AAV GENE THERAPY: IMMUNOLOGY AND IMMUNOTHERAPEUTICS

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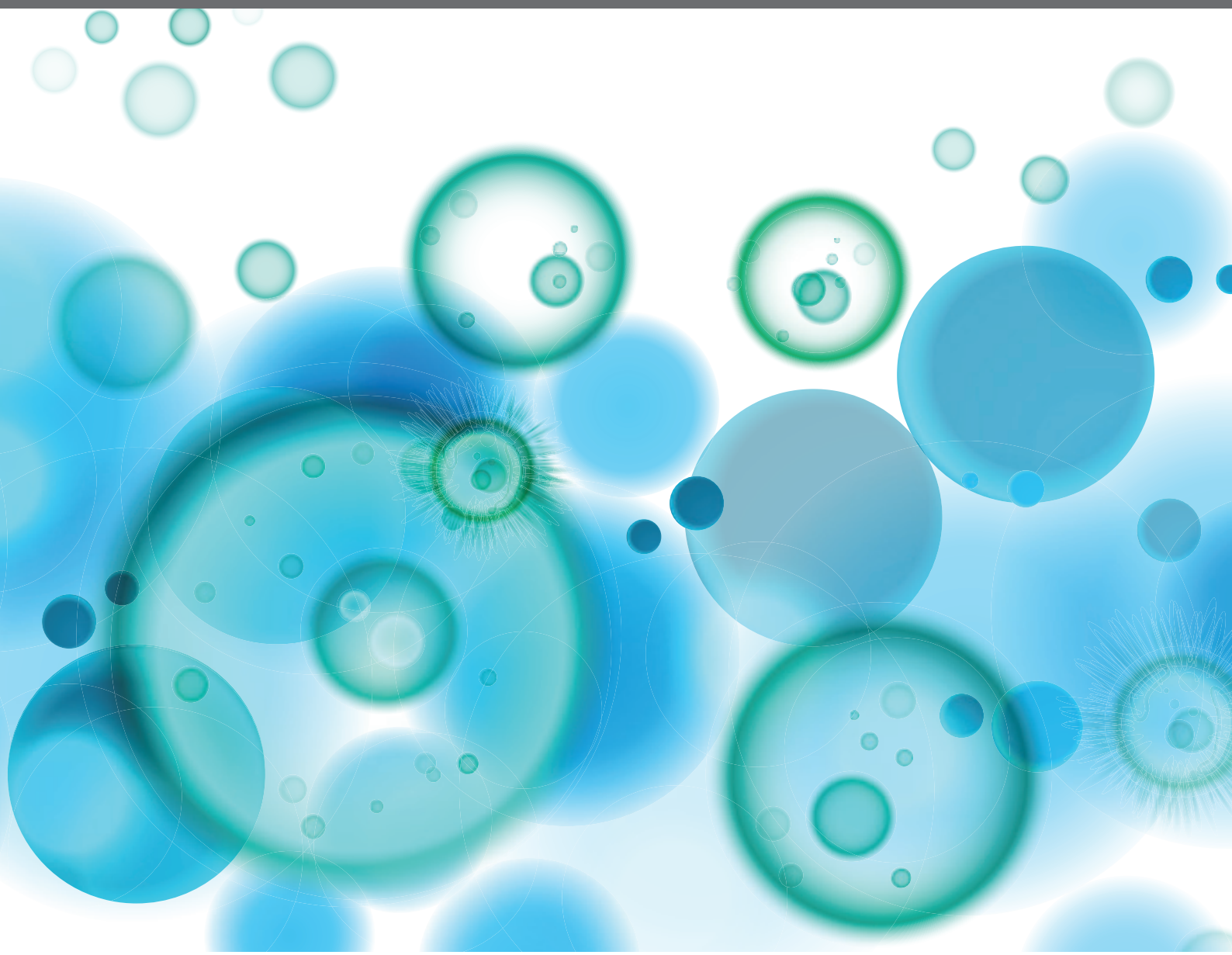
PUBLISHED IN: Frontiers in Immunology



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frontiers

ISSN 1664-8714
ISBN 978-2-88974-306-3
DOI 10.3389/978-2-88974-306-3

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AAV GENE THERAPY: IMMUNOLOGY AND IMMUNOTHERAPEUTICS

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Citation: Martinez-Navio, J., Paulk, N. K., Gao, G., eds. (2022). AAV Gene Therapy: Immunology and Immunotherapeutics. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-306-3

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frontiers in Immunology

EDITORIAL
published: 21 December 2021
doi: 10.3389/fimmu.2021.822389

Editorial: "AAV Gene Therapy: Immunology and Immunotherapeutics"

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Keywords: AAV, gene therapy, immunogenicity, capsid antibodies, cellular responses, innate immune activation, immunotherapeutics

Editorial on the Research Topic

AAV Gene Therapy: Immunology and Immunotherapeutics

Adeno-associated virus (AAV) has become the vector of choice for current gene therapy approaches. AAV is a small, single-stranded DNA virus which effectively infects humans and other vertebrates without causing disease. AAV is highly infectious but naturally replication-defective in the absence of a helper virus, and its genome is simple to manipulate. To generate a recombinant AAV (rAAV) vector, the viral genes are replaced with a transgene expression cassette, while the flanking inverted terminal repeats (ITRs) required for encapsidation, are retained. Virion capsid proteins for encapsidation of the vector DNA are provided in *trans* and the resultant rAAV is subsequently purified. The safety profile of rAAV vectors is well established from decades of research and over 200 clinical trials to date. Many more are forthcoming with numerous investigational new drug applications in various stages of review. AAV-mediated gene transfer has benefited numerous individuals with genetic diseases by mediating long-term expression of the transgene. However, some hurdles remain, such as pre-existing immunity to the rAAV capsids and unwanted immune responses to the transgene product. A total of 13 articles in this Research Topic examine current immunological barriers in the field of rAAV-based gene therapy and immunotherapeutics, evidencing the latest discoveries on approaches on how best to overcome them.

Pre-existing neutralizing antibodies to the AAV capsid are found in a significant percentage of the human population from exposure to circulating wild-type AAV. These antibodies substantially reduce the transduction efficiency of rAAV and can prevent successful delivery of the transgene in those individuals. In this Research Topic, Weber reviews this topic, including descriptions of what the prevalence of such antibodies are in the general population, and the difficulties associated with measuring these antibodies in a way that is predictive of therapeutic outcomes. Importantly, the author also discusses potential solutions to these issues. In line with this, an approach that holds great promise to overcome natural pre-existing immunity is the rational design of engineered AAV capsids. In a perspective article, Wec et al. discussed how machine learning and high-throughput screening can expand the landscape of engineered capsids, which can make AAV therapies safer and more broadly applicable.

While rAAV vectors have been traditionally seen as non-immunogenic, immune responses can be generated to the AAV itself and/or to the transgene product. In a perspective article,

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *25 November 2021*
Accepted: *07 December 2021*
Published: *21 December 2021*

Citation:
Martinez-Navio JM, Paulk NK and Gao G (2021) Editorial: "AAV Gene Therapy: Immunology and Immunotherapeutics". Front. Immunol. 12:822389. doi: 10.3389/fimmu.2021.822389

Hamilton and Wright discuss the inherent immunogenicity of the rAAV vectors, and how they can trigger both innate and adaptive immune responses (the latter including both cellular and humoral responses) and mediate complement activation. Innate immune responses can be triggered by the rAAV capsid, but also by the vector's DNA genome, which can trigger toll-like receptor 9 (TLR9) activation. TLR9 recognizes unmethylated CpG motifs (cytosine guanine dinucleotides) and is expressed in different sets of immune cells, including dendritic cells, macrophages, and other antigen presenting cells. Activation of TLR9 triggers the secretion of pro-inflammatory cytokines and the recruitment and activation of cytotoxic T-cells which can, in turn, mediate elimination of transduced cells. Bertolini et al. have reported a deimmunization strategy that consists of removing these CpG motifs from the vectors. In a rodent model of hemophilia B, the authors have shown markedly reduced cytotoxic T-cell infiltration after intramuscular administration of CpG-depleted rAAV. Importantly, this approach resulted in improved preservation of transduced cells. Immune-mediated rejection and clearance of rAAV-transduced cells is a crucial issue during gene therapy. In a review focused on cellular responses, Ertl has discussed how CD8 T-cells can recognize and destroy rAAV-transduced cells. Notably, ITRs flaking the transgene cassette in rAAV can be sensed by DNA damage response proteins in transduced cells. Dudek and Porteus have reviewed the current understanding of DNA damage response and innate immune activation to both genomes and capsids during early steps of vector transduction. As reviewed by Chu and Ng, current strategies to prevent or ameliorate unwanted host immune responses during gene therapy include an array of potential pharmacological immunosuppressive and immunomodulatory regimens.

Post-translational modifications were recently discovered on rAAV capsids and could be contributing to the undesired immunogenicity seen in some gene-therapy recipients. Recent concerns regarding immunogenic responses to high-dose intravenous administration of rAAV has highlighted the need for extensive analysis of the preparations in order to identify and catalog potentially immunogenic vector lot components. In this Research Topic, Rumachik et al. have described a mass spectrometry workflow to thoroughly analyze and characterize post-translational modifications in rAAV capsids and on potential host cell protein impurities carried over in the vector preparation. Extensive rAAV characterization promotes enhanced batch-to-batch consistency and is crucial for the development of safer and more reliable rAAV vectors.

Viral tropism, or the ability to infect a specific tissue or cell type, is a key factor to consider when selecting the most appropriate rAAV serotype for gene delivery. Viral tropism is largely determined by the rAAV capsid, and a variety of different capsid serotypes have been identified. When rAAV vectors are produced in the laboratory, an AAV2 ITR genome is engineered to contain the transgene of interest and the resulting recombinant genome is encapsidated in the preferred capsid. Depending on the desired target cells or tissues, different serotypes may be preferred. Brown et al. have developed an experimental and computational approach based on single-cell RNA sequencing to characterize *in vivo* viral tropisms and uncover targeting biases. An additional potential source for immunogenicity against the rAAV-expressed transgene product is related to off-target delivery. Inadvertent transduction of antigen presenting cells (APCs) can result in presentation to the immune system, and trigger an immune response. To prevent this, Muhuri et al. have used miRNA biding sites to efficiently block transgene expression in APCs. miRNAs are small non-coding RNAs that function in RNA silencing. By using miRNA binding sites specific for miRNAs found in APCs, but not in target cells, expression can be carefully ablated in APCs while sparing target tissues. rAAV vectors containing this detargeting strategy strongly inhibited cytotoxic T-cell activation and the generation of antibodies against the transgene. This approach mediated higher levels of transgene expression *in vivo*.

Although mostly used to treat monogenic diseases, gene-transfer mediated by rAAV can also be exploited to deliver immunotherapeutics, such as monoclonal antibodies. The coding sequence of properly characterized protective/neutralizing antibodies against a pathogen of interest can be delivered *via* rAAV, thus aiming to prevent or treat infectious diseases and confer long-lasting immunity. This strategy passively bypasses the immune system as no immune response to an immunogen is required. Zhan et al. have reviewed this topic, highlighting how rAAVs can become game changers in our fight against transmissible diseases, including HIV, dengue, influenza and others. Interestingly, antigens of interest can also be delivered in rAAV vectors with the aim of conferring protection to a disease in the recipient. Shahnaij et al. have reported a vectored vaccination regimen against malaria that conferred full protection to malaria-parasite challenge in a rodent model. The vaccination regimen consisted of a prime with human adenovirus and a booster inoculation with AAV8, both encoding the *Plasmodium falciparum* circumsporozoite protein (a protein of the sporozoite's surface).

As depicted by Rapti and Grimm in their review article, there is a continuous immunological and molecular race between AAV and its human host. Current and upcoming advances in rAAV gene therapy as described in the compelling articles of this Research Topic, promise safer and more broadly applicable therapies in the future. We invite you to read each of these enlightening papers and reflect on the Research Topic as a whole.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of Interest: GG is a scientific co-founder of Voyager Therapeutics, Adrenas Therapeutics and Aspa Therapeutics and holds equity in the companies. NP and GG are inventors on patents related to AAV-based gene therapies, some of which have been licensed to commercial entities.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Immunology

MINI REVIEW
published: 17 March 2021
doi: 10.3389/fimmu.2021.658399

Anti-AAV Antibodies in AAV Gene Therapy: Current Challenges and Possible Solutions

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Adeno-associated virus (AAV) vector-based gene therapy is currently the only *in vivo* gene therapy approved in the US and Europe. The recent tragic death of three children in a clinical trial to treat X-Linked Myotubular Myopathy by delivering myotubularin with an AAV8 vector notwithstanding, AAV remains a highly promising therapeutic gene delivery platform. But the successful use of AAV vectors to treat an increasing number of diseases also makes establishing protocols to determine therapeutically relevant titers of pre-existing anti-AAV antibodies and approaches to deplete those antibodies more urgent than ever. In this mini review, I will briefly discuss (i) our knowledge regarding the prevalence of anti-AAV antibodies, (ii) the challenges to measure those antibodies by methods that are most predictive of their influence on therapeutic efficacy of AAV gene transfer, and (iii) approaches to overcome the formidable hurdle that anti-AAV antibodies pose to the successful clinical use of AAV gene therapy.

Keywords: gene therapy, aav, antibodies, antibody assays, removal of antibodies, removal of inhibitors, adeno-associated virus, prevalence of antibodies

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *25 January 2021*
Accepted: *23 February 2021*
Published: *17 March 2021*

Citation:
Weber T (2021) Anti-AAV Antibodies in AAV Gene Therapy: Current Challenges and Possible Solutions. Front. Immunol. 12:658399. doi: 10.3389/fimmu.2021.658399

INTRODUCTION

Thirty years ago, the Chicago Tribune published a front-page article entitled "Gene therapy poised to reinvent medicine." While, after three humbling decades of intense research, we have not achieved this lofty goal, it is safe to say that we made tremendous progress toward establishing gene therapy as an important tool to treat both inherited as well as acquired diseases. Among the currently available gene therapy platforms, vectors based on adeno-associated virus (AAV) have clearly emerged as one of the most promising gene delivery vehicles.

In fact, only three *in vivo* gene therapy treatments have been approved by regulatory agencies for clinical use (1). Notably, all three of these therapies are based on AAV vectors. Whereas the treatment of lipoprotein lipase deficiency with an AAV-based vector to deliver the defective protein was the first to be approved by the European Medicines Agency (EMA) in 2012, it is currently no longer on the market owing to a lack of demand. In contrast, Luxturna (2) to treat the early childhood blindness disease Leber's congenital amaurosis type 2 is in clinical use in both the US and Europe (1), whereas Zolgensma (1) to treat spinal muscular atrophy (SMA)—a horrific disease that causes the death of most children afflicted by it before age 2—has been approved by the US Food and Drug Administration (FDA) in 2019 (1).

The success of AAV as the leading gene delivery modality is based on a multitude of factors: (i) Even wild-type AAV has not been shown to cause any disease, (ii) In contrast to other currently available gene delivery methods, in non-dividing or very slowly dividing cells AAV results in the

long-term expression of the therapeutic payload, even though the AAV vector DNA persists mostly as extrachromosomal episomes (3), (iii) AAV vectors are not strongly immunogenic, most likely because the only viral elements in a therapeutic AAV genome are the two inverted terminal repeats. As a result, AAV vectors do not express any viral proteins, which dramatically reduces the presentation of viral peptides on MHCI complexes thus strongly reducing the incidence and intensity of a cellular immune response against transduced cells (4).

Until recently, AAV gene therapy had an unblemished safety profile (5). Sadly, this unparalleled safety record has recently been shattered by the tragic death of three children in a trial (NCT03199469) aimed at treating X-linked myotubular myopathy (XLMTM) with an AAV8 vector promoting the expression of functional MTM1. This trial is currently on hold, and the cause of the deaths of the three children is under active investigation [Wilson and Flotte (6) and references cited therein].

One of the drawbacks of AAV is its limited cargo capacity of ∼5 kb. Even though the median size of a human protein is only 375 amino acids (7), the coding sequences of larger proteins naturally harbor more mutations. Consequently, many proteins associated with inherited diseases cannot be expressed with a single AAV vector because the size of an expression cassette for a functional protein exceeds the packaging capacity of AAV.

As mentioned above, wild-type AAV is an apparently non-pathogenic virus. However, infection with wild-type AAV is very common and depending on the serotype (and assay used, vide infra) 30–60% of all individuals harbor antibodies that neutralize AAV transduction (8). Interestingly, however, even when using the same assay, significant differences in the prevalence of neutralizing antibodies (NAbs) exist (9, 10). In fact, Greenberg et al. (9) showed that within the US the prevalence of antibodies against AAV1 varied from 32% in Wisconsin to 67% in South Carolina, and in Europe 48% of people in Sweden harbor NAbs against AAV1 in contrast to 79% NAb positive people in Poland and Hungary. Another challenge for the use of AAV gene therapy in patients with NAbs against a specific AAV serotype is the fact that there is substantial cross-reactivity among the AAV serotypes (8, 10, 11).

ASSAYS MEASURING NEUTRALIZING AND TOTAL ANTI-AAV ANTIBODY LEVELS

In the vast majority of past or ongoing AAV gene therapy clinical trials using AAV vectors the presence of NAbs (determined in an *in vitro* assay) is/was one of the exclusion criteria. While this makes inherent sense, the absence or presence of NAbs is ill defined. The *in vitro* assays employ an AAV reporter vector (usually carrying a luciferase expression cassette or GFP) of the serotype used in the respective clinical trial. Pre-treatment patient serum/plasma samples are then serially diluted and incubated with an AAV reporter virus with a pre-determined number of vector genomes. After addition of the virus to cells (most often HEK293 cells) and incubation for a defined amount of time, the expression of the reporter protein is measured for each serum dilution as well as samples that did not receive any patient serum. Expression levels of each serum dilution sample are then normalized to the no-patient serum control. In general, the neutralizing titer is defined as the highest dilution that reduces transduction by ≥50%, although other levels of reduction have been used as well [e.g., 29% (12)]. However, the assay conditions vary widely not only for preclinical work (8) but also for clinical trials. For instance, for their hemophilia A trial (NCT02576795) (13), Biomarin only enrolled patients that had no neutralizing antibodies against AAV5. Their NAb assay used 25,000 vector genome containing particles per cell (14). Uniqure, on the other hand, used a GFP based assay for their hemophilia B trial (NCT02396342) (15) without reporting the MOI. For another hemophilia B trial (NCT03489291) (16) they used a highly sensitive luciferase assay (12) with an MOI of 378.4. So, even if all assay conditions were absolutely identical, the mere use of an MOI 25,000 and 378.4 would yield dramatically different (>66-fold) NAb titers.

Unfortunately, the AAV dose/MOI is not the only parameter that can influence NAb titers. Two other critically important parameters are the purity of the vector preparation, especially as it relates to the absence or presence of monomeric or oligomeric capsid proteins. Moreover, depending on the vector production method and purification scheme, the final vector preparations can also contain empty viral particles, particles with truncated vector genomes or particles with plasmid or genomic DNA. All of those particles are essentially inactive virus, and their presence in an NAb assay will artificially lower the NAb titer (17). It is also noteworthy to point out that so-called "neutralizing antibody assays" also incorporate inhibition by other factors in the serum [e.g., galectin-3 binding protein (18)] that inhibit transduction. Hence, the terms neutralizing or inhibitory factors is more appropriate. Without doubt, the most critical parameters affecting NAb titers is the AAV dose and serum volume. Therefore, I propose that, in the future, the field should report the presence of NAbs not as NAb titers but rather report the number of AAV particles that are neutralized per μl serum (or plasma). Not only would this facilitate the comparison of NAb assay results among different labs, it would also include non-antibody inhibitory factors. Of course, reporting the number of AAV particles that can be neutralized per μl serum (or plasma) doesn't eliminate variations obtained with different neutralizing assays. However, in the absence of a *very* detailed description of the methods and materials used for NAb assays in every publication, this is the most accurate unit to report neutralizing factors in serum (or plasma).

There are several reports that use an *in vivo* assay to determine neutralization by human or non-human primate (NHP) sera [e.g., (19–21)]. These assays, which rely on the passive immunization of mice with human or NHP sera, appear to be much more sensitive than most of the currently used *in vitro* neutralizing assays (21). However, *in vivo* experiments are inherently more cumbersome and expensive and are not useful for the screening of large potential patient populations.

Currently, AAV gene therapy is rapidly expanding with more than 200 ongoing or completer clinical trials (www.clinicaltrials.gov). In my view, going forward, it will be essential that regulatory agencies such as the FDA and the

EMA will create standardized *in vitro* assays for each AAV serotype/variant. These assays should be sensitive and rely on laboratory equipment that is commonly available in academic laboratories. Ideally, these assays will be validated *in vivo* in mice and NHPs. Importantly, the assays should include commercially available reagents, such as a neutralizing, monoclonal antibody, to allow the validation of the assay in each laboratory using it. Only standardized assays will allow a valid comparison of NAb levels reported by different groups.

ELISA assays can be used to measure the total levels of antibodies against a specific serotype, whether these antibodies are neutralizing or not. In general, there appears to be a good correlation between total anti-AAV antibody levels and neutralizing antibody (factor) levels (11, 22). However, intravenous IgG (IVIG) appears to also contain non-neutralizing antibodies against AAV that can, in fact, enhance transduction, although the precise mechanism needs to be elucidated (23). As such, the value for using ELISA to determine eligibility for treatment with AAV gene therapy needs to be established fully.

APPROACHES TO OVERCOME THE HURDLE THAT ANTI-AAV ANTIBODIES POSE TO AAV GENE THERAPY

In theory, the most appealing approach to overcome the challenge of pre-existing NAbs is to introduce mutations into the AAV capsid that prevent the binding of NAbs. However, the significant cross-reactivity among AAV serotypes (11) shows that this is not an easy feat to achieve. Nonetheless, it has been demonstrated that the introduction of point mutations into the AAV2 capsid can attenuate the sensitivity of these mutant viruses to neutralization (24). However, for most viruses, and presumably for AAV as well, of all the neutralizing antibodies against the virus a large fraction is directed against the receptor binding domain(s). Consequently, mutating residues in the receptor binding region(s) might be best to ameliorate neutralization, but mutation in the receptor binding region(s) are also very likely to affect viral tropism and/or transduction efficiencies.

"Traditional" plasmapheresis, which removes all immunoglobulins, has shown some promise in depleting most NAbs from patient sera, albeit only in patients with low NAb titers (25). Moreover, removing all immunoglobulins has its own drawbacks. More recently Bertin et al. (26) and Orlowski et al. (27) have demonstrated that neutralizing antibodies/factors can be removed *in vitro* by incubating IVIG or human sera with beads that have AAV particles covalently coupled to them. Moreover, my colleagues and I were able to demonstrate that performing hemapheresis with such beads can fully restore liver transduction in animals with NAb titers that without hemapheresis show none to negligible transduction (27). Restoration of transduction of cardiac and especially skeletal muscle was more modest, likely due to rebound of NAbs from the extracellular fluid into the bloodstream. Unfortunately, technical limitations prevented us from performing multiple rounds of hemapheresis over several days (27). Multiple rounds of hemapheresis over the span of several days can easily be performed in humans (25), so it is highly likely that the rebound limitation in rats could be easily overcome in humans. Future tests in large animal models, particularly in non-human primates, could provide proof of principle for the utility of this approach.

Recently, Mingozzi and colleagues took advantage of imlifidase (IdeS), a streptococcal cystein protease, that can cleave IgG into $F(ab')_2$ fragments and Fc (28, 29). Treatment of IVIG with IdeS resulted in the complete digestion of total IgG and anti-AAV8 IgG after a 24-h incubation period. In addition, when Leborgne et al. passively immunized mice with human IVIG and 1 day later injected them with AAV8 encoding either secreted Gaussia luciferase or human FIX (hFIX), this completely abrogated Gaussia luciferase or hFIX expression. If, on the other hand, the mice were injected at day 0 first with human IVIG and 30 min. later with IdeS, followed 1 day later by injection of AAV8 encoding secreted Gaussia luciferase or hFIX, the expression levels of the luciferase or hFIX in the blood were indistinguishable from levels in naïve mice (28). Strikingly, not only did IdeS treatment allow transduction of NHPs with pre-existing neutralizing antibodies, it even allowed vector *re-administration* with the same AAV variant (AAV-LK03) (28).

Using IdeZ, a homolog of IdeS produced by a different streptococcal strain, Asokan and colleagues (30) also could demonstrate that administration of IdeZ allows transduction of mice that have been passively immunized with IVIG. Furthermore, they also reported successful transduction of an NHP with pre-existing neutralizing antibodies that had been pre-injected with IdeZ (30).

DISCUSSION

Pre-existing anti-AAV antibodies are a vexing problem for AAV gene therapy because they can severely limit the patient population that could benefit from AAV gene therapy. This becomes all the more frustrating as the field rapidly advances and many more AAV therapeutics will become available to treat an increasing number of genetic diseases. Nonetheless, over the last couple of years, significant progress has been made in overcoming this formidable obstacle and to extend therapy to patients with pre-existing neutralizing antibodies, either as a result of infection by wild-type AAV or prior treatment with an AAV vector. Because of the ease of use, the IgG cleaving proteases IdeS and IdeZ are particularly attractive to use in AAV gene therapy patients with pre-existing anti-AAV antibodies (28, 30). However, while these proteases cleave all IgGs, they do not remove other transduction inhibitors such as galectin 3-binding protein (18). Plasmapheresis to remove specifically anti-AAV8 antibodies as well as other inhibitory factors (26, 27) is a promising alternative, and only clinical trials will ultimately answer the question, which of the currently available strategies to mitigate the negative effects of anti-AAV antibodies is best for any given therapeutic application. It would, however, be interesting to test with identical human sera whether the addition of IdeS/IdeZ (28, 30) or incubation of the sera with "AAV-beads" (26, 27) has a more positive effect on transduction.

Because of significant differences in assay conditions, a comparison of neutralizing titers obtained by different laboratories is currently impossible. As the use of AAV gene therapy rapidly expands it will, in my view, be absolutely essential that validated assays that are approved by regulatory agencies for any given AAV serotype or variant will be used to determine the amount of neutralizing factors in sera. Until such assays are validated and approved, I urge my colleagues to report very detailed descriptions of their assays used and to report the neutralizing activity of sera/plasma samples as AAV particles neutralized per μl serum/plasma. While imperfect, this should allow at least a "reasonable" comparison of results obtained by different laboratories.

Clearly, a significant amount of future research will be required to "solve" the problem that neutralizing antibodies and factors pose to the expansion of the eligible patient population. Nonetheless, recent developments offer a great deal of optimism that we are on the way to being able to include patients into clinical trials and treatment regimens that owing to the presence of neutralizing antibodies and factors are currently excluded from treatment with AAV gene therapy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was funded in part by the NIH (HL117505 to TW).

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 01 April 2021
doi: 10.3389/fimmu.2021.658038

Immunomodulation in Administration of rAAV: Preclinical and Clinical Adjuvant Pharmacotherapies

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Recombinant adeno-associated virus (rAAV) has attracted a significant research focus for delivering genetic therapies to target cells. This non-enveloped virus has been trialed in many clinical-stage therapeutic strategies but important obstacle in clinical translation is the activation of both innate and adaptive immune response to the protein capsid, vector genome and transgene product. In addition, the normal population has pre-existing neutralizing antibodies against wild-type AAV, and cross-reactivity is observed between different rAAV serotypes. While extent of response can be influenced by dosing, administration route and target organ(s), these pose concerns over reduction or complete loss of efficacy, options for re-administration, and other unwanted immunological sequalae such as local tissue damage. To reduce said immunological risks, patients are excluded if they harbor anti-AAV antibodies or have received gene therapy previously. Studies have incorporated immunomodulating or suppressive regimens to block cellular and humoral immune responses such as systemic corticosteroids pre- and post-administration of Luxturna® and Zolgensma®, the two rAAV products with licensed regulatory approval in Europe and the United States. In this review, we will introduce the current pharmacological strategies to immunosuppress or immunomodulate the host immune response to rAAV gene therapy.

Keywords: immunomodulation, immunosuppression, immune response, gene therapy, adeno associated virus, pharmacotherapies

OPEN ACCESS

Edited by:
Nicole K. Paulk, University of California, San Francisco, United States

Reviewed by:
Chengwen Li, University of North Carolina at Chapel Hill, United States
Roland W. Herzog, Indiana University, United States

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *25 January 2021*
Accepted: *05 March 2021*
Published: *01 April 2021*

Citation:
Chu WS and Ng J (2021) Immunomodulation in Administration of rAAV: Preclinical and Clinical Adjuvant Pharmacotherapies. Front. Immunol. 12:658038. doi: 10.3389/fimmu.2021.658038

INTRODUCTION

Adeno-associated virus (AAV) is a 26nm, non-enveloped virus of *Parvoviridae* family. It is 4.7kb single-stranded DNA genome containing 4 open reading frames (ORFs) (rep, cap, aap, and MAAP) flanked by inverted terminal repeats (ITRs) (1, 2). In therapeutic gene delivery, the viral ORFs are replaced by the desired transgene expression cassette and referred as recombinant AAV (rAAV). It has emerged as a leading vector to deliver genetic therapies due to its ability to transduce diverse cell types and safety profile.

A significant obstacle in clinical delivery of rAAV is host immune response triggered by rAAV capsid, genome, and therapeutic protein produced (3). Although AAV infection is non-pathogenic in humans, initial exposure induces humoral and cellular anti-capsid response that are reactive to

rAAV due to capsid similarity (4, 5). Pre-existing neutralizing antibody (NAb) can effectively block rAAV transduction even at low levels (1:5) (6). Most rAAV clinical trials exclude seropositive patients; given the high seroprevalence (60% for AAV2), limiting patients suitable for rAAV therapy (7, 8). Furthermore *ex vivo* studies have shown predominantly pre-existing memory phenotype cytotoxic T lymphocytes (CTL), following exposure to rAAV can undergo expansion and potentially lead to elimination of transduced cells (9, 10).

After rAAV administration, capsid-derived epitopes can be presented by professional antigen presenting cells (APC) *via* major histocompatibility complex (MHC) class I pathway and activate CTL (11). The activation of CTL can result in targeted destruction of transduced cells, as observed in rAAV2 hemophilia B clinical trial (12). Despite initial stable therapeutic factor IX (FIX) expression (>10% activity) for 4 weeks, FIX levels gradually declined to baseline (<1%). This was associated with asymptomatic, self-limiting transaminitis, and corresponding changes in capsid-specific CTL population (5). In the subsequent study using AAV8, administration of steroids was able to negate this response and maintain therapeutic FIX levels albeit a 50-70% decline from peak levels (13). Moreover, transgene protein product-specific CTL was observed in human rAAV trials for Duchenne's Muscular Dystrophy (14) and α-1-antitrypsin (15). Regulatory T cells (Treg) modulate immune tolerance towards transgene product and capsid that are vital to durable expression of therapeutic protein (16, 17). Although the full clinical significance of innate response to rAAV is unclear (18), unmethylated CpG motifs in rAAV vector genome interact with toll-like receptor (TLR) 9 present in plasmacytoid dendritic cells and Kupffer cells, releasing type I interferons activating cellular and humoral responses in mouse models (19, 20), and has been suggested as the cause of loss of expression in a rAAV8 hemophilia B trial (21). Furthermore, rAAV capsid-targeting TLR2, various DNA sensors, and complement activation may also play a role (22).

Different pharmacotherapies have been used to modulate immune responses in current *in vivo* rAAV studies. Here, with a particular focus on licensed agents, we discuss the pharmacology of each drug (**Figure 1**), and their applications in enabling safe and long-term expression of rAAV gene therapies (**Table 1**).

IMMUNOMODULATION TO FACILITATE rAAV GENE THERAPY DELIVERY

Global Effects

Corticosteroids

Corticosteroids (CCS; methylprednisolone, prednisolone and prodrug prednisone) bind to glucocorticoid receptors modifying diverse downstream transcriptional signaling. This includes annex I, MAPK phosphatase 1, and NF-κB resulting in anti-inflammatory and immunosuppressive properties (63). They have broad inhibitory effects on both innate and adaptive immune cells by reducing pro-inflammatory cytokine and chemokines, T- and to a lesser extent, B-cells production (64). CCS are used short-term in conjunction with systemically delivered gene therapies to negate transaminitis and associated CTL-induced injury transgene loss (30, 65), and reduce T-cell infiltrates in muscular fibers in non-human primates (NHP) (33). They are also adopted in approved gene therapies for inherited retinal dystrophy (25) and spinal muscle atrophy (SMA) (26).

Subsequently increasing doses of systemic rAAV have been delivered in preclinical and clinical studies with significant hepatic sequelae. High dose intravenous AAV9 (2×10^{14} vector genomes (vg)/kg) in NHP resulted in marked transaminitis and acute liver failure (66), posing concerns over dosage related hepatotoxicity (67). Furthermore, clinical phase II trial for X-linked myotubular myopathy delivered intravenous rAAV8.AT132 (NCT03199469) 3×10^{14}vg/kg in high dosage group, with 16-weeks of prednisolone commencing 1 day prior to dosing. Three patients with pre-existing intrahepatic cholestasis (68) experienced severe hepatobiliary complications culminating in death. The exact mechanisms of the hepatotoxicity remain to be elucidated. These studies however build evidence that short-course CCS alone is likely to be insufficient to inhibit formation of capsid-reactive T cells (13) and rAAV-mediated immune response with systemic high dosages. Therefore, the addition of other immunosuppressive agents maybe beneficial. In a AAVrh10-microRNA study delivering 4.2×10^{14} vg intrathecally into two adult patients, the first developed meningoradiculitis after intrathecal infusion despite corticosteroids (IV methylprednisolone on day 0 and oral prednisone tapered over 4 weeks). In the second patient, the addition of rituximab and rapamycin to the regimen resulted in a lower increase of NAb and T-cell response (29) and these drugs are further discussed.

Rapamycin (Sirolimus)

Rapamycin is a macrolide immunosuppressant that binds to the same intracellular target (immunophilin) as tacrolimus; however, rapamycin/FKPB12 complex inhibits a crucial cell-cycle kinase known as mammalian target of rapamycin (mTOR). Beneficial downstream effects include Treg generation, suppressing CTL and T helper (T_H) activation and at higher doses, B-cell proliferation and differentiation (69–71).

Rapamycin has beneficial effects on circumventing existing antibodies and studied in current hemophilia gene therapy trials. Hemophilia patients develop inhibitors (antibodies) to clotting factor replacement and another cause for exclusion in gene therapy trials. In a murine hemophilia A model, rapamycin (4mg/kg three times a week) was given in addition to B-cell depleting anti-CD20 antibodies to suppress T_H and Treg response suppressing inhibitor development (37). Intraperitoneal prednisolone with rapamycin was shown to inhibit B-cell activation in murine spleen and bone marrow, reducing pre-existing anti-capsid immunoglobulin G (IgG) by up to 93% after 8 weeks (72). Additionally, co-administrating AAV vectors with rapamycin encapsulated in synthetic vaccine particles (SVP [Rapa]) enabled re-dosing of AAV8 at 4×10^{12}vg/kg in mice and NHP (39). SVP [Rapa] provided sufficient reduction of B and T cell activation in an antigen-selective manner, inhibited

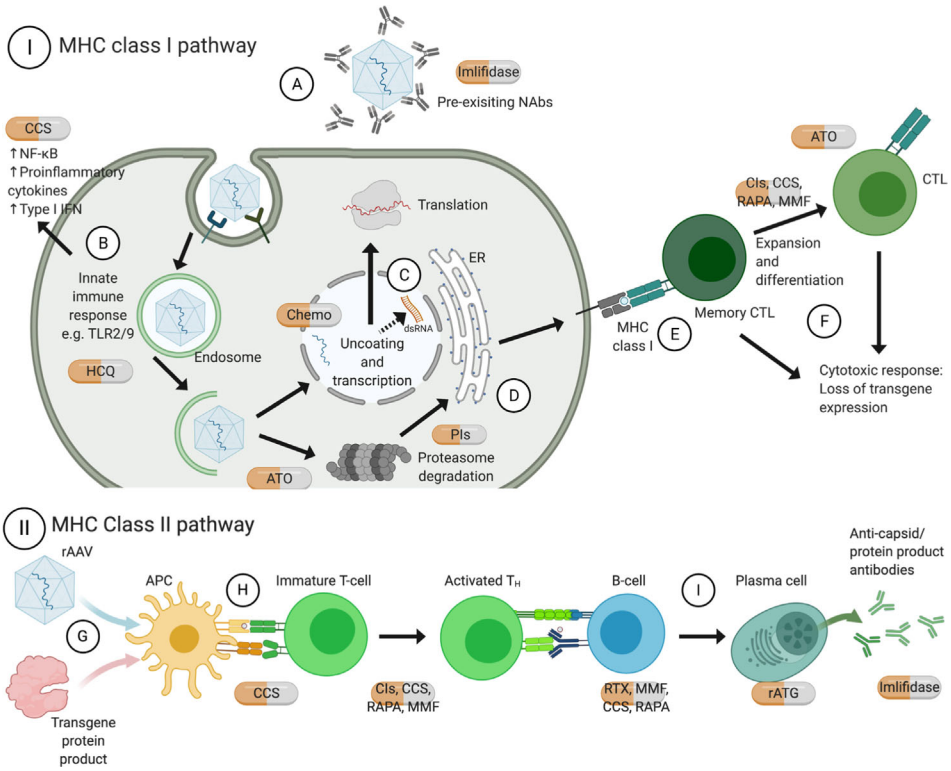

FIGURE 1 | Mechanisms of action of approved pharmacotherapies for immunomodulation with rAAV gene therapy. Pre-existing NAb can inhibit receptor-mediated endocytosis thus transduction of rAAV **(A)**. TLR9 recognizes CpG motifs, and TLR2 on cell surface or endosomal membrane recognizes vector capsid, both of which lead to release of pro-inflammatory cytokines **(B)**. Recent evidence shows that ITRs facilitate bidirectional transcription to form dsRNA, which triggers cytosolic MDA5 and downstream type I interferon response **(C)**. Upon endosomal escape, rAAV can be degraded by proteasome and loaded on MHC class I by the endoplasmic reticulum **(D)**. Recognition by memory CTL **(E)** leads to expansion and differentiation into CTL, and both can commence effector functions leading to loss of transgene expression **(F)**. On the other hand, rAAV can also transduce APC, for instance dendritic cells, and transgene protein product can be phagocytosed **(G)**. They are processed by proteasomes and endosomes respectively and the antigens can be presented on MHC class II molecules **(H)**, leading to downstream activation of T_H and B-cells; among other actions, B cells would differentiate into plasma cells and produce antigen-specific antibodies **(I)**. Created with BioRender.com. APC, antigen presenting cells; ATO, arsenic trioxide; CCS, corticosteroids; Chemo, chemotherapeutics; CIs, calcineurin inhibitors; CTL, cytotoxic T lymphocytes; dsRNA, double-stranded ribonucleic acid; HCQ, hydroxychloroquine; IFN, interferon; IL, interleukin; ITR, inverted terminal repeats; MHC, major histocompatibility complex; MMF, mycophenolate mofetil; NAbs, neutralizing antibodies; NF-κB, nuclear factor kappa B; PIs, proteasome inhibitors; RAPA, rapamycin; rATG, rabbit anti-thymocyte globulin; RTX, rituximab; TH, T helper cells; TNF, tumor necrosis factor; TLR, toll-like receptor.

