model.** Female C57BL/6 recipients ($n = 5$) were adoptively transferred with $1 \times 10^7$ retrovirally transduced B cells. One week later, the mice were actively immunized for EAE induction using $MOG_{35-55}$ (200 μg)/CFA. The mice were also received 200 ng Pertussis toxin in 0.2 ml PBS via i.p. injection at the same day and 2 days after immunization. The clinical score was monitored daily using a 0–5 scoring system. Mean disease score **(A)** and the disease incidence **(B)** were shown. As the mice that received MOG–Ig transduced B cells from wt C57BL/6 donors (left panels), the mice that received MOG–Ig transduced IL-10 KO B cells (right panels) also had ameliorated disease symptoms and decreased disease incidence, as compared with the control.

day 21 by testing *in vitro* T-cell response and antibody titer. As expected, control mAb treatment has no effect on the hyporesponsiveness induced by OVA–IgG B cells (**Figures 3A,B**). Mice that received OVA–IgG transduced B cells and control mAb treatment exhibited reduced T-cell and antibody responses against OVA protein. However, in anti-IL-10R treated mice, tolerance induction was completely abrogated, suggesting that IL-10 produced by the host is required for tolerance induction.

To confirm these results, we used another model in which mOVA transgenic B cells were used as APCs. We have found that both resting and LPS-activated mOVA B cells are tolerogenic *in vivo* (Su et al., in preparation). In this study, we first transferred LPS-activated mOVA B cells to wt mice and challenged these recipients 1 week later. The recipients were treated with anti-IL-10R or control mAb four times on day −3, 0, 7, and 14. We assayed T-cell and antibody responses on day 21. Again, the tolerogenicity of mOVA B cells was abrogated by *in vivo* IL-10R blockade (**Figure 4**). Together, our data in both systems suggest that host IL-10 production is required for genetic engineered B-cell mediated tolerance induction.

## DISCUSSION

Adoptive transfer of target-antigen expressing B cells has been demonstrated to be able to induce antigen-specific tolerance in various animal models such as EAE, allergy, and hemophilia. In this study, we defined the role of IL-10 in an established B-cell based gene therapy system for tolerance induction. We demonstrated that antigen-specific tolerance does not need IL-10 generated by peptide–IgG transduced tolerogenic B cells. Rather, IL-10 produced by the host is required for tolerance induction.

B cells play an important role in controlling autoimmunity. For example, B-cell deficient mice lose the ability of recovery from clinical disease symptoms after EAE induction (Wolf et al., 1996). It has been found that IL-10 produced by B cells is critical at

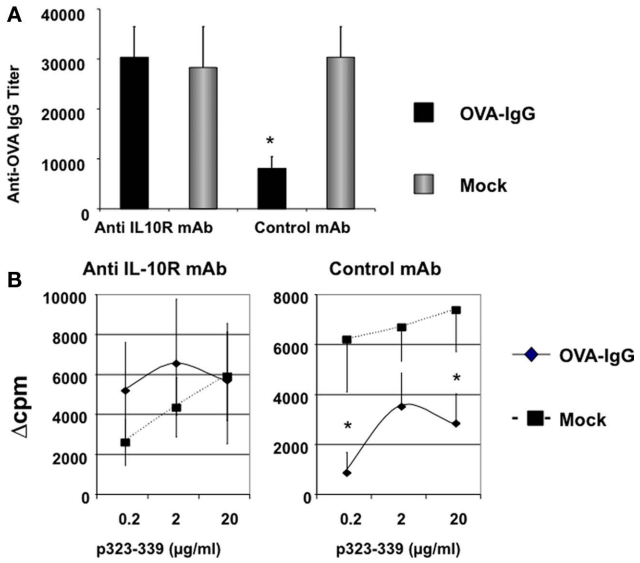


**FIGURE 3 | IL-10 produced by the host is required for tolerance induction by peptide–IgG transduced B cells.** LPS-activated wt B cells were transduced with OVA–IgG or a mock control vector (PLP) and $1 \times 10^7$ B cells per mouse were injected i.p. into naïve C57BL/6 recipients on day 0. Seven days later, mice were immunized with 25 μg OVA protein emulsified in CFA via a hind footpad and the base of tail. Two weeks after immunization, mice were sacrificed and assayed for $CD4^+$ T-cell and antibody responses. On day −3, 0, 7, and 14, mice were injected i.p. four times with 1 mg/mouse of anti-IL-10R (1B1.3a, rat IgG1) or control mAb (GL113, anti-*E.coli* β-galactosidase, rat IgG1). The antibody **(A)** and T-cell responses **(B)** were assayed and presented as described in **Figure 1** legend. Values represent mean ± SE of antibody titers or mean Δcpm ± SE. ($n = 4$). Data are representative of two independent experiments.

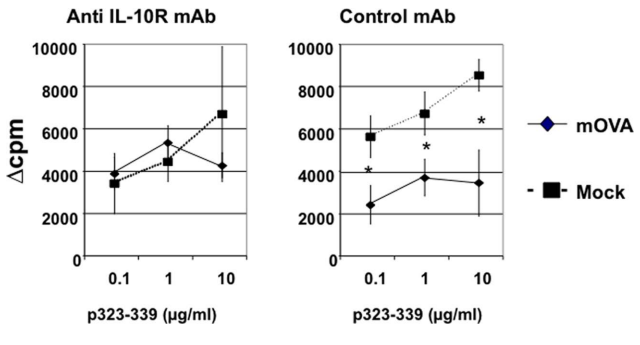


**FIGURE 4 | IL-10 produced by the host is required for tolerance induction by transgenic tolerogenic B cells.** One $\times 10^7$ B LPS-activated mOVA or wt B cells were injected into wt C57BL/6 mice. Tolerance induction protocol, anti-IL-10R treatment, and assay method are same as described in **Figures 1** and **3** ($n = 4$). Data are representative of two independent experiments.

the remission stage of EAE as B-cell restricted IL-10 deficiency results in mice that fail to recover from EAE (Fillatreau et al., 2002). Recently, Carter et al. (2011) demonstrated that in an arthritis model, IL-10-producing B-cell deficient mice exhibited an exacerbated disease symptom. In these IL-10-producing B-cell deficient mice, a decrease of FoxP3 + Tregs and an increase of Th1 and Th17 cells were found. These findings indicate that IL-10-producing B cells might have a regulatory cell phenotype and the IL-10 produced by B cells plays an essential role in regulation of autoimmunity.

Several years ago, we demonstrated that bone marrow cells from IL-10 KO mice could be transduced to be tolerogenic for a cognate antigen (El-Amine et al., 2000). In addition, Frommer et al. (2008) showed that IL-10 produced by B cells is not required for tolerance induction in an EAE model. In that study, EAE resistant mice that express MOG on B cells ($B^{MOG}$) were crossed with IL-10 deficient mice to generate IL-10 KO MOG expressing B cells. They found that IL-10 KO transgenic mice were tolerant and were resistant to MOG-induced EAE. Recently, Calderon-Gomez et al. (2011) validated this result using lentiviral transduction of B cells. In sum, these results suggested that transduced or transgenic B cells tolerize T cells in an IL-10 independent manner.

In contrast, Ahangarani et al. (2009) recently found that IL-10 produced by target-antigen expressing B cells is critical for tolerance in an allergic model. This is of our particular interest because the B-cell gene therapy protocol used in this study is similar as ours. In the allergy model, B cells that express a fusion protein of an endosomal targeting sequence (gp75) and a major T-cell epitope of a common allergen, Der p2, induced tolerance to Der p2 in mice upon peptide immunization. Although detailed mechanisms were not fully elucidated, it was found that genetically modified B cells induced type 1 regulatory T cells (Tr1) in an IL-10 dependent manner (Ahangarani et al., 2009). In a follow-up study, Ahangarani et al. (2011) demonstrated that retroviral particles could bind and activate Toll-like receptor 2 on transduced B cells and in turn, activation of Toll-like receptor 2 results in upregulated production of IL-10 through STAT3 signaling pathway.