CTL liver infiltration, and efficiently blocked memory T cell response. Potential of intramuscular rAAV9 re-administration is currently investigated for Pompe disease (NCT02240407) (73), by attenuating T and B cell response with rapamycin and rituximab respectively. Preliminary results were successful in preventing formation of anti-capsid and anti-transgene antibodies (38), with aims to enable rAAV re-administration and maintain effectiveness in different underlying mutations.

Mycophenolate Mofetil

Inosine monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme for guanosine nucleotide synthesis, and type II IMPDH is upregulated in activated lymphocytes. Mycophenolate mofetil (MMF), prodrug of mycophenolic acid, preferentially inhibits type II IMPDH, suppressing T and B cells proliferation (74). In mice MMF reduced rAAV transduction efficiency by depleting guanosine triphosphate required for vector genome second strand synthesis (75), but this was not observed in higher animals. No difference in AAV8-hFIX transgene expression was observed when administered with tacrolimus in NHP (6), highlighting the difficulties of recapitulating human immune system in mouse models.

T-Cell Specific

Calcineurin Inhibitors

Ciclosporin and tacrolimus are immunosuppressants that inhibit calcineurin, a key signaling phosphatase, by binding to their respective immunophilins - cyclophilin and FKBP12 (76). A major downstream effect is suppression of interleukin (IL)-2 transcription, thereby inhibiting T cells differentiation, survival, and subsequent antibody production and CTL activities *via* effector T_H cells. Daily systemic administration of tacrolimus (0.06mg/kg/day) has been shown to prolong rAAV8 and rAAV9 expression in NHP skeletal muscle, up to 42 weeks from 8 and 16 weeks respectively (47). No generalized toxicity was reported but T-cell and macrophages infiltrations were observed.

TABLE 1 | Licensed pharmacotherapies used in preclinical and clinical studies as adjuvant to AAV gene therapies.

Drug	Licensed indication(s)	Significant adverse effects in humans	Example AAV serotype trialed	Type of study
Corticosteroids (23, 24)	Anti-inflammatory and immunosuppressive properties are used in most areas of medicine - Autoimmune diseases e.g. rheumatoid arthritis, systemic lupus erythematous (SLE) - Systemic and local inflammation - Acute exacerbation of asthma and inflammatory bowel disease	Short term treatment: adrenal suppression, hyperglycemia Long term treatment: osteoporotic fracture, insulin resistance, Cushingoid features, cataracts/glaucoma, neuropsychiatric disturbances, cardiovascular risks, muscle and skin atrophy In children: growth suppression, Cushing's syndrome, medication-induced diabetes	AAV2 (25), scAAV9 (26)	Approved
			AAV2 (27), AAV5 (28), AAVrh10 (29), AAV-Spark100 (30), scAAV2/8 (13), scAAV5 (31)	Clinical
			AAV1 (32)	Clinical as combination
			AAVrh74 (33)	Preclinical
Rapamycin (34, 35)	Prophylaxis of organ rejection after transplantation	Thrombocytopenia, dyslipidemia, mucositis, impaired wound healing, proteinuria	AAV1 (36), AAV8 (37), AAV9 (38), AAVrh10 (29)	Clinical as combination
			AAV8 (39)	Preclinical
			AAV2 (40), (41), AAV9 (42)	Preclinical as combination
Mycophenolate mofetil (43, 44)	Prophylaxis of organ rejection after transplantation	Gastrointestinal toxicity (requiring dose reduction/discontinuation in 40-50% transplant patients), myelosuppression, infection, genotoxic	AAV8 (6), AAV2 (40) (41)	Preclinical as combination
Calcineurin inhibitors (45, 46)	Prophylaxis of organ rejection after transplantation	Narrow therapeutic index - nephrotoxicity, neurotoxicity, infection, gastrointestinal toxicity, malignancy	AAV1 (32)	Clinical as combination
			AAV8, AAV9 (47)	Preclinical
			AAV8 (48)	Preclinical as combination
Rituximab (49)	Rheumatoid arthritis, Non-Hodgkin's lymphoma	Infusion reaction including cytokine release syndrome, infection, febrile neutropenia, myelosuppression, cardiotoxicity	AAV2 and 5 NAb (50)	*Ex vivo* human serum
			AAV1 (36), AAV9 (38), AAVrh10 (29)	Clinical as combination
			AAV8, AAV6 (51); AAV9 (42)	Preclinical as combination
Imlifidase (52)	Pre-transplant desensitization in highly sensitized, crossmatch positive renal transplant patients	Infection (pneumonia, sepsis), infusion site reaction, hepatic dysfunction, headache	AAV8, AAV-LK03 (53)	Preclinical
Proteasome inhibitors (54, 55)	Multiple myeloma	Peripheral neuropathy, myelosuppression (especially thrombocytopenia), cardiovascular events, herpes reactivation	AAV2 (56), AAV8 (57)	Preclinical
Arsenic trioxide (58)	Acute promyelocytic leukemia	Hyperleukocytosis, gastrointestinal toxicity, skin lesions, hepatic dysfunction	AAV8 (59)	Preclinical
Hydroxychloroquine (60)	Rheumatoid arthritis, SLE	Gastrointestinal effects, retinopathy, myopathy, QT prolongation (at high dosage)	AAV2 (61)	Preclinical
Rabbit anti-thymocyte globulin (62)	Prophylaxis of graft-versus-host disease or organ rejection after transplantation	Infusion reaction including cytokine release syndrome, opportunistic infection/ reactivation	AAV2 (41)	Preclinical as combination

Indications, adverse effects observed in recommended dosages, and example of AAV studies are listed below. MnTBAP and Teniposide are excluded as they are not or no longer licensed in Europe and the US.

The first approved gene therapy in Europe, alipogene tiparvovec (Glybera), incorporated 12-week immunosuppression regimen with ciclosporin (3mg/kg/day) and MMF (2g/day) (32). In the initial regimen, 9/14 subjects showed humoral and cellular response against rAAV1 (77). Subsequent study (AMT-011-02) modified the regimen to commence ciclosporin and MMF from day -3 with additional methylprednisolone on day 0 resulting in transient cellular responses without clinical sequalae (78).

Ciclosporin and tacrolimus were found to inhibit Treg proliferation and activity *in vitro* (79), and similar effects were observed in tacrolimus-treated allograft patients *ex vivo* (80); this could be detrimental in inhibiting the development of peripheral tolerance following rAAV administration. However, preclinical delivery of ciclosporin and non-depleting CD4 receptor antibody (NDCD4) have been shown to induce antigen-specific Treg, enabling AAV intravenous re-administration after 3 months (48).

B-Cell Specific

Rituximab

Rituximab (RTX) is a chimeric mouse/human monoclonal antibody targeting CD20 present in pre-B and mature B cells except plasma cells. It depletes B cells by inducing apoptosis, antibody dependent cell-mediated cytotoxicity and complement dependent cytotoxicity, thereby limiting antibody production and epitope presentation *via* MHC class II to T_H cells (81).

A preclinical model for hemophilia B showed RTX with ciclosporin dampened NAb response to human FIX and capsid without affecting Treg (51). As ciclosporin inhibits T_H cell, this further improves B-cell inhibition profile. Variable responses have been observed in RTX's effect on reducing pre-existing AAV NAb. A small group of patients with rheumatoid arthritis were treated with combination of methotrexate and RTX, lowering anti-AAV2 and anti-AAV5 NAb in a subset of patients with variable magnitudes (50). For AAV2, 9/28 patients showed at least a half-log reduction, and inferred individuals with NAb titer ≤1:1000 were more likely to respond to RTX but the contribution of methotrexate is unknown. Considering the supportive evidence from previous AAVrh10-microRNA with RTX (29), further study in RTX application is warranted.

IgG-Degrading Cysteine Proteinase

Imlifidase (Idefirix, Hansa Biopharma) is a IgG-degrading cysteine protease derived from *Streptococcus pyogenes* (IdeS), which specifically cleaves opsonizing IgG at the lower hinge region of the heavy chains, resulting in a $F(ab')_2$ and a non-functioning dimeric Fc fragment (82). It could potentially overcome a limitation of RTX and cleave existing capsid-specific IgG. Using a laboratory version of IdeS with rAAV8, significant reductions in anti-AAV8 IgG and NAb levels, with enhanced liver transduction and transgene expression and observed in passively immunized murine models and naturally immunized NHP (53). Notably, the study also explored rAAV re-administration with IdeS pre-treatment in NHPs. In the first study (n=1), no induction of anti-capsid IgG and NAb, along with lower IgM and increased transgene level was observed for 21 days after second rAAV8-hFIX administration. However, this was not replicated in a larger cohort (n=5) immunized with rAAV-LK03, that developed anti-capsid IgM and IgG. Further studies are required as the IdeS dosing regimen differed between studies, and two rAAV-LK03 vectors (expressing GAA and hFVIII) were used in the latter study.

Other Pharmacological Agents

Proteasome Inhibitors

Proteasome inhibitors (PIs) are licensed for multiple myeloma. Second-generation carfilzomib is irreversible and more specifically inhibits chymotrypsin-like activity than bortezomib, the reversible first-generation inhibitor, which also inhibits lysosomal and calcium-activated cellular proteases (54, 83). After endosomal escape, rAAV particles either enter the nucleus for transgene expression, or become ubiquitylated then degraded by proteasome (84). The latter pathway results in unsuccessful transduction, and capsid-derived peptides are presented to CTL by MHC class I molecules, provoking elimination of transduced cells and loss of transgene expression (85). In addition, these inhibitors may have immunomodulatory role in suppressing dendritic cells function and downstream T-cell stimulation (86).

PIs have been investigated in preclinical models for their ability to increase rAAV availability and reduce CTL responses. Bortezomib has been shown to dose-dependently decrease cell surface MHC class I antigen presentation and inhibit CTL-mediated lysis after rAAV administration *in vitro* (87). Moreover, a single bortezomib dose given with rAAV8 dosing enhanced transgene expression by >50% for one year (compared to ~10%) in hemophilia A mice, and longer in-range clotting time for at least 10 months in hemophilia A dogs (57). Both bortezomib and carfilzomib enhance rAAV2 transduction *in vitro*, but bortezomib is more efficacious than carfilzomib *in vivo* when administered by retro-orbital injection with rAAV2 (56). Although no toxicity was found in the animal models, peripheral neuropathy and myelosuppression are adverse effects observed in humans (54). Emerging evidence showing variations in PI effectiveness across cell types and AAV serotypes (88), which warrants further study.

Chemotherapeutics

Second strand synthesis after capsid uncoating in nucleus is long-recognized as the rate-limiting step of rAAV transduction (89); an improvement in such efficacy could allow rAAV administration at lower dose. As traditional chemotherapeutics directly or indirectly induce DNA damage, thereby initiating DNA damage response (DDR) to repair lesions (90), it has been postulated that these repair mechanisms could increase conversion of rAAV genome into dsDNA (91), or divert DDR proteins that would otherwise impede dsDNA production (92). Several chemotherapy agents were evaluated previously (91, 93) and a high throughput screening study identified teniposide, a type II topoisomerase inhibitor pharmacologically similar to etoposide, as a potent transduction enhancer (94). Tail vein injection of rAAV2-Luc with teniposide (at doses of 1×10^{11}vg and 20mg/kg respectively) resulted in bioluminescence 2-log

higher 48 hours post-administration without hepatotoxicity. This difference reduced to ~1 log at 8 days post-administration (study endpoint). Further study is required to determine whether the effect is sustained, and evaluate potential long-term effects of non-tissue-selective chemotherapy.

Agents Affecting Oxidative Stress

Oxidizing agents, such as arsenic trioxide (ATO) (59), and antioxidants, such as manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) (95), have been evaluated. Intraperitoneal ATO 5μg/g/day from day -2 to 2 showed 3.9-fold increase in luciferase assay 12 days after rAAV8 retro-orbital injection, with dose-dependent increase of intracellular reactive oxygen species that inhibit vector degradation pathways (59). Intraperitoneal MnTBAP 80mg/kg/day from day 0-4 reversibly downregulated CD4 on T cells, inhibiting T cell priming and humoral responses to initial rAAV1 dosing, and allowing re-administration of rAAV1 *via* a different route 28 days later (95).

Anti-Malarials

Hydroxychloroquine is an anti-malarial that interferes with TLRs and cyclic GMP-AMP synthase (cGAS), dampening downstream pro-inflammatory cytokine and type I IFN production (60). A study injected hydroxychloroquine subretinally (18.75μM) with rAAV2, resulting in 5.9-fold improvement in photoreceptor transgene expression (61). However, endosomal acidification is essential for rAAV escape (84), and hydroxychloroquine increases endosomal and lysosomal pH (60), this effect may not be replicated or consistent with systemic application.

Combination Therapy

Triple T-Cell Directed Therapy

This study highlights importance of pharmacotherapy choice. rAAV2-hFIX (8×10^{12}vg/kg) was delivered intrahepatically to NHP alongside 2-drug regimen of MMF and rapamycin compared to 3-drug adding Daclizumab (40). The addition of daclizumab resulted in decreased $CD4^+CD25^+FoxP3^+$ Treg and consistent formation of inhibitory antibodies to hFIX; this was not observed in the 2-drug group. Daclizumab is a humanized monoclonal antibody targeting CD25 present on interleukin-2 receptor commonly found in activated T cells and $CD4^+CD25^+FoxP3^+$ (96). This indicates careful selection of immunosuppressive agents is necessary as Treg play a critical role in regulating immune response to rAAV products, particularly observed in liver and muscle gene transfer (97).

Triple T-Cell Directed Therapy: Delayed rATG

Timing of T cell immunosuppressant regimen was evaluated with liver-directed rAAV2-hFIX, at 7.5×10^{12}vg/kg *via* hepatic artery in NHP (41). Rabbit anti-thymocyte globulin (rATG), a rabbit polyclonal IgG, causes T-cell and plasma cell depletion and modulation of other immune effectors (98). Used with MMF (25 mg/kg) and rapamycin (4mg/kg, then 2mg/kg), a 35-day delay in rATG administration prevented formation of anti-transgene humoral response compared to commencing immunosuppression on day 0 (41). Neither group had cellular response to capsid or transgene, and 2 of 3 NHP in the delayed rATG group did not develop anti-capsid antibodies. It is possible by postponing rATG lowers the Th17/Treg ratio, allowing peripheral tolerance to the transgene product (41).

B and T Cell-Directed Therapy

This intensive immunosuppressive therapy included T-cell-targeting ATG and tacrolimus, B-cell targeting rituximab, with MMF and methylprednisolone to deliver global immunosuppression (99). This 5-drug regimen with rAAV5-PBGD 1×10^{13}vg/kg infusion resulted in reduced T-cell response in NHP, but did not prevent NAb emergence following regimen removal. This suggests that drug selection, initiation and duration of suppression, and role of global immunosuppression are important considerations.

DISCUSSION

AAV gene therapy has the potential to be durable and transformative treatment for previously incurable, life-limiting genetic diseases. However, human immune responses to the viral vector, transgene, and protein product determine the therapeutic efficacy and possibility of re-administration. Studies showed cross-reactive anti-capsid NAb present at 15 years (100), CTL and Treg infiltrates at injection site after 5 years (101); and in NHP adverse effects related to high-dosage (42, 66). With the increasing applications of systemic rAAV at higher dosages in clinical trials, further understanding of innate and adaptive immune responses to rAAV gene therapies is essential to safe and efficacious treatment.

Multiple approaches are being developed to evade the host immune response such as evaluating effects of empty capsids (102), capsid engineering guided by antigenic footprints (103), and plasmapheresis (104). The use of existing licensed medications for their immunosuppression and immunomodulation properties offers the advantages of flexibility (by allowing variations of drug combinations, dose, and duration of immunosuppressive course), accessibility, and well-documented pharmacological and safety profiles. As summarized above, a range of pharmacological agents have been used in clinical and preclinical studies, and the timing of immunomodulation, duration, and drug regimen itself have all contributed to treatment efficacy. Corticosteroids are the most commonly used agents to resolve transaminitis, however, its relationship with resolution by corticosteroids and T-cell response are not always clear as observed in a hemophilia A trial (28, 105). Also, rAAV vectors and patients' characteristics must be thoroughly evaluated to optimize safe delivery of high-dose systemic rAAV or re-dosing.

To better design immunomodulation regimens, thorough considerations of the underlying immunological mechanisms are essential. Peripheral tolerance mediated by Treg to counteract CTL responses in hepatic AAV studies remains an important area of development (106). Reports on Treg in liver and their persistence in muscle fibers after intermuscular

delivery (17) further emphasizes the need for Treg-sparing therapies. Moreover, binding (non-neutralizing) antibodies in mice seemed to have a different biodistribution profile than NAb and higher efficacy in liver transduction (107). A proposed late-phase innate response triggered by ITRs' inherent promoter activity that generates dsRNA that activates cytosolic MDA5 sensors and releases type I interferons as demonstrated in mice xenografted with human hepatocytes (108), poses further questions as to the ideal immunosuppression regimen. Lastly, the lack of fully predictive animal models (3, 109), and possibility of alternative, non-immune-mediated toxicity such as dorsal root ganglion toxicity with AAV9 (110), continue to represent challenges in safety and efficacy evaluation.

CRISPR-Cas9 is a promising therapeutic tool that allows genetic target-specific cleavage and editing (111). The first clinical trial is currently underway for Leber's congenital amaurosis 10 (NCT03872479), EDIT-101, consists of Staphylococcus aureus Cas9 (SaCas9) and two guide RNA packaged in AAV5 vector for subretinal redelivery. One concern is that the prevalence of anti-SaCas9 antibodies and T-cell in humans are reported to be 78% (111). Studies showed pre-existing SaCas9 immunity in mice resulted in increased CTL response leading to hepatocyte apoptosis and loss of transgene (112). Although no adaptive immune response towards SaCas9 was reported (113), the eye is a relatively immunoprivileged site, these data will not necessarily predict immune response in humans or systemic administration. By gaining a precise understanding of the immune mechanisms, drug repurposing (for instance JAK inhibitors for type I interferon signaling, anti-interleukin-6 human monoclonal antibodies), alongside with how and when to immunomodulate around rAAV dosing and required duration, will help to fully maximize gene therapy safety and efficacy.

AUTHOR CONTRIBUTIONS

WC researched on and prepared the draft. JN reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

JN received funding from UK Medical Research Council MR/K02342X/1, MR/R015325/1, Great Ormond Street Hospital Children's Charity (V1284), the Rosetrees Trust, Robert Luff Foundation and John Black Foundation (M576).

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Conflict of Interest: JN has sponsored research agreements with AskBio Europe and Rocket Pharma.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

METHODS
published: 01 April 2021
doi: 10.3389/fimmu.2021.657795

VectorMOD: Method for Bottom-Up Proteomic Characterization of rAAV Capsid Post-Translational Modifications and Vector Impurities

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Progress in recombinant AAV gene therapy product and process development has advanced our understanding of the basic biology of this critical delivery vector. The discovery of rAAV capsid post-translational modifications (PTMs) has spurred interest in the field for detailed rAAV-specific methods for vector lot characterization by mass spectrometry given the unique challenges presented by this viral macromolecular complex. Recent concerns regarding immunogenic responses to systemically administered rAAV at high doses has highlighted the need for investigators to catalog and track potentially immunogenic vector lot components including capsid PTMs and PTMs on host cell protein impurities. Here we present a simple step-by-step guide for academic rAAV laboratories and Chemistry, Manufacturing and Control (CMC) groups in industry to perform an in-house or outsourced bottom-up mass spectrometry workflow to characterize capsid PTMs and process impurities.

Keywords: AAV (adeno-associated virus), PTM (post-translational modification), proteomics, mass spectrometry - LC-MS/MS, bottom-up approach, glycosylation, immunogenicity, adverse drug reaction

OPEN ACCESS

Edited by:
Fabio Bagnoli, GlaxoSmithKline, Italy

Reviewed by:
Ronzitti Giuseppe, Genethon, France
Arun Srivastava, University of Florida, United States

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *24 January 2021*
Accepted: *03 March 2021*
Published: *01 April 2021*

Citation:
Rumachik NG, Malaker SA and Paulk NK (2021) VectorMOD: Method for Bottom-Up Proteomic Characterization of rAAV Capsid Post-Translational Modifications and Vector Impurities. Front. Immunol. 12:657795. doi: 10.3389/fimmu.2021.657795

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is becoming the most widely used viral vector for gene delivery and genome editing, as it is naturally replication-incompetent, non-lytic, non-pathogenic, and largely non-immunogenic. It exhibits high transduction efficiency in nearly all tissues *in vivo* and can express payloads stably from unintegrated episomes in non-dividing tissues (1). Additionally, it can target integration in actively dividing tissues when homology arms are included in the payload construct (2). Despite decades of use in the clinic, new basic biology of this virus continues to be uncovered. In the last two years alone, we have seen two dogma-changing papers re-shape the AAV textbooks. First, despite the genome's notoriously small size, yet another new rAAV gene, MAAP, has been found using machine learning (3). MAAP codes for a protein that appears to be involved in rapid extracellular secretion from host cells during production. Second, we reported that during vector production, rAAV genomes are methylated and capsids acquire PTMs (4, 5). These PTMs include acetylation, O-linked glycosylation, phosphorylation, and methylation (in addition to potential degradation products like

deamidation and oxidation). Numerous groups have now also independently identified and validated our AAV capsid PTM discoveries (6, 7).

It is known that PTMs on any therapeutic protein can elicit immune responses by inducing aggregates (8) (a known problem with high concentration rAAV), altering stability or functional activity, or by altering antigen processing and presentation. Despite historically being considered largely non-immunogenic, recent systemically-administered rAAVs at high doses have led to various unwanted immunogenic responses (9–12). It remains unclear what critical quality attributes of rAAV vectors may be contributing to these effects; thus, additional product characterization and preclinical modeling is warranted. Among PTMs, glycosylation specifically can act as a strong modulator of immunogenicity (13). A concern for the rAAV manufacturing space is the potential risk for immunotoxicity from producing vector within insect cells like *Spodoptera frugiperda* in the baculovirus-*Sf9* platform. Humans can have acute allergic responses to non-mammalian N-glycans (14), as well as any N-glycan with an α1,3-fucose or β1,2-xylose linkage on the basal N-acetyl glucosamine (GlcNAc), both of which are modifications found on insect glycoproteins (15). Thus, insect glycoprotein process impurities in baculovirus-*Sf9* produced rAAV vector lots could pose potential immunogenicity risks. As we demonstrated previously (4, 5), rAAV vectors produced in human cells are more potent than vectors produced in insect cells, allowing for lower, potentially safer, doses to be administered. Interestingly, while insect glycoforms can trigger negative human immune responses, conserved mammalian glycoforms (like those that would occur when producing vector in HEK293, HeLa, Vero, or BHK cells) generally enhance product solubility and reduce undesirable immune reactions and aggregation. Glycosylation can also shield potentially immunogenic protein epitopes from the immune system (16). The FDA has existing guidance on what protein PTMs they focus on when reviewing other therapeutic protein products (8, 17, 18), and while these do not yet apply to rAAV, they suggest where the agency may focus as we learn more about the impact these chemical modifications have on rAAV. At present, the FDA lists rAAV capsid PTM assessment as a recommended extended characterization assay (19). Thus, here we provide a detailed protocol for assessing rAAV vector lot PTMs on capsids and host protein impurities (**Figure 1**) based on our years of developing these methods.

MATERIALS AND EQUIPMENT

Reagent List (Vendor, Catalog #)

Protein LoBind 1.5 mL microcentrifuge tubes (Eppendorf Cat#0030108116)

Acetone, liquid chromatography (LC) grade (Thermo Fisher Scientific Cat#AA22928)

Protease Max trypsin enhancer, 1 mg (Promega Cat#V2071)

Ammonium bicarbonate (ABC) (Sigma Cat#09830-500G)

Dithiothreitol (DTT) (Thermo Fisher Scientific Cat#R0861)

Iodoacetamide (Sigma Cat#87-51-4)

Endoglycosidase-H (Endo-H) (Promega Cat#PRV4875)

Sequencing Grade Modified Trypsin (Promega Cat#V5111)

Formic acid, 1 mL ampules (Thermo Fisher Cat#A11710X1-AMP)

C18 MonoSpin SPE columns (GL Sciences Cat#5010-21701)

Acetonitrile (Fisher Scientific Cat#A955-4)

BCA protein assay kit (Pierce Cat#23227)

Bovine serum albumin standard ampules, 2 mg/mL (Pierce Cat#23209)

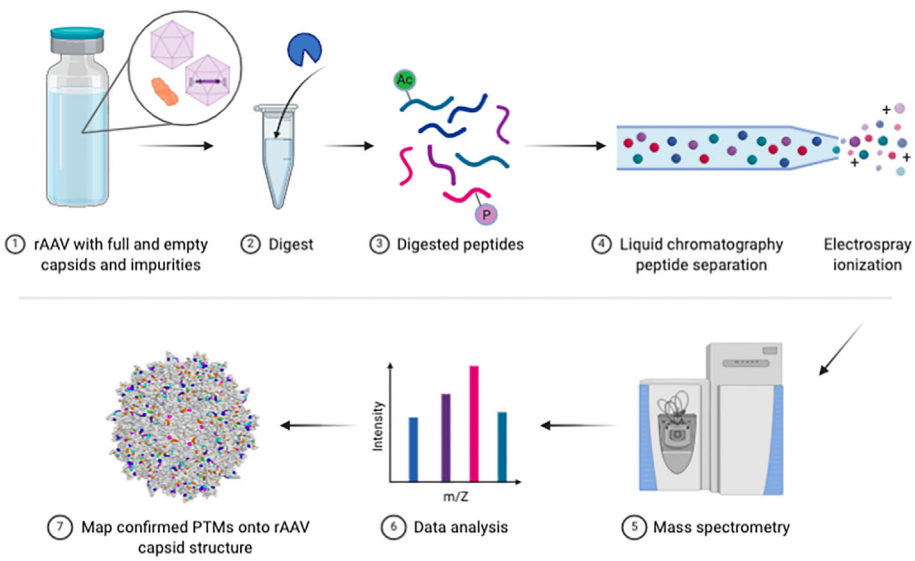

FIGURE 1 | Bottom-up rAAV proteomic workflow. Schematic of key steps in the vector preparation for LC-MS/MS and subsequent data analysis steps.

Millex-GP syringe filter unit 0.22 µm (EMD Millipore Cat#SLGP033RS)

Parafilm (Fisher Scientific Cat#S37441)

Amicon ultra centrifugal filter units, 50 kDa MWCO, 0.5 mL (MilliporeSigma Cat#UFC505008)

Angiotensin I Human Acetate Hydrate (Sigma Aldrich Cat#A9650-1MG)

Vasoactive intestinal peptides fragment VIP 1-12 MS standard (Anaspec Cat#AS-24217)

Software List

Preview™ and Byonic™ software (Protein Metrics)

XCalibur™ software (Thermo)

Windows-based PC with multi-core processors (8-core AMD) and 8GB of RAM minimum

Equipment List

Refrigerated benchtop centrifuge (any manufacturer)

Centrifugal vacuum concentrator (SpeedVac or any manufacturer)

Microcentrifuge (any manufacturer)

ACQUITY UPLC M-Class System (Waters Corporation)

Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific)

Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific)

75 µm x 150 mm EASY-Spray™ column 2 µM C18 beads (Thermo Scientific Cat#ES904)

Autosampler (Thermo Fisher Scientific)

Vortexer (any manufacturer)

Thermomixer (Eppendorf)

Solution Formulations

Solutions to Prepare in Advance and Store

100 mM ammonium bicarbonate (ABC)

Combine all reagents listed and bring to a final volume of 200 mL. Sterile filter with a 0.22 µm filter and store at room temp.

Reagent	[Final]	Amount needed
NH_4HCO_3 (MW: 79.06 g/mol)	100 mM	1.58 g
Distilled water	–	As needed (~198 mL)
Total Volume	–	200 mL

Solutions to Prepare Fresh

100-mM dithiothreitol (DTT)

Combine all reagents listed and bring to a final volume of 100 mL. Sterile filter with a 0.22 µm filter and store at 4°C.

Reagent	[Final]	Amount needed
$C_4H_{10}O_2S_2$ (MW: 154.25 g/mol)	100 mM	1.54 g
Distilled water	–	As needed (~98 mL)
Total Volume	–	100 mL

***CAUTION:** Dithiothreitol (DTT) is a harmful chemical and should be handled with appropriate safety precautions in a chemical fume hood. When handling DTT stock, wear appropriate PPE including a lab coat, goggles/face shield, closed toe shoes and gloves. Dispose of materials that contact DTT in a labeled solid waste container (weigh boats, pipette tips, etc.).

METHODS

Proteolytic Digests of rAAV Samples for LC-MS/MS

Time Required: 2.5 Days

1. Determine the protein concentration of your rAAV samples. We strongly recommend using the colorimetric bicinchoninic acid (BCA) assay as it is fast, accurate, and has the largest relevant dynamic range (20-2,000 µg/ml) suitable for AAV concentrations compared to other common assays like Bradford, Lowry, and NanoOrange. We prefer the Pierce BCA kit and recommend following the manufacturer's instructions for the 'Standard' assay rather than the 'Enhanced' assay for a more relevant working range of the bovine serum albumin controls. Read all assay results simultaneously on a microplate reader, rather than one-by-one on a spectrophotometer, given the rapid colorimetric change and large number of controls, samples, and replicates.

   ***Stop point:** you can keep your thawed AAV at 4°C until you are ready to continue. Although AAV is stable at 4°C short term, we recommend preparing your samples for digestion as soon as possible after thawing and performing the BCA assay.

2. The desired amount of total protein per rAAV sample is 50 µg, but we've successfully run samples with as little as 10 µg. Whatever concentration you choose, if you are running multiple rAAV samples with the intention of comparing the results, prepare an equal amount of total protein for each sample and place each in a labeled protein low-binding 1.5 mL microcentrifuge tube.

3. Precipitate each rAAV sample in 4X the volume of LC-grade acetone overnight at -80°C with the tube wrapped in parafilm.

4. To separate out the precipitated rAAV proteins, centrifuge at 12,500 *x g* for 15 min at 4°C. Decant and discard the supernatant and dry the rAAV protein pellet within a chemical safety hood for 30 min with the tube lid open.

5. Resuspend the dried pellet in 100 µL of 0.2% Protease Max surfactant trypsin enhancer*, 50 mM ammonium bicarbonate, and reduce the disulfide bonds with dithiothreitol to a final concentration of 10 mM at 55°C for 30 min in a thermomixer. This step loosens the protein secondary structure to make the full-length polypeptide accessible for enzymolysis in the next step.

   * **Note:** Protease Max surfactant helps solubilize proteins. This will help your AAV stay in solution, but it is not strictly necessary for digestion, should it be difficult to acquire.

Reagent	[Stock]	[Final]	Volume needed (µL)
Dried total protein pellet with rAAV	–	10-50 µg	Dried pellet
Protease Max trypsin enhancer	1%	0.2%	20
Ammonium bicarbonate (ABC)	100 mM	50 mM	50
Dithiothreitol (DTT)	100 mM	10 mM	10
Distilled water			As needed (~20)
Total volume	–	–	100

   ***CAUTION:** Dithiothreitol (DTT) is a harmful chemical and should be handled with appropriate safety precautions in a chemical fume hood. When handling DTT stock, wear appropriate PPE including a lab coat, goggles/face shield, closed toe shoes and gloves. Dispose of materials that contact DTT in a labeled solid waste container (weigh boats, pipette tips, etc.).

6. Following reduction of the disulfide bonds, alkylate the now-free sulfhydryl groups with an alkylating agent (e.g. 20 mM propionamide or iodoacetamide) at 25°C for 30 min in the dark. This step permanently prevents the reformation of disulfide bonds, keeping the polypeptide accessible.
7. To digest full-length rAAV polypeptides into short peptides, add 500 ng of Trypsin, wrap the tubes in parafilm to prevent evaporation, and allow the solution to digest overnight for ~18 h at 37°C in a thermomixer. Other digestion enzymes can be used in place of or in combination with Trypsin for varied digestion specificity (e.g. Chymotrypsin, etc.).
8. To stop the protease activity, add 1% total volume formic acid to the reaction in a chemical fume hood.

   ***CAUTION:** Formic acid is a harmful chemical and should be handled with appropriate safety precautions in a chemical fume hood. When handling formic acid at any concentration, wear appropriate PPE including a lab coat, goggles/face shield, closed toe shoes and gloves. Dispose of materials that contact formic acid in a labeled solid waste container (weigh boats, pipette tips, etc.). Store formic acid at 4°C.

   ***Stop point:** you can keep your quenched AAV peptides at -20°C for long term storage or 4°C for clean up the next day.
9. Purify the digested peptides by running the solution through a C18 MonoSpin SPE column, and then dry the peptides to completion in a speed vac. The speed vac time needed will vary with the elution volume. For example, a 300 µL solution with 80% acetonitrile can take 4-6 h.

Liquid Chromatography Tandem Mass Spectrometry

Time Required: 1.5 h

1. Reconstitute your dried rAAV peptides in 0.1% formic acid in ultrapure water and then inject onto an HPLC system such as a Dionex Ultimate 3000.

   ***CAUTION:** Formic acid is a harmful chemical and should be handled with appropriate safety precautions in a chemical fume hood. When handling formic acid at any concentration, wear appropriate PPE including a lab coat, goggles/face shield, closed toe shoes and gloves. Dispose of materials that contact formic acid in a labeled solid waste container (eppendorf tubes, pipette tips, etc.).

   ***CAUTION:** Acetonitrile is a highly dangerous chemical that is flammable in both liquid and vapor phases and can ignite with moist air or water. Is harmful if swallowed, inhaled, or absorbed through the skin. May cause skin and respiratory tract irritation. It is metabolized to cyanide in the body, which may cause headache, dizziness, weakness, unconsciousness, convulsions, coma and possibly death. Use only in explosion-proof chemical fume hoods equipped with proper grounding procedures to avoid static electricity.
2. We recommend running rAAV samples on a Thermo Fisher Fusion™ Tribrid™ Series or Q-Exactive™ mass spectrometer to improve proteome coverage, detection limits, peptide spectra acquisition, and identification rates. Unlike a linear hybrid model, which combines an ion trap and an Orbitrap, a parallelized tribrid combines a quadrupole, Orbitrap, and linear ion trap. We prefer the Orbitrap Fusion™ Tribrid™ or the Orbitrap Fusion™ Lumos™ Tribrid™ to acquire PTM data. Set up the instrument to acquire data in a dependent fashion using higher-energy collisional dissociation (HCD). If you are going to be analyzing labile modifications such as phosphorylation or glycosylation, we recommend also using electron-transfer dissociation (ETD) (20). The instrument parameters we suggest are as described below.
3. Generate HCD data with the precursor mass resolution set to 60,000 at full width at half maximum 400 *m/z*, a mass range of 350-1,500 *m/z*, and sample charge states 2-6. Set the precursor automated gain control (AGC) settings to 3e5 ions, and use the "fastest" mode in the MS/MS ion trap. Set the isolation window for HCD to 1.6 Da and the collision energy to 30. Enable dynamic exclusion with a repeat count of 3, repeat duration of 10 sec, and an exclusion duration of 10 sec. MS2 spectra should be generated at top speed for 3 sec.
4. If you are analyzing labile PTMs such as glycosylation, set up a product-dependent HCD-triggered-ETD method. Here, perform ETD if: (a) the precursor mass is between 300 and 1000 *m/z* and (b) in the HCD spectrum, 3 of 7 glyco-fingerprint ions (126.055, 138.055, 144.07, 168.065, 186.076, 204.086, 274.092, 292.103) are present at +/- 0.1 *m/z* and greater than 5% relative intensity. Set ETD parameters as

follows: calibrated charge-dependent ETD times, 2e5 reagent target, and precursor AGC target 1e4. Read out fragment ions in the ion trap in a centroid fashion.