In our system, the mechanisms of tolerance induction appear to be different. We previously demonstrated that transduced B cells tolerize recipient animals in a MHC class II dependent manner (Litzinger et al., 2005; Su et al., 2008) and that tolerance induced by peptide–IgG expressing B cells depends on activation of natural $CD4^+CD25^+$ Tregs and/or generation of inducible Tregs (Lei and Scott, 2005; Soukhareva et al., 2006; Skupsky et al., 2010). Depletion of $CD4^+CD25^+$ Tregs completely abrogates the tolerance induction by peptide–IgG transduced B cells (Lei and Scott, 2005; Soukhareva et al., 2006). We also demonstrated that peptide–IgG transduced B cells could induce an increase of $FoxP3^+$ Tregs in recipient animals and a reduction of the $CD4^+$ effector T-cell population (Skupsky et al., 2010). Moreover, we recently observed by two-photon microscopy that tolerogenic B cells tightly conjugate with target $CD4^+$ T cells and this B:T conjugation is CTLA-4/B7 dependent (Su et al., in preparation). Taken together, these observations suggest that in our system, B cells induce tolerance by induction of antigen-specific $CD4^+CD25^+$ Tregs through direct B:T contact. In addition, the IgG heavy chain might also play a role in induction/activation of $CD4^+CD25^+$ Tregs. We previously showed that the IgG heavy chain enhances the degree of tolerance and promotes the hyporesponsive state induced by B-cell gene therapy (Kang et al., 1999; Lei et al., 2005).

Recently, several highly promiscuous major histocompatibility complex class II regulatory T-cell epitopes (Tregitopes) in the

conserved regions of IgG were identified (De Groot et al., 2008). Indeed, we suggest that these IgG epitopes may enhance the tolerogenic properties of the fusion constructs utilized in our B-cell gene therapy system. We found that *in vivo* administration of Tregitopes can stimulate natural Tregs in DO11.10 transgenic mice and delivery of these Tregitopes with OVA protein by B cells also protect mice from antigen challenge (Su et al., in preparation). Based on these observations, we propose that the exogenous IL-10 produced by tolerogenic B cells is not required for inducing/activating $CD4^+CD25^+$ Tregs in the early stage and maintaining tolerance. Thus, we believe that the distinct roles of IL-10 played in these two studies (our work and Ahangarani et al., 2009) are likely due to the different model systems used, i.e., whether the fusion construct expresses IgG, whether natural Tregs or Tr1 cells are induced, or whether an endosomal targeting sequence is utilized.

Nevertheless, our data suggest that the endogenous IL-10 produced by the host is essential for tolerance induction. We have previously shown that both Th1 and Th2 responses in host mice are reduced by peptide–IgG B-cell gene therapy (Kang et al., 1999). Therefore, it is unlikely that the *in vivo* IL-10R blockade has skewed the Th1 and Th2 populations directly. Instead, we suggest that the IL-10R blockade affects the activation or function of natural $CD4^+CD25^+$ Tregs or inducible Tregs, which we have shown to be integral for the efficacy of B-cell gene therapy. The endogenous IL-10 could be produced by different subsets of lymphocytes including regulatory T cells. One of the suppressive mechanisms of Tregs is through producing IL-10. IL-10 produced by APCs is also important for induction of Tregs. For example, IL-10 produced by macrophages in the lamina propria is critical for conversion of Tregs from convention T cells in mucosal immunity (Denning et al., 2007; Murai et al., 2009; Hadis et al., 2011). In addition, it has been demonstrated recently that transitional two marginal zone B cells, which are IL-10-producing and have regulatory phenotypes, play an important role in inducing FoxP3 Tregs and suppressing Th1 and Th17 cells (Carter et al., 2011). Therefore, although in our system exogenous IL-10 from donor B cells is dispensable for inducing tolerance, blocking endogenous IL-10 from the host interrupt tolerance induced by peptide–IgG B cells possibly due to at least two mechanisms: influence the induction or conversion of Tregs from conventional T cells or interfere the function of Tregs.

In summary, several groups have utilized B cells to deliver tolerance via gene therapy. Here we demonstrate that host IL-10 is critical for the tolerogenicity of B-cell based peptide–IgG gene therapy *in vivo* and that IL-10 produced by B cells themselves is not required for tolerance induction.

## CONTRIBUTION

Yan Su and Ai-Hong Zhang designed and performed research, analyzed data, and wrote the manuscript; Nancy Noben-Trauth supervised writing of the manuscript; and David W. Scott designed research and supervised data analysis and writing of the manuscript.

## ACKNOWLEDGMENTS

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# Manipulating immune tolerance with micro-RNA regulated gene therapy

***Kevin Scott Goudy*[1], *Andrea Annoni*[1], *Luigi Naldini*[1,2] *and Maria-Grazia Roncarolo*[1,2]***

[1] San Raffaele Telethon Institute for Gene Therapy, Department of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Institute, Milan, Italy
[2] Universitá Vita-Salute San Raffaele, Milan, Italy

***Edited by:***
*Roland W. Herzog, University of Florida, USA*

***Reviewed by:***
*Ashley Thomas Martino, St John's University, USA*
*Thierry Vanden Driessche, Vrije Universiteit Brussel, Belgium*

***Correspondence:**
*Maria-Grazia Roncarolo, San Raffaele Telethon Institute for Gene Therapy, Via Olgettina 58, Milan, Italy 20132.*
*e-mail: roncarolo.mariagrazia@hsr.it*

The success of *in vivo* gene therapy greatly depends on the ability to control the immune response toward the therapeutic transgene. Over the last decade several vector-based and pharmacological approaches have been explored to control the immune-mediated clearance of transgene-expressing cells after viral delivery. One important outcome from these studies is the concept that expression of a transgene in tolerance-promoting organs, such as the liver and tolerogenic antigen-presenting cells, can help safeguard transgene-expressing cells from immune-mediated clearance. Gene therapists are now manipulating vectors to target naturally occurring tolerogenic properties of the body by: (i) incorporating tissue/cell specific promoters for targeted expression, (ii) using viral-capsid engineering to alter tropism and avoid pre-existing immunity, and (iii) regulating cell and activation dependent expression by including micro-RNA (miR) targets into expression cassettes. The combination of these three layers of vector regulation greatly enhances the targeting of tolerogenic cells and limits off-target expression of the transgene, which can lead to the induction of transgene-specific pathogenic effector T cells. In this review, we discuss the application of using miR transgene regulation to generate tolerogenic responses and speculate on possible mechanisms used by the liver to induce the transgene-specific regulatory T cells.

**Keywords: Treg, micro-RNA, gene therapy, hepatocytes, tolerance, transgene, regulatory cells, liver**

## INTRODUCTION

A major obstacle for achieving therapeutic efficacy when using *in vivo* gene therapy is the development of an immune response toward the transgene since pathogenic immune responses can lead to the clearance of transgene-expressing cells. While long-term transgene expression has been achieved in several clinical trials using direct viral-vector administration (Kaplitt et al., 2007; Bainbridge et al., 2008; Hauswirth et al., 2008), targeting immune-privileged sites like the eye, brain, and testis as was done in these trials suggests that selective transgene expression in immune-privileged tissues (for review see Lowenstein et al., 2007) may diminish but not necessarily abolish the need for vector modification and/or pharmacological help to maintain transgene expression. Unfortunately for many individuals in need of gene therapy, disease correction requires delivery of a vector to immune-competent organs and requires robust local or systemic expression of a therapeutic protein. To combat the potential loss of transgene expression due to immune complications, gene therapists are modifying gene delivery platforms and using therapeutic regimens (such as immunosuppressive drugs) to promote tolerance toward the transgene. In this review, we focus on the use of vector modification with micro-RNA (miR) targets to regulate transgene expression and how they can be used with other layers of vector modification to promote transgene tolerance.

## IMMUNE TOLERANCE

Tolerance is a naturally occurring process that uses one or more immune mechanisms to achieve immunological homeostasis. One means to achieve a tolerogenic state toward an antigen (Ag) is through passive tolerance, which is a quiescent immunological state where T cells do not come into contact with their cognate Ag or where effector T cells (Teff) are deleted or anergized upon Ag recognition, resulting in immune ignorance. Active tolerance, on the other hand, is a dynamic process whereby FoxP3-expressing regulatory T cells (Treg) work to suppress inflammatory immune responses in Ag-specific and non-specific ways (Fehervari and Sakaguchi, 2004a; Sakaguchi et al., 2008). Among the Treg subpopulations are the naturally occurring Treg (nTreg), which are differentiated in the thymus, and the induced Treg (iTreg) which are induced in the periphery, are phenotypically similar and have similar regulatory properties; however, the relative contribution of iTreg and nTreg in regulating immune responses in different disease states is subject to ongoing discussions. Nevertheless, Treg induction and expansion have become a focal point by some gene therapists for developing tolerance to therapeutic proteins because of their potent regulatory properties (Luth et al., 2008; Nayak et al., 2009).