5. Prior to running your rAAV samples, we recommend running calibration standards to ensure the instrument is within mass tolerance. We prefer to use Angiotensin I and Anaspec VIP (1–12) MS standards. Reconstitute to 500 fmol/µL and inject 1 µl to assure that calibration is within 5 ppm prior to injecting your rAAV samples.
   Angiotensin I standard: monoisotopic molecular weight of 1296.6848 Da
   VIP (1-12) standard: monoisotopic molecular weight of 1425.6393 Da
6. Load samples *via* autosampler and inject using a flow rate of 0.3 µL/min onto a 75 µm x 150 mm EASY-Spray column containing 2 µm C18 beads. Hold columns at 40°C using a column heater in the EASY-Spray ionization source.
   Note: This setup can be variable and tailored to fit your rAAV sample. The exact HPLC and column type can be adjusted and optimized for your sample separation.
7. Chromatographically separate the sample using a 90 min gradient and a 140 min instrument method. Solvent A should be 0.1% formic acid in ultrapure water, and Solvent B should be 0.1% formic acid in acetonitrile. Setup the gradient profile as follows: (minute:%B):

Minute	% Solvent B
0	3
3	3
93	35
103	42
104	98
109	98
110	3
140	3

8. Following your final rAAV sample, re-run your calibration standards as before (see Step #1) to ensure the instrument is still within tolerance at the end of your rAAV sample run.

ANTICIPATED RESULTS

Mass Spectra Data Analysis Using Byonic

1. Prepare a concatenated FASTA file containing the relevant protein sequences for your rAAV capsid serotype and host proteome(s): *Homo sapiens* for HEK293 and HeLa cells, *Spodoptera frugiperda* for *Sf9* cells, *Chlorocebus aethiops* for Vero cells, *Mesocricetus auratus* for BHK cells, *Autographa californica multiple nuclepolyhedrovirus* for baculovirus, and *Adenoviridae* or *Herpesviridae* if using Adenovirus or HSV-1 to rescue stable lines, *etc.* You can also prepare an exclusion list for common contaminants (21, 22) that typically originate from the user (e.g. keratin from hair and skin), and from common reagents (e.g. Trypsin) used in sample preparation.
2. Search your raw files with 10–12 ppm mass tolerances for precursor mass ions, with 10–12 ppm or 0.1–0.4 Da fragment mass tolerances for HCD and ETD fragmentation, respectively. Allow up to two missed cleavages per peptide and semi-specific, N-ragged tryptic digestion. Use a 1% false discovery rate using standard reverse-decoy techniques. Methionine oxidation (common 2), asparagine deamidation (common 2), and N-term acetylation (rare 1) should be set as variable modifications with a total common max of 3, rare max of 1. Depending on the goals of your experiment, you may also want to add phosphorylation, methylation, acetylation, and/or glycosylation as variable modifications. Note that the search time will increase exponentially with additional modifications, so it may be advantageous to search these modifications separately.
3. After the search is complete, open the.RAW file in the ProteinMetrics Preview software to view the trypsin digestion efficiency (**Figure 2**). The resulting identified peptide spectral matches and assigned proteins should then be exported for further analysis and validated using custom tools to provide visualization and statistical characterization.
4. PTM mass spectra should be manually validated by an expert. Do not simply trust PTM reports produced by the software. If you are not skilled in MS analysis of PTMs, seek help from an expert. Here we refer readers to an excellent manuscript summarizing common errors (23) we've seen among groups incorrectly interpreting PTMs in rAAV vector lots:
   a. Assigning the wrong PTM to a peptide
   b. Assigning the correct PTM to the wrong protein
   c. Assigning a PTM to the wrong residue on a correctly identified peptide
   d. Missing modified peptides because of a flawed database search strategy

De Novo Glycan Identification

1. All potential glycopeptide sequences should be validated by *de novo* manual interpretation of HCD and ETD mass spectra. For a thorough guide on this subject, please see Malaker et al. (24). Briefly, generate extracted chromatograms for all MS2 spectra containing the "HexNAc fingerprint," which consists of a 204.0867 *m/z* ion and 5 additional fragment ions.
2. First, use the HCD spectrum containing the HexNAc fingerprint to identify glycan structures. Distinguish whether the glycopeptide is modified by an N- or O-glycan by analyzing the ratio of 138 *m/z* to 144 *m/z* ions (**Figure 3**). Then, calculate the intact mass of the glycopeptide using the high-resolution MS1 spectrum. From the intact mass, you will see sequential glycan losses that will lead to the largest peak in the spectrum. For N-glycopeptides, this will be the mass of the peptide modified by one HexNAc. For O-

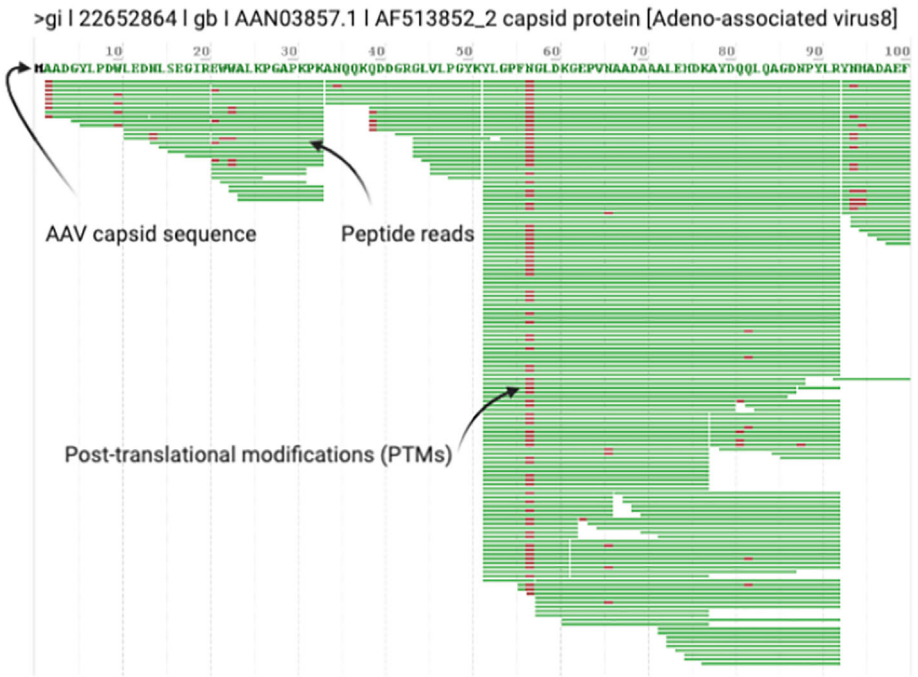

FIGURE 2 | Example Digestion Efficiency Validation. Trypsin cleaves peptides on the C-terminal side of lysine and arginine residues (unless followed by a proline). All rAAV capsid serotypes have regular Lys and Arg amino acids throughout VP1 so you should see regular cleavage that produces peptides ~10-45 amino acids in length (shown in Preview as green bars with PTMs noted in red) throughout the entire length of VP1. Good digestion efficiency and MS/MS runs should exhibit >90% sequence coverage of rAAV VP1. This screenshot highlights typical results for the first 100 amino acids of a prototypical rAAV8 run.

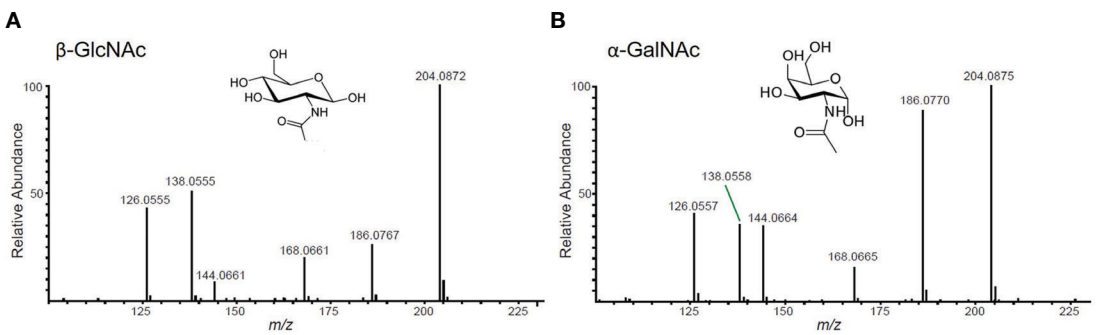

FIGURE 3 | Representative HexNAc fingerprints. **(A)** GlcNAc-containing glycopeptides will have a higher abundance of the ion at 138 *m/z* than the ion at 144 *m/z*. This fingerprint will be present in peptides with N-glycans, O-GlcNAc, and/or GlcNAc-containing core 2 O-glycans. **(B)** GalNAc-containing glycopeptides will have nearly equal abundance of 138 *m/z* and 144 *m/z* ions. This fingerprint will be present in mucin-type glycopeptides that do not contain GlcNAc.

glycopeptides, this will be the mass of the naked peptide backbone. From this, you can calculate the glycan monosaccharide composition. Finally, sequence the peptide backbone using techniques described in detail elsewhere (25).

3. Next, use the ETD spectrum to site-localize glycan modifications. This is especially important for O-glycopeptides, due to the labile nature of this modification. A detailed tutorial for manual interpretation of ETD spectra is available here (26).

TROUBLESHOOTING

1. **Your purified input rAAV is not sufficiently concentrated to achieve 10-50 µg in 50 µL volume.**
   If your purified rAAV production has a low titer, below ~1e12 vg/mL, you will likely not have sufficient rAAV capsid protein concentration for the initial digestion and subsequent steps. You can concentrate your rAAV vector with an Amicon™ 50 kDa spin column. We recommend a 20-30

minute spin at 1,500 *x g* for ~10-20X concentration as suggested by the manufacturer for purified AAV (27). You will need to remeasure your protein concentration by BCA assay after completing the spin concentration, do not assume you will get a certain fold improvement. Make sure to use the 50 kDa Amicon™ columns for optimal rAAV retention as intact rAAV capsids are 20-24 nm in diameter (28), as shown below.

MWCO (kDa)	Pore Size (nm)	Min Retention Diameter (nm)	Max Retention Diameter (nm)
3	0.3	1	1.5
10	1	3	9
30	3	9	15
50	**5**	**15**	**30**
100	10	30	90

2. **Difficulty re-solubilizing your rAAV proteins.**
   As noted above, surfactant may not be necessary for protein resolubilization; however, if rAAV protein samples are insoluble in ABC buffer, two distinct steps can be followed to improve sample solubility. First, add a mass spectrometry friendly surfactant, such as Protease Max, to the samples and gently mix by pipette action. Incubate the samples at room temperature for 20 min before re-mixing. If this does not solve the problem, samples can be heated using a heating block set to 95°C for up to 5 min.
3. **AAV sample degradation from freeze/thaw.**
   For long term native AAV storage, samples should be kept at -80°C, however digested rAAV peptides can be stored at -80°C. This will prevent rAAV VP1/2/3 peptide degradation and help to retain labile PTMs. For short-term storage once thawed, samples can be kept at 4°C; however, it is important to avoid repeated freeze/thaw cycles, as the extreme temperature fluctuation can lead to more rapid sample degradation. We typically avoid more than 1-2 freeze/thaw cycles per sample prior to analysis.
4. **Contaminating peptides obscuring your results.**
   Perhaps one of the biggest complicating factors in analyzing rAAV samples is the potential presence of contaminating peptides in your sample. Notably, these peptides might also have PTMs that can interfere with the analysis of your rAAV sample. Contaminant peptides can come from a variety of sources, including sample handling, host cell impurities, and vector purification inefficiencies. Therefore, it is crucial to manually validate all search results to ensure the correct site-localization of PTMs on the proper protein. We recommend searching for not only the sequence of interest (i.e. rAAV of the appropriate serotype), but also the proteomes of potentially contaminating species, such as *Sf9* or other sources involved in vector production. In our hands, upon manual validation we found several hundred N-glycopeptides from ferritin proteins that were initially assigned as N-glycopeptides of rAAV. This underscores the importance of carefully manually validating automated search results by the methods outlined above.
5. **If you think your sample has glycopeptides but you are having trouble detecting them.**
   Following the reduction of disulfide bonds in Step 6, treat with Endo-H. Heat your reduced rAAV sample to 95°C for 5 min, then briefly chill on ice to reduce the temperature and add 5 µL of the supplied Endo-H reaction buffer and 5 µL Endo-H. Deglycosylate for 4 h at 37°C in a thermomixer.
   ***Note:** Endo-H is a recombinant glycosidase which hydrolyses the bond connecting the two GlcNAc groups modifying Asn within the chitobiose core, leaving a single GlcNAc covalently bound to Asn for mass spectrometry detection.
6. **Sample analysis challenges and limitations.**
   With all PTM assignments using LC-MS/MS, it is important to remember that absence of a peptide with a PTM does not mean that no PTM was present. Labile modifications like phosphorylation and glycosylation can be lost during sample preparation or ionization prior to detection. It is also important to note that quantifying PTM frequency is challenging. In many cases, PTMs are not site-localizable, which can dramatically alter the accuracy of quantitative site occupancy evaluations. Additionally, different peptides from an individual protein can differ in cleavage efficiency.

DISCUSSION

Preclinical investigators and existing clinical stage gene therapy companies should catalog and track potentially immunogenic vector lot components. These include capsid PTMs and PTMs on host cell protein impurities, especially given the recent concerns around immunogenicity with systemic high dose rAAV. The FDA currently lists rAAV capsid PTM assessment as a recommended extended characterization assay (19). The methods detailed above provide a powerful platform to easily interrogate the proteomic and PTM landscapes of rAAV vectors. As improvements to both vector process/product development and analysis by mass spectrometry continue, the quality, complexity, and utility of these data will continue to grow. These methods can be extended to other viral gene therapy vectors by changing the appropriate search parameters and optimizing the initial proteolytic digest conditions (for example, enveloped viruses will need a detergent step to remove the envelope to allow proteases to digest the capsid proteins). While we regularly detect common PTMs on rAAV capsids and host impurities (N-terminal start methionine acetylation, serine/threonine/tyrosine phosphorylation, lysine acetylation, arginine methylation, O-linked glycosylation, and asparagine deamidation), other modifications, if present, can also be detected with this method.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: exemplary datasets analyzed for this method are available from the corresponding authors on reasonable request. Requests to access these datasets should be directed to NKP, nicole.paulk@ucsf.edu.

AUTHOR CONTRIBUTIONS

NKP conceived the study. NGR, SAM, and NKP designed experiments. NGR, SAM, and NKP generated reagents, protocols, performed experiments, and analyzed data. NGR, SAM, and NKP wrote the manuscript. SAM and NKP generated the figures. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants to NKP from the NIH (K01-DK107607, U01-HL145795), an American Society of Gene & Cell Therapy Career Development Award and the Sandler Family Foundation; and funds to SAM from the Yale Science Development Fund. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the funding bodies, or the respective universities and organizations.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 13 April 2021
doi: 10.3389/fimmu.2021.666666

T Cell-Mediated Immune Responses to AAV and AAV Vectors

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Adeno-associated virus (AAV)-mediated gene transfer has benefited patients with inherited diseases, such as hemophilia B, by achieving long-term expression of the therapeutic transgene. Nevertheless, challenges remain due to rejection of AAV-transduced cells, which in some, but not all, patients can be prevented by immunosuppression. It is assumed that $CD8^+$ T cells induced by natural infections with AAVs are recalled by the AAV vector's capsid and upon activation eliminate cells expressing the degraded capsid antigens. Alternatively, it is feasible that AAV vectors, especially if given at high doses, induce *de novo* capsid- or transgene product-specific T cell responses. This chapter discusses $CD8^+$ T cell responses to AAV infections and AAV gene transfer and avenues to prevent their activation or block their effector functions.

Keywords: $CD8^+$ T cells, memory, immunosuppression, effector functions, animal models, clinical trials

OPEN ACCESS

Edited by:
Nicole K. Paulk, University of California, San Francisco, United States

Reviewed by:
Ronzitti Giuseppe, Genethon, France
Romain Hardet, INSERM U1234 Physiopathologie, Autoimmunité, Maladies Neuromusculaires et THErapies Régénératrices (PANTHER), France

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *10 February 2021*
Accepted: *22 March 2021*
Published: *13 April 2021*

Citation:
Ertl HCJ (2021) T Cell-Mediated Immune Responses to AAV and AAV Vectors. Front. Immunol. 12:666666. doi: 10.3389/fimmu.2021.666666

INTRODUCTION

The goal of gene therapy is to permanently replace a missing or faulty gene and thereby through sustained production of the transgene product achieve a functional cure. Various methods have been explored to insert genes *in situ* into specific cells (1, 2). One of the most promising gene transfer vectors are AAV vectors, which in initial preclinical studies achieved sustained expression of their transgene product in mice (3), dogs (4), and nonhuman primates (5) without any overt serious adverse events. In humans clinical trials targeting Leber's congenital amaurosis, a congenital form of blindness, by small doses of AAV injected into the subretinal space reported long-term improvement of vision (6, 7). In contrast, the first clinical trial for hepatic AAV-mediated transfer of factor (F)IX for correction of hemophilia B accomplished initial increases in F.IX levels, which were followed a few weeks later by a subclinical transaminitis and loss of F.IX (8). Additional studies showed that patients developed concomitantly with rises in liver enzymes circulating $CD8^+$ T cells to AAV capsid antigens (9). This led to the still valid but nevertheless unproven hypothesis that patients had AAV-capsid-specific memory $CD8^+$ T cells, which were reactivated by the gene transfer and then eliminated the vector-transduced hepatocytes (10).

This opened a slurry of pre-clinical experiments that aimed to recapitulate the findings of the clinical trial. Although the animal experiments allowed the field to gain valuable knowledge of the intricacies of anti-AAV capsid T and B cell responses (11–13), in the end the studies confirmed what we have known for long – mice are not humans (14) and neither mice nor larger animals are overly informative about the presumably immune-mediated rejection of AAV-transduced cells.

Clinical AAV-mediated gene transfer trials by reducing vector doses and using various immunosuppressive regimens at least in part overcame immunological barriers and achieved treatment benefits or even cures for their patients (15, 16). Nevertheless, transfer of genes with high

doses of AAV remains a crapshoot especially in 2020/21 during a global pandemic with a potentially fatal virus that is especially dangerous for immunocompromised humans (17). Immune responses to AAV gene transfer are complex involving both the innate and adaptive immune systems. Here we discuss what is known from pre-clinical models as well as clinical trials about $CD8^+$ T cells to AAV gene transfer.

AAV VIRUS AND IMMUNE RESPONSES TO NATURAL INFECTIONS

AAVs are single-stranded DNA viruses of the parvovirus family. As dependoviruses they only replicate in presence of a helper virus such as an adenovirus. AAVs do not cause any known disease. The ~4,700 base pair long AAV genome, which is flanked by inverse terminal repeats (ITRs), has two open reading frames, one for rep proteins needed for viral replication, and the other for the capsid proteins vp1, vp2 and vp3, which are produced by differential splicing and therefore only differ in their N-terminus (18). Capsid proteins distinguish serotypes of AAV. Thus far 12 human serotypes of AAV have been identified (19). They differ in their tropism (20) and in the prevalence, with which they circulate in humans (21). AAV genomes persist mainly episomally in the nucleus of infected cells although they can integrate into a specific site of human chromosome 19 (22).

Humans, who become naturally infected with AAVs, mount adaptive immune responses, which presumably are in part driven by innate responses to the helper virus (23). Prevalence rates of neutralizing antibodies to different serotypes of AAVs, which serve as indicators for previous infections, vary in part depending on age and country of residency (21, 24–31). Some studies report strikingly different prevalence rates even when they tested similar populations. This likely reflects that AAV neutralization assays are not standardized and therefore differ in their sensitivity. Overall trends are similar. Prevalence rates of neutralizing antibodies to AAV increase with age and they are higher for AAV2 or AAV8 than for example AAV5 or AAV6.

T cell responses have been studied less well. We reported that about 50% of healthy human adults have detectable frequencies of circulating AAV capsid-specific $CD8^+$ and/or $CD4^+$ T cells when tested by intracellular cytokine staining (ICS); 50% of these $CD8^+$ T cells belong to the central memory subsets and 25% each to the effector and effector memory subsets. AAV capsid-specific $CD4^+$ T cells belong mainly to the central memory subset (32). Non-human primates tested by the same method showed that 5 out of 6 have AAV capsid-specific $CD8^+$ T cells while 6/6 have $CD4^+$ T cells of that specificity. In monkeys, $CD8^+$ T cells are strongly biased towards effector cells (32). For these assays we used a peptide panel that reflected the capsid sequence of AAV2 but would like to point out that many of the T cell epitopes are highly conserved. Nevertheless, unlike in humans AAV-mediated gene transfer achieves long-lasting transgene product expression in nonhuman primates, which may reflect that their T cells potentially due to high levels of persisting AAVs are functionally exhausted (32). Overall, not only prevalence but also frequencies of AAV capsid-specific T cells are higher in non-human primates than in humans. Testing additional non-human primates by an ELISPOT assay, which is the assay that is primarily being used by gene therapists to monitor T cell responses to AAV capsid upon AAV-mediated gene transfer, showed lower prevalence rates of AAV capsid-specific T cells of ~50% (32).

Using a proliferation and cytokine secretion assays another group reported that peripheral blood mononuclear cells (PBMCs) of less than 10% of humans mount a response (29) although it should be pointed out that these assays lack sensitivity. Another group using ELISPOT assays as well as ICS showed with either assay that ~30% of health human adults respond to AAV1 capsid (33). A study using a very sensitive method based on pre-selection of AAV8-specific $CD8^+$ T cells with a specific tetramer showed that all tested humans have circulating effector memory $CD8^+$ T cells against AAV8 capsid (34). Human circulating AAV capsid-specific $CD8^+$ T cells are functional, they secrete cytokines (32, 34) and lyse target cells expressing their cognate antigen (33). T cell epitopes are conserved between several AAV serotypes (9) and several studies reported no correlations between antibody and $CD8^+$ T cell responses (32, 35). One study showed that peripheral blood mononuclear cells from AAV2 seronegative donors mount a robust IFN-g-secreting natural killer cell response to *in vitro* culture with an AAV capsid peptides while those from seropositive individuals showed activation of tumor necrosis factor-α producing $CD8^+$ T cells (36).

Overall, these data demonstrate that AAV infections are highly prevalent and cause sustained immunological memory that can presumably be recalled upon re-infection or transfer of an AAV vector.

AAV VECTORS

Production and purification methods for AAV vectors are well established (37). In AAV vectors the viral genes but for the inverted terminal repeats (ITRs) are replaced with an expression cassette for a therapeutic protein. A variety of promoters have been used, some of which drive ubiquitous expression while others are specific for selected cell types. For some applications, such as hemophilia B, a variant transgene with improved functions compared to protein encoded by the wild-type gene has been used to allow for dose-sparing.

AAV vectors are in general generated by triple transfection of a cell line, such as HEK 293 cells, which carry the E1 gene of adenovirus. One plasmid expresses additional adenoviral genes to promote AAV production. A second plasmid carries the AAV cap and rep genes. The AAV2 rep gene is used for most AAV vectors while the cap gene determines the serotype of the vector. The third plasmid carries the transgene expression cassette flanked by the ITRs, again most commonly of AAV2. Vectors are then released from the transfected cells and purified by various methods such as gradient centrifugation, column

purification and others (38). The type of purification may affect levels of empty AAV particles within the preparation, which in turn can influence the induction of immune responses or reduce the inhibitory effects of AAV neutralizing antibodies.

AAV vectors can also be produced in the baculovirus expression system, which is more amenable for scale-up than mammalian expression systems (39). AAVs produced in mammalian cells or insect cells show differences in post-translational modification, genome methylation and levels and types of host cell contaminations which affect their immunogenicity and their performance in clinical trials (40, 41).

CD8$^+$ T CELLS

CD8$^+$ T cells are uniquely capable to eliminate virus-infected or vector-transduced cells by direct lysis mediated by the release of perforin and granzyme. They also secrete anti-viral cytokines such as interferon (IFN)-γ. Activation of naïve CD8$^+$ T cells, which reside in lymphatic tissues and circulate in blood, requires presentation of antigen-derived peptides bound to major histocompatibility complex (MHC) class I molecules by professional mature antigen-presenting cells. Peptides can be generated from *de novo* synthesized misfolded proteins that upon degradation by proteasomes are transported by the transporter associated with antigen presentation (TAP) into the endoplasmic reticulum (ER), where they bind to MHC class I molecules, which are then transported to the cell surface. This classical presentation pathway would apply to vector-encoded transgene products. Antigen-presenting cells are able to cross-present protein such as those of the AAV capsid that are taken up by pinocytosis, phagocytosis or endocytosis. In the so-called cytosolic pathway, the particles are degraded in phagosomes and peptides are released into the cytoplasm from where they can be transported into the ER; there they can bind to MHC molecules (42). In the vacuolic pathway, proteins are degraded within endosomes. They escape into the cytoplasm upon acidification of the endosomes or upon reactive oxygen species-mediated lipid peroxidation of endosomal membranes (43).

The antigenic peptides displayed by MHC class I antigens on the cell surface bind to T cell receptors, which triggers a signaling cascade that through the adaptor molecule zeta-chain-associated protein kinase 70 (ZAP70) induces activation of calcineurin leading to the activation of nuclear factor of activated T-cells (NFAT). Full activation of T cells furthermore requires interactions with co-stimulators most commonly CD80 and CD86 or CD40 on antigen-presenting cells, which interact with CD28 or CD40L on T cells, respectively. This amplifies T cell receptor signaling and through phosphoinositide 3-kinase (PI3K) induces the mechanistic target of rapamycin (mTOR)/protein kinase B (Akt) pathway which modifies the T cells' metabolism to provide energy and building blocks for rapid proliferation. Dendritic cells, the main cell type that presents antigens to naïve T cells, are immature when they are released from bone marrow. At this stage they do not express co-stimulators and are therefore unable to activate an effector CD8$^+$ T cell response but rather induce tolerance. Maturation of dendritic cells into profession antigen-presenting cells is driven by pathogen-associated molecular patterns (PAMPs), such as CpG motifs within the genome, which are common in bacteria and viruses but largely absent in mammalian cells. PAMPs interact with pathogen recognition receptors (PRR) such as Toll-like receptors (TLRs) and others expressed in different cellular compartments (44). Binding of a PAMP to a PRR causes activation of numerous pathways, such as the nuclear factor kappa B (NK-κB) and interferon regulatory factor (IRF)3 pathways. Induction of these pathways, which can also be activated by type I interferons (IFN) or members of the tumor necrosis factor (TNF) family, involves a number of molecules such as TIR domain containing adaptor protein (TIRAP), myeloid differentiation primary response 88 (MyD88), inhibitor of NF-κB kinase (IKK)-γ, or interleukin-1 receptor-associated kinase (IRAK)-4, all of which can be targeted by drugs to block inflammatory responses. Once NF-κB or IRF-3 are activated they induce pro-inflammatory cytokine responses, which initiate or increase production of molecules that are essential for antigen processing and presentation. Upon stimulation, CD8$^+$ T cell proliferate very rapidly and then migrate to sites of infection where they assume effector functions. Recognition of foreign antigen is exquisitely sensitive and can be triggered by as few as 2-3 MHC-peptide complexes on the surface of a cell (45). CD8$^+$ T cell differentiation requires help from CD4$^+$ T cells (46) belonging to the T helper (Th)1 subset. Once the antigen has been removed most of the effector CD8$^+$ T cells die, some will differentiate into memory cells, which can be recalled rapidly. Re-activation of memory CD8$^+$ T cells does not require professional antigen presenting cells and is less dependent on co-stimulation. Effector CD8$^+$ T cells can differentiate into different type of memory cells, i.e., effector, central memory or tissue resident memory T cells. Effector memory CD8$^+$ T cells circulate. They do not proliferate extensively after re-exposure to antigen and can assume functions instantly. Over time in absence of antigen they differentiate into central memory cells or die. Central memory CD8$^+$ T cells reside in lymphatic tissues. They do not exhibit functions. Upon reencounter of their antigen they proliferate vigorously before they assume effector functions; this may take several days. Central memory CD8$^+$ T cells are maintained at steady numbers potentially throughout the lifespan of an individual. Tissue resident memory CD8$^+$ T cells are also very long-lived but they remain at sites of previous infections. Upon local reinfection they can immediately release cytolytic enzymes and initiate an inflammatory reaction. If the antigen is not removed but continues to persist at high levels, T cells will differentiate towards exhaustion by gradually losing function, increasing expression of co-inhibitors such as programmed cell death protein 1(PD-1) and eventually undergoing apoptosis (47).

How does this apply to AAV vectors? To activate naïve CD8$^+$ T cells one would expect that the vector would be phagocytosed by immature dendritic cells. PAMPs within the vector genome or on the capsid would interact with PRRs on or within the cells.

This would trigger an inflammatory reaction and maturation of the dendritic cells, which would then migrate to draining lymph nodes. Within cells antigens encoded by the AAV vector would enter the classical presentation pathway while antigens of the AAV capsid would be processed and presented by either the cytosolic or vacuolic pathway. Dendritic cells presenting antigen bound to MHC class II molecules would activate specific $CD4^+$ T cells which would then facilitate stimulation followed by expansion of $CD8^+$ T cells by antigen displayed by MHC class I antigen. Subsequently T cells would migrate out of lymph nodes and circulate till they find their cognate antigen. Cells displaying this antigen would be killed rapidly within minutes and then the T cell would find its next target. This process would continue till all of the antigen is removed, which in case of AAV particles may take months. Memory $CD8^+$ T cells generated in response to a natural infection can be stimulated by cells other than dendritic cells and depending on subset they can act immediately once they see their antigen displayed on MHC class I. If they belong to the tissue resident memory $CD8^+$ T cell subset they may not even proliferate, which raises the question if screening for increases in circulating T cells is adequate to predict immune-mediated rejection of AAV-transduced cells. Due to higher numbers of precursors memory T cell responses are more potent and come up more rapidly.

It is assumed that the increases of circulating AAV capsid specific $CD8^+$ T cells reflect recall of memory cells that had initially been activated by a natural infection. This may be the case for some patients but activation of naïve $CD8^+$ T cells should not be ruled out. The very slow increases of AAV capsid-specific T cells in some AAV vector recipients would be more typical for primary than secondary responses. As stimulation requirements and thereby sensitivity to immunosuppressive drugs differ for naïve and memory $CD8^+$ T cells further studies are needed to elucidate what T cell subsets respond to AAV gene transfer.

$CD8^+$ T CELL RESPONSES TO AAV VECTORS

Viral vectors can induce $CD8^+$ T cell responses to their own antigens as well as to a transgene product. In the case of AAV vectors, which have been stripped of genes that encode AAV proteins, any effector T cell response to the viral proteins would be limited to the time frame till all the vector particles have been completely degraded. T cell responses to the transgene product on the other hand could continue till all antigen-producing cells have been removed or till immunosuppressive mechanisms such as T cell exhaustion or regulatory T cells turn off the T cells.

Initial studies reported that AAV vectors did not induced $CD8^+$ T cell responses to the transgene product and this was attributed to lack of activation of innate responses, which resulted in immunological ignorance (48). Additional studies contradicted these results and reported that AAV vectors can induce transgene product-specific $CD8^+$ T cell responses in experimental animals (49–51). It was also shown that AAV vectors elicit albeit weak and transient innate responses that are largely driven by TLR9 activation through CpG motifs within the vector genome (52) or TLR2 activation by capsid components (53). Naturally immunogenic transgene products, such as antigens from another pathogens, induce depending on the vector's serotype and it's genome structure such robust immune responses that AAV vectors were explored as vaccine carriers (50, 51). The magnitude of the immune responses depends on vector dose, the AAV serotype, the transgene, the type of promoter, the target tissue and the vectors' genome structure (54). Self-antigens, such as F. IX with point mutations, are non-immunogenic while the same mutant F.IX in a mouse with a genetic F.IX deletion induces cellular and humeral responses (55). Comparing vectors with single-stranded and double-stranded DNA genomes, showed that the latter are more immunogenic presumably by inducing more potent innate responses (56). Some studies showed that $CD8^+$ T cell responses induced by an AAV vector-encoded transgene are defective in mice: T cells do not proliferate upon re-exposure to their antigen *in vivo*, they only produce low levels of cytokines and they fail to protect against a surrogate pathogen (57, 58). Others showed that the effectiveness of transgene product-specific hepatic $CD8^+$ T cell responses is dependent on vector dose; intermediate doses of vector lead to a delayed $CD8^+$ T cell response that eliminates antigen-producing hepatocytes. High doses of vector induce multiple immunosuppressive pathways that block induction of transgene product-specific $CD8^+$ T cells (59).

AAV vectors can induce capsid-specific $CD8^+$ T cell responses, especially if highly immunogenic T cell epitopes are incorporated into the capsid (60, 61). This process is likely driven by cross-presentation of capsid proteins and not only requires plasmacytoid and conventional dendritic cells but also help from $CD4^+$ T cells (62, 63). Nevertheless, in mice specific $CD8^+$ T cells induced by AAV gene transfer fail to eliminate AAV-transduced cells (64), which can be achieved by adaptive transfer of *ex vivo* expanded capsid-specific $CD8^+$ T cells suggesting defects at the level of T cell differentiation *in vivo* that could be overcome in tissue culture (65).

Although they fail to reject AAV-transduced cells, mice have been useful to study the duration of capsid degradation, which in the end dictates how long gene transfer recipients are at risk to lose treatment benefit due to AAV capsid-specific $CD8^+$ T cells. Experiments using proliferation of capsid-specific $CD8^+$ T cells, which were adoptively transferred into AAV-injected mice as a read-out, showed that T cells proliferated in their hosts even if transferred 6 months after AAV injection, which is reflective of the very slow degradation of AAV capsid (66).

Clinical trials using different serotypes of AAV vectors over a large range of doses have been completed, are ongoing or planned for a number of diseases. For ocular diseases such as choroiderma, an X-linked form of progressive vision loss (67), achromatopsia or color blindness (68), X-linked retinitis pigmentosa (69), or Leber's congenital amaurosis (70) AAV vectors encoding the therapeutic protein are injected at modest doses into the subretinal space, which similar to the central

nervous system is an immunoprivileged site (71) that contains high levels of transforming growth factor (TGF)-ß and is shielded by a physical barrier from blood. In addition, parts of the eye, such as the ocular chamber, actively induce immune tolerance through a process called anterior chamber-associated immune deviation (ACAID) (72). It should be noted though that this pathway does not induce systemic tolerance after subretinal injection of an AAV vector (73). While some trials for correction of ocular diseases reported stable transgene product expression for years without evidence for induction of T cell responses (74, 75), others observed stimulation of adaptive immune responses combined in some cases with loss of therapeutic benefits (76). Pre-clinical studies indicate that induction of immune responses to ocular injection of AAV vectors depends on vector dose, the promoter, route of application and the transgene (77), which may in part explain discrepancies of results.

Many AAV-mediated gene transfer trials focus on hemophilia where circulating coagulation factors offer an easy read-out for transgene product expression. Vectors that were or are being explored for hemophilia B include AAV8, AAV5, AAVrh10 or AAVs with genetically engineered capsids expressing either wild-type F.IX or the 5 time more potent F. IX Padua variant. Vectors either contain a single-stranded or self-complementary genome. They were or are given at doses ranging from $2x10^{11}$ to 2×10^{12} vg/kg achieving post-infusion levels of F.IX depending on the trial and the vector dose from ~1-40% of normal. Many trials use codon-optimized vectors to increase expression and/or vectors in which most CpG motifs are modified to minimize TLR9 activation. Some trials, such as one using AAV5 expressing the Padua variant of F.IX failed to observe any post-infusion transaminitis (78), which would be indicative of an anti-AAV immune response while others using the same vector reported transient increases in liver enzymes, which were not accompanied by detectible T cell responses (79). Using an AAV vector with a bioengineered capsid expressing F.IX Padua or a self-complementary AAV8 vector expressing wild-type F.IX other groups reported in some patients increases in transaminases combined with increases of circulating AAV capsid-specific T cells (80, 81). Data have been published for one hemophilia A trials, which used a baculovirus-derived AAV5 vector. The study reported increases in liver enzymes but failed to detect concomitant rises in capsid-specific circulating T cells (80). Circulating FVIII levels tended to decrease gradually by 2-3 years after gene transfer most likely reflecting hepatocyte turn-over rather than an immune-mediated rejection (16). A number of additional trials that are ongoing for correction of hemophilia A have reported therapeutic benefits (82) but not yet released potential problems with immune-mediated rejection.

Overall transfer to the liver, a unique microenvironment that favors immunosuppression (83), can induce in a dose-dependent manner capsid-specific $CD8^+$ T cell responses, which have been implicated to eliminate AAV-transduced cells. Thus far transgene product-specific $CD8^+$ T cells have not yet been observed upon hepatic transfer of AAV vectors. This may in part reflect that trials mainly enrolled patients with point mutations in their coagulation factors, which destroy their biological activity but fail to prevent induction of immunological tolerance. Most patients had also received factor replacement therapy, which would further promote tolerance. It would be expected that AAV vectors will induce F.VIII- or F.IX-specific T cell responses in patients with large deletion mutations although such responses might potentially be dampened or blocked by concomitant induction of regulatory T cells (59).

For treatment of other diseases AAV vectors are given into the muscle. For example, for treatment of Pompe disease, caused by glycogen storage in muscle and motor neurons due to lack of lysosomal alpha-glucosidase, an AAV9 vector was injected at a high dose into the diaphragm in children with progressive respiratory failure requiring ventilation and enzyme replacement therapy (84). T cell responses to the vector or transgene product were not detected. Several trials are exploring intramuscular injection of AAV vectors expressing dystrophin for treatment of Duchenne's muscular dystrophy (85, 86) or a1-antitrypsin (AAT) deficiency (87, 88). An AAT trial using an AAV-1 vector reported that vector-derived AAT levels were sustained for several months although all treatment recipients developed T cell responses to the capsid proteins of AAV1 and 1 of the 9 patients had at one timepoint a positive T cell response to AAT. A follow-up study conducted about a year later showed sustained presence of the AAV genome in the injected muscle and a marked reduction in inflammatory cells in year 1 compared to months 3 biopsy samples. A substantial portion of the muscle infiltrating lymphocytes were regulatory T cells suggesting that they had suppressed vector-induced effector T cell responses and thereby prevented loss of AAV-transduced muscle cells (87, 88). A further follow-up study conducted 5 years after gene transfer detected Tregs and AAV capsid-specific $CD8^+$ T cells within the injected muscle (89).

For AAV-mediated correction of Duchenne's muscular dystrophy one trial reported that patients developed no or only very weak T cell responses to the viral capsid but instead some generated robust transgene product-specific $CD8^+$ T cell responses (90, 91). AAV1-mediated transfer of the alpha-sarcoglycan gene for correction of limb-girdle muscular dystrophy reported a detectable AAV capsid-specific T cell response in 3 patients. In one patient this response came up very rapidly on day 2 after gene transfer and may have contributed to his loss of AAV-transduced muscle cells (92). Additional studies are needed to further determine the risk induction of T cells upon intramuscular injection of AAV vectors. It will be especially important to further elucidate the role of regulatory T cells in preventing immune-mediated destruction of AAV-transduced muscle cells.

AAV vectors are directly infused into the central nervous system to treat neurological diseases such as Alzheimer (93), Parkinson's disease (94–97), infantile neuronal ceroid lipofuscinosis (98), Canavan disease, a N-acetylaspartate storage disease of the brain caused by mutations of the aspartoacylase gene (99) and others. Studies did not assess AAV capsid or transgene product-specific T cell responses following AAV gene transfer. Some trials analyzed serum

antibody responses to AAV and reported that they increased in some patients suggesting that enough vectors had leaked from the injection sites to trigger a peripheral B cell response.

Considering that the central nervous system is an immunoprivileged site that lacks the lymphatic structures needed for activation of immune responses it is unlikely that enough vector will leak into the periphery to activate AAV capsid or transgene-product-specific $CD8^+$ T cells which could then cross the blood brain barriers and attack vector-transduced cells. Nevertheless, it would be prudent to monitor patients for AAV-induced T cell responses.

Intracoronary application of an AAV vector expressing SERCA2a to modulate calcium metabolism reported transient increases in AAV capsid-specific T cell frequencies in 1 out of 9 patients without any clinical consequences or further changes in blood chemistry (100). Intravenous application of an AAV9 vector expressing the survival motor neuron 1 gene for treatment of spinal muscular atrophy reported increases in liver enzymes in some of their patients (101). As T cell responses were not analyzed, the etiology of this observation remains uncertain.

As one would expect, AAV vectors induce adaptive immune responses especially if given at high doses to peripheral sites. It remains unclear how common T cells to AAV capsid or the encoded transgene product interfere with sustained therapeutic benefits as many trials still fail to test for T cell responses. Injections of even large doses of AAV does not inevitably lead to detectable T cell responses with the obvious caveat that T cell assays have sensitivity limits and may not reveal small responses. Furthermore, the activity of tissue resident memory $CD8^+$ T cells may not be spotted by monitoring increases in circulating AAV capsid- or transgene product-specific T. cells. It will be important for gene therapist that base their therapeutics on AAV to further define factors that promote $CD8^+$ T cell responses. Vector dose and route of administration clearly play roles. CpG content within the vector genome may affect responses as was shown in mice (98). The capsid itself may have an effect. Age can have an effect; younger people tend to mount better immune responses, but older people are more likely to have immunological memory to AAVs. The underlying disease especially if it causes local inflammatory reaction can affect treatment outcome. The HLA type of the gene therapy recipient will affect if and how many epitopes of the capsid can be recognized by T cells. The type of the transgene and its similarity to endogenous proteins will determine if the host is tolerant or responsive.

After the initial AAV gene transfer trials for hemophilia B indicated that AAV-induced $CD8^+$ T cells may cause loss of cells producing F.IX (8), one of the next trials very carefully monitored serum F.IX and transaminase levels following AAV gene transfer. The two patients that received the highest vector dose showed by weeks 7 or 9 modest subclinical increases in aspartate aminotransferase and alanine aminotransferase and decreases in F.IX. They were treated for 9 or 4 weeks, respectively with prednisolone, which reduced transaminase levels and stopped further declines in F.IX (5). Steroids, such as prednisolone, are in general well tolerated if given for a short period of time. They are widely used in transplantation medicine, to treat auto-immune diseases or conditions caused by overwhelming inflammatory responses. They affect multiple aspects of T cell responses resulting in reduced cytokine production and decreased proliferation. Specifically, they dampen T cell activation by reducing phosphorylation of key signaling molecules of the T cell receptor (102). They induced production of anti-inflammatory cytokines and they promote proliferation of regulatory T cells (103). Although steroids are not suitable as a monotherapy to prevent transplant rejection they have been used successfully by Nathwani (5) and others (79, 104, 105) to prevent loss of AAV-transduced cells upon hepatic gene transfer. Nevertheless, in other trials steroids did not avert gradual loss of the transgene product (106–108). The optimal regimen for steroid therapy remains to be established (109). While most trials carefully monitored serum enzyme levels and started steroid treatment once transaminases increased others gave steroids to all patients immediately or shortly after treatment. The former approach is not only cumbersome as it requires weekly testing but also carries the risk that treatment could be initiated too late. The latter approach has the disadvantage that patients, who would not mount immune-mediated rejection are treated unnecessarily. It also remains unclear how long steroids should be given and if this time frame depends on the type of vector or differs for each patient. As steroids have not always prevented loss of transgene-expressing cells additional immunosuppressives need to be explored. In a trial with an AAV1 vector for correction of lipoprotein lipase deficiency, a 12 week course of methylprednisolone together with cyclosporin and mycophenolate mofetil was started shortly before AAV transfer; the drugs did not prevent increases in AAV capsid-specific T cells, but T cells appeared to be functionally impaired and failed to achieve removal of vector-transduced muscle cells (110). Cyclosporin inhibits T cell activation by blocking signaling through NFAT transcription factors, which are regulators of $CD8^+$ T cell functions (111) as well as the NF-κB and activator protein 1 (AP-1) pathways. Cyclosporin is also being explored in pre-clinical models for dampening of AAV capsid-specific neutralizing antibody responses (112). Nevertheless, cyclosporin depending of timing of antigen exposure *versus* drug treatment was shown in a cardiac allograft rat model to prevent activation of regulatory T cells (113), while in other systems cyclosporin augmented the activity of this immunosuppressive cell subset (112, 113). Mycophenolate mofetil inhibits inosine monophosphate dehydrogenase, the rate-limiting enzyme for synthesis of guanosine nucleotides which are essential for cell cycle progression of proliferating lymphocytes (114).

Other drugs that are being considered are TLR antagonists or drugs that target downstream molecules of PRR signaling to inhibit activation of the inflammatory responses that are essential to drive activation of naïve T cells (115). Such drugs could block numerous steps of PRR activation pathways such as TIRAP, MyD88, IKK-γ, NF-κB, or IRAK-4. Inflammatory responses could also be dampened by blocking TNF-α signaling through monoclonal antibodies such as Remicade, Humira, Cimzia, the receptor fusion protein Enbre or the small molecule inhibitor pentoxifylline (116). Drugs are also available to block type I IFN signaling by inhibiting IRF-3 or JAK1 which would affect T cell maturation (117). All of

these drugs would impair primary T cell responses but may not block recall responses. Induction of $CD4^+$ T cells could be inhibited by preventing antigen presentation through the MHC class II pathway through chloroquine, which prevents acidification of endosomes (118), cyclosporin A and tacrolimus, which both inhibit calcineurin (119) or blockers of the lysosomal protease cathepsin S, such as morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl (120). Some of the drugs such as cyclosporin A and tacrolimus also block MHC class I antigen presentation, which can also be inhibited by proteasome inhibitors such as lactacystin (121) or inhibitors of TAP (122). T cell activation is inhibited by some of the drugs that are already being used such as steroids, cyclosporin A and mycophenolate mofetil. Others could be tried. These include belatacept, a fusion protein of CTLA-4 and the Fc portion of IgG-1, which binds CD80 and CD86 and thereby blocks CD28 signaling (123). Dapirolizumab pegol is an antibody against CD40L, which blocks another co-stimulatory pathway. This drug is currently undergoing phase III trials for treatment of systemic lupus erythematosus (124, 125). Rapamycin was tested preclinically and was shown to block humoral and cellular immune responses to AAV and to increase induction of regulatory $CD4^+$ T cells (126–128). Rapamycin is an mTOR inhibitor that blocks cell cycle progression. When used during T cell activation it prevents generation of effector $CD8^+$ T cells but promotes memory formation (129).

Immunosuppression although potentially essential for some patients to ensure therapeutic benefits of AAV-mediate gene transfer comes at a cost. Immunosuppressive drugs have side effects that are unrelated to inhibition of immune responses. To name a few, prednisolone can cause intestinal ulcers, tacrolimus can result in headaches and muscle pain, belatacept has been linked to intestinal problems as has mycophenolate mofetil. Immunosuppression by its very nature robs an individual of its ability to fight off pathogens thus heightening the risks of more serious infections associated with more severe disease and increased shedding of the pathogen.

SUMMARY

The immune system evolved to respond to components of pathogens including those of viral vectors. This system, which is essential for survival of an organism, is built on redundancy to counter rapidly mutating pathogens that have come up with multiple ways to dodge immune-mediated destruction. Modifying AAV vectors by removing parts that induce inflammatory responses or provide epitopes for T cell recognition may at best blunt responses. Nevertheless, modern medicine has developed a multitude of drugs that prevent activation of the immune system to overcome rejection of organ transplants. This in turn provides blueprints for drugs that can effectively block destructive T cell responses. Organs have been transplanted successfully since 1954 while AAV was not discovered until the mid-1960s. It then took another 30 years before an AAV vector was tried in a human gene therapy trial. By 2008, AAV-mediated gene therapy reported clinical benefits for a congenital blindness and then by 2011 systemic AAV transfer to the liver showed clinical benefits for hemophilia B patients. AAV-mediated gene transfer has thus progressed from its infancy to a stage of adolescence where hopefully remaining problems such as immune-mediated rejection can be solved in the near future.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

Wistar Institute.

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Conflict of Interest: The author has in the past been the recipient of a Sponsored Research Agreement by Spark Therapeutics.

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 27 April 2021
doi: 10.3389/fimmu.2021.674021

Overcoming Immunological Challenges Limiting Capsid-Mediated Gene Therapy With Machine Learning

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A key hurdle to making adeno-associated virus (AAV) capsid mediated gene therapy broadly beneficial to all patients is overcoming pre-existing and therapy-induced immune responses to these vectors. Recent advances in high-throughput DNA synthesis, multiplexing and sequencing technologies have accelerated engineering of improved capsid properties such as production yield, packaging efficiency, biodistribution and transduction efficiency. Here we outline how machine learning, advances in viral immunology, and high-throughput measurements can enable engineering of a new generation of de-immunized capsids beyond the antigenic landscape of natural AAVs, towards expanding the therapeutic reach of gene therapy.

Keywords: gene therapy, protein engineering, immune evasion, machine learning, AAV capsid design

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *28 February 2021*
Accepted: *09 April 2021*
Published: *27 April 2021*

Citation:
Wec AZ, Lin KS, Kwasnieski JC, Sinai S, Gerold J and Kelsic ED (2021) Overcoming Immunological Challenges Limiting Capsid-Mediated Gene Therapy With Machine Learning. Front. Immunol. 12:674021. doi: 10.3389/fimmu.2021.674021

INTRODUCTION

Recently approved AAV-based therapeutics and numerous therapeutic candidates in advanced clinical development (1) have demonstrated the transformative and life-saving potential of viral capsids as vectors for gene therapy (GT). The demands on viral capsids to deliver gene replacement and gene editing tools will continue to increase as our understanding of genetic diseases reveals new therapeutic opportunities. Development of next generation capsids that enable more precise, efficient, and durable gene delivery will be key to improving the effectiveness and safety of such therapies. In this perspective, we explore how high throughput (HT) measurement and characterization methods can be combined with machine learning (ML) approaches to identify such capsids by efficiently optimizing capsid sequences for both improved transduction and reduced immunogenicity. Combining these technologies will generate capsid-mediated gene therapies with broader therapeutic uses that are accessible to all individuals in need.

THE NEED TO OPTIMIZE NATURAL AAV CAPSIDS FOR THERAPEUTIC DELIVERY

Most recombinant AAV capsids used clinically today are closely related, or even identical, to naturally occurring AAVs in their amino acid sequences and biological properties. As natural selection did not optimize such capsids for therapeutic use, they display limited specificity of cell

targeting and low overall *in vivo* transduction efficiency in many target tissues, particularly following intravenous administration. Improving *in vivo* transduction of target cells and organs would enable gene therapies to more effectively treat diseases, to perdure, and to address new therapeutic applications. Importantly, pre-existing humoral and cellular immunity against natural AAV capsids limits patient eligibility for therapies as well as their therapeutic efficacy (2). Furthermore, capsids possess inherent immunogenicity — the propensity to activate immune responses — which can impact safety and efficacy, as well as the potential for redose. The challenges of evading both pre-existing immunity and *de novo* adaptive immune responses against AAV vectors are made especially difficult by the heterogeneous nature of patient immune responses and immune histories. Thus, discovering capsids that circumvent the immune system is a significant hurdle facing developers of next generation GT vectors (2).

Established approaches for obtaining novel capsids include mining the naturally-occurring sequence diversity of capsids, rational design and directed evolution (3–5). Each methodology has contributed valuable capsids to the available catalog of GT vectors, but limitations related to speed and throughput of discovery persist because the total number of possible capsids far exceeds the capacity of current screening approaches. Directed evolution methods often take advantage of ultra-high diversity generated by random mutagenesis in an attempt to overcome the barrier of low discovery yield (i.e. success per individual design). In contrast, rational design approaches rely on expert knowledge and focus on a higher likelihood of success per design, but are relatively low throughput (and overall low yield) as a result. ML approaches offer a promising new option that may mitigate the trade-off between yield and throughput (**Figure 1A**). ML can be used in combination with these established approaches, or as a stand-alone technique to open new avenues of discovery through high-throughput direct synthesis (6).

The set of desired properties that a capsid should possess in order to be therapeutically transformative can collectively be termed a *capsid profile*, in other words the target of optimization efforts. Capsids that embody every therapeutically desirable property

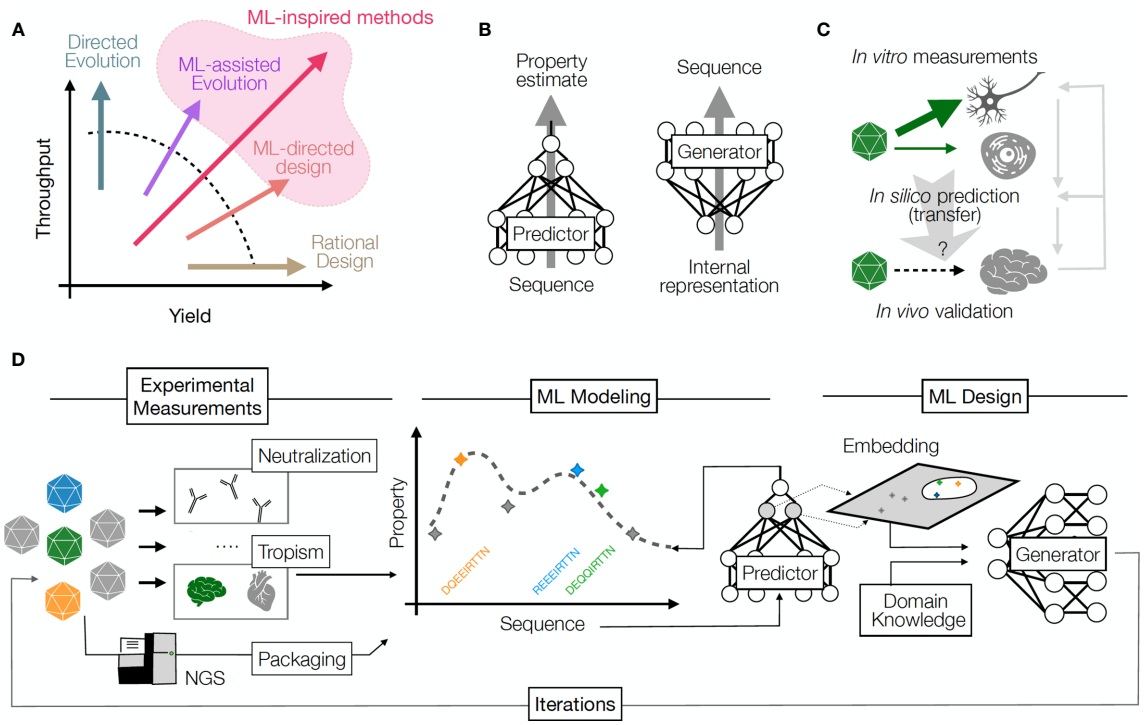

FIGURE 1 | **(A)** A comparison of throughput (number of samples) and yield (fraction of successful samples generated per attempt) for multiple protein design approaches. Rational design increases yield, directed evolution leverages throughput, and ML methods increase the likelihood of success by balancing yield and throughput. **(B)** Predictive ML models map sequences to their functional properties, while Generative methods can turn an internal data representation back into sequences, producing desirable samples. **(C)** An example of transfer learning whereby a model *transfers* information across cell types and experimental contexts: a model learns based on *in vitro* capsid performance in diverse cell transduction experiments (including neurons), then is applied to predict the result of *in vivo* transduction in the brain neurons, when such experimental data is sparse or missing. Information from *in vivo* validation of the predicted capsid performance is used to refine model performance and understand the relationship between *in vivo* and *in vitro* assays. Right grey arrows illustrate the iterative power of this approach, which refines predictive and generative models over time. **(D)** The design cycle starts with HT screening and measurements of several AAV capsid variant properties. These properties are then used to train predictive models that can impute the property for unseen sequences (predictor model) and can be used to build helpful representations (embeddings), which can then be integrated with auxiliary input (e.g., domain knowledge) to propose a batch of new sequences (generator model). The design process can be repeated in multiple iterations until desired capsids are discovered.

outlined above have eluded discovery despite years of effort. Despite the vast number of possible capsid sequences, it is reasonable to assume capsids which achieve these desired profiles, if they exist, are extremely rare in sequence space (7, 8). Reducing the number of required properties in the context of a particular therapeutic application may increase the chance of finding a candidate capsid, but this may come at the cost of failure in later stages of clinical development. The therapeutic usefulness of a given capsid and our ability to find it are therefore fundamentally in tension. In this perspective, we share how new approaches to immunological data gathering, combined with analysis and design approaches powered by ML, are overcoming this tension towards discovery of capsids that are more therapeutically useful.

KEY CONCEPTS FOR APPLYING MACHINE LEARNING TO ENGINEER NOVEL CAPSIDS

Recent advances in ML enable new solutions to problems inherent to designing immune-evasive capsids. ML is a collection of algorithmic approaches that allow for automatic learning. These approaches are capable of learning rules for predicting the outcome of complex processes directly from input data. Larger and richer datasets pose a challenge for traditional methods of rational design but are the environment in which ML methods thrive (9). ML models can be considered mathematical approximators of physical processes we have measured, and oftentimes have yet to understand mechanistically (10–12). In the context of biological design, ML models can replace labor- or resource-intensive experiments with *in silico* screening. With increasing amounts of data, these approximations can become very accurate, and their rapid and cost-effective application enables the identification of biological designs which would not be accessible by experimentation alone. Importantly, mechanistic knowledge need not be wasted in this approach — biological insights can be incorporated into ML architectures in a way that bolsters model robustness, allowing for more accurate models trained by less data. Additionally, ML can simplify how we represent and understand high-dimensional and high-throughput data, allowing us to substantially improve the experiments themselves. Finally, while many mechanistic details of AAV gene therapy remain poorly understood, ML models trained on empirical data that can predict capsid functions are sufficiently useful for engineering better capsids despite the models being agnostic to mechanism, and in some cases querying such models can guide or improve our mechanistic understanding.

Key ML concepts illustrate the potential for this approach to transform capsid engineering. First, ML algorithms can learn arbitrary *sequence-to-function* relationships. These relationships can be learned automatically from large datasets of capsid sequences and their measured properties. A model can predict one or multiple properties at once. For instance, models can be trained to learn the relationship between the capsid sequence and its ability to produce a viable capsid (6) or its tropism to the liver (13). These training schemes, termed *supervised*, require collecting data labels (measurements) of the kind we are intending to predict. However, it is also possible to train models solely based on a set of good examples without additional measurements. For instance, training models on the rapidly growing set of publicly available protein sequences to learn relationships among them has shown promise in protein structure and function prediction (12, 14–17). This type of training is known as *unsupervised*. Both supervised and unsupervised training schemes can yield *predictive models* that output property values given an input sequence, or alternatively *generative models* that produce novel sequences given desirable property values as inputs (**Figure 1B**). It is noteworthy that building models with good generalization ability, i.e. ability to predict accurately on samples far from those in the training data, requires care in experimental design and training schemes. Otherwise, models may *overfit* to the training data available, where they perform well on samples similar to their training data, but unexpectedly poorly in novel settings.

Second, effective machine learning methods often make use of internal *latent representations*, also known as *embeddings*, which attempt to represent the information contained in raw inputs in a way that is more amenable to human understanding. One such simple and widely applied method is principal component analysis (PCA), in which a linear transformation of input data allows for the identification of data elements that contribute most to the variance in the data set. PCA and other more complex non-linear dimensionality reduction methods transform high-dimensional raw input data to a lower-dimensional representation (a latent space) that is easier to interpret, visualize, and optimize (14, 18–21). If these and other methods can be applied to the problem of AAV capsid engineering, AAV variant sequences with similar properties to each other would be close together in latent space after being transformed into their latent representations, even if they are far apart in sequence space. A similar strategy was recently used to predict the emergence of escape mutations in multiple viruses (22).

Finally, modern ML can utilize auxiliary data to make inference about domains where information is sparse, a process known as *transfer learning* (**Figure 1C**) (23, 24). An illustrative conceptual example for this technique in machine vision involves "style-transfer" where particular painting styles are learned from an artist's work, and can then be applied to any new image, converting the style to that of the original artist (25). This type of learning can be used in many contexts in biology (23, 26). For instance, predictive models around AAV serotypes for which little data is available could be improved by training them on data available from other related serotypes or even a larger set of related proteins. Similarly, population level data for immunity profiles of specific patient groups could be used to reduce the amount of data required to make inferences for individual patients. Along with the ability to integrate information from multiple modalities, transfer learning can rapidly accelerate the application of ML models in areas where data is limited, and open new domains for prediction and design.

An example of a ML-driven design pipeline is illustrated in **Figure 1D**. These concepts will be useful for designing immune-evasive capsids, as we explain below.

SAFE AND EFFECTIVE TREATMENT AT LOWER DOSES

Among all capsid properties that could be improved, increased tissue-specific transduction is key to enabling safe and effective gene therapies. Improving this attribute would allow for a higher proportion of injected capsids to deliver their payloads to the intended cells, reducing the dose needed for effective treatment. This in turn would make treatment safer by reducing activation of the innate immune responses and of B and T cell responses, which increase in magnitude relative to the amount of antigenic stimulus (vector dose) delivered (27).

Making viral vectors safer and more effective will require optimization towards multi-property capsid profiles. However, many capsid properties are intrinsically coupled to one another and efforts to optimize or re-direct any single attribute often result in capsids that fail basic tests of functionality, such as capsid assembly and genome packaging. ML models can greatly reduce the burden of multi-property optimization through *in silico* screening of variants (28), ensuring that optimization toward one property does not break other desired functions (29, 30), shifting the engineering burden away from experimental approaches (28). For instance, four supervised models can be trained to learn sequence-to-function maps between capsid sequences and their ability to (i) transduce the liver, (ii) bypass off-target organs, (iii) evade neutralization, and (iv) produce at high yield. The first model can be used in an *in silico* search for variants with better transduction, and the other models can be used to eliminate sequences proposed by the first model that do not meet the specificity, immune evasion and capsid production requirements. A significant body of work in the interface of ML and biology is focused on algorithms that use such supervised models to optimally design protein sequences (31). Notably, while non-human primates are at present the industry-preferred model for measuring transduction, the ability for ML to integrate diverse sources of information may increase the utility of data from other animal models (including transgenic animals with humanized immune systems), as well as human cell culture models, for predicting transduction patterns in human patients and lead to better rates of clinical translation. Capsids optimized towards a profile of improved and specific transduction, reduced immunogenicity, and production efficiencies equivalent to natural AAV capsids would already be transformative relative to currently available vectors.

PERDURING GENE THERAPY

In an ideal therapeutic scenario, a single dose of GT would provide a durable, curative effect throughout a recipient's lifetime. In practice, this goal has been difficult to realize as therapeutic transgene expression from current vectors decays over time (32). Waning transgene expression can result from silencing of the viral genome through epigenetic mechanisms, from cell division, or from transduced cell death, among other factors. One mechanism underlying the loss of transduced cells observed in a number of clinical studies (33–35) was the induction of cytotoxic $CD8^+$ T lymphocyte (CTL) responses against cells presenting capsid antigens, for which immunosuppression is the primary clinically viable remedy.

Engineering capsids that reduce or even eliminate CTL responses will facilitate perduring therapeutic gene expression. Transduced cells process viral capsids through the intracellular proteolytic machinery and present capsid-derived peptides on their surface though the major histocompatibility (MHC) class I molecules (33, 34). $CD8^+$ T cells recognize presented peptides *via* their highly specific T cell receptors, which in turn determines cell stimulation, proliferation and cytotoxic activity. CTL activation results in killing of transduced cells as well as generation of immunologic memory that poses a barrier for vector redosing. Unlike B cells, which interact with surface exposed capsid epitopes, T cells can in theory sample the full peptidome of an AAV capsid, including buried capsid sequences that drive assembly or disassembly, and which may be more difficult to alter by conventional engineering approaches. Extensive mapping of $CD8^+$ T cell epitopes within AAV capsid proteins and evaluation of their propensity to activate T cell responses would identify the key sequences which must be modified to de-immunize AAV capsids. The large diversity of HLA alleles among people and distinct patterns of peptide presentation and recognition determined by them makes this challenging. While it is currently not possible to exhaustively assess peptide presentation by all variants of MHC class I found in humans, emerging ML methods in peptide presentation and immunogenicity prediction (36, 37) will increase the accuracy of these predictions compared to tools available today. Recently developed strategies of experimental immunopeptidome characterization using mass spectrometry (38, 39) will provide a rich source of data for training such models.

Understanding the determinants of capsid antigen presentation (40) and their effect on CTL activation will provide the foundations for ML models to engineer capsids that evade them. The rules of peptide presentation are shared across the entire proteome based upon an individual patient's HLA alleles (41). This means that ML models can benefit from all existing datasets that catalog $CD8^+$ T cell epitopes and learn general properties that influence which peptides tend to be presented in particular genetic backgrounds (17). Through transfer learning, such general models could be tuned toward more accurate models that predict $CD8^+$ T cell epitopes for AAV capsid variants specifically. This would require relatively small amounts of additional data that is specific to AAV capsids and would enable engineering of capsids depleted of T cell-activating peptides. While predictions of MHC class I presentation have advanced significantly, meaningful annotation of peptide immunogenicity that enables more accurate models for immunogenicity prediction will require development of HT

functional assays and remains an open challenge for the field of T cell biology.

GENE THERAPY FOR ALL: OVERCOMING PRE-EXISTING ANTI-CAPSID ANTIBODIES

A majority of prospective GT recipients have pre-existing antibodies against one or more natural AAV serotypes, often excluding them from treatment (42–44). Pre-existing antibodies accelerate vector clearance, redirect vector biodistribution, and can directly inhibit capsid-mediated cell entry (33). To overcome these activities of antibodies, it is critical to identify capsids that cannot be efficiently bound and neutralized by them – in other words, capsids with surface-exposed sequence and structural features not previously encountered by the adaptive immune response. Altering antibody recognition of capsids in a therapeutically meaningful way is challenging because serum antibody responses are highly diverse and can target the entire capsid surface (45, 46). Antibodies bind both linear and discontinuous epitopes on the capsid exterior surface, sometimes spanning across neighboring capsid subunits, making rational approaches to altering these sites challenging. Moreover, neutralizing antibodies often target capsid regions involved in critical functions such as cell receptor recognition, meaning that mutations which prevent antibody binding can also adversely affect vector transduction (47).

Much remains to be learned about how human antibodies bind to and neutralize capsids, however several technologies now enable high-throughput mapping of antibody responses at the monoclonal level. The study of both serum antibodies and antibodies encoded by memory B cells in donors with recent AAV exposures can reveal key characteristics of human anti-capsid antibody responses and provide a more complete picture of anti-capsid antibody immunity. While serum antibodies are maintained at steady state by long lived plasma cells, the memory B cell repertoire approximates the antibody repertoire that will be mobilized on AAV re-encounter and their characterization is methodologically useful as a means of identifying anti-capsid antibody sequences for in depth functional studies. For example, efforts in the infectious diseases therapeutic space have yielded multiple approaches to fine mapping of *de novo* and memory B cell responses, where hundreds or even thousands of virus-specific antibodies encoded by B cells can now be routinely sequenced, cloned and produced (48). Epitopes of such antibodies can be characterized using HT competition assays (49, 50) and correlations can be derived between binding site location and neutralization activity. Recently developed approaches utilizing cryo-electron microscopy (51, 52) and high resolution, quantitative, proteomics-based approaches (53–55) enable serum antibody specificities to be characterized in unprecedented detail, to inform their identities and their binding sites. These and other studies revealed for a number of pathogens that just one class of antibodies can contribute the majority of neutralizing activity in the serum despite the overall high diversity of antibody responses (56–58). Identifying any dominant human neutralizing antibody types against AAVs would inform the sites where capsid engineering can be most effectively applied.

Data with resolution at the individual antibody level would enable ML models to learn how antibody responses target a particular capsid and how to predict their effect on other (designed) capsids. Models can serve as *in silico* evaluators of capsids before they are administered to patients with pre-existing antibodies based on characterization using the methods described above. Through sequencing of capsid-specific B cells and characterization of serum antibodies, a personal 'immunological fingerprint' can be created with the aid of ML models, which could also be used to find general patterns in human anti-capsid antibody responses (59). For instance, unsupervised models can directly learn from genetic data to predict immune profile responses. Supervised models could use patient serum data together with other measurements [e.g. sequencing of immune repertoires (59) or genome scanning antibody profiling (60)] to predict likelihood of therapeutic success, or to help select vector administration options. With such models in hand, panels of antibody-evading AAV capsids could be recommended based on a patients' pre-existing antibody repertoire to maximize the chance of effective antibody evasion.

Many gaps remain in our understanding of how anti-capsid antibodies can be evaded. Serology studies with naturally occurring AAVs have been useful in defining population-level prevalence of anti-AAV immunity but such bulk-level measurements have had limited value for engineering antibody-evading capsids. Some monoclonal antibodies isolated from mice have been characterized in detail (46, 61) providing important insights about the antigenic sites on AAV capsids targeted by neutralizing antibodies. However, it remains a challenge to generalize these results to human antibody responses, which are encoded by distinct germline genes, are more diverse (62), and are shaped in response to a distinct set of natural AAVs endemic in humans. An in-depth large-scale characterization of human antibodies targeting capsids would facilitate our ability to engineer capsids with maximal therapeutic impact.

One such promising approach would be to measure the activity of serum antibodies against highly diverse libraries of capsid variants using immune human serum samples. Such data would enable ML models to learn the quantitative relationship between AAV capsid sequences and their abilities to evade pre-existing antibodies, and to learn commonalities in anti-capsid antibody responses among people. Similarly, intravenous immunoglobulin (IVIg) preparations containing antibodies from thousands of donors may be useful in such screens for identifying the predominant patterns in human antibody responses. Recent work characterizing B cell and antibody responses to a number of important human pathogens (56, 63–65) reveal common features of antibody responses elicited by a given pathogen across donors. If similar shared antibody types arise against AAV capsids, resurfacing the epitopes they target would allow engineering of capsids that more broadly

evade antibody activity, towards the goal of creating *universal capsids* capable of treating all patients.

FUTURE DIRECTIONS

ML-powered capsid design and engineering will transform the landscape of GT delivery modalities, however non-capsid improvements are also relevant from an immunological perspective and can also increase therapeutic effectiveness. Reducing the activation of innate immunity by engineering the vector genome (66, 67), co-administration with targeted immune-modulators to induce tolerance toward the vector (68) or depletion of pre-existing anti-capsid antibodies (69) should work in synergy with engineered capsids to pave a path for repeat vector administration, while further increasing the safety and tolerability of next generation GTs.

As we have outlined, ML approaches to engineer improved AAV capsids have multiple applications: enabling gene therapies that are effective in a lower dose regimen, removing capsid peptides which elicit cytotoxic T cell responses thereby leading to longer lasting gene expression, and resurfacing capsid exteriors allowing potentially universal treatment of all patients. While these goals are ambitious and each individually worthy of study, combining all such properties in a single capsid would be transformative for the field. ML approaches will facilitate this goal by incorporating information from diverse experimental systems and improving the efficiency of multi-trait capsid optimization. We are optimistic that safe, efficient, target-specific, non-immunogenic and universal capsids will one day enable gene therapy to reach its full potential by delivering therapeutic DNA to cure, treat and prevent disease and even to improve overall health for all patients. Interdisciplinary collaborations focused on combining HT measurements with ML-powered sequence design algorithms will dramatically accelerate progress towards achieving these goals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AW, KL, JK, SS, JG and EK conceptualized, wrote and edited the manuscript. AW and SS prepared figures. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank George Church, Jakub Otwinowski, Sam Wolock, Alexander Brown, Sylvain Lapan, Adrian Veres and Tomas Björklund for their helpful discussions and comments on the manuscript.