## TOLEROGENIC NATURE OF THE LIVER

Liver architecture creates a unique immunological site where circulating Ags and immune cells can meet. In a healthy state, natural tolerogenic mechanisms of the liver ensure that immunity against innocuous Ag (gut-derived nutrients and damaged/aged cells) and potentially immunogenic Ag (gut flora-derived) are kept in check. The means by which the liver induces this tolerance is partly attributed to the composition and diversity of antigen-presenting

cells (APC) and the presence of regulatory cytokines. The liver accommodates a variety of cells equipped to engage T cells, including canonical APC like liver-resident macrophages (Kupffer cells, KC) and conventional- and plasmacytoid-dendritic cells (cDC, pDC respectively), and non-canonical APC like liver sinusoidal endothelial cells (LSEC), and stellate cells (SC) and hepatocytes that are able to process and present Ag, secrete regulatory cytokines, and provide co-stimulatory molecules (Knolle et al., 1999; Limmer et al., 2000; Warren et al., 2006). Furthermore, all of these cells, with the exception of hepatocytes [which are deficient in MHC-class (MHC) – II expression] have been implicated in the generation of Treg and reported as one of the important biomarkers for predicting successful liver engraftment without immunosuppression (Seyfert-Margolis and Turka, 2008).

The tolerogenic properties of liver APC can be ascribed to the consistent expression of anti-inflammatory cytokines and other tolerogenic molecules found in a hepatic micro-environment. The liver is a rich source of IL-10 and TGF-β, both of which are potent modulators of APC differentiation, trafficking, and function (Lau and Thomson, 2003). In the absence of the *IL-10* gene or blocking IL-10 with a recombinant IL-10R fusion protein, hepatic tolerance is not established in a ConA induced model of hepatitis, signifying the central role of this cytokine in maintaining tolerance (Erhardt et al., 2007). In the Erhardt et al. study, KC were shown to be the key source of IL-10, but more importantly, the lack of IL-10 expression by Treg was a critical factor for resolving inflammation, suggesting that IL-10 is needed by APC and T cells to maintain tolerance. TGF-β was originally recognized as a growth factor for non-immune cells and only later as an inhibitory signal for activation of immune cells, preventing activation of macrophages and maturation of DC and modulating effector functions of T and B cells (Chen and Konkel, 2010). Chen and colleagues showed that TGF-β signaling coupled with TCR stimulation convert $CD4^+CD25^-Foxp3^-$ into iTreg. Thus, hepatic architecture, position, and micro-environment encourage interactions between non-parenchymal cells and circulating lymphocytes in the presence of immunoregulatory cytokines. Additionally, 50% of hepatic Treg are also Ki-$67^+$ (unpublished data by our group) supporting the hypothesis that the liver could be a preferential site for the expansion of Treg. Overall, these features distinguish the liver as a potential site for the development and expansion of acquired peripheral tolerance.

## PROMOTER AND VECTOR MODULATION TO REDUCE TRANSGENE IMMUNITY

Tissue-specific promoters that restrict transgene expression to a targeted cell type have been widely employed in gene therapy to avoid immune responses. The basic concept for using tissue-specific promoters is to drive expression of the therapeutic transgene to target cells while preventing its expression in APC in order to avoid the priming of Teff (Vandendriessche et al., 2007). Despite the specificity of tissue-specific promoters, their use as a regulatory layer does not necessarily prevent immune responses toward the encoded transgene. We among others have found that the use of promoters specific for tolerogenic cells such as hepatocytes can still lead to transgene-specific neutralizing antibodies and immune-mediated clearance of transgene-expressing cells (Brown et al., 2004; Follenzi et al., 2007; Herzog, 2007; Haribhai et al., 2011; Mingozzi and High, 2011). These results may be due to non-specific activity of the promoter, cross-priming, or in the case of integrating vectors, promoter-enhancer trapping at insertions near active regions of transcription (De Palma et al., 2005). Taken together, tissue-specific promoters can foster transgene stability by avoiding immune responses and enhancing tissue targeting in many cases; however, the use of tissue-specific promoters does not guarantee that transgene tolerance will be achieved in all immunological settings.