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Conflict of Interest: AW, KL, JK, SS, JG and EK are employees and shareholders in Dyno Therapeutics Inc.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 April 2021
doi: 10.3389/fimmu.2021.674242

Novel Combinatorial MicroRNA-Binding Sites in AAV Vectors Synergistically Diminish Antigen Presentation and Transgene Immunity for Efficient and Stable Transduction

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Recombinant adeno-associated virus (rAAV) platforms hold promise for *in vivo* gene therapy but are undermined by the undesirable transduction of antigen presenting cells (APCs), which in turn can trigger host immunity towards rAAV-expressed transgene products. In light of recent adverse events in patients receiving high systemic AAV vector doses that were speculated to be related to host immune responses, development of strategies to mute innate and adaptive immunity is imperative. The use of miRNA binding sites (miR-BSs) to confer endogenous miRNA-mediated regulation to detarget transgene expression from APCs has shown promise for reducing transgene immunity. Studies have shown that designing miR-142BSs into rAAV1 vectors were able to repress costimulatory signals in dendritic cells (DCs), blunt the cytotoxic T cell response, and attenuate clearance of transduced muscle cells in mice to allow sustained transgene expression in myofibers with negligible anti-transgene IgG production. In this study, we screened individual and combinatorial miR-BS designs against 26 miRNAs that are abundantly expressed in APCs, but not in skeletal muscle. The highly immunogenic ovalbumin (OVA) transgene was used as a proxy for foreign antigens. *In vitro* screening in myoblasts, mouse DCs, and macrophages revealed that the combination of miR-142BS and miR-652-5pBS strongly mutes transgene expression in APCs but maintains high myoblast and myocyte expression. Importantly, rAAV1 vectors carrying this novel miR-142/652-5pBS cassette achieve higher transgene levels following intramuscular injections in mice than previous detargeting designs. The cassette strongly inhibits cytotoxic CTL activation and

OPEN ACCESS

Edited by:
Ingo Drexler, Heinrich Heine University, Germany

Reviewed by:
Chengwen Li, University of North Carolina at Chapel Hill, United States
Paulo Bettencourt, Independent Researcher, Lisbon, Portugal

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *01 March 2021*
Accepted: *07 April 2021*
Published: *28 April 2021*

Citation:
Muhuri M, Zhan W, Maeda Y, Li J, Lotun A, Chen J, Sylvia K, Dasgupta I, Arjomandnejad M, Nixon T, Keeler AM, Manokaran S, He R, Su Q, Tai PWL and Gao G (2021) Novel Combinatorial MicroRNA-Binding Sites in AAV Vectors Synergistically Diminish Antigen Presentation and Transgene Immunity for Efficient and Stable Transduction. Front. Immunol. 12:674242. doi: 10.3389/fimmu.2021.674242

suppresses the Th17 response *in vivo*. Our approach, thus, advances the efficiency of miRNA-mediated detargeting to achieve synergistic reduction of transgene-specific immune responses and the development of safe and efficient delivery vehicles for gene therapy.

Keywords: adeno-associated virus vectors, microRNA, miR-BS, miR-142, miR-652-5p, miR-223-3p, antigen presenting cells, gene therapy

INTRODUCTION

Adeno associated virus (AAV) vector-mediated gene therapies have emerged as the platforms of choice for the treatment of monogenic diseases. First isolated from adenovirus preparations in the 1960s (1, 2), AAV is a non-pathogenic dependoparvovirus that is able to transduce a wide range of cell types. Recombinant AAVs (rAAVs) have been proven to confer long-lasting and safe transgene expression in a variety of human tissues (3–6). rAAVs have also achieved sustained therapeutic effect for a variety of inherited diseases, including Leber's congenital amaurosis type 2 (7, 8), hemophilia B (9), M-type a-1 antitrypsin deficiency (10, 11), and lipoprotein lipase deficiency (12, 13). Two AAV-based drugs (Luxturna and Zolgensma) to date have been approved by the FDA, and >100 clinical trials using AAV-based therapies are in progress (5, 6, 14, 15).

AAV vectors are known to possess a weak immunological footprint, in part, because of their relative inability to transduce antigen presenting cells (APCs). However, there have been multiple reports of vector-related toxicities that have compromised transgene product expression (3, 16, 17). Human clinical trials have also demonstrated how B and T cell immune responses directed against the AAV capsid, likely arising after natural infection with wild-type AAV, might potentially impact gene transfer safety and efficacy in patients (3). Moreover, AAV-delivered transgene products are often presented as foreign antigens that can stimulate host immune responses, and can lead to the generation of transgene-specific antibodies and cytotoxic T lymphocytes (CTL) (18, 19). The quality and intensity of humoral and cellular immune responses can vary depending on the transgene DNA composition, vector tropism to APCs, route of administration, and the tissue target.

Skeletal muscle is considered an important target tissue for AAV-mediated vector gene transfer. Intramuscular administration of rAAV-encoding therapeutic transgenes enables muscle to serve as a bio-factory for the sustained production of secreted proteins (20–25). Notably, AAV serotype 1 (AAV1) is known for its tropism to skeletal muscle cells and also has a limited system-wide biodistribution profile. Intramuscular administration of rAAV1 in humans has been used previously for the exogenous expression of therapeutic proteins (26–28). However, targeting muscle tissues can result in immune reactions, and anti-transgene responses have been mostly documented in gene therapy trials involving intramuscular delivery of rAAV vectors (28, 29). One of the primary reasons attributed to anti-transgene toxicity is the unwanted transduction of APCs. rAAV transduction of professional APCs, like dendritic cells (DCs), macrophages, and B cells leads to the presentation of transgenic peptides on MHC class I molecules, culminating in cytotoxic T cell-mediated clearance of transfected cells. Importantly, rAAV1 has also been shown to transduce APCs to elicit transgene immunity (30). The use of muscle-specific promoters in rAAV expression cassettes have shown limited success in controlling leaky expression in DCs (31, 32). Alternatively, microRNA (miRNA)-mediated detargeting *via* posttranscriptional control has been successfully demonstrated to restrict cell type-specific transgene expression with lentiviral gene transfer to the mouse liver (33, 34).

Detargeting transgenes from specific cell types *via* endogenously expressed miRNAs can also be used to enhance tissue-specificity by excluding spurious transgene expression from non-target cells (35–39). miR-142 is regarded as a hematopoietic-specific miRNA and is expressed at high levels in APCs (33, 40). In the absence of miR-142, DCs show reduced production of proinflammatory cytokines and the ability to activate T cells in mice (41). We have previously shown that miR-142-mediated APC detargeting boosts transgene levels and inhibits antibody formation and blunts the cytotoxic T cell response (42). Incorporation of two or three miR-142 binding sites achieved detargeting from APCs to levels that enable sufficient stable transgene expression (30, 42) following intramuscular injections. However, CD8+ T cell infiltrates were still observed at early treatment timepoints (two weeks post-injection), suggesting that full APC detargeting and maximal transgene expression may not have been achieved with miR-142BS cassettes alone.

In this study, we have identified two miRNAs, miR-223-3p and miR-652-5p, whose expression is enriched in immune cell populations in mice. miR-652 and miR-223 are expressed in cells of the myeloid lineage, including monocytes and granulocytes (43). Incorporation of binding sites for miR-223-3p and miR-652-5p, in combination with miR-142, can effectively detarget expression of the chicken ovalbumin (OVA) transgene from APCs following intramuscular administration. The novel combinatorial microRNA-binding site (miR-BS) designs effectively improve transgene expression, blunt antibody response against the transgene, and reduce the activation of T cells. Furthermore, the miR-142/652-5pBS cassette confers the lowest capacity for triggering OVA-specific cytotoxic CTL activation and inhibits the activation of Th1 and Th17 cells more effectively than miR-142BS on its own. This unique miR-BS design therefore confers a global immunosuppressive milieu that is specific to the transgene. Our findings not only reiterate the therapeutic potential of miRNA-mediated detargeting

cassettes, but also demonstrate that a combination of different miR-BSs might have an additive or synergistic effect on inhibition of transgene immunity.

MATERIALS AND METHODS

Vector Plasmid Construction and rAAV Production

rAAV expression cassette was made by inserting full-length OVA cDNA between the chicken β-actin (CB) promoter and rabbit β-globin (RBG) polyA signal to generate the pAAV.*CB.OVA cis* plasmid (42). For pAAV.*CB.OVA*.miR-BS constructs, two copies of the miR-BS sequence, individually or in combination with miR-142BS, were inserted between the *OVA* cDNA and *RBG* polyA signal. The sequences of the miR-BS are listed in **Table 1**. All expression cassettes were verified by Sanger sequencing. rAAV1 vectors were produced by the Viral Vector Core at the University of Massachusetts Medical School as previously described (42, 65, 66).

In Vitro Screening of OVA Constructs

OVA expression plasmids with or without the miR-142BS elements were transfected into mouse myoblast C2C12 cells (ATCC, CRL-1772) and the macrophage cell line RAW264.7 (ATCC, TIB-71) using jetPRIME transfection reagents (Polyplus-transfection SA) according to the manufacturer's instructions. C2C12 and RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (Hyclone, SH30022) with 20% and 10% fetal bovine serum, respectively (FBS, Hyclone, SH30071), and 1% penicillin/streptomycin (Hyclone, SV30010). C2C12 cells were differentiated by culturing the cells in DMEM containing 2% horse serum (HyClone) and 1 μM insulin (Sigma-Aldrich). Mouse dendritic cells (JAWS II; ATCC, CRL-11904) were cultured in α minimum essential medium (MilliporeSigma, M8042) with ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate, and 20% FBS with 5 ng/mL murine GM-CSF. JAWS II were transfected by Nucleofection. Briefly, 2.0×10^6 cells were collected and resuspended in 100 μL Nucleofector Solution (Lonza, V4XP-4024) at room temperature. Plasmids were then added, mixed, and transferred into Nucleocuvette Vessels. The P4 HF program for immature mouse DCs was selected and ran. Then, 2 mL of medium was added, and cells were split into a 24-well plate (500 μL/well) (Corning, CLS3527). Three days after transfection, supernatants were collected for OVA ELISA. A Gaussia luciferase expression plasmid was transfected along with OVA expression plasmids to account for transfection variabilities. Transfections were done in triplicate for each round.

Mice

C57BL/6 mice were purchased from The Jackson Laboratory and maintained at the University of Massachusetts Medical School. Mice were housed under specific pathogen-free conditions. Six- to eight-week-old male mice were injected unilaterally into tibialis anterior (TA) muscles with 1.0×10^{11} genome copies (GCs) of rAAV1 diluted in sterile phosphate-buffered saline (PBS). Blood samples were collected *via* facial vein by using an animal lancet (Goldenrod) and BD Microtainer tubes with serum separator additive (Becton Dickinson and Company). All animal

TABLE 1 | List of candidate miRNA binding sites shortlisted for *in vitro* screening.

miRNA	Cell types enriched with miRNAs	Sequences of binding sites	References
miR-106	Monocyte	$(CTACCTGCACTGTTAGCACTTTG)_2$	(44, 45)
miR-126a	pDC	$(CGCATTATTACTCACGGTACGA)_2$	(46, 47)
miR-142	DC	$(TCCATAAAGTAGGAAACACTACA)_2$	(40, 41, 48)
miR-16	B	$(CGCCAATATTTACGTGCTGCTA)_2$	(49, 50)
miR-17	B, T, Monocyte	$(CTACCTGCACTGTAAGCACTTTG)_2$	(45)
miR-18	B, T, Monocyte	$(CTATCTGCACTAGATGCACCTTA)_2$	(44, 51)
miR-19a	B, T, Monocyte	$(TCAGTTTTGCATAGATTTGCACA)_2$	(44, 51)
miR-19b	B, T, Monocyte	$(TCAGTTTTGCATGGATTTGCACA)_2$	(44, 51)
miR-20	B, T, Monocyte	$(CTACCTGCACTATAAGCACTTTA)_2$	(44, 52)
miR-21a	B, Monocyte, MF	$(TCAACATCAGTCTGATAAGCTA)_2$	(53)
miR-223	Myeloid	$(TGGGGTATTTGACAAACTGACA)_2$	(43, 54)
miR-24-3p	DC	$(CTGTTCCTGCTGAACTGAGCCA)_2$	(48)
miR-29a	T	$(TAACCGATTTCAGATGGTGCTA)_2$	(55, 56)
miR-29b	T	$(AACACTGATTTCAAATGGTGCTA)_2$	(55)
miR-29c	T	$(TAACCGATTTCAAATGGTGCTA)_2$	(55)
miR-302a-3p	MF	$(TCACCAAAACATGGAAGCACTTA)_2$	(57, 58)
miR-30b	DC	$(AGCTGAGTGTAGGATGTTTACA)_2$	(48)
miR-33-5p	MF	$(TGCAATGCAACTACAATGCAC)_2$	(59)
miR-34a	B, DC	$(ACAACCAGCTAAGACACTGCCA)_2$	(53)
miR-424	Monocyte	$(TTCAAAACATGAATTGCTGCTG)_2$	(60, 61)
miR-652-3p	DC	$(AATGGCGCCACTAGGGTTGTG)_2$	(43)
miR-652-5p	DC	$(GAATGGCACCCCCTCCTAGGGTTG)_2$	(43)
miR-9-3p	MF	$(ACTTTCGGTTATCTAGCTTTAT)_2$	(62, 63)
miR-9-5p	MF	$(TCATACAGCTAGATAACCAAAGA)_2$	(61, 62)
miR-92a	B, T, Monocyte	$(CAGGCCGGGACAAGTGCAATA)_2$	(45)
miR-99b-5p	MF, DC	$(CGCAAGGTCGGTTCTACGGGTG)_2$	(52, 64)

procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Experiments were conducted in accordance with relevant guidelines and regulations.

ELISAs

Serum levels of OVA and anti-OVA IgG were determined by ELISA. Briefly, 96-well Nunc Maxisorp Immunoplates (Thermo Fisher Scientific) were coated with 2 μg/mL of rabbit anti-OVA polyclonal antibodies (AB1225, MilliporeSigma) or OVA protein (MilliporeSigma) in 100 μL coating buffer (KPL) per well. After an overnight incubation at 4°C, plates were washed with 0.05% Tween-20 in PBS, followed by incubation with blocking buffer (KPL) for two hours at room temperature. For OVA detection, the samples were diluted 100-fold with ELISA diluent (KPL), and OVA protein standards (Bioworld) were two-fold serially diluted with 1% normal mouse serum starting from 50 ng/mL. Then, 100 μL of sample or standard was added to plates and incubated for one hour at room temperature. After washing four times, peroxidase-conjugated rabbit anti-OVA polyclonal antibody (200-4333-0100, Rockland Immunochemicals) (1:5,000 diluted) was added and incubated for one hour at room temperature. For anti-OVA IgG1 detection, samples were diluted 1:200, and the mouse anti-OVA IgG1 (sc-80589, Santa Cruz Biotechnology) was used as the standard. After a one-hour incubation in OVA-coated plates, wells were washed, HRP-conjugated goat anti-mouse IgG1 (sc-2060, Santa Cruz Biotechnology) was added, and plates were incubated for another hour at room temperature. Plates were then washed four times and incubated with 100 μL of ABTS HRP Substrate (KPL). Optical density at 410 nm was measured using a Synergy HT microplate reader (BioTek). Standard curves for OVA and IgG1 were generated by using the 4-parameter logistic regression with Gen5 software (BioTek).

ELISA quantification of secreted cytokine levels was performed using customized ProcartaPlex Immunoassays (Thermo Fisher Scientific) following manufacturer's instructions. Briefly, the samples were incubated in a 96-well plate (Corning) with magnetic beads conjugated to antibodies against desired cytokines for two hours at room temperature with shaking. Wells were then washed thrice with wash buffer, using a magnetic plate washer (Bio-Rad). This step was followed by incubation with detection antibody for one hour at room temperature with shaking. Following three washes, the samples were incubated with Streptavidin-PE for 30 mins at room temperature with shaking. The samples were finally resuspended in 1X reading buffer after three washes. Plates were read in a MAGPIX® System instrument (Luminex Corporation). Standard curves were generated, and the levels of each cytokine were calculated using the 4-parameter logistic regression using GraphPad Prism 8.

Isolation of Immune Cells From Liver and TA Muscle

Mice were anesthetized and perfused with PBS by transcardial perfusion. Livers and injected TA muscles were harvested from perfused mice and stored temporarily in RPMI media on ice. Tissues were minced with a razor blade followed by enzymatic digestion (0.4% Collagenase type II (Sigma-Aldrich) and 300 μg/mL DNase I (Millipore Sigma) for 30 min at 37°C. Dissociated livers were strained through a 70 μm cell strainer (Falcon) and washed twice with 1X processing buffer (5% FBS in PBS). Cell pellets were resuspended in 40% Percoll (GE Healthcare) and carefully overlaid onto 70% Percoll followed by centrifugation for 25 mins at 400 g, with the brakes off. Leukocytes that band at the 40-70% interphase are removed with a pipette onto a fresh tube and washed thrice with 1X processing buffer to prepare them for staining.

Minced TAs were incubated with 0.5 mg/mL DNase I and 0.25 mg/mL Liberase TL (Roche) in processing buffer for two hours at 37°C. The digested pieces were pooled and strained through a 70 μm cell strainer. Cell suspensions were washed at 1,500 rpm for 7 min in complete RPMI followed by resuspension of the cell pellet in processing buffer for staining.

Flow Cytometry

Cells were suspended in 100 μL PBS with 5% FBS and washed once in PBS. For live/dead staining, cells were resuspended in PBS containing Fixable viability dye eFluor 506 (Thermo Fisher Scientific; 1:1000 dilution) and incubated for 30 min at 4°C in the dark. Following one wash with FACS buffer (2% FBS in PBS), the cells were blocked with anti-CD16/32 (2.4G2) mAb (BD Biosciences, catalog 553141; 1:100 dilution) for 15 min at 4°C. After blocking, the corresponding antibodies were added at 1:100 dilution for 30 min at 4°C in the dark. Following antibody staining, cells were washed twice in FACS buffer. Flow cytometry analyses were performed on an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Data were analyzed using FlowJo (Tree Star).

For intracellular staining, cells were permeabilized in a 1X solution of fixation/permeabilization solution (BD Biosciences) for 30 min at 4°C after blocking/cell surface staining. Thereafter, the cells were washed thrice in 1X Perm/Wash buffer (BD Biosciences). Antibody dilutions (1:100) are prepared in 1X Perm/Wash buffer and cells were resuspended in the antibody containing solution and incubated for 30 min at 4°C. Following antibody staining, cells were washed once in 1X Perm/Wash buffer and resuspended in FACS buffer for analyses by flow cytometry. The list of antibodies used for staining are provided in **Supplementary Table 3**.

qPCR and RT-qPCR

Mouse tissue DNA was isolated using the QIAamp genomic DNA kit (QIAGEN) following manufacturer's instructions. Detection and quantification of vector genomes in extracted DNA were performed by real-time qPCR as described previously (67, 68). Total RNA was isolated from mouse tissues or cells using Trizol (Life Technologies). cDNA preparation for miRNA quantification was done using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) following manufacturer's instructions. qPCR to quantify expression levels of miR-142-3p, miR-652-3p, miR-652-5p, miR-223-3p, and miR-33-5p were done using TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific). Real-time qPCR was performed using the ViiA 7 real-time PCR system (Life Technologies). All other reagents, primers, and probes were purchased from Life Technologies.

Immunohistochemistry

Mouse tissues were fixed in 10% buffered formalin (Fisher Scientific, catalog SF100-20) overnight and embedded in paraffin. Sections (8 μm thick) were stained with H&E. Images were acquired on a TissueFAXS Whole Slide Scanning System (TissueGnostics) using a 20x objective. Nuclei quantification was performed with Image J.

For immunofluorescence staining, muscle sections were de-paraffinized in xylene and rehydrated using a graded ethanol series culminating with PBS. Following antigen retrieval using a programmable pressure cooker with "target retrieval solution", pH 6.0 (Dako), tissue sections were blocked with 10% goat serum in PBS. The slides were then stained for CD8 (1:500, D4W2Z, Cell Signaling Technology), granzyme B (1:40, AF1865, R&D Systems), F4/80 (1:100, MCA497R, Bio-Rad), and OVA (1:500, AB1225, Millipore Sigma) for 16 hours at 4°C. Species-specific secondary antibodies conjugated to Cy5 or Cy7 fluorophores were used and incubated for one hour at room temperature in the dark. Sections were washed, counterstained with DAPI (100 ng/ml) and mounted using FluorSave (Calbiochem) mounting medium. Images were acquired on a Leica SP8 laser scanning confocal microscope using a 40x oil-immersion objective. Quantification of the fluorescent signals of the respective markers was performed using QuPath (69).

Statistics

All data are shown as mean ± SD. Unpaired Student's t tests (two-tailed), one-way ANOVA and two-way ANOVA, with or without *post hoc* testing, were calculated using GraphPad Prism 8. Differences were considered significant when p values were less than 0.05.

RESULTS

In Vitro Screening of Candidate miR-BSs in DCs, Macrophages, and Muscle Cells

To perform functional validation of miR-BS-mediated detargeting, two copies of miR-BSs were engineered into the 3′-UTR of the highly immunogenic chicken OVA cDNA. OVA is used as a model immunogen for studying antigen-specific immune responses in mice. The occurrence of immune responses against the OVA transgene, and the resulting loss of OVA protein expression, following intramuscular delivery by rAAVs have been described previously by us and others (30, 42). Transgene expression is driven by the strong and ubiquitous CMV enhancer/chicken β-actin promoter to achieve ubiquitous transcription, irrespective of cell type (70). These expression cassettes were then subcloned into rAAV vectors (**Figure 1A**). In the absence of publicly available databases that display cell type-specific miRNA expression, a list of candidate miRNAs whose expression levels were reported in the literature to be enriched in hematopoietic lineage cells (DCs, monocytes, B and T cells) was generated. Two copies of binding sites for these miRNAs were individually cloned into the rAAV expression cassettes to generate a library of 26 vectors (**Table 1**).

The inhibitory effects of individual miR-BSs on OVA expression was first evaluated in mouse immature DCs (JAWS II), mouse macrophage cells (RAW264.7), and the mouse C2C12 skeletal myoblast cell line. Expression plasmids were transfected into the aforementioned cell types along with a *Gaussia* luciferase (GLuc) expressing plasmid to account for transfection variability. The conditioned media from the transfected cells were harvested 72 hours post-transfection for measuring secreted OVA levels by ELISA. The levels of OVA were then normalized to GLuc levels for each transfection. Transfected C2C12s were also differentiated under serum starvation conditions to examine the effects of the miR-BS in myocytes (dC2C12). An ideal miR-BS candidate is expected to retain high OVA expression in myoblasts and myocytes indicating specific transgene expression in muscle cell types, but exhibit reduced expression in DCs and macrophages, reflecting translational inhibition in APCs (**Figure 1B**). The results of the *in vitro* screen revealed that the miR-142BS element in dC2C12s conferred levels of OVA expression that were equal to those conferred by the construct that lacks miR-BSs, and significantly reduced OVA levels in JAWSII and RAW264.7 cells (**Figure 1C**). This is consistent with previously reported studies where miR-142BSs were shown to successfully detarget rAAV-delivered transgenes from APCs and to suppress anti-transgene immunity in mice (30, 42). We also note that despite the fact that APCs are enriched with these miRNAs as described in the literature, the design of some cognate miR-BS, namely miR-126aBS and miR-19aBS, failed to reduce transcript expression in JAWSII and RAW264.7 cells. These results demonstrate that not all 3'-UTR modifications were capable of reducing transcript stability.

Combinatorial miR-BS Designs in rAAV-OVA Vectors Increase Transgene Expression With Negligible Anti-OVA Antibody Responses

Several miR-BS candidates maintained high muscle expression of OVA while conferring detargeting from immune cells, albeit to slightly lesser degrees than what was achieved by miR-142BS. Three of these: miR-223-3pBS, miR-652-5pBS, and miR-33-5pBS were further selected for combinatorial designs with miR-142BS. Two copies of each miR-BS were cloned along with two copies of miR-142BS in the 3'-UTR of the OVA transgene. The resulting miR-BS expression vectors were then screened for OVA expression in JAWSII, RAW264.7, and C2C12 cells. The most promising miR-BS combinations to emerge from this round of screening were miR-142/223-3pBS and miR-142/652-5pBS (**Figure 1D**).

To provide support for the notion that the miR-BS cassettes are operating through endogenously expressed cognate miRNAs, we quantified the levels of these miRNAs in cells from immunological and non-immunological lineages to demonstrate their levels of enrichment in APCs. Interestingly, miR-223-3p was found to be approximately 500- to 15,000-fold higher in cell types of the immunological lineage (RAW264.7, JAWSII, bone marrow derived macrophages (BMDM), and Kupffer cells) than in C2C12 and differentiated C2C12 cells

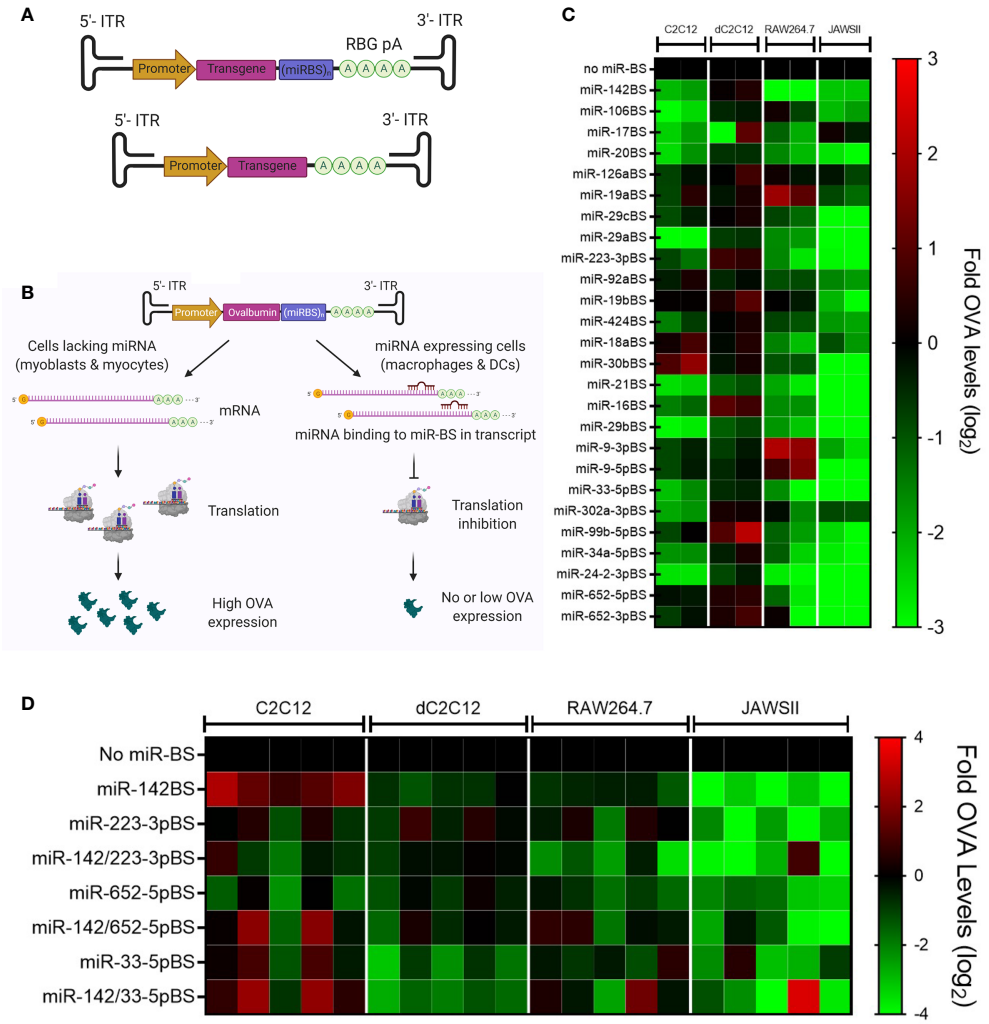

FIGURE 1 | Selection and *in vitro* screening of candidate miRNA binding sites (miR-BS). **(A)** Schematic illustration of rAAV.*OVA* expression vectors. The expression of the OVA transgene is driven by the CB6 promoter. Two copies of miR-BSs are cloned between the transgene and the rabbit β-globin poly A (RBG pA). The OVA expression cassette is flanked by inverted terminal repeats (ITRs) at both ends. **(B)** An ideal miR-BS candidate, upon delivery to myocytes and myoblasts, which do not express the corresponding miRNA, is expected to undergo transcription and translation to produce high levels of transgene. In contrast, when this vector is delivered to miRNA-expressing immune cells, like macrophages and DCs, miRNA binding to the transgene mRNA leads to translational inhibition and transcript degradation, resulting in minimal transgene protein production. **(C)** Summary of the *in vitro* screening of individual miR-BS candidates represented as a heat map with OVA expression denoted as fold change (log_2) with respect to expression vectors lacking any miR-BS. **(D)** The *in vitro* screening of miR-BS combinations summarized as a heat map with relative OVA levels represented as fold change (log_2) (n = 5). C2C12, mouse myoblasts; dC2C12, mouse myocytes; RAW264.7, mouse macrophages; JAWSII, mouse DCs.

(**Figure 2A**). Expression of miR-652-5p in RAW264.7 and JAWSII cells was about 2- and 8-fold higher than levels observed in C2C12 cells, respectively. On the other hand, no significant enrichment of miR-652-3p and miR-33-5p was seen in immune cell types (**Figure 2A**).

We next aimed to assess the function of miR-223-3p and miR-652-5p binding sites *in vivo*. We therefore packaged the rAAV-OVA expression cassettes into AAV1 capsids with or without the individual miR-223-3p or miR-652-5p binding sites, or in combination with miR-142BS elements. Produced vectors were then injected into TA muscles of adult mice. Mice administered with rAAV1 empty capsids or PBS (mock) were used as controls. Animals injected with rAAV1.*OVA*.miR-BS vectors generated increasingly high and sustained levels of OVA expression in circulation, with a negligible anti-OVA antibody response (IgG1). In contrast, animals treated with rAAV1.*OVA* without miR-BSs showed baseline levels of OVA after eight weeks (**Figures 2B, C**). These animals also generated the highest levels of anti-OVA antibodies, which were substantially greater than the anti-OVA IgG levels produced in mice injected with rAAV1.OVA.miR-BS vectors (**Figures 2B, C** and **Supplementary Tables 1, 2**). Interestingly, the combination of *miR-142* and *652-5p* binding sites (miR-142/652-5pBS), or miR-652-5pBS alone, conferred the highest serum OVA levels. The differences in OVA expression levels

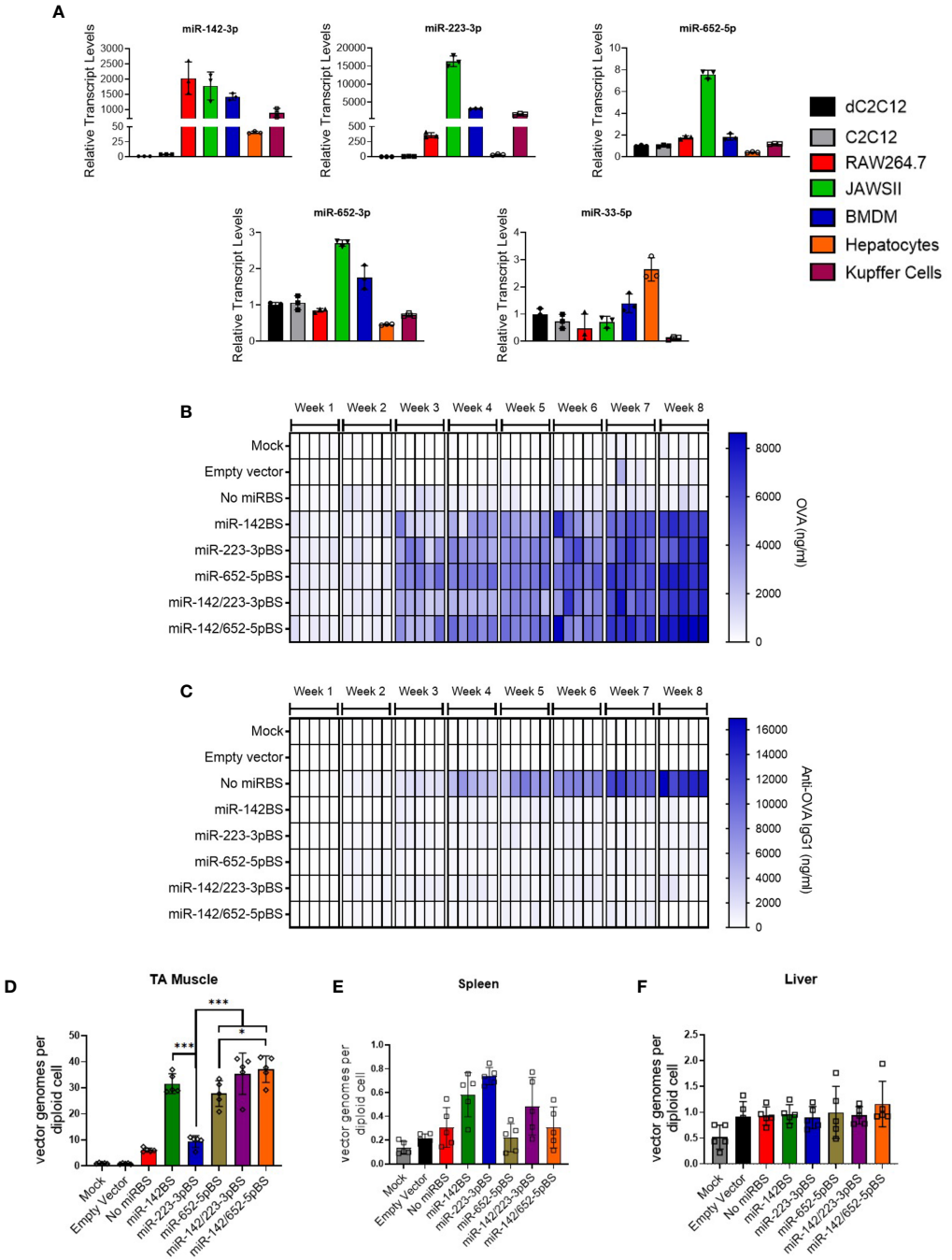

FIGURE 2 | Incorporation of miR-223BSs and miR-652BSs boosts *in vivo* OVA production and suppresses antibody development. **(A)** Endogenous miRNA expression levels in cultured mouse myoblasts (C2C12), myocytes (dC2C12), macrophages (RAW264.7), DCs (JAWSII), bone marrow derived macrophages (BMDM), primary mouse hepatocytes, and Kupffer cells as quantified by reverse transcription quantitative PCR (RT-qPCR) (n = 3). rAAV1 expression vectors were injected by i.m. on day 0 followed by serum collection every week for an eight-week period. **(B, C)** ELISA quantification of circulating OVA expression **(B)** and anti-OVA IgG1 **(C)** (1×10^{11} GCs/mouse, $n = 10$). Single gradient heat map representing respective analyte levels ($n = 5$). **(D–F)** ddPCR detection of rAAV vector genome copies in injected skeletal muscle **(D)**, spleen **(E)**, and liver **(F)** at eight weeks post-injection ($n = 5$). Values represent mean ±SD. $^{*}p < 0.05$, $^{***}p < 0.001$, one-way ANOVA with Tukey's *post hoc* test.

between this combination and other miR-BS designs were most pronounced at eight weeks post-injection (p <0.001) (**Supplementary Figure 1A** and **Supplementary Table 1**). However, anti-OVA antibody production in mice injected with any of the miR-BS expressing vectors was not significantly different from each other, indicating that incorporation of any individual or combination of the tested miR-BSs led to similar levels of anti-transgene antibody suppression (**Supplementary Figure 1B** and **Supplementary Table 2**). With the exception of miR-223-3pBS, the levels of vector genomes detected in TAs eight weeks after injection were high (**Figure 2D**). Interestingly, there was a greater than five-fold increase in the abundance of vector genomes in muscles treated with miR-BS-containing vectors, than in muscles treated with the rAAV-*OVA* construct that lacks miR-BSs (**Figure 2D**). Consistent with immune clearance of transduced muscle fibers and loss of vector genomes, rAAV1.*OVA*.miR-142/652-5pBS-transduced muscle tissues showed at least a six-fold increase in vector genomes at eight weeks as compared to rAAV-*OVA* with no miR-BSs (**Figure 2D**). As expected, vector genome counts in the spleen and liver were at near background levels of detection (**Figures 2E, F**).

miR-142BS and Other Novel miR-BS Designs Downregulate Macrophage Activation and Costimulatory Signals in DCs

Although there were no clear differences in the anti-OVA antibody levels conferred between the miR-BSs cassette designs, we wondered whether any underlying immune responses against the vector and/or the transgene product might still preclude efficient OVA transduction, which can be overcome by optimizing APC detargeting. To address this notion, we analyzed immune effector cell activation following vector treatment. Mice injected intramuscularly with rAAV1 vectors with or without miR-BSs were sacrificed at four weeks post-injection and cells were isolated from injected TAs.

The antigen-specific T cell receptor (TCR) binds foreign peptide antigen-MHC complexes, and the CD28 receptor binds to B7 (CD80/CD86) costimulatory molecules expressed on the surface of APCs, a process that is vital to initiating and maintaining the proliferation of T cells (71). Immunophenotyping of isolated cells by flow cytometry revealed an overall depletion of macrophages and CD80/CD86-positive DCs (CD11c+ cells) in mice injected with vectors carrying miR-BS at the four-week time point (**Figures 3A, E**). The greatest repression was achieved with vectors harboring the miR-652-5pBS or miR-142/652-5pBS cassettes. Notably, there was also a remarkable decrease in overall activated DCs in TA muscles across different vectors (**Supplementary Figure 2A**).

Lymph nodes are secondary lymphoid organs where different immune cell populations coordinate both the innate and adaptive arms of the immune response. Therefore, we also examined the number of CD80/CD86-positive DCs in draining lymph nodes of the injected limb at two and four weeks post-injection. We observed a significant reduction in the population of activated macrophages, CD80/CD86-positive DCs in the animals treated with vectors carrying miR-BSs (**Figures 3B, F** and **Supplementary Figure 2B**, **Supplementary Figures 3A–C**). However, the suppression in macrophage and DC activation did not significantly vary among the different miR-BS designs.

In our previous report, we demonstrated that miR-142BS-mediated APC detargeting leads to a reduction of co-stimulatory molecule expression in isolated splenocytes (42). To further confirm this effect, we isolated splenocytes from injected mice and stained them for macrophage, DC, and DC co-stimulatory markers. All miR-BS-containing vectors significantly suppressed DC activation, macrophage activation, and CD80/86-positive DCs in splenocytes (**Figures 3C, G** and **Supplementary Figure 2C**). While most miR-BS designs mediated weak suppression at two weeks post-injection, indicating no change in the activation state of macrophages and DCs, miR-142BS-, miR-652-5pBS-, and miR-142/652-5pBS-containing vectors inhibited CD80/86 expression as early as two weeks following administration (**Supplementary Figures 4A–C**).

We also isolated immune cells from the livers of injected mice and found that miR-142/652-5pBS-containing vectors mediated the strongest reduction of activated macrophages, DCs, and CD80/86-positive DCs (**Figures 3D, H** and **Supplementary Figure 2D**).

Finally, to determine the activation status of circulating immune cells, we immunophenotyped peripheral blood lymphocytes (PBLs) isolated from the blood of treated mice four weeks post-injection. We did not observe any differences in the levels of activated macrophages, DCs, and CD80/86-positive DCs in the presence of miR-BSs (**Supplementary Figures 5A–C**); indicating that immune cell activation occurred within different tissue compartments, not systemically.

miR-BS-Mediated APC Detargeting Downregulates OVA-Specific T Cell Activation

We previously established that miR-142BS-mediated APC detargeting achieves circumvention of adaptive immunity by blunting OVA-specific CD8+ T cell response, resulting in sustained transgene expression (42). To assay the ability of rAAV1.*OVA*-miR-BS vectors to engage the adaptive immune response, recruitment of CD4+ and CD8+ T cells was measured four weeks following vector administration in the injected TA muscles, lymph nodes proximal to the injection site, the spleen, and the liver. Analyses of the overall CD8+ T cell populations showed that all of the tested miR-BS designs significantly repressed CD8+ T cell response to the vector in all tissues (**Figures 4A–D**).

Although CD8+ T cell responses in tissues were significantly repressed with groups treated with vectors harboring miR-BS cassettes, they were not indicative of the transgene-specific CD8 + T cell activation status. We therefore assessed whether there was any reduction in CD8+ T cell response specific to OVA protein. The ovalbumin SIINFEKL peptide fragment is recognized by the MHC class I molecule (H-2Kb) of T cells in mice. Therefore, OVA-specific CD8+ T cells can be identified by staining cells with H-2Kb/SIINFEKL MHC Tetramers and quantified by flow cytometry. At four weeks post-injection, we observed that the levels of activated OVA-specific CD8+ T cells were reduced by all

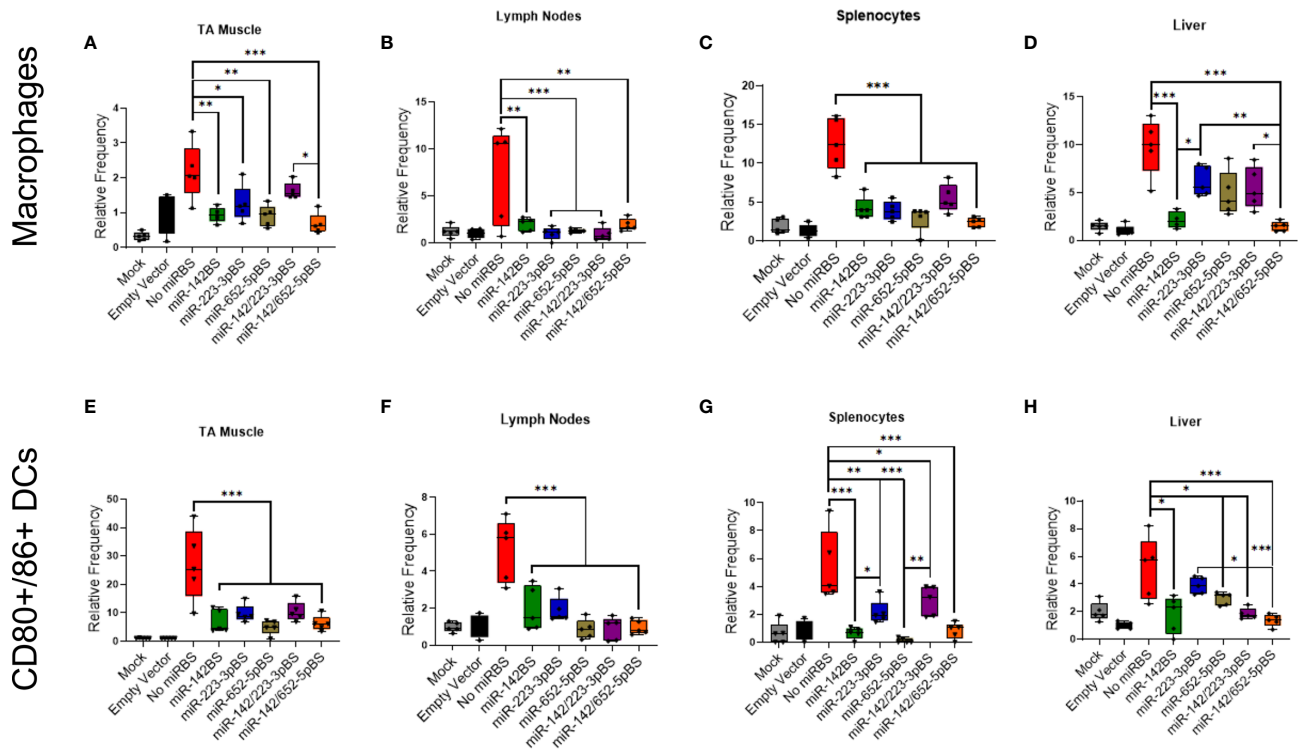

FIGURE 3 | Incorporation of miR-142BS, miR-223BS, and miR-652BS into rAAV1.*OVA* transgene vectors reduces macrophage and DC activation. rAAV1.*OVA* expressing vectors with or without miR-BSs (1×10^{11} GCs/mouse) were delivered by i.m. injections into C57BL/6 mice. Four weeks after injection, cells were isolated from TAs, lymph nodes, spleens, and livers and stained for macrophage markers (CD11b+, F4/80+) and activated DCs (CD11c+, CD80+, CD86+) followed by flow cytometry analysis. Relative frequencies of macrophage populations **(A–D)** and activated DCs **(E–H)** are represented as box plots with means, first and third quartile boundaries, and whiskers indicating max and min values. ($n = 5$). Mock = AAV1.empty capsid. p values were determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

miR-BS designs. Notably, vectors carrying miR-652-5pBS and miR-142/652-5pBS appeared to confer the strongest reduction (**Figures 4E–H**). The extent of reduction in lymph nodes and splenocytes were nearly equal to those conferred by PBS and empty capsid treatments. Interestingly, the miR-142BS and miR-223-3pBS cassettes were not as sufficient as other miR-BS designs in reducing OVA-specific CD8+ T cell responses in the liver (**Figure 4H**). Furthermore, reduced activation of CD8+ T cells and OVA-specific CD8+ T cells was observed in lymph nodes and spleens as early as two weeks post-injection (**Supplementary Figures 3D, E** and **Supplementary Figures 4D, E**), but not in TA muscle and liver (data not shown). Significant differences in OVA-specific CD8+ T cells were not observed between the different miR-BS combinations, except in lymph nodes at two weeks post-injection (**Supplementary Figure 3E**). Immunophenotyping of PBLs at four weeks post-injection revealed a significant reduction in circulating OVA-specific CD8+ T cells, but not overall CD8+ T cells for vectors containing miR-652-5pBS, miR-142/223-3pBS, and miR-142/652-5pBS combinations as compared to the vector without miR-BSs (**Supplementary Figures 5A, B**).

We next tested the effect of vector injection on the CD4+ T cell population. A significant reduction in CD4+ T cell counts was seen in the injected TAs across all vectors containing miR-BSs. Consistent with other cell types, miR-652-5pBS and miR-142/652-5pBS cassettes were the most efficient in suppressing CD4+ T cell activation (**Supplementary Figure 6A**). A similar decrease in CD4+ T cell numbers was also observed in lymph nodes, the spleen, and the liver (**Supplementary Figures 6B–D**). An overall reduction in the CD4+ T cell population in PBLs was also seen in treatment groups receiving vectors containing any combination of miR-BSs with no notable differences when compared to each other (**Supplementary Figure 5C**). Taken together, we were able to ascertain that incorporation of miR-652-5pBS in the rAAV expression cassette mediated efficient suppression of macrophage, DC, CD4+, and CD8+ T cell activation and a decrease in the expression of co-stimulatory markers. In certain cases, this effect was enhanced when miR-652-5pBS was combined with miR-142BS.

miRBS-Mediated APC Detargeting Downregulates OVA-Specific Th1 Response, Inflammatory Cytokine Production, and Memory T Cells

Previous studies have shown that TNF-α and IFN-γ are two principal pro-inflammatory cytokines produced in response to

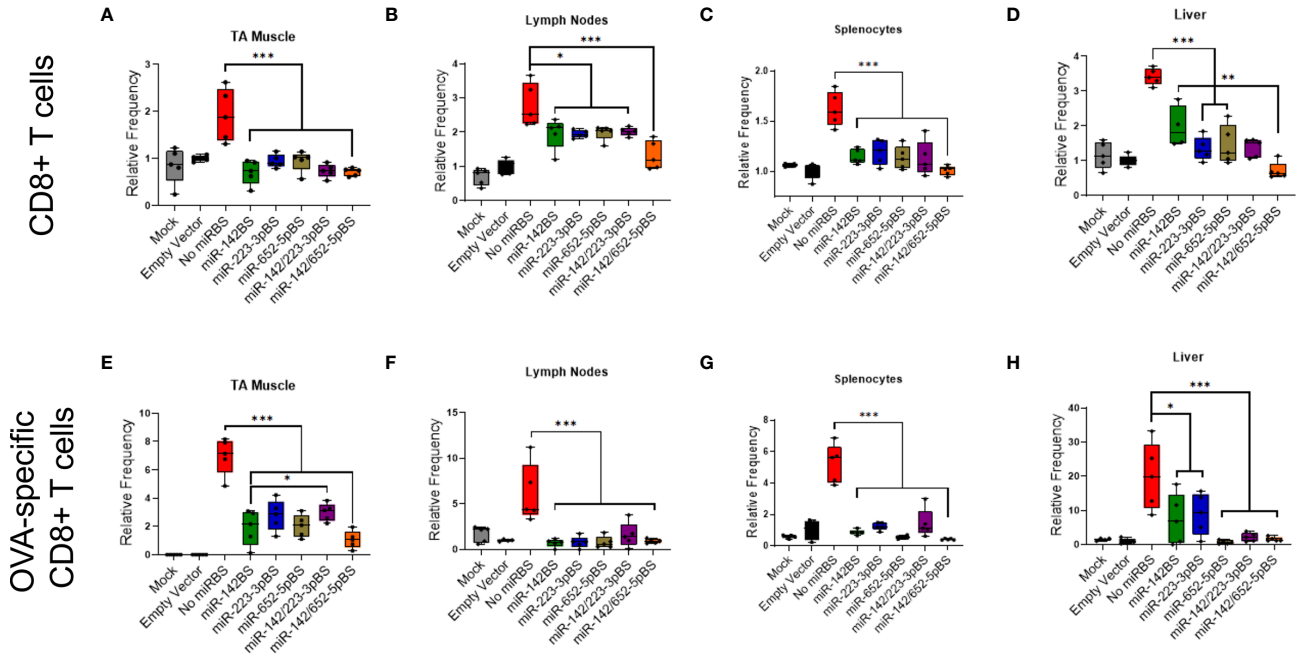

FIGURE 4 | miR-142BS-, miR-223BS-, and miR-652-mediated detargeting suppresses OVA specific CD8 T cell response. C57BL/6 male mice, six weeks old, were i.m. injected with mock, empty capsid, or rAAV1.*OVA* vectors with or without miR-BSs (1×10^{11} GCs/mouse). Mice were sacrificed four weeks after treatment and cells were isolated from TAs, lymph nodes, spleens and livers. Cells were then stained for CD8 T cell markers or with anti-CD8a/H-2Kb SIINFEKL tetramer and quantified by flow cytometry ($n = 5$). Relative frequencies of CD8+ T cells **(A–D)** and OVA-specific CD8+ T cells **(E–H)** are depicted as box plots with means, first and third quartile boundaries, and whiskers indicating max and min values ($n = 5$). p values were estimated by one-way ANOVA with Tukey's *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

rAAV transduction (72). TNF-α is produced by DCs and other immune cells and is involved in both innate and adaptive immune responses (71, 73). IFN-γ is the classic cytokine secreted by Th1 cells and promotes phagocytosis and upregulates microbial killing. We therefore sought to determine TNF-α and IFN-γ response to APC-detargeted vectors. We isolated and cultured splenocytes from mice that were injected with rAAV1.*OVA* vectors with or without miR-BSs at both two- and four-week timepoints. Upon OVA stimulation, splenocytes from mice treated with rAAV1.*OVA* with no binding sites secreted high levels of TNF-α and IFN-γ. In contrast, vectors carrying miR-BSs attenuated cytokine responses to levels that were on average greater than two-fold reduced, which is comparable to cytokines secreted by the splenocytes from mice that received PBS and empty capsids (**Figures 5A, B**). To further validate the suppression of Th1 response, the OVA stimulated splenocytes were stained for IFN-γ producing CD4 T cells and analyzed by flow cytometry. OVA-specific Th1 cell counts were high in rAAV1.*OVA* splenocytes as early as two weeks post-injection (**Figures 5C, D**). However, the reductions in the Th1 response became significant at two and four weeks post-injection only when miR-142BS, miR-652-5pBS, or both elements were incorporated into vectors. Interestingly, splenocytes from rAAV1.*OVA*.miR-223-3pBS-treated mice showed no change at week 2 in IFN-γ-producing Th1 cells as compared to splenocytes from rAAV1.*OVA*-treated animals. Addition of miR-142BS to these vectors reduced responses to an extent at week 2 (**Figure 5C**). Interestingly, miR-223-3pBS-containing vectors (in combination with or without miR-142BS) seems to completely suppress Th1 activation by week 4 (**Figure 5D**). This may suggest a possibility that the suppression of immune cell activation mediated by miR-223-3pBS incorporation follows kinetics that are slower than those conferred by miR-652-5pBS-mediated suppression.

The effects of miR-BS APC detargeting was also gauged by the activation of CD4+ and CD8+ memory T cells. In mice, CD4 and CD8 T cells can be further categorized into memory and naïve phenotypes, based on CD62L (L-selectin) and CD44 expression. $CD44^{low}CD62L+$ populations are considered naïve (T_N) cells, $CD44^{high}CD62L+$ populations are considered central memory (T_{CM}) cells, and the $CD44^{high}CD62L^{neg}$ populations are considered effector and/or effector memory ($T_{E/EM}$) cells. It is known that CD4 and CD8 T cells differ in their distribution of these subsets in lymphoid and peripheral organs (74, 75). We therefore evaluated OVA-stimulated splenocytes for CD4 and CD8 memory T cell activation (both T_{CM} and T_{EM}) at two weeks post-rAAV injection. While there was a substantial reduction in T_{CM} and T_{EM} populations among CD4+ and CD8+ T cells, with incorporation of miR-652-5pBS and miR-142/652-5pBS elements, no differences were noticed in the naïve T cell population. This may suggest that the phenotypic changes observed in T cell populations can be attributed to the different

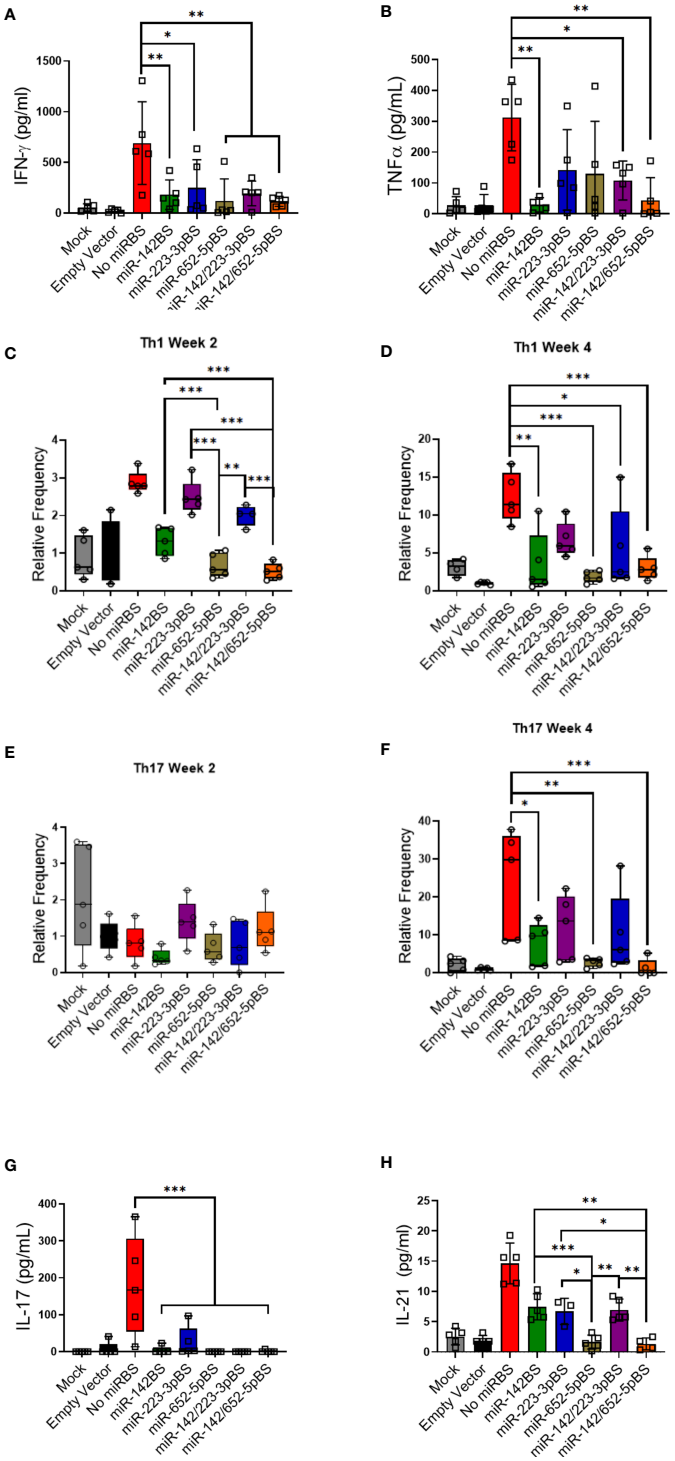

FIGURE 5 | miR-BS incorporation diminishes transgene-specific Th1 and Th17 inflammatory responses. **(A, B)** Estimation of IFN-γ and TNF-α response to OVA stimulation (5 μg/mL) by splenocytes isolated from mice four weeks after vector injection. Three days after treatment, supernatants were collected and quantitated by ELISA (mean ± SD, $n = 5$). p values were determined by ANOVA with Tukey's *post hoc* test. Splenocytes isolated two and four weeks post-AAV1 injection were stimulated for 24 hours with OVA (5 μg/mL) and stained for detecting either Th1 population (CD4+) **(C, D)** or Th17 cell population (CD4+IL-17A+) **(E, F)** and analyzed by flow cytometry (Box plots with means, first and third quartile boundaries, and whiskers indicating max and min values; $n = 5$). **(E, F)** Levels of secreted Th17-specific cytokines, IL-17 and IL-21- were assessed after stimulation of splenocytes (harvested four weeks post-injection) for 72 hours with OVA (5 μg/mL) by ELISA (mean ± SD, $n = 5$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. p values were estimated by one-way ANOVA with Tukey's *post hoc* test.

extents of OVA-specific activation by different vector designs, and not due to the naïve T cell populations that were not stimulated by OVA (**Supplementary Figure 7**). However, the reduction in T_{CM} and T_{EM} populations was not maintained at four weeks post-injection (data not shown).

The miR-142/652-5pBS Combination Effectively Suppresses Th17 Response

One pathway that lacks study with respect to rAAV transgene immunogenicity is the involvement of Th17 cells. Th17 cells are a recently discovered cell type that secrete IL-17 as their primary effector cytokine and belong to the CD4+ T cell family (76, 77). We hypothesized that these pro-inflammatory cells might be involved in mounting an anti-transgene immune response (78, 79). To investigate their contribution towards anti-transgene immunity, OVA-stimulated splenocytes at two and four weeks post-injection were quantified for IL-17A expressing CD4+ T cells by flow cytometry. While no OVA-specific Th17 response was observed at two weeks, rAAV1.*OVA* splenocytes showed elevation in the number of Th17 cells at four weeks (**Figures 5E, F**). With the exception of miR-223-3pBS, inclusion of the candidate miR-BSs in vectors significantly downregulated Th17 activation, with maximal repression imparted by miR-652-5pBS and miR-142/652-5pBS. This outcome was similar in fashion to Th1 responses under these treatments. Both IL-17 and IL-21 are Th17 cell-secreted cytokines that accentuate the protective effects of Th17-mediated immune response. Therefore, Th17 activation was further confirmed by measuring IL-17 and IL-21 production from OVA-stimulated splenocytes. Consistent with the flow cytometry data, splenocytes from mice treated with rAAV1.*OVA* lacking miR-BSs produced high levels of IL-17. In contrast, vectors carrying miR-BSs conferred a significant reduction in Th17 activation, and hence a concomitant decrease in IL-17 production (**Figure 5G**). Incorporation of miR-652-5pBS and miR-142/652-5pBS seemed to significantly suppress IL-21 secretion in stimulated splenocytes as well (**Figure 5H**). Our data thus suggests that transgene-specific Th17 response might play a critical role in the suppression of transgene expression over time. Incorporation of miR-BSs in expression cassettes blunts this response, and in turn, boosts the levels of transgene expression.

miRNA-Mediated Detargeting Does Not Activate Regulatory T Cells to Enable Immunosuppression

The use of miR-142BS elements in lentiviral vectors induces immunologic tolerance and activates regulatory T cells (Tregs) (80). To investigate if this effect is reproduced in rAAV-delivered transgenes containing miR-BSs, we isolated immune cells from TAs, lymph nodes, and spleens at two and four weeks post-injection and stained them for Treg-specific markers. The Treg population can be identified as CD4+ T cells that are also double-positive for CD25 and FOXP3. None of the miR-BS containing vectors lead to an increase in the Treg cell numbers in any of the analyzed tissues (**Figures 6A–C** and **Supplementary Figures 8A, B**). Additionally, stimulated splenocytes from treated animals did not reveal any elevation of the anti-inflammatory cytokines IL-10 and TGF-β (**Supplementary Figures 8C, D**). Therefore, the incorporation of miR-BSs into AAV vectors does not induce Treg activation nor induce tolerance by the suppression of other immune cell types as observed with lentiviral vectors.

miRBS-Mediated Suppression of CD8+ T Cell Response Reduces Clearance of Transduced Cells and Boosts Transgene Expression

Our previous study demonstrated that inclusion of miR-142BS elements in the rAAV transgene cassette reduced infiltration of CD8+ T cells and subsequent clearance of transduced muscle fibers (42). We aimed to determine whether our novel miR-BS cassettes have the same capacity to repress cytotoxic T cell recruitment and tissue clearance. Histopathology and immunohistopathology analyses of the injected TA muscle tissue was performed two weeks after rAAV treatment. H & E imaging showed that rAAV1.*OVA*-injected TAs had a high degree of cellular infiltration. Tissues injected with AAV vectors containing miR-BSs showed reductions in infiltrates (**Figure 6D**; top panels). Quantification of the number of nuclei in TA cross-sections showed significant decreases in cellular infiltration in mice treated with vectors carrying miR-BSs. Notably, the miR-142/652-5pBS cassette conferred the lowest abundance of immune cell infiltrates (**Figure 6E**). Moreover, H&E-stained cross-sections of TA muscles from animals treated with rAAV1.*OVA* and rAAV1.*OVA*.miR-223-3pBS vectors revealed a high degree of centrally located myonuclei. In healthy muscle fibers, myonuclei are located at the periphery of the muscle fiber. Centrally located myonuclei are indicators of myofiber regeneration following damage (81). These results are thus indicative of active clearance of transduced myofibers and muscle turnover. Notably, centrally located nuclei are absent in the TAs of mice treated with vectors carrying the miR-652-5pBS element (**Figure 6E**).

We also performed immunohistochemical staining of the treated muscle sections for CD8+ T cells, granzyme B, F4/80, and OVA. Granzyme B is a marker for activated cytotoxic T cells and F4/80 is a cell surface marker for macrophages. We observed robust CD8+ T cell (CTL) infiltration in muscle samples from rAAV1.*OVA*-injected animals, and about one-third of infiltrates were positive for granzyme B (**Figure 6D**; bottom panels and **Figures 6F, G**). A significantly lower degree of CTL infiltration was observed among muscles injected with vectors bearing miR-142BS, miR-652-5pBS, miR-142/223-3pBS, and miR-142/652-5pBS elements, of which a very small portion expressed granzyme B. While the number of CD8+ T cells was reduced at least three-fold, there was a two-fold reduction observed in granzyme B expression with the incorporation of these miR-BSs (**Figures 6F, G**). Although miR-223-3pBS-containing vectors showed relatively higher numbers of CD8+ T cells, granzyme B expression was considerably lower, indicating reduced CTL activity. CTL infiltration was also accompanied by macrophage infiltration in the injected muscle tissues of rAAV1.*OVA*-treated mice (**Supplementary Figures 9A, B**). Tissues with a high abundance of CTLs and macrophages also have relatively low

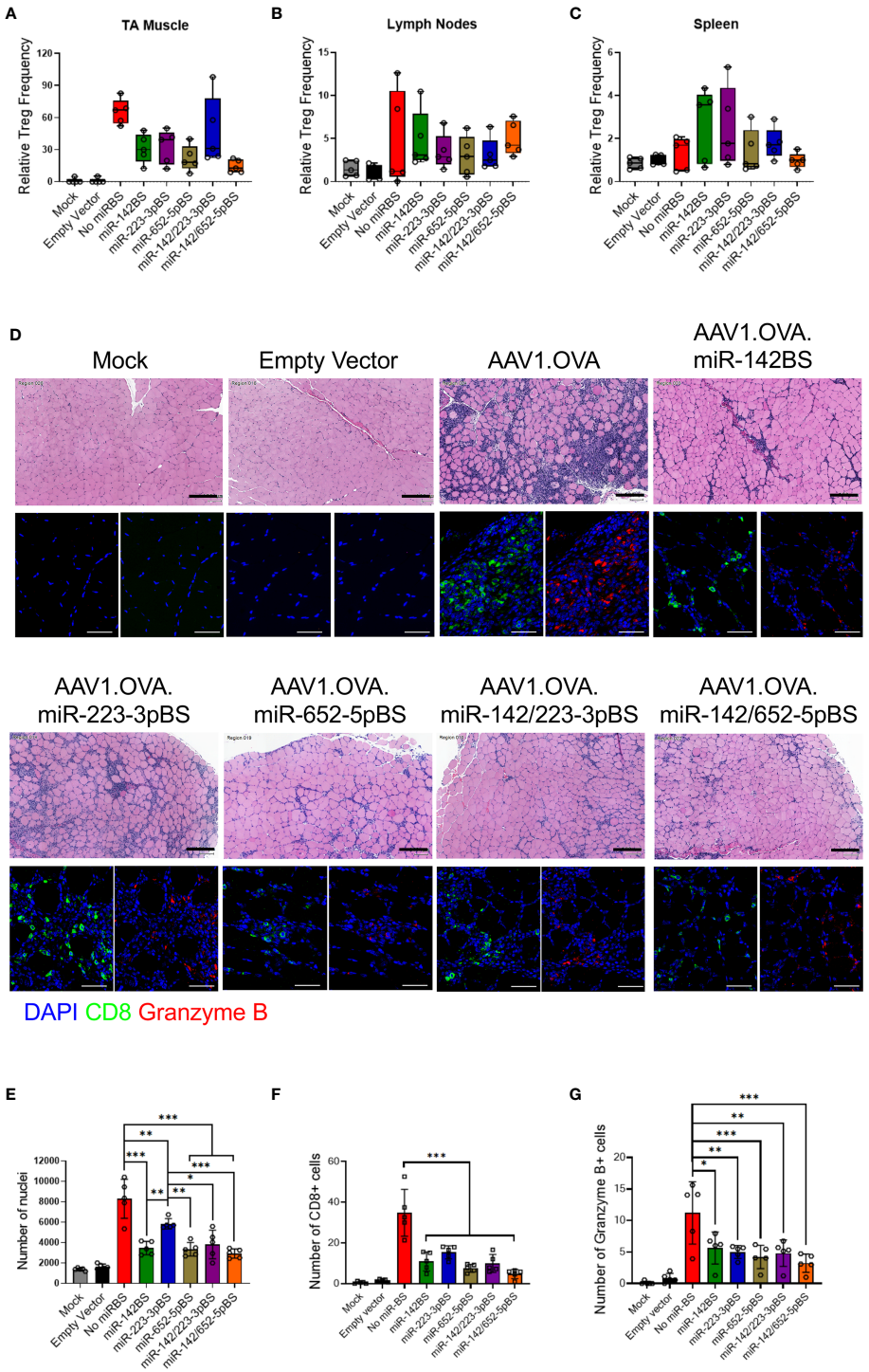

FIGURE 6 | miRNA-mediated detargeting acts independent of Treg immunosuppression and reduces tissue clearance by downregulation of OVA-specific CTL response. **(A–C)** Cells from TA muscle, lymph nodes and spleen were harvested from rAAV1 injected C57BL/6 mice four weeks after treatment and stained for Treg markers (CD4+, CD25+, FOXP3+). The frequencies of Tregs were quantified by flow cytometry and displayed as box and whisker plots (n = 5). **(D)** C57BL/6 male mice, six weeks old, were i.m. injected with rAAV1.OVA vectors with or without miRBS (1×10^{11} GCs/mouse, n = 5) and sacrificed two weeks after injection to harvest muscles. Tissue sections were stained for H&E (upper panels, original magnification: 20x), CD8 and granzyme B (lower panels: DAPI, blue; red, granzyme B, red; and CD8, green; original magnification: 40x). Scale bars = 200 μm (H&E images), 50 μm (fluorescence images). **(E)** Quantification of H&E images for nuclear infiltrates in whole tissue section at original magnification of ×20. **(F, G)** Quantification of CD8 **(F)** and granzyme B **(G)** images in four fields at original magnification of 40x. p values were estimated by one-way ANOVA with Tukey's *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

OVA expression (**Supplementary Figures 9A, B**). Tissues of mice treated with OVA vectors that harbor miR-BS elements express high levels of OVA and confer reduced levels of macrophage activation (~two-fold reduction). Consistent with CTL infiltration, miR-652-5pBS-bearing vectors showed the lowest degree of infiltration into injected TAs (more than four-fold reduction) by macrophages. These findings indicate that inclusion of miR-BS elements in the OVA transgene led to a reduction in cytotoxic CD8+ T cell and macrophage infiltration, resulting in reduced clearance of transduced muscle fibers, and ultimately efficient and stable transgene expression in the tissue.

DISCUSSION

Owing to their safety and efficacy in preclinical and clinical studies, rAAVs are promising gene therapy vectors for a variety of genetic diseases (5, 6, 82–84). Skeletal muscle is an attractive target tissue for rAAV-mediated gene therapy for neuromuscular diseases, metabolic disorders, and hemophilia (8, 82, 85–87). The easy accessibility of skeletal muscles makes them ideal for vector administration and the extensive vascular blood supply provides an efficient transport system for secreted therapeutic proteins. However, host immune responses against rAAV1-encoded transgene products after intramuscular delivery have been reported to cause the clearance of transduced fibers and loss of transgene expression (88–91). The induction of these immune responses can be attributed to the undesirable transduction of APCs, such as DCs, which lead to transgene expression and antigen presentation on these cell types. These events, in turn, activate T and B cells (31, 92–94). Therefore, the prevention of transgene-specific immune responses is particularly crucial to the success of rAAV gene therapy in muscles.

miRNA-mediated regulation of transgene expression by engineering miR-BSs in rAAV expression cassettes has proven to reduce transgene-specific immune responses. Binding sites against *miR-122*, *miR-199*, *miR-1*, *miR-183*, and *miR-206* can successfully detarget transgene expression from tissues like liver, heart, dorsal root ganglia and skeletal muscles (35, 36, 95–98). We previously demonstrated that miR-142BS elements can blunt CTL activation and can confer sustained transgene expression in transduced mouse TA muscle (42). *miR-142* is a hematopoietic-specific miRNA whose expression levels are high in APCs, which is central to the effectiveness of miR-142BS-mediated detargeting. Little attempt has been made to identify additional miRNAs that can be utilized to repress transgene expression in APCs and restrain immune response activation. In this study, we identified two miRNAs, miR-223-3p and miR-652-5p that are enriched in macrophages and DCs and have the potential for enhancing APC detargeting. Through *in vitro* and *in vivo* screening of individual miR-BSs and in combination with miR-142BS across several cell types, miR-142/223-3pBS and miR-142/652-5pBS were determined to be potent elements in transgene detargeting from APCs.

DCs are the most potent professional APCs that form an essential link between the innate and adaptive immune responses. Due to their unique aptitude for stimulating T cells, activated DCs play a major role in determining immunological outcome (99, 100). Activation of DCs leads to a significant upregulation of MHC and expression of costimulatory molecules, which play crucial roles in initiating or promoting T cell priming and proliferation. These responses are needed for effective immunity (99). Following AAV infection, both conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs) are required for the cross-priming of CD8+ T cells, while the other APCs seem to be dispensable for this process (101). miR-BS-mediated detargeting hinders MHC-dependent presentation of the transgenic peptides on the surface of DCs by degradation of the mRNA transcript. In the absence of antigen presentation, the ensuing T and B cell activation and the inflammatory responses are suppressed. Our findings with respect to the efficacy of these novel miR-BSs in promoting transgene detargeting would provide flexibility and alternatives in vector design strategies and increase the safety profile of rAAV-based therapeutics.

Durability and memory are important hallmarks of the adaptive immune system that arise from clonal expansion and differentiation of antigen-specific lymphocytes. Memory CD4+ and CD8+ T cells have low activation thresholds and confer protection in peripheral tissues by responding to antigens upon re-encountering them in secondary lymphoid organs (102, 103). Effector memory T cells (T_{EM}) migrate to inflamed peripheral tissues and display immediate effector functions. T_{EM} can be stimulated by antigen presented by nonprofessional APCs in a milieu that does not favor stable cell-cell interactions (104, 105). On the other hand, central memory T cells (T_{CM}) have no effector function but readily migrate, proliferate, and differentiate into T_{EM} upon antigenic challenge (106). T_{CM} are more sensitive to antigenic stimulation and less dependent on co-stimulation when compared to naïve T cells and thus provide more effective feedback to stimulation from DCs and B cells (75). Here, we have demonstrated for the first time that rAAV-delivered transgenes can increase both CD4+ and CD8+ memory T cell populations (T_{CM} and T_{EM}) in spleen. Importantly, we show that the addition of miR-BSs, specifically miR-652-5pBS and miR-142/652-5pBS significantly suppress memory T cell activation. We previously reported that miR-142BS-mediated detargeting can suppress anti-transgene responses following a second dose of vector to enable successful vector re-administration (42). Further research to determine if redosing of rAAV-delivered transgenes can be enhanced by the inclusion of miR-223-3pBS and miR-652-5pBS elements is warranted.

Another frequently overlooked and under-explored cell type of the CD4+ family are Th17 cells. Th17 cells have been implicated in anti-transgene immune responses in certain preclinical and clinical trials (107, 108). An increase in pro-inflammatory Th17 cells was also observed in non-human primates treated with rAAV-delivered human factor IX (*hF.IX*) (109). It has been hypothesized that a skewed Th17/Treg balance towards increased Th17 values results in higher intensities of anti-transgene immune responses (109). Consistent with this

idea, an increase in OVA-specific Th17 cell population was seen in rAAV1.*OVA* vectors, but not in vectors containing miR-652-5pBS or miR-142/652-5pBS cassettes in our present study. To investigate the hypothesis that sufficient Treg activity might be induced to offset the pro-inflammatory Th17 activity by miR-BS inclusion, we quantified the Treg population in TA muscle, lymph nodes, and liver. However, we did not find any difference in the activation status of Tregs, nor an increase in anti-inflammatory cytokine secretion. This finding indicated that tolerance to the transgene protein is likely not mediated by Treg activation, but rather by blunting OVA-specific Th1 and Th17 responses, and inhibition of CTL response.