Alternating viral-capsid proteins to avoid pre-existing immunity and/or the development of immunity toward the vector and transgene is another approach by which gene therapists can maximize transgene expression. Viral capsids are immunogens, which can trigger innate and adaptive immune responses and lead to the development of neutralizing antibodies toward the vector as well as capsid-specific-Teff responses. In fact, it is believed that the first clinical trial for hemophilia B using adeno-associated vector (AAV) to deliver the factor IX (FIX) transgene failed due to pre-existing anti-AAV immune responses that led to the clearance of the transduced cells (Prasad et al., 2001; Li et al., 2007; Mingozzi et al., 2007). The use of pseudocapsids or particular serotypes with viral gene therapy can avoid existing antibody or cellular immune responses to the vector if the Ags of the introduced capsid protein(s) are naïve/less immunogenic to the immune system. In addition, swapping or novel capsid proteins can alter tropism of the vector and de-target APC. Kang et al. (2005) showed that in the case of long-term factor VIII (FVIII) expression the use of a gp64 capsid protein in lieu of the canonical VSV-G capsid protein in a feline immunodeficiency vector was able to partially correct disease in hemophilia A mice. However, when a gp64 lentiviral (LV) vector was used in the same mouse model, Lillicrap and colleagues were unable to achieve long-term FVIII expression unless the expression was combined with another layer of gene regulation (Matsui et al., 2011). While these results suggest that tissue-specific promoters and viral pseudotyping can improve transgene expression and long-term outcome of gene therapy with viral vectors, they also suggest that more regulation may be required to achieve transgene-specific tolerance in more stringent immunological settings.

## miR TRANSGENE REGULATION

Molecular engineering of viral-vector cassettes with miR targets is the latest attempt at regulating expression in gene therapy. miR are a family of endogenously expressed, small, non-coding RNA of ∼22 nucleotides that are post-transcriptional regulators of gene expression and inhibit expression by binding to miR targets on mRNA to repress transcription or to promote mRNA degradation. Similar to the way miR regulates the expression of genes naturally encoding miR targets, vectors engineered with miR targets can prevent transgene expression in the transduced cells provided the target cells express the particular miR matching the miR target encoded in the vector (Brown and Naldini, 2009). Since miR are intimately involved in a variety of immunological processes, including B and T cell development, hematopoietic cell differentiation, apoptosis, and proliferation (Landgraf et al., 2007; Kelly

and Russell, 2009), the incorporation of miR target sequences in vectors makes it possible to direct expression to or away from particular cells of the immune system by exploiting their natural miR profile. miR expression profiles vary considerably from being constitutively expressed or induced to shut off, depending on cell type and maturation state (Petriv et al., 2010). This variability suggests that by incorporating miR targets in vectors, it is possible to target transgene expression not only to certain cell types, but also to certain stages of cell differentiation or activation. The ability to regulate gene expression at a state-specific level is of particular interest to gene therapists because it adds a new dimension of regulating gene expression that cannot be achieved by other vector modifications.

## miR TO GENERATE IMMUNE TOLERANCE

One attractive approach to generate immune tolerance is to direct transgene expression toward APC in a tolerogenic state. The immature state of some APC is considered to have tolerogenic properties because they lack the necessary molecules needed to induce robust immune responses. Our group among others has shown that immature dendritic cells (iDC) are a potent inducer of Treg and, therefore, have become targets for inducing Treg (Wakkach et al., 2003; Levings et al., 2005; Yamazaki and Steinman, 2009). The mechanisms by which iDC promote Treg induction is through the expression of tolerance-promoting cytokines, such as IL-10 (Gregori et al., 2010) and TGF-β (Wakkach et al., 2003; Fehervari and Sakaguchi, 2004b) and the surface expression of tolerogenic co-stimulatory molecules such as PDL-1 (Unger et al., 2009); however, a major caveat for targeting iDC is the short window of opportunity for maximizing their tolerogenic abilities since activated DC produce inflammatory cytokines and up-regulate co-stimulatory molecules that promote the development of Teff. Therefore, in the scenario of exploiting the tolerogenic state of APC it is vital to limit Ag expression to the tolerogenic state of the cell and turning off expression once the APC is activated. Using miR targets in the vector can take advantage of the unique state-specific miR expression of APC to accomplish this goal. iDC, for example, do not express miR-122, -146a, or -155 in the immature state, however, upon activation by cytokines or TLR ligands these miR are induced and become highly expressed by the DC (Taganov et al., 2006; Jurkin et al., 2010). Therefore, using a vector regulated by miR targets encoding for miR-122, -146a, or -155 targets could eliminate transgene expression of transgene in DC upon activation. Brown et al. (2007b) showed that state-specific regulation is possible with a LV.GFP miR-155 target encoded construct, where GFP is selectively expressed in the immature state of human peripheral blood-derived DC, while GFP expression was dramatically reduced upon LPS activation of the DC. While this approach has been proven feasible for selective targeting of iDC, more work needs to be done to determine the immunological consequence of this approach and whether it can be used *in vivo*.

An established strategy for generating transgene tolerance with miR-containing vectors is to de-target transgene expression of the hematopoietic system while expressing the transgene in hepatocytes. All cells of the hematopoietic system highly express miR-142; therefore, vectors including miR-142 targets can repress transgene expression in all cells of this linage including APC. The effectiveness of using miR-142-targeted vectors to eliminate expression in APC and generate transgene tolerance was first achieved by Naldini and colleagues who showed that including miR-142 target repeats in a LV.GFP vector reduced GFP expression in mouse macrophages and DC to undetectable levels while maintaining GFP expression in non-hematopoietic tissues (Brown et al., 2006). The same group went on to show that hemophilia B can be cured when FIX deficient mice receive a LV expressing human FIX under the regulation of miR-142 targets, whereas the animals receiving the same LV without miR-142 targets cleared the transgene-expressing cells (Brown et al., 2007a). Interestingly, the original belief that miR-142-targeted vectors promote transgene tolerance through immune ignorance due to de-targeting of APC recently changed when we showed induction of Treg using a LV. GFP, miR-142-targeted vectors with liver-specific promoters mediate transgene tolerance (Annoni et al., 2009). To prove that active tolerance is at play in regulating transgene expression with miR-142 vectors, we showed that in a collaborative effort that mice injected with LV encoding the Ag ovalbumin (OVA), liver-specific promoter, and miR-142-targeted sequences along with naïve, OVA-specific T cells induces OVA-specific Treg, suggesting that Ag-specific iTreg are regulating transgene tolerance (Matrai et al., 2011). The concept that Ag-specific Treg are regulating tolerance is supported by the fact that upon pDNA re-challenge with the transgene, tolerized mice showed little response to the Ag, while mice that cleared the transgene-expressing cells showed increased reactivity (Annoni et al., 2009; Matrai et al., 2011).

An important point to consider in most of the experimental conditions used to generate transgene tolerance with miR targets is the preferential targeting of the transgene to the hepatocytes. It is important to note, however, that targeting expression to hepatocytes and de-tartgeting APC do not guarantee transgene persistence. Osborn et al. (2011) showed in a mucopolysaccharidosis type I mouse model that hydrodynamic injection of minicircle DNA expressing α-L-iduronidase (IDUA) regulated by liver-specific promoter and miR-142 targets mice do not tolerate IDUA and must receive immunosuppression in order to achieve long-term transgene expression. In this study it was unclear why IDUA expression was lost; however, it was speculated that immunogenicity of the transgene and/or the acute inflammation from the hydrodynamic injection lead to the clearance of the transgene. This result stresses the importance of selecting a vector delivery platform/method that has the least amount of immunogenicity and selecting or modifying the transgene in order to reduce the immune response. Despite the failure of this protocol, targeting hepatocytes still remains one of the best options for gene delivery because of their ability to secrete high levels of protein and the innate tolerogenic properties; however, the exact mechanism(s) the liver uses to induce tolerance still remains a mystery. To better understand how hepatocyte-targeted gene therapy with miR regulated vectors generates transgene-specific Treg after gene therapy we are addressing the tolerogenic properties particular to the liver-resident cells that promote tolerogenic responses.