Taken together, we have provided additional lines of evidence and novel mechanistic insights into the immune cell populations involved in rAAV transgene-specific immune responses. The effect of rAAV administration on Th17 population and memory T cells had yet to be documented. Inclusion of miR-BSs seems to inhibit the activation of these immune cell types. miRNA-mediated detargeting is an approach that has been successfully employed to achieve tissue-and cell type-specific expression by restricting spurious transcription conferred by the wide-tropism profiles inherent to contemporary rAAV platforms. *miR-223-3p* and *miR-652-5p* are enriched in cells of the myeloid lineage and hence serve as ideal candidates for miRNA-mediated regulation. Synergistic action of two miR-BSs has been shown for hepatocyte detargeting (110). Our present study is the first attempt at identifying, screening, and investigating the combinatorial effects of miRNAs for transgene detargeting from APCs following intramuscular injections. Although *miR-652-5p*-mediated transgene regulation seems to be more effective from our findings, miR-223-3pBS incorporation is also effective at transgene detargeting. Interestingly, our results indicate that the kinetics of immune cell suppression by miR-223-3pBS elements is slower and starts becoming evident usually four weeks after vector injection. Additional investigation into whether miR-223-3pBS elements can contribute to immunological suppression in later stages of the therapeutic window may further improve APC-detargeting cassette designs. Finally, in the course of this study, we revealed that some miR-BS cassette designs conferred increases in transgene expression in a cell type-specific manner. Unfortunately, the mechanisms by which these elements increased transgene expression *in vitro* was not investigated. Whether some of these 3'-UTR modifications can act to stabilize transcripts is indeed intriguing and begs further investigation.

The safety of these design elements also need to be closely investigated. miR-652-5p has been identified as a disease-associated miRNA that is dysregulated in various pathological processes like esophageal cancer, bladder cancer, osteosarcoma, gastric cancer, and breast cancer (111–116). miR-223-3p expression has also been shown to be aberrant in gastric cancers, osteosarcoma, glioblastoma, squamous cell carcinoma, breast cancer, neuroblastoma, and myocardial infarction (117–123). Their function as cancer-related miRNAs warrants caution as these elements might trigger unintended consequences in related cell types. Nevertheless, it is worth noting that in an earlier report, miR-BS-mediated post-transcriptional detargeting by AAV vectors did not disturb endogenous miRNA profiles (35).

Importantly, combinatorial miR-BS designs is not just used to alleviate the immunogenic effects, they may also be used for multi-tissue detargeting. The expression profiles of multiple microRNAs can be exploited concomitantly to reshape rAAV tropism to achieve tissue specific expression. Given the small size of these miR-BS elements, they can be combined with other targeting strategies to overcome other roadblocks in rAAV transduction. However, transgene immunogenicity represents a major hurdle in the efficacy of rAAV gene therapy. Continued vector engineering efforts are key to expanding rAAV gene therapy to a wider set of human conditions. In conclusion, we report a post-transcriptionally regulated transgene delivery system where the transgene expression is selectively eliminated from APCs by three miRNAs whose expression is enriched in these cell types. We demonstrate that these miRNAs individually or synergistically are capable of blunting transgene-specific immune response and enable sustained transgene expression over time.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

AUTHOR CONTRIBUTIONS

MM, and WZ executed the flow cytometry workflows and analyses, with initial guidance from KS and AMK, YM, JL, AL, JC, ID, MA, TN, and PT helped with tissue harvests and sample preparation described in the study. SM, RH and QS produced the vectors used in the study; MM and WZ wrote the initial draft of the manuscript. WZ, MM, PT, and GG revised and finalized the manuscript. MM, PT, and GG developed the study design and interpreted the data. PT and GG supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partially supported by a Sponsored Research Grant from Voyager Therapeutics and supported by grants from the University of Massachusetts Medical School (an internal grant) and

by the NIH (R01NS076991-01, 4P01HL131471-02, UG3 HL147367-01, R01HL097088, U19 AI149646-01).

ACKNOWLEDGMENTS

We thank Christina Baer and the Sanderson Center for Optical Experimentation (SCOPE) light microscopy core facility at University of Massachusetts Medical School, which provided access to TissueFAXS Whole Slide Scanning System and Leica SP8 laser scanning confocal microscope and for quantitative imaging advice. We also thank JX, MN, and JH for helpful discussions and guidance during the drafting of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.674242/full#supplementary-material

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Conflict of Interest: GG is a scientific co-founder of Voyager Therapeutics, Adrenas Therapeutics, and Aspa Therapeutics, and holds equity in these companies. GG is an inventor on patents with potential royalties licensed to Voyager Therapeutics, Aspa Therapeutics, and other biopharmaceutical companies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 11 May 2021
doi: 10.3389/fimmu.2021.673699

Vectored Immunotherapeutics for Infectious Diseases: Can rAAVs Be The Game Changers for Fighting Transmissible Pathogens?

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Edited by:
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Health Canada, Canada

Reviewed by:
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Chen Ling,
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 28 February 2021
Accepted: 23 April 2021
Published: 11 May 2021

Citation:
Zhan W, Muhuri M, Tai PWL and Gao G (2021) Vectored Immunotherapeutics for Infectious Diseases: Can rAAVs Be The Game Changers for Fighting Transmissible Pathogens? Front. Immunol. 12:673699. doi: 10.3389/fimmu.2021.673699

Conventional vaccinations and immunotherapies have encountered major roadblocks in preventing infectious diseases like HIV, influenza, and malaria. These challenges are due to the high genomic variation and immunomodulatory mechanisms inherent to these diseases. Passive transfer of broadly neutralizing antibodies may offer partial protection, but these treatments require repeated dosing. Some recombinant viral vectors, such as those based on lentiviruses and adeno-associated viruses (AAVs), can confer long-term transgene expression in the host after a single dose. Particularly, recombinant (r)AAVs have emerged as favorable vectors, given their high *in vivo* transduction efficiency, proven clinical efficacy, and low immunogenicity profiles. Hence, rAAVs are being explored to deliver recombinant antibodies to confer immunity against infections or to diminish the severity of disease. When used as a vaccination vector for the delivery of antigens, rAAVs enable *de novo* synthesis of foreign proteins with the conformation and topology that resemble those of natural pathogens. However, technical hurdles like pre-existing immunity to the rAAV capsid and production of anti-drug antibodies can reduce the efficacy of rAAV-vectored immunotherapies. This review summarizes rAAV-based prophylactic and therapeutic strategies developed against infectious diseases that are currently being tested in pre-clinical and clinical studies. Technical challenges and potential solutions will also be discussed.

Keywords: adeno-associated virus, vectors, immunotherapy, gene therapy, vaccines

INTRODUCTION

Infectious diseases are among the biggest threats to our society. They range from ancient maladies, such as malaria and influenza, to modern illnesses, such as human immunodeficiency virus (HIV)-1 and the coronavirus disease of 2019 (COVID-19) pandemic. Many strategies have been developed to cure patients with these diseases and to eradicate the related pathogens. Most vaccines today function by introducing either an inactivated form of the pathogen, a live-attenuated strain, or a protein subunit of the pathogen into the body. This exposure stimulates antigen-specific adaptive

immune responses and immunological memory, which can protect the host or mitigate the severity of the infection (1). Vaccines have led to the eradication of small pox and have reduced the global incidence of many other diseases (2). However, certain pathogens have been able to evade vaccine-induced sterilizing immunity *via* various mechanisms. RNA viruses, such as HIV, hepatitis C virus (HCV), and influenza exhibit high genetic variation among diverse strains across different geographical locations. Viral polymorphism can persist even within the same infected individual, thus limiting the utility of vaccines that are based on a single strain (3, 4). In addition, retroviruses have rapidly mutating genomes that permit their escape from adaptive immunity (5). Many pathogens actively suppress inflammation and immunological memory by infecting immune cells and inducing T cell exhaustion, further preventing the formation of sterilizing immunity (5, 6). Broadly neutralizing antibodies (bNAbs) can offer protection against multiple strains. Alternatively, blocking the essential primary host cell receptor with monoclonal antibodies (mAbs) can limit infection by multiple strains. For example, ibalizumab, a CD4-targeting mAb, has received United States Food and Drug Administration (FDA) approval for use in multidrug-resistant HIV-1 infections (7). In addition, many mAbs are under development for the targeted treatment of Ebola, Zika, and COVID-19, among others (8). However, these drugs typically require repeated dosing through intravenous injections and have high production costs (9). Therefore, their practical value is limited, especially in underdeveloped areas of the world. Moreover, the processes involved in generating inactivated virus might damage the native conformation of the antigen; while in the case of subunit vaccines, the antigen is often produced using cell lines of non-human origin, which may have distinctive post-translational modification patterns not native to the virus (10, 11). Collectively, these issues may create antigens with altered conformations, resulting in antibodies that are produced which do not neutralize the real pathogen. These low-potency antibodies also run the risk of enhancing virus entry by assisting viral attachment to the host cell in a phenomenon termed antibody-dependent enhancement (ADE). One of the biggest concerns with the recent COVID-19 pandemic, which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the possibility of vaccine-mediated ADE effect, since similar outcomes have been observed with other coronavirus infections (12). To counter this, Pfizer/BioNTech and Moderna have each developed a vectored strategy, in which mRNA encoding the coronavirus spike (S) protein packaged in lipid nanoparticles (LNPs) is delivered to the body to mediate *de novo* synthesis of the S protein, so that the conformation and topology of the antigen best resembles the native protein that is decorated on SARS-CoV-2 (13, 14). This strategy achieved astounding efficacies of 94% or more in phase III trials (ClinicalTrials.gov Identifier NCT04368728 and NCT04470427).

The ability of viral gene transfer systems to deliver functional genes in the host greatly expands the number of strategies that can be used to fight infectious diseases. One such strategy is to use a recombinant virus as a vector to deliver genes encoding therapeutic molecules, such as neutralizing antibodies (NAbs), bNAbs, therapeutic mAbs, and immunoglobulin (Ig)-related derivatives, for direct expression from the host's tissues, thus negating the necessity of repeated dosing. Viral vectors may also be used to deliver antigen-encoding genes for vaccination against the antigen. Vectors based on adeno-associated viruses (AAVs) are by far the most popular choice for *in vivo* gene delivery, as a result of their relatively low immunogenicity, high safety profile, broad tropism to a range of tissue types, and their propensity to maintain long-term gene expression. This review will cover recombinant (r)AAV-based immunotherapeutic strategies used to combat infectious diseases. An overview of other viral vectors used in vaccines and immunotherapeutics will be introduced, followed by a general biology of AAV. Subsequently, we will discuss technical challenges and potential solutions to rAAV-vector approaches. Finally, the numerous prophylactic and therapeutic strategies that have been developed over the years for various infectious diseases will be highlighted.

Overview of Viral Vector Gene Delivery Systems

Viral gene delivery systems take advantage of natural viruses' inherent ability to evade host defense mechanisms and to transfer genetic cargos inside the cell. In general, viral systems offer better delivery efficiencies to the nucleus than non-viral systems, such as LNPs, naked DNAs, or various polymeric complexes. At the same time, viral vectors are more immunogenic, which can be desirable or unwanted, depending on the specific application (15). Many viral vector platforms based on adenovirus, lentivirus, AAV, Sendai virus, poxvirus, measles virus, baculovirus, and herpes simplex virus vectors, to name a few, have been explored for gene delivery, with the first three being the current most common (16–20).

Adenoviral vectors (AdVs) have a packaging capacity of up to 34 kb, mediate rapid gene expression, can potently activate innate immune responses, and can induce strong Th1-polarized adaptive immunity against transgene products (21–23). These features make AdVs attractive for vaccination against infectious disease outbreaks. Human adenovirus type 5 (Ad5), type 26 (Ad26), and chimpanzee adenovirus ChAdOX1 have been explored as delivery vectors for the coronavirus S gene, with the latter achieving 70% efficacy in a phase III trial (NCT04400838) (24–26). The SARS-CoV2-Ad26 vaccine developed by Johnson & Johnson, recently received from the FDA an emergency use authorization approval. At the same time, the strong immunogenicity of AdVs, as well as pre-existing immunity against Ad5 among various human populations, can negatively affect transduction efficacy, transgene longevity, and can cause untoward side effects (27). Ad5 vectors encoding mAbs or Ig derivatives against respiratory syncytial virus (RSV) and H5N1 influenza A virus hemagglutinin (HA) demonstrated short-term (4 to 14 days) protection in mice against these respective viruses (28, 29). Ad5 delivery of mAbs against anthrax (*Bacillus anthracis*) showed initial protection from a bacterial toxin challenge, but was lost within six months (30). Recombinant AdVs that express HIV-1 proteins to elicit vaccination of patients

against HIV-1 infection have been explored, but lacked efficacy in human trials (31–33).

Lentiviral vectors (LVs) utilize the capacity of lentiviruses, most notably HIV-1, to mediate semi-random integration of DNA into the host cell genome to enable long-term transgene expression (34, 35). By deleting the genes that are non-essential to vector production or expressing them in *trans*, LVs can accommodate up to 10 kb of sequence and have the potential to transduce both mitotic and postmitotic cells (23, 36). LVs are commonly used for *ex vivo* gene delivery, most prominently in hematopoietic stem cells (HSCs) and T cells, and are an essential production component in the two FDA-approved chimeric antigen receptor (CAR)-T cell therapies (37). Current LVs have limited tropism profiles, which is a bottleneck for *in vivo* gene delivery in preclinical trials (23, 38–41). However, pseudotyping with envelope proteins from other viruses can enhance tissue targeting (42). Other issues for LVs are their potential for genotoxicity and immunogenicity. Integrase-defective LVs may reduce genotoxicity and have been used for episomal delivery with varying degrees of success (43, 44). Alternatively, packaging LVs with nucleases as protein cargos inside the virion might reduce long-term genotoxicity concerns (45, 46).

Characteristics of AAV and Their Recombinant Vector Counterparts

Wild-type (wt)AAVs are non-enveloped dependoparvoviruses with a relatively simple architecture (**Figure 1**). Each virion consists of a total of 60 monomers of the capsid proteins (cap), VP1, VP2, and VP3. These proteins respectively assemble at a roughly 1:1:10 ratio into an icosahedral virion that is approximately 25 nm in diameter and 3.9 MDa in size. Multiple AAV serotypes, that differ in cap proteins, are being tested as gene therapy vectors in numerous clinical trials; with each serotype characterized by their unique tissue tropism profiles. Thirteen different AAV serotypes (AAV1-13) and more than 100 natural variants have been reported so far, and more are being discovered. AAV1, AAV2, and AAV9 have been approved for clinical use by the FDA or European Medicines Agency (EMA), and more are being tested in phase I and phase II trials (23, 47, 48). Additionally, the AAV capsid can accommodate various modifications, such as amino acid substitutions, post-translational processing, and

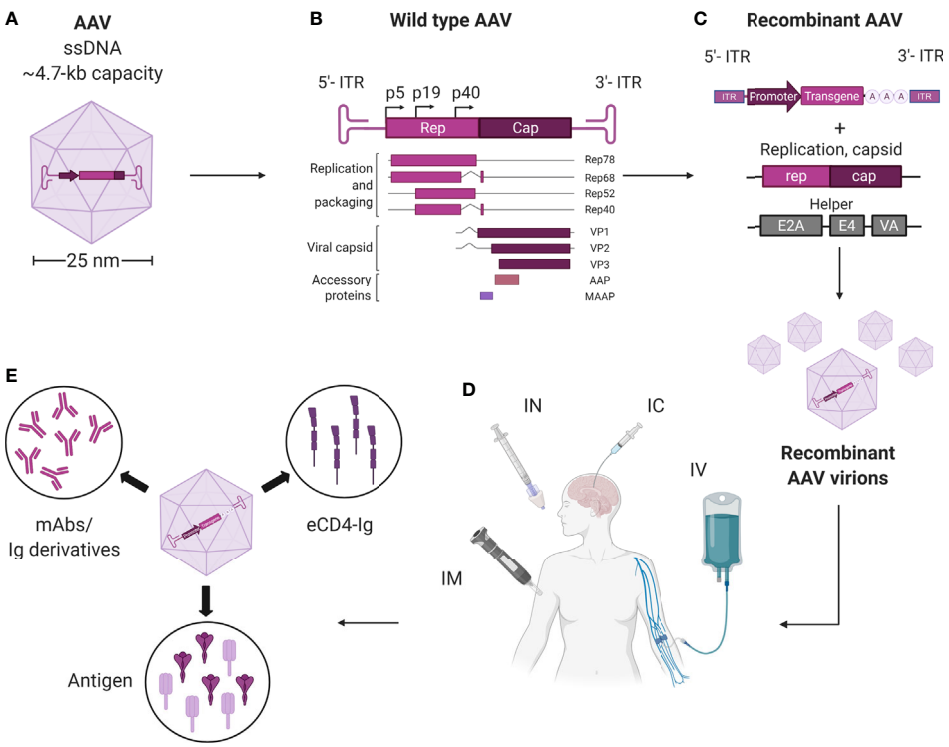

FIGURE 1 | Overview of AAV vectors harnessed for vectored immunoprophylaxis and therapeutics. **(A)** AAVs are small (~25 nm), non-enveloped viruses and have a 4.7-kb, single-stranded, linear DNA (ssDNA) genome encoding four open reading frames. **(B)** *rep* encodes the four genes required for genome replication (Rep78, Rep68, Rep52, and Rep40) and *cap* encodes the structural proteins of the viral capsid (VP1, VP2 and VP3). A third gene, which encodes assembly activating protein (AAP), is embedded within the cap coding sequence in a different reading frame and has been shown to promote virion assembly. The fourth ORF encodes for the recently discovered membrane associated accessory protein (MAAP). The role of MAAP is yet to be clearly defined. **(C)** Providing *rep* and *cap* in trans enables a transgene of interest to be packaged inside the capsid to generate a replication-incompetent vector (recombinant AAV; rAAV). **(D)** rAAVs may be delivered *via* intramuscnular (IM), intranasal (IN), intracerebral (IC), and intravenous (IV) routes. **(E)** rAAVs expressing mAbs, eCD4-Ig and pathogenic antigens can be administered *via* different routes for therapeutics and immunization against infectious diseases. Created with BioRender.com.

chemical alterations (38). This versatility enables the selection of AAV vectors that are better at avoiding pre-existing immunity, targeting the desired tissue, or modulating immune responses.

The encapsidated AAV genome consists of a 4.7-kb linear, single-stranded (ss)DNA that harbors four known open reading frames: *rep*, which encodes for the replication proteins; *cap*, which encodes VP1-3; assembly-activating protein (*AAP*), which promotes capsid assembly within the host cell nucleus; and membrane-associated accessory protein (*MAAP*), whose function is not completely known. The genome is flanked by two T-shaped hairpin structures called inverted terminal repeats (ITRs) (23, 48, 49).

To create rAAV vectors, the genome of wtAAV, save for the ITRs, can be deleted to free up to 4.6 kb of space for transgene cassette packaging (**Figure 1**). Following rAAV transduction, the linear ssDNA genome is converted into circular double-stranded (ds)DNA episomes that reside within the nucleus and are shielded from exonucleases. These episomes are thus highly stable, non-genotoxic, and can be continuously transcribed to enable long-term transgene expression (50–52). For instance, the transgene for the blood coagulation factor IX (F.IX) in one clinical trial patient persisted for more than ten years (53). Alternatively, integration of the rAAV-delivered DNA into the host genome may occur at low frequencies (54, 55). Self-complementary (sc)AAV vectors, in which the sense and the anti-sense sequences of the transgene are packaged inside the vector, can bypass the ssDNA to dsDNA conversion step (23, 48). This allows faster transgene transcription and higher transduction efficiency, but reduces the cargo size by half.

On their own, wtAAVs are non-replicating and require helper viruses, such as adenovirus or herpesvirus to complete their life cycle, while rAAV vectors as a single formulation can deliver transgenes to cells both *in vitro* and *in vivo*. In vitro, rAAV is able to transduce many types of primary human and animal cells, as well as cell lines. This ability is highly dependent on the AAV serotype and the cell type (56). Multiple studies have used rAAV vectors to modify cells ex vivo, whereby the cells are isolated from a patient, modified by vectors, and then transplanted back to the host (57, 58). Natural AAV infections are typically asymptomatic in humans. Nonetheless, several recent reports and clinical trials have demonstrated that immune responses were mounted by the host against rAAV vectors and their transgene products. These findings have been covered by multiple comprehensive reviews describing the known immune pathways triggered by rAAV administration (59–61). But overall, rAAV vectors are non-pathogenic with relatively low immunogenicity profiles, making them attractive for *in vivo* transgene delivery. In the context of infectious diseases, rAAV vectors have been used to deliver mAbs and Ig derivatives to achieve prophylactic and therapeutic benefits. Additionally, rAAV vectors have been used as antigenic gene delivery vehicles; because unlike adenoviruses, rAAVs can confer strong immune response against the transgene with minimal response towards the delivery vehicle (62, 63). In certain cases, rAAVs can induce greater and more sustained antibody responses than other vaccination approaches (62). The different modalities for immunotherapeutics, including non-vectored, vectored, and rAAV-vectored approaches, are summarized in **Figure 2**.

Using rAAVs for *De Novo* Antigen Expression and Induction of Active Immunity

Unlike inactivated, live-attenuated, or subunit vaccines, all of which deliver foreign immunogens in the form of proteinaceous antigens directly to the host, nucleic acids-based vaccines deliver the gene that encodes the antigen for *de novo* synthesis in the host's cells (1, 2). This strategy simulates the antigen expression process during a natural infection, thus preserving the conformation, topology, multimerization, and glycosylation features of the natural antigen. Conceptually, this should allow the generation of high-quality antibodies that are highly specific to the functional target antigen, and reduce the risk of cross-reactivity and ADE (12). If mutations were to arise during an ongoing infection, the genetic sequence carried by the vaccine vector can be quickly modified without dramatic changes in the manufacturing process; while in the case of inactivated vaccines, the production of variants usually requires many more steps of re-optimization. Despite these advantages, nucleic acids-based vaccines do come with an important concern: the delivery of exogenous antigens into the host may lead to inflammation-mediated toxicity against the transduced tissue. In such cases, vectors can be specifically engineered to target only select tissues without affecting others, *via* cell type-specific expression cassette designs.

Nucleic acids-based vaccine strategies can be broadly categorized into DNA- and RNA-based vaccines. DNA-based vaccines tend to allow longer and more stable antigen expression, but present risks for host genome integration and genotoxicity. In contrast, RNA-based vaccines do not interfere with the genome, but are less stable and may require additional support for optimal function, such as cold-chain storage and booster shots (2, 13, 14). rAAVs are DNA vectors that exhibit relatively low genotoxicity and high stability (64). The first use of an rAAV as a vaccine carrier was in an intramuscular delivery of the herpes simplex virus (HSV)-2 glycoprotein B (gB) or glycoprotein D (gD) antigen to induce active immunity against HSV-2 in mice (65). This treatment resulted in antigen-specific cytotoxic T lymphocyte (CTL) responses and induction of humoral immunity that was more effective than treatments with protein subunits of the antigen or antigen-encoding plasmid DNA. This proof-of-concept study opened a new avenue for rAAV-based genetic vaccines.

Using rAAVs for Enhancing Humoral Immunity

Strategies for rAAV vectors to deliver therapeutic antibodies or Ig-derivatives to enhance humoral immunity are under development. Humoral immunity is mediated by antibodies, which are highly functional molecules that play critical roles in the host defense against pathogens (66, 67). Each antibody monomer is a Y-shaped molecule with two antigen-binding fragment (Fab) arms and one Fc tail that is capable of host cell ligation *via* Fc receptors (FcR). Upon first encounter with the pathogen, antibodies of the IgM isotype are released from activated B cells. This isotype has a

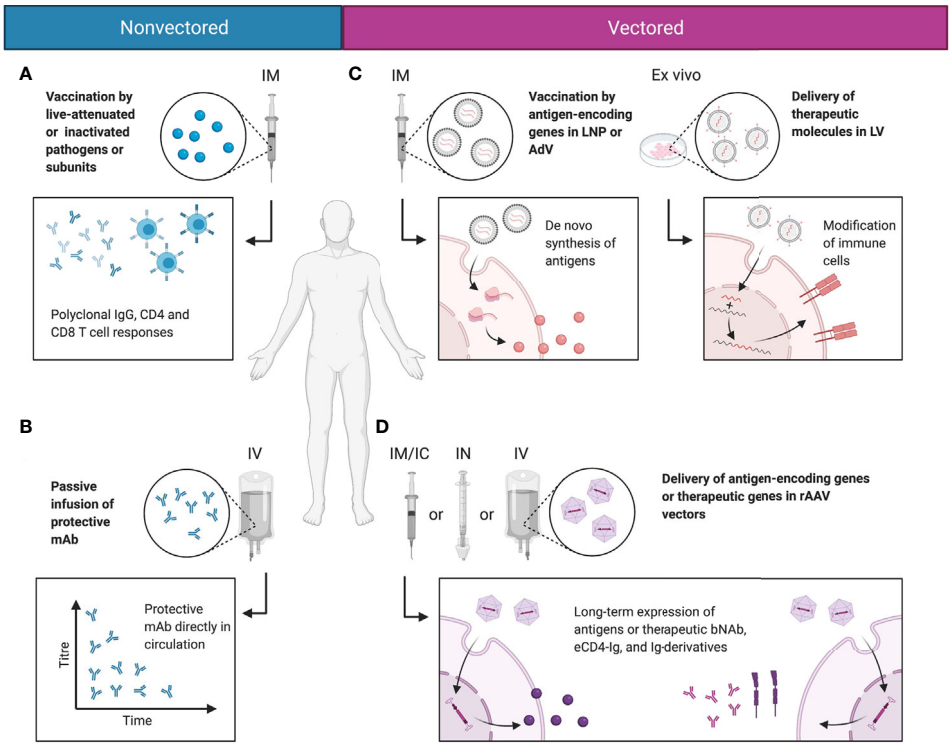

FIGURE 2 | Comparisons between non-vectored and vectored immunotherapeutic strategies. **(A)** Most vaccines today function by delivering antigen in the form of live-attenuated or inactivated pathogens or antigen subunits. This induces polyclonal humoral and cellular responses and immunological memory that protect the host from infections. **(B)** In rapidly mutating pathogens that evade normal vaccine-induced immunity, protective mAbs may be directly delivered to the blood stream *via* passive infusion to mediate protective function. **(C)** Alternatively, genetic material may be delivered in LNPs or viral vectors, such as AdV or LV. This mediates *de novo* synthesis of the antigen in the natural conformation, or allows the modification of immune cells into stronger effector cells. **(D)** rAAV vectors deliver the genetic material of the encoded antigen or therapeutic molecule putatively to the nucleus, as persistent episomes for gene expression. This enables long-term expression of the antigen or therapeutic molecule in a native conformation and topology, and reduces the need for drug redosing. Created with BioRender.com.

pentameric structure with a total of ten antigen-binding sites that enables high-avidity, low-affinity binding for quick antigen recognition. Activated B cells then undergo clonal selection and somatic hypermutation to release antibodies of the IgG isotype, which exhibit high-affinity binding of the invading pathogen. The vast majority of prophylactic and therapeutic mAbs developed to date are IgG antibodies (68).

Under ideal situations, the above processes eventually lead to the production of potent IgGs that are capable of neutralizing the pathogen, targeting it for processing and degradation in antigen-presenting cells (APCs). In addition, certain IgG-producing B cells gain a memory phenotype that confers long-term immunity against reinfections (69). However, this ideal situation is not always achievable in certain infections, as a result of various pathogen-specific issues. In some cases, the pathogen has highly variable antigens, which may be due to their rapid mutation rates or large geographical distributions. For these pathogens, such as HIV-1 and influenza, rAAV vectors can be used to mediate long-term expression of therapeutic recombinant bNAbs (70, 71). If successful, these approaches would be especially useful for the protection of elderly and immunocompromised people, whose humoral response is impaired.

PRACTICAL CONSIDERATIONS IN DEVELOPING rAAV-VECTORED IMMUNOTHERAPIES

rAAV vectors are showing great potential for treating patients with genetic diseases, but if rAAVs are to be deployed for the prevention and treatment of infectious diseases, additional factors need to be considered. Namely, animal studies of current rAAV-based immunotherapeutics have also revealed many issues to be solved before deployment in a clinical setting. Some of these issues, such as rAAV packaging size and pre-existing immunity against the rAAV capsid, are limitations that are inherent with rAAV-based gene delivery systems. Other challenges, such as anti-drug antibodies (ADAs), mAb-mediated toxicity, and issues related to long-term antigen expression in non-target tissues, are unique challenges for rAAV-based immunotherapy platforms. Advancements in AAV vectorology and virology research may help overcome some of these concerns.

rAAV Cargo Size

Using rAAVs to deliver mAbs is a well-explored rAAV-mediated immunotherapy modality. A full-length mAb, which is

composed of two identical heavy chains (~450 aa) and two identical light chains (~220 aa), requires a ~2-kb packaging space minimum for the cDNA alone. Together with promoter sequences, cis-regulatory elements, and other components, each rAAV vector may only accommodate one full-length mAb (72). This may present a therapeutic challenge, since pathogens like HIV-1 optimally require co-delivery of several bNAbs to better suppress escape mutants (73, 74). Regarding rAAV-vectored bNAb delivery, co-injection of three rAAV1 vectors, each encoding a distinctive bNAb, was explored in rhesus macaques (71). Sustained expression of two out of three bNAbs was observed in one rhesus macaque with low ADA.

If scAAV vectors are used for faster mAb generation, the heavy and light chains of the mAb would need to be packaged into more than one rAAV vector (75). When dealing with cargoes that exceed the packaging capacity of AAV vectors, several strategies can be used (76, 77): 1) once inside the cell nucleus, the vector DNAs may reconstitute into the full-length transgene through homologous recombination; 2) the mRNAs from each cargo can be engineered to undergo trans-splicing into a single mRNA transcript for full-length transgene expression; and 3) the polypeptide products of the cargos can be joined into full-length proteins *via* intein-mediated trans-splicing. The DNA and mRNA recombination-based methods would require co-transduction of the same cell by two rAAV vectors, which can be efficiency-limiting. Intein-mediated protein trans-splicing can presumably work extracellularly and eliminates the need for co-transduction of the same cell (78). Given that mAbs are secreted, intein-based methods may be more appropriate in the context of rAAV-mediated immunotherapy. Alternatively, smaller Ig-derivatives, such as bivalent single-chain variable fragments (scFvs), immunoadhesins, or chimeric Ig-like molecules that combine the functional domain with the Fc domain, may suffice as therapeutic surrogates for full-length mAbs (79–82).

ADAs

Passively administered mAbs and AAV-delivered mAbs are subject to reduced potencies by ADAs, which come in the form of host antibodies that target exogenous mAbs (83–85). The clinical sequela of ADAs developed against infused mAbs tend be mild. Patients with ADAs may require higher therapeutic mAb doses. But in extremely rare cases, ADA may be associated with anaphylactic shock (85, 86). For AAV-vectored mAb delivery, ADAs are almost universally detected in preclinical studies that looked for them and are considered the main reason for declining therapeutic mAb titers over time. ADAs can appear as early as two weeks post-AAV transduction and animals with higher levels of ADAs usually present with lower levels of therapeutic mAbs in the blood stream (71, 87, 88). On the plus side, no severe adverse events have been documented in preclinical and clinical studies when ADAs against rAAV-delivered mAbs were detected (89).

Developing a generic solution to ADAs is challenging because ADA development is still an enigmatic process. Therapeutic mAbs might be prone to inducing ADAs, as they are large proteinaceous foreign antigens. However, not all hosts receiving the same mAbs develop ADA. This phenomenon is true for passively administered mAbs and rAAV-delivered mAbs in humans (NCT01937455) (71, 87, 89, 90). Also, new mAbs are constantly generated *via* V(D)J recombination and somatic hypermutation during natural immune responses, but no ADAs against endogenous mAbs have been documented. One important difference among endogenous mAbs and rAAV-delivered mAbs is that their site of production is vastly different. The former is endogenously produced in B cells, while the latter is exogenously expressed in other tissues, most commonly the muscle (**Table 1**). Differences in post-translational processing, like the glycosylation of mAbs with non-native cell types might introduce novel epitopes that can potentiate the development of ADAs (125). One study demonstrated that rAAV8-delivered mAbs presented different glycosylation patterns than mAbs derived from infection of cells *in vitro* (88). If these findings hold true in clinical settings, then one possibility of reducing ADAs is to use B cell-targeting rAAV vectors, such as those based on AAV6, for preferential mAb expression *via* gene-edited B cells (126, 127).

However, several lines of evidence suggests that ADA development, to a large extent, is dependent on the primary sequence of the rAAV-delivered mAb. First, ADA responses are not broad-spectrum, but selective (71). In a triple vector system in which rAAV encoding three different mAbs were delivered to the same non-human primates (NHPs), animals with low ADA response to one mAb can have high ADA response towards the other mAb (71). Second, eCD4-Ig, a modified fusion of CD4 and human IgG Fc, was markedly less immunogenic compared to rhesus-optimized anti-HIV bNAbs (95, 96). ADA responses against eCD4-Ig appeared around four weeks post-transduction and quickly reverted to baseline levels in three out of four NHPs by week 14, while ADA responses against bNAbs persisted until the bNAbs were cleared from the serum. And third, the magnitude of ADA responses in rhesus macaques was positively associated with the degree of sequence divergence from germline V-genes and J-genes (87).

The Fc portion in therapeutic mAbs from non-human origin is thought to drive immunogenicity in human patients (128). However, humanized or fully human mAbs are also immunogenic during passive infusion. Similarly, rAAV-delivered rhesus-optimized anti-HIV bNAbs are immunogenic in rhesus macaques (95, 96). In one study, two murine mAb clones and one human mAb clone were packaged into the same rAAV construct and expressed by the same promoter (116). In recipient mice, the human mAb was expressed at much higher levels than the murine mAbs. While the reason for this observation is unclear, it is possible that the murine mAb-Fc portion might have caused an immune activation against itself, while the human mAb-Fc portion was not as reactive. Alternatively, the Fc portion of an mAB may also potentially lead to complement activation and lysis of the rAAV-transduced cell (93, 129, 130). Nevertheless, there is no experimental evidence that such events happen *in vivo*.

To mediate better bNAb expression, cyclosporin A, an immunosuppressant commonly used during transplantation, was administered to NHPs from 9 days to 28 days after rAAV8-bNAb administration (94). This approach significantly increased average peak bNAb expression from 5 μg/mL to 38 μg/mL, while preserving the capacity of bNAbs to prevent HIV-1 infection. Another method may be to take advantage of liver-induced

TABLE 1 | List of AAV-vectored immunotherapeutic strategies.

Pathogen	AAV serotype	Animal	Injection route	Therapeutic mode	References
HIV		Mouse	IM	bNAb	Lewis 2002 (91)
	AAV2/8	Mouse	IM	bNAb	Balazs 2011 (92)
	AAV1	Rh.M.	IM	bNAb-derived immunoadhesins	Johnson 2009 (93)
	AAV1	Rh.M.	IM	bNAb	Fuchs 2015 (70); Martinez-Navio 2019 (71)
	AAV8	Rh.M	IM	bNAb	Welles 2018 (88); Saunders 2015 (94)
	AAV1	Human	IM	bNAb	Priddy 2019 (89)
	AAV1	Rh.M.	IM	eCD4-Ig	Gardner 2015 (95); 2019 (96)
Flu	AAV2/8	Mouse	IM	bNAb	Balazs 2013 (97)
	AAV9	Mouse, Ferret	IN	bNAb	Limberis 2013a (98), 2013b (99); Adam 2014 (100)
	AAV9	Mouse	IN	Multi-domain Ab	Laursen 2018 (101)
	AAV8	Mouse	IM	Nanobody	Del Rosario 2020 (102)
	AAV8	Mouse	IV	2CARD-MAVS	Nistal-Villán 2015 (103)
	AAV9	Mouse	IN	HA/antigen	Demminger 2020 (104)
SARS-CoV-1/2	AAV2	Mouse	IM	RBD/antigen	Du 2006 (63)
	AAV2	Mouse	IN	RBD/antigen	Du 2008 (105)
	AAVrh32.33		IM	Spike/antigen	Vandenberghe and Freeman
	Undisclosed		IN	bNAb	Wilson
Malaria	AAV1, AAV3	Mouse	IM	MSP4/5/antigen	Logan 2007 (106)
	AAV8	Mouse	IM	mAb	Deal 2014 (107)
	AdHu5/AAV1	Mouse	IM	PfCSP/Pfs25/antigen	Yusuf 2019a (108), 2019b (109)
	AAV8	Mouse	IV	miR-155	Hentzschel 2014 (110)
HCV	AAV8	Mouse	IV	NS5B/antigen	Mekonnen 2020 (111)
	AAVrh32.33	Mouse	IM	NS3/4/antigen	Zhu 2015 (112)
	AAV8, AAVrh32.33	Mouse	IM	E2/antigen	Zhu 2019 (113)
HPV/Cervical cancer	AAV1	Mouse	IM	E7/antigen	Zhou 2010 (114)
	AAV5, AAV9	Rh.M.	IN	L1/antigen	Nieto 2012 (115)
Ebola	AAV9	Mouse	IM, IN	mAb	Limberis 2016 (116)
	AAV9	Mouse	IV, IM, IN	mAb	Robert 2017 (117)
	AAV6.2FF	Mouse	IM	mAb	van Lieshout 2018 (118)
Dengue	AAV1	Rh.M.	IM	mAb	Magnani 2017 (119)
	AAVrh32.33, AAV8	Mouse	IM	79E/antigen	Li 2012 (120)
	AAV6, AAV9	Mouse	SC, IM	EDIII/antigen	Slon-Campos 2020 (121)
Prion	AAV2	Mouse	IC	mAb-derived scFv	Wuertzer 2008 (82)
	AAV2	Mouse	IC	mAb-derived scFv	Zuber 2008 (122)
	AAV9	Mouse	IC	mAb-derived scFv	Moda 2012 (123)
Rabies		Mouse	IM	G/antigen	Liu 2020 (124)
Anthrax	Ad5/AAVrh.10	Mouse	IV/Intrapleural	mAb	De 2008 (30)
RSV	Ad5/AAVrh.10	Mouse	IV/Intrapleural	mAb	Skaricic 2008 (28)

IM, intramuscular; IN, intranasal; IV, intravenous; SC, subcutaneous; IC, intracerebral; Rh.M., rhesus macaques.

tolerance of the transgene (131), which primarily results from nonconventional antigen presentation in the liver, causing T cell anergy, apoptosis, and T regulatory (Treg) cell expansion (132). A circumstantial piece of evidence that this strategy may work is that mAb delivery using rAAV8, a serotype with strong tropism in liver, demonstrated less frequent ADA formation than mAb delivery using rAAV1 (~20% ADA-positive in rAAV8 vectors vs. > 50% ADA-positive in rAAV1 vectors) (87, 88). However, there are too many experimental variables between the two studies and direct comparisons are not meaningful.