## HOW TARGETING AG EXPRESSION TO HEPATOCYTES BY MIR-REGULATED VECTOR GENERATES TOLERANCE: A POSSIBLE MECHANISM

Based on our data, we have developed a hypothesis explaining the mechanisms leading to tolerance using liver-directed, miR-142 regulated LV gene therapy. So far, we have learned that LV containing miR-142 targets (LV.142T) and conventional LV have similar transduction efficiency and immunogenicity toward vector components. In fact, the immune response to LV.142T vector RNA compared to conventional LV vectors in infected pDC results in comparable levels of type 1 IFN release after TLR7 triggering (Brown et al., 2007c). However, since LV.142T suppresses transgene expression in APC, the encoded Ag avoids presentation by IFN α-β-activated professional APC, thus circumventing the induction of a robust transgene-specific $CD8^+$ Teff cell responses. Nevertheless, $CD8^+$ T cell priming occurs among LV.142T infected hepatocytes due to their expression of MHC-I molecule and co-stimulatory molecules; however, Teff priming by hepatocytes is short-lived due to the low levels of MHC-I expression and lack of key co-stimulatory molecules needed to sustain Teff activation and expansion. Therefore, in this scenario miR-142 regulation indirectly causes alterations to T cell signal 1 (MHC-pep) and signal 2 (co-stimulation) that are necessary for optimal T cell priming. In addition, very little is known about signal 3 (cytokine milieu) at the time of Ag-presentation in this model, but given the reduced priming and Teff memory formation using LV.142T, it is likely that altered levels of IL-7, IL-15, and TGF-β in the liver micro-environment play a role in reducing the Teff response (Sawa et al., 2009). Thus, despite de-targeting APC with LV.142T, transgene-specific $CD8^+$ Teff do occur, but since they are suboptimally primed by the hepatocytes, there is a premature contraction phase and reduced cytotoxic effects.

It is difficult to interpret how the generation of Treg occurs using LV.142T vectors since the primary expressing cell (hepatocytes) inefficiently express MHC-II necessary for priming $CD4^+$ T cells, and the transgene is not expressed in the professional APC. Our hypothesis is that $CD4^+$ T cells are primed by APC that have engulfed the Ag expelled from transduced dead or dying hepatocytes and/or by engulfing the dead cells and cross-presenting the Ag. After engulfment and presentation by APC, the Ag can prime naïve $CD4^+$ T cells under a tolerogenic cytokine milieu (signal 3) that promotes the induction of transgene-specific Treg cells. Since the liver micro-environment is rich in TGF-β which is required to convert naïve $CD4^+$ T cells into iTreg (Chen et al., 2003), it is likely the low level of Ag-presentation by APC combined with the presence of TGF-β provided by the hepatocytes that evokes iTreg generation. In addition, we have demonstrated that active tolerance to transgene expressed via integrase defective (ID) IDLV.142T platform outlasts the window of transgene expression (Matrai et al., 2011), suggesting that tolerance induction and Tregs conversion are related to tolerogenic Ag-presentation rather than Ag-persistence.

## CONCLUSION

In summary, transgene immunity can be overcome when using *in vivo* gene therapy by exploiting vectors that have been designed to take advantage of the tolerogenic properties of the body. The recent incorporation of molecular de-targeting techniques achieved with miR targets combined with tissue-specific promoters and capsid engineering provides a potent regulatory scheme that, when designed correctly, can induce Treg capable of controlling transgene-specific-Teff and prolong transgene expression. While miR targeting of viral vectors has not been tested in humans, the transition of miR targets into clinical vectors is very appealing since most clinical trials will be challenged by the problem of maintaining immune tolerance. However, before miR targeted vectors can be widely accepted, they must be tested in large-animal models for efficacy, safety and confirmation that the approach of targeting the liver to induce tolerance is similar in rodents and non-human primates. In due time, it is likely that miR regulation will become commonplace in the clinical arena given its efficiency, potential tolerogenic properties, and the broad spectrum of miR that are naturally expressed.

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