Transgene Toxicity

The delivery of antigens in the form of codon-optimized genes *via* a vectored approach will undeniably result in foreign antigen expression in injected or target tissues. Hypothetically, this may lead to inflammation-mediated immunotoxicity against the transduced host tissue. For LNP-vectored mRNAs, this concern is alleviated because mRNAs are inherently unstable and rapidly degraded (2). However, rAAVs are DNA vectors that are capable of mediating long-term antigen expression. To prevent potentially undesirable toxicity, the rAAV vector may be engineered to target specific cell types, such as muscle, without affecting others.

Another major risk is that mAbs are immunologically active molecules with the propensity to activate multiple inflammatory pathways that can mediate tissue damage. When they bind non-specifically, they may cause unanticipated adverse events. Preclinical testing, such as passive administration and human tissue binding studies, can help to avert most of these issues. However, if off-target effects do occur *in vivo*, there is currently no effective method to control antibody transgene expression. As implicated in animal studies, rAAV-mediated mAb expression can persist for life following a single administration of rAAVs (71). In the event that rAAV-mediated mAb is causing off-target toxicity, designing transgene expression cassettes with regulatory elements that can act as on/off switches might solve this problem. Several studies in mice and monkeys demonstrated that rAAV transgene expression levels may be controlled *via* small-molecule drugs (133–135). For example, the mAb may be placed under a

rapamycin-regulated promoter (133). Another possibility is to inactivate rAAV-delivered transgenes by destroying the vector DNA. This has been explored in the context of rAAV-mediated CRISPR-Cas9 delivery, in which a second rAAV encoding anti-Cas9 gRNA was able to reduce Cas9 expression (136).

Pre-Existing Anti-rAAV Immunity

In the context of infectious diseases, successful prevention programs rely on the establishment of herd immunity, whereby a large enough proportion of the population is immunized to cut off the spread of infections. However, pre-existing immunity against AAV capsids significantly limits the pool of eligible recipients (137). Circulating NAbs against AAV serotypes can occur immediately following the disappearance of maternal antibodies (138, 139). Even low titers of pre-existing NAbs can negatively impact vector transduction (140–142). Capsid-specific T cell responses represent another major obstacle for rAAV vector transduction. Prior AAV infections have been proposed to elicit memory T cell responses against the virus (143, 144). These memory $CD8^+$ T cell responses are more easily triggered than naïve $CD8^+$ T cells and were shown to eliminate host hepatocytes that were successfully transduced by rAAV vectors (141), resulting in reduced or absent transgene expression in clinical trials of rAAV gene therapies for muscle and liver-related genetic diseases (143, 145, 146).

Some direct immunity-inhibiting strategies have been developed to overcome pre-existing immunity against the rAAV capsid, such as using plasmapheresis or IgG-degrading enzymes to remove pre-existing anti-AAV antibodies, or using rapamycin nanoparticles to reduce rAAV immunogenicity (147–149). Unfortunately, these strategies may not be suitable for rAAV-mediated immunotherapy for infectious diseases, because these platforms require an intact immune system. Alternatively, structural modification of NAb recognition sites, directed evolution or rational engineering to generate novel capsids, rAAV epitope masking, chemical modifications, and injecting empty AAV capsids to act as decoys, may be viable evasion strategies against pre-existing immunity. These strategies have been described and reviewed elsewhere (23, 150–163). One potential complication is that, antigen-specific $CD8^+$ T cells do not recognize the whole virion but only a short peptide sequence (typically 8 to 14 amino acids in length) presented by major histocompatibility molecule class I (164). Thus, a memory $CD8^+$ T cell clone that was induced by one AAV capsid might be cross-reactive against another AAV capsid, if both capsids share the same peptide antigen recognized by the $CD8^+$ T clone. Such possibilities, however, have only been demonstrated ex vivo (143). Lastly, administration of rAAV vectors *via* intramuscular, intranasal, or intracerebral routes might be able to reduce or prevent vector encounter with anti-capsid NAbs and T cells (**Table 1**).

Immune Activation Against rAAV Transgenes

In addition to the anti-AAV NAb and ADA mechanisms discussed above, adaptive immunity against rAAV-vectored immunotherapies can also be encountered in the form of anti-transgene $CD4^+$ and $CD8^+$ T cell responses, which may result in inflammatory toxicities and other adverse events (165–173). On the other hand, immune activation might be a prerequisite if rAAV vectors are used as a delivery vehicle for vaccination against the delivered antigen.

Innate immunity against rAAVs is remarkably subdued as compared to other viral vectors, but the transgene cargo can be recognized by various pattern-recognition receptors (PRRs) (61). Toll-like receptor (TLR)9 recognizes unmethylated CpG DNA motifs in the genome cargo of rAAV vectors when they are exposed in endosomes and lysosomes, while TLR2 recognizes the AAV capsid. Both of these sensors lead to the activation of type 1 interferons *via* the MyD88 signaling pathway and play vital roles in shaping immune responses (174–182). To reduce TLR9 detection, CpG motifs may be removed from the rAAV cargo (176). More recently, short-noncoding DNA sequences that antagonize TLR9 activation were engineered into the vector genome to prevent the detection of the transgene DNA (183).

To control transgene-specific T cell responses, miRNA-mediated regulation may be exploited to prevent transgene expression from antigen-presenting cells (APCs). For example, binding sites for myeloid-specific miRNA-142 can be engineered into the 3'-untranslated region of the transgene, so that its transcript is destroyed in APCs but not in other cell types (184, 185). Conversely, vectors based on AAVrh.32.33 can induce a robust CD8 T cell response to the transgene product and has been used to express antigens for vaccination purposes (112, 113, 120, 186). Additionally, scAAV vectors induce stronger CD8 T cell responses and humoral responses against the transgene compared to corresponding ssAAV vectors (187).

Manufacturing and Storage

When deploying rAAV vectors to combat infectious diseases in a large portion of the world's total population, large production pipelines that can yield consistent quality will be required (188, 189). In addition, current manufacturing processes are prone to introducing empty and partially packaged vectors, thus reducing the purity and efficacy of the rAAV drug, while running the risk of causing deleterious immune activation (190). A standard for rAAV purity should be established, while major innovations in rAAV manufacturing is required. Once rAAV vectors are manufactured, they need to be properly stored to ensure their stabilities. Compared to LNP-mRNA vectors, which currently require stringent storage conditions (-20°C to -80°C) (13, 14), rAAV vectors are generally stable for short periods at room temperature under typical laboratory conditions (64, 186). When distributed as vaccines or mAb carriers, rAAV vectors must confront shipment and handling hazards. Nevertheless, the stability of rAAV vectors might confer an advantage in areas of the world where cold-chain transport facilities are lacking.

rAAV-BASED IMMUNOTHERAPIES IN DEVELOPMENT

HIV-1

Despite the advent of antiretroviral therapies, HIV-1 continues to be a major threat to public health, with an estimated 38 million

people living with HIV-1 infection and an additional 33 million deceased to date (191). HIV-1 is a lentivirus belonging to the *Retroviridae* family. It harbors two copies of its single-stranded RNA genome, enclosed inside an inner capsid structure and an outer lipid bilayer (192). This lipid bilayer is decorated with HIV envelope glycoproteins gp120/gp41, which use human CD4 as the main receptor, and either CCR5 or CXCR4 as coreceptors for virion attachment and membrane fusion (193). The error-prone nature of reverse transcription, coupled with host-restriction factor APOBEC3-mediated guanosine-to-adenosine mutations, and genomic recombination with other HIV strains, numerous mutations can be introduce into the HIV genome, resulting in antigenic drift, with the potential to evade immune recognition and impart drug resistance (194, 195). No vaccination regimen for HIV has been able to successfully induce bNAb responses. Additionally, HIV specifically targets CD4 T cells and CD4-expressing monocytes, macrophages, dendritic cells, and microglial cells *via* its use of CD4, CCR5, and CXCR4 as receptors/coreceptors. The loss of these essential immune cell populations greatly impairs antiviral response. Finally, HIV can lay dormant inside the host genome without active gene transcription, thus, evading peptide presentation on MHC molecules and detection by T cells (192).

Developing protective humoral immunity against HIV-1 has been particularly challenging. Only bNAbs or antibody clones that recognize multiple strains of HIV-1 have the potential to overcome their high genomic variability (196). Second, each HIV-1 virion has only 4 to 35 glycoprotein Gp120/Gp41 trimer spikes on its surface, while a similarly sized influenza virus has ~450 spikes per virion (197). This means that the monomeric IgG may likely only binds to a single Gp120/Gp41 molecule *via* one of its two Fab arms (197). Thus, the bound IgG is easily detached as a result of its low avidity (197). To stabilize IgG-Gp120/Gp41 binding, the unbound Fab arm must bind to another molecule, usually the virion lipid bilayer or a host transmembrane protein that is incorporated into the envelop during budding and viral egress. This requires the HIV-1-bound IgG to be polyreactive, and in fact, many bNAbs do exhibit a certain degree of polyreactivity and self-reactivity (196). And third, HIV-1 directly impairs CD4 T cells, whose help is critical to proper B cell and antibody development (198). As a result, bNAbs only occur in a minority of HIV-1-infected individuals and only after several years into the infection. Passive infusion using a combination of bNAbs has been able to suppress viremia in HIV-infected patients and offers prophylaxis in simian models (199–201). However, different bNAbs have varied circulatory half-lives and repeated dosing is required, which is impractical. With combinatorial treatments using two bNAbs, the diminishing titer of one bNAb can drive virus evolution towards resistance to the other bNAb (200, 202).

The development of rAAV-based HIV immunotherapies has had mixed, yet promising success. For example, to achieve sustained HIV-1/SIV inhibition in a rhesus macaques model, simian Gp120-specific chimeric immunoadhesins 4L6 and 5L7 (made by fusing select Fabs to the Fc portion of simian IgG2) were packaged into rAAV1 vectors, and tested by IM delivery (93). Four weeks after rAAV transduction, the macaques were challenged intravenously with a lethal dose of SIVmac316. Three out of three with rAAV1-*4L6* and one out of three with rAAV1-*5L7* were completely protected from infection. In another study, macaques were dosed with rAAV1 packaged with *4L6-IgG1* or *5L7-IgG1* transgenes, and then received six intravenous challenges at escalating doses with another SIV strain, SIVmac239 (70). Interestingly, one rAAV1-*5L7-IgG1*-tranduced animal resisted all six challenges, while the other animals presented lower viral loads and slower progression to peak viral loads, indicating that rAAV1-*5L7-IgG1* was partially protective against SIVmac239. This partial efficacy could be explained by the fact that 5L7 conferred strong NK-dependent and antibody-dependent cellular cytotoxicity (ADCC) (70). In another study, rAAV8 vectors carrying anti-SIV mAbs ITS01 and ITS06.02 transgenes were co-administered into macaques by intramuscular injection in a dual vector approach. Animals later received repeated low-dose intrarectal challenge with SIVsmE660 (88). Approximately 90% protection was achieved by these vectors. In a groundbreaking study, a cocktail of three different AAV1 vectors, each encoding a human HIV bNAb (10E8, 3BNC117, or 10-1074), was given to four macaques with preexisting SHIV (SIV-HIV amalgamation) viremia (71). Remarkably, this led to the complete suppression of viremia in a single animal within weeks of rAAV administration. This outcome was maintained throughout the course of the 240-week study, suggesting that a functional cure was achievable. These partial or complete successes in animal models have led to a phase I trial (NCT01937455), in which the HIV-1 bNAb PG9 was packaged into the AAV1 capsid and given to healthy humans at 4×10^{12} to 1.2×10^{14} vector genomes per subject (89). This approach was well-tolerated, with only mild to moderate side effects that resolved without intervention. However, PG9 could not be detected in the volunteer sera, while anti-PG9 ADA was readily detected in 10 out of 16 volunteers. Only 2 out of 16 volunteers demonstrated neutralization activity against a small number of HIV-1 isolates.

Since CD4 is the universal host receptor for all Gp120/Gp41 variants, one potential solution to counter all HIV-1 strains is to overexpress soluble forms of CD4, which act as decoys to saturate the CD4-binding site of Gp120/Gp41. One such attempt combined parts of CD4 to CCR5mim1, a sulfopeptide that binds CCR5- and CXCR4-tropic viruses. These domains are then fused to the human IgG1 Fc (95). This fusion protein, named eCD4-Ig, or its variants can neutralize a wide panel of HIV isolates, as well as several SIV isolates *in vitro*. The rhesus form of the transgene (*rh-eCD4-Ig*) was delivered intramuscularly by an AAV1 vector (AAV1-*rh-eCD4-Ig*) into macaques. Upon repeated IV challenge with SHIV-AD8, none of the vector-transduced animals became infected, even at the highest dose (95). AAV1-*rh-eCD4-Ig*-transduced macaques were also protected from infection by SIVmac239, of which the gp120/gp41 complex is highly divergent from SHIV-AD8 (96). Escalating doses of SIVmac239 eventually infected all AAV-*rh-eCD4-Ig*-transduced macaques, but the viruses also developed escape mutations that came with fitness costs. Nonetheless, the research for rAAV-based HIV vaccines is ongoing and further advancements are necessary to ensure these therapies are more efficacious.

Influenza

Influenza infections are the seventh leading cause of death in the United States, with more than 20,000 fatalities recorded in the last year (203). Illnesses range from mild to severe, and even death. Hospitalization and death occur mainly among high risk groups. Individuals with a reduced capacity to mount an immune response upon infection have an increased susceptibility to influenza infections and complications, which include fatal pneumonia and acute respiratory distress syndrome (ARDS) (204). Vaccines are pivotal for influenza prevention, but their efficacies are substantially reduced in the elderly (205–207).

Unlike HIV-1, protective humoral immunity is easily generated against the flu virus by vaccination. However, the flu genome can undergo antigenic shift, a unique influenza virus-associated phenomena that poses additional difficulties (208). The influenza virus is made up of eight segmented negative-sense RNA strands enclosed inside a lipid bilayer that is studded by glycoproteins hemagglutinin (HA) and neuraminidase (NA). When the same organism is infected by multiple influenza subtypes, the RNA segments can be reshuffled to produce novel subtypes in a process called antigenic shift. In addition, influenza RNA polymerase is also highly error-prone, leading to the accumulation of mutations *via* antigenic drift (209).

Most antibodies produced in response to seasonal flu vaccines target the receptor binding site (RBS) within the globular HA-head region (210). Although functional against the vaccinated subtype, they can be rendered less effective by HA mutations and reshuffling. High-affinity bNAbs that can bind a broad array of influenza viruses have been isolated (211–214). They can offer protection by inhibiting fusion of the viral and cellular membranes (215–217), or by Fc receptor (FcR)-mediated mechanisms *via* ADCC (218). However, bNAbs usually target the more conserved regions of HA, which are less accessible than the globular head region and are thus more difficult to vaccinate against (215–217). Variable regions from the heavy and light chains of F10 and CR6261 bNAbs packaged into rAAV2/8 have been tested by administration into mice by intramuscular injection (97, 214, 217, 219). This treatment led to bNAb expression in mice within one week of rAAV administration, and protected mice against lethal influenza challenges with different H1N1 strains (97). Another study made a recombinant antibody-like molecule by fusing alpaca-derived single domain antibody (nanobody) to Fc domains (102). This construct named R1a-B6-Fc was delivered using an rAAV8 vector *via* intramuscular injection, resulting in high-level transgene expression in sera for at least six months, and conferred complete protection against lethal H1N1 and H5N1 challenges.

Given that the respiratory tract is the primary target of influenza virus, the possibility of applying rAAV vectors intranasally has also been explored (104, 220). This administration route is thought to be superior. Intranasal delivery of rAAVs and its transgene product are targeted to nasal epithelia, and has the ability to circumvent pre-existing anti-AAV immune responses, while providing passive immunization. As a proof-of-concept, a potent bNAb against various influenza A subtypes (FI6) (98, 99), was designed into an rAAV9 vector and delivered into mice and ferrets *via* intranasal distillation (98, 100). This afforded protection against several clinical isolates of H5N1 and H1N1, and provided partial protection in mice against clinical isolates of H7N9 (99). An alternative design packaging a humanized, multidomain recombinant antibody (MD3606) into an rAAV9 vector, protected mice against mouse-adapted influenza H1N1, H3N2, and B viruses following intranasal instillation (101). Investigation with NHPs and humans in clinical trials should provide insight into whether these approaches will be efficacious and more effective than conventional vaccines.

Dengue

Dengue viruses (DENVs) are members of the *Flaviviridae* family and are comprised of four distinct serotypes (DENV1-4) (221, 222). DENVs are enveloped and have a plus-stranded RNA genome. DENV infections may be asymptomatic or characterized as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). There are approximately 3.9 billion people who are at risk of dengue virus infection around the world. Up to 390 million people are infected with dengue virus annually in over 100 endemic countries, with 70% of the actual burden being in Asia (223, 224). DENV is primarily caused by the spread of mosquito vectors and the growth of worldwide travel, and represents a significant global public health problem. Development of safe and effective immunotherapeutics and vaccines are thus a top priority that have yet to be met.

The major challenge for humoral immunity against dengue viruses (DENVs) is avoiding ADE, where the presence of specific antibodies actually enhances DENV pathogenesis (225). The DENV coat is decorated with viral envelope (E) and membrane (M) proteins (221, 222). Without pre-existing DENV antibodies, DENV E proteins bind to specific host cell receptors, leading to virus uncoating and genome release. Infection by one DENV serotype leads to production of antibodies that are cross-reactive with other serotypes. These cross-reactive antibodies do not neutralize DENV, but instead tag the virion for FcR-mediated endocytosis and productive infection (226). Several lines of evidence suggest that these cross-reactive antibodies can increase DENV disease severity. First, mAbs induced by DENVs can increase virus infection of FcR-bearing cells (227). Second, passive immunization of mice with DENV antibodies produced higher viremia following DENV infection, resulting in the death of the infected animals (228). And third, in human populations with high rates of DENV re-infections, the presence of low-titer DENV-specific antibodies is associated with a higher risk of severe disease when patients are infected with a different serotype (229, 230). These outcomes pose significant challenges to DENV vaccine development. As a matter of fact, Dengvaxia, the only licensed DENV vaccine, is only recommended in populations with prior DENV infections, as it is thought to increase dengue disease severity in naïve individuals (231). On the other hand, re-infection with the same DENV serotype leads to efficient neutralization by pre-existing antibodies (232–235).

Given the nature of ADE in DENV immunotherapy, it may be feasible that a cocktail consisting of four AAV-mAb constructs, each encoding a recombinant antibody that can

specifically neutralize one of the four DENV serotypes, may protect the host against all serotypes. In addition, the mAbs inside AAV-mAb constructs may be selected *a priori*, for those that lack ADE capacity. This precise strategy has not been attempted. Instead, anti-DENV3 NAb P3D05 was packaged in rAAV1 and delivered to macaques *via* intramuscular injection (119). Despite the high P3D05 expression that lasted for months, DENV3 infection, replication, and the development escape mutants were unaffected. Better understanding of the interactions between NAbs and DENVs, and screening of stronger NAbs are necessary to improve vector design and transduction outcomes.

SARS-CoV-2

COVID-19 emerged during the fall and winter of 2019 and was declared a global pandemic by the World Health Organization on March 11, 2020 (236, 237). As of February 27th, 2021, more than 100 million people worldwide have been infected with SARS-CoV-2 and more than two million deaths have been reported (238). SARS-CoV-2 is a coronavirus composed of a positive-sense RNA genome that is enclosed inside a lipid membrane, which is heavily studded by transmembrane S proteins (239). The S protein contains the receptor binding domain (RBD) and directly interacts with the host angiotensin-converting enzyme 2 (ACE2) receptor, which is essential for virus attachment and cell invasion (239, 240). Current vaccine development has focused on inducing antibody responses against the S protein. However, previous studies on SARS-CoV, a virus with ~80% sequence identity with SARS-CoV2, suggests that ADE induction may be a concern. Some studies have demonstrated that anti-S antibodies, or certain vaccine compositions, could increase virus uptake in macrophages, elevate pulmonary infiltration of proinflammatory cells, and exacerbate lung injury. Other studies have shown that immunization reduces viral load and protects the lung from severe damage (241–244).

After examining the available evidence from SARS-CoV and DENV studies, it appears that the key to avoiding ADE, while achieving antibody-mediated protection, is to generate highly specific NAbs at high doses without inducing low-potency antibodies that bind, but do not neutralize (12). To achieve this outcome, the vaccinating antigen must resemble the native antigen; hence, the majority of COVID-19 vaccine designs use the S-2P recombinant protein that contains two proline insertions to stabilize the vaccinating antigen (240). Two such designs, one by Pfizer/BioNTech and another by Moderna, demonstrated greater than 94% efficacy in phase III trials and are currently being distributed in the United States under emergency use (13, 14). The underlying mechanisms for achieving such high efficacies in these two vaccine compositions are still being investigated, but NAbs against SARS-CoV-2 are thought to play an important role (245–247).

Recently, an rAAV platform called AAVCOVID, which borrows from the same principle of expressing the S protein antigen by packaging the SARS-CoV-2 S gene within an AAV capsid called AAVrh32.33 is being explored as an experimental vaccine (186, 248). The basis of the strategy relies on two previous findings. First, AAVrh32.33, a hybrid of two AAV natural capsid sequences isolated from rhesus macaque, has been shown to induce high antibody titers and potent CD8+ T cell responses in mice and non-human primates (249–251). And second, rAAV2 vector expressing the RBD of SARS-CoV, the virus responsible for the 2002 SARS epidemic, can induced sufficient neutralizing antibody against SARS-CoV infection with a single intramuscular injection (63). In addition, intranasal instillation can induce a strong local humoral response, and elicited stronger systemic and local specific cytotoxic T cell responses than intramuscular injections. Nevertheless, the protection against SARS-CoV challenge was comparable for both modes of administration (105).

Another immunotherapy strategy is to directly deliver a cocktail of neutralizing mAbs (casirivimab and imdevimab) that target different regions of the RBD, in order to prevent further viral spread in patients (252–254). This method was recently granted an emergency use authorization by the FDA in certain high-risk patients with mild to moderate COVID-19 (255), and a phase I-III trial is ongoing to analyze its safety and efficacy (NCT04425629). The interim results suggest that intravenous and subcutaneous administration of the cocktail resulted in mAb presence for a month or more, was generally safe, and reduced viral loads in COVID-19 patients. Based on these preliminary results, research is underway to investigate whether intranasal administration of rAAVs carrying casirivimab and imdevimab can confer long-term mAb expression in the nasal mucosa for prophylaxis against COVID-19 (256).

Prion Disease

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are caused by the misfolding of the normal cellular prion protein (PrP^C) into the abnormal pathogenic scrapie isoform (PrP^{Sc}), or simply prions (257). Unlike PrP^C, prions are resistant to protease digestion and are capable of converting normal PrP^C into more prions. Prion accumulation primarily affects the central nervous system (CNS) and leads to a series of neurological degenerative disorders that inexorably ends in death. Drug development for prion diseases is challenging, because in order to gain access to prions, the drugs must cross the blood brain barrier to efficiently reach the CNS. It has been demonstrated that anti-PrP^C mAbs could protect the normal PrP^C from interacting with the pathogenic prion and slow disease progression (258, 259). However, these antibodies and other compounds typically show limited efficacy in long-term animal studies. This failure is likely due to host tolerance to endogenous PrP^C and poor antibody diffusion into neuronal tissues (260–264).

The natural tropism of certain AAV serotypes towards the CNS presents a huge advantage for rAAV-vectored mAb delivery (265). To this end, several studies have tested rAAV vectors that deliver scFv proteins *via* the intracerebral route (82, 122, 123, 266). These scFvs were made by fusing the variable regions of the heavy and light chains of an mAb clone, which preserved the antigen-recognition capacity of the original mAb, but lacks Fc-mediated function. Intracerebral delivery of rAAV2 or rAAV9-packaged scFvs that binds to PrP^C can delay prion disease onset in mice (82, 123). An rAAV2 vector that delivers scFvs against the laminin receptor, which interacts with both PrP^C and prions, also delayed onset of prion pathogenesis and decreased PrP^{Sc}

burden in the CNS of mice (82, 122, 123, 266). Further studies are needed to assess the translatability of the platform in humans.

Filoviruses

Filoviruses, which include Ebola virus, Marburg virus, and others, are notable for their capacity to cause highly lethal infections in humans (267). A live attenuated vaccine, trade-named Ervebo, is available for *Zaire ebolavirus*, a highly pathogenic strain of Ebola (268). This vaccine consists of a pseudotyped vesicular-stomatitis virus (VSV) that expresses *Zaire ebolavirus* glycoprotein (GP) and has near 100% efficacy. However, no vaccines are yet available for other filoviruses, which tend to cause sporadic outbreaks and lack exhaustive study (269). Furthermore, no targeted treatments are available for patients with symptomatic infections.

Several studies have demonstrated that mAbs raised against the surface glycoprotein (GP) of Zaire ebolavirus are capable of protecting experimental animals from lethal Ebola virus infections within a therapeutic window of three days pre-challenge to five days post-challenge (270–273). In light of these findings, several groups examined whether rAAV vectors can be used to deliver long-lasting Ebola-specific mAbs for durable prophylaxis and therapeutics. *Zaire ebolavirus* GP-recognizing mAbs were designed into rAAV9 vectors and delivered into mice *via* intramuscular, intravenous, or intranasal routes. Vector-treated animals showed rescue and longer survival from lethal Ebola challenges than animals subjected to mAb infusion (116, 117). Injection by intravenous and intramuscular routes offered better protection against Ebola virus as compared to intranasal administration, and the level of protection significantly increased with vector dose. Notably, an AAV6 variant, called AAV6.2FF, was used to package Ebola-specific mAbs (118). In this study, treatment with a single vector rAAV6.2FF-mAb clone presented 83% to 100% efficiency, while a dual therapy using two vectored mAb clones resulted in complete protection. The protective effect lasted for at least five months after a single rAAV6.2FF-mAb administration. However, treatment with a seven-day lead time was required for complete protection, suggesting that this method of mAb delivery may be too delayed for treating patients with symptoms, since the entire disease course spans only 14 to 21 days (274).

Anthrax and RSV

Some pathogens require a rapid response to block progression of the disease. To promote rapid transgene expression, one strategy is to use a dual-vector approach with an initial AdV treatment to mediate rapid expression, followed by an rAAV vector to mediate long-lasting expression. This formulation is explored in treating infections by *B. anthracis*, a potential bioweapon in terrorist attacks (275), and in RSV, a common cause of respiratory disease in infants and adults (276).

Antibodies against anthrax protective antigen (PA) are effective at inhibiting anthrax lethal toxin (LT)-mediated damage (277). To achieve rapid inhibition of toxin, a dual-vector platform comprising of recombinant anti-PA antibody packaged into Ad5 and AAVrh.10 vectors was developed (30). The Ad5-anti-PA and AAVrh.10-anti-PA vectors were then administered into mice *via* intravenous and intrapleural injection routes, respectively. While Ad5-anti-PA conferred complete protection against LT challenge between one day and eight weeks post-injection, the AAVrh.10-anti-PA conferred complete protection against LT challenge from two weeks to 26 weeks. When both vectors were given together, complete protection was observed from day 1 to week 26 post-injection.

The dual-vector strategy for RSV utilizes the murine form of the anti-RSV drug palivizumab (28). While intravenous injection of Ad5-anti-RSV mAb produced high mAb titers at three days post-administration, intrapleural injection of AAVrh.10-anti-RSV mAb took four to eight weeks to reach high mAb titers in the serum, but reduced RSV viral load for at least 21 weeks post-injection.

Malaria

Malaria presents many unique challenges for vaccine development (278). Malaria is caused by many species of parasites within the genus *Plasmodium*. These parasites, particularly *P. falciparum*, undergo complex life cycles that transmit between humans and mosquitos, and between different host tissue types (279). All of these features increase antigen diversity and pose difficulties for vaccine target selection. For reasons not completely understood, memory B cells against malaria do not persist very long in either natural infections or in vaccination attempts, resulting in the lack of persistent humoral immunity and repeated infections (280–282).

To overcome the inability for humans to retain humoral immunity towards *P. falciparum*, rAAV vectors can be used to mediate long-term expression of the malarial antigens, thus enabling continued stimulation of the immune system. Given that AAVs naturally have low immunogenicity, AdV formulations may be added to improve the immune response raised against the parasite. For example, in a dual-vector strategy, Ad5 and rAAV1 were co-packaged with the *P. falciparum* circumsporozoite protein (PfCSP) and its sexual stage P25 protein (Pfs25). PfCSP is relatively conserved across different *Plasmodium* species, while immunity against Pfs25 is thought to block transmission (283). The Ad5-*Pfs25-PfCSP* vector was given to mice *via* intramuscular injection, followed by AAV1-*Pfs25-PfCSP* vector six weeks later (108). The dual-vectored Ad5-prime/AAV1-boost regimen was highly effective in mice for both full protection and transmission-blocking activity against transgenic *P. berghei* parasites expressing the corresponding *P. falciparum* antigens (108). Remarkably, antibody responses raised in this manner were sustained for over nine months after boosting, and maintained high levels of transmission-reducing activity (108, 109).

Alternatively, rAAV vectors are capable of mediating mAb expression outside of B cells, thus escaping malaria-mediated B cell suppression. To show this ability, rAAV2 vectors expressing human mAbs against PfCSP were delivered to mice *via* intramuscular injection (107). This method offered sterile immunity against the rodent *Plasmodium berghei* strain *via* mosquito bites in all mice that expressed 1 mg/mL or more of the mAb. Expression of mAb also lasted for 8 to 11 weeks, or at the end points of the experiment. It remains to be determined whether rAAV-delivered recombinant mAbs can persist longer than naturally formed anti-malaria mAbs.

HCV

HCV is a single-stranded, positive-sense RNA virus that uses an error-prone RNA polymerase for genome replication (284). This feature results in high inter-host and intra-host genetic variability that limits the effectiveness of humoral immunity. The non-structural proteins of HCV contain several highly conserved regions with potential to act as vaccine epitopes (285). Intravenous injections of rAAV expressing NS5B in mice elicit strong and durable intrahepatic, NS5B-specific, CD8 T cell activation (111). Additionally, rAAVrh32.33 packaged with the HCV E2 envelope protein can elicit a strong antibody response, while rAAVrh32.33 packaged with HCV NS3 or NS3/4 can elicit both antibody and cellular responses (112, 113). Further studies are needed to assess whether these strategies are effective in protecting against HCV infection in NHP models and human subjects.

Rabies

Rabies is a severe infection of the CNS that is always nearly fatal. It is caused by zoonotic viruses that belong to the Lyssavirus genus, the most common member being rabies virus (RABV). RABV is responsible for the majority of rabies infections in humans (286). Vaccines and antisera are effective at preventing disease onset following RABV exposure (287). However, in areas where rabies is still endemic, awareness and accessibility to these preventative measures are limited (288). Furthermore, no cure is available once symptoms start to appear. Hence, there is an urgent need to develop prophylactic and treatment regimen against RABV.

Hypothetically, the strong tropism of certain rAAV serotypes toward the nervous system might allow rAAV-mediated delivery of therapeutic mAbs to the RABV-affected tissues. This possibility has not been extensively explored for the treatment of rabies. An attempt to use rAAVs for immunization against RABV was performed in mice (124). In this work, rAAVs containing glycoproteins from various strains of RABV (rAAV-G) were administered *via* intramuscular injection. All rAAV-G treatments induced higher neutralizing antibody titers and cytokine responses than the attenuated RABV vaccine LBNSE-GMCSF. Furthermore, rAAV-G protected mice from intracerebral challenges of RABV for nine months post-administration. It is unclear whether rAAV-G can offer protection against RABV post-exposure.

Human Papillomavirus (HPV)

Chronic infection by HPV subtypes 16 and 18 are the strongest risk factors for cervical cancer development (289, 290). While vaccines are available to prevent new infections, no cure is present for existing infections. HPV-mediated oncogenesis is driven by the viral proteins E6 and E7. They inhibit tumor suppressor proteins p53 and Rb, leading to the arrest of proliferating cells at the DNA synthesis and growth phases of the cell cycle (291–293). This action increases the risk of genomic instability and malignant transformation of host cells (290). Thus, HPV E6 and E7 are being used as vaccination targets. Intramuscular administration of rAAV1 packaged with the E7 and heat shock protein 70 (hsp70) fusion protein (rAAV1-1618E7hsp70) completely protected mice from challenge by E7-expressing tumor cell lines at 5, 12, and 24 weeks post-treatment (114). In addition, when the rAAV1-E7 vector was given eight to ten days after tumor inoculation, the size of the pre-established tumor was significantly reduced. An alternative strategy is to target the HPV major capsid protein L1. rAAV5 and rAAV9 packaged with HPV16 L1 was administered to rhesus macaques *via* intranasal delivery (115). By using rAAV5-*HPV16-L1* as the prime vector and rAAV9-*HPV16-L1* as the boosting vector, neutralizing antibodies were elicited in four out of six animals and mediated protection for seven months post-immunization. This effect was achieved even in the presence of pre-existing anti-AAV9 antibodies.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The ability to deliver gene expression cassettes *in vivo* has greatly expanded the toolbox to combat infectious diseases. With rAAV-mediated gene delivery, expression of antigens or therapeutic molecules may be achieved and maintained for long periods. Current studies demonstrated that this approach could prophylactically prevent new infections, with the additional possibility of eliminating existing infections in animal models. However, rAAV-mediated immunotherapy is still in its infancy, with many issues to be solved. Future studies are warranted to address several key questions. First, ADAs toward therapeutic mAbs and Ig derivatives are currently the biggest obstacle to long-term mAb expression from the host, but the underlying mechanisms of ADA development are largely a mystery. Elucidating these mechanisms will be critical to the design of rAAV immunotherapeutics that are capable of overcoming ADAs. Second, pre-existing immunity to the rAAV capsid and the transgene prevents a large portion of the world's population from gaining access to AAV-vectored immunotherapies. Strategies to evade pre-existing anti-rAAV immunity without compromising anti-pathogen immunity need to be devised. Third, given the fact that rAAV can mediate long-term transgene expression, even when the transgene is no longer desired, regulatable immunotherapies ought to be developed to further ensure patient safety. Fourth, novel capsids should be explored in the context of immunotherapy. And finally, the manufacturing process requires major improvement for deploying rAAV vectors in global distributions efforts.

AUTHOR CONTRIBUTIONS

WZ and MM wrote the initial draft of the manuscript. WZ, MM, PT, and GG revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the University of Massachusetts Medical School (an internal grant) and by the NIH (R01NS076991-01, P01HL131471-02, UG3HL147367-01, R01HL097088, U19 AI149646-01).

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Conflict of Interest: GG is a scientific co-founder of Voyager Therapeutics and Aspa Therapeutics, and holds equity in these companies. GG is an inventor on patents with potential royalties licensed to Voyager Therapeutics, Aspa Therapeutics, and other biopharmaceutical companies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 17 May 2021
doi: 10.3389/fimmu.2021.675897

Challenges Posed by Immune Responses to AAV Vectors: Addressing Root Causes

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Host immune responses that limit durable therapeutic gene expression and cause clinically significant inflammation remain a major barrier to broadly successful development of adeno-associated virus (AAV)-based human gene therapies. In this article, mechanisms of humoral and cellular immune responses to the viral vector are discussed. A perspective is provided that removal of pathogen-associated molecular patterns in AAV vector genomes to prevent the generation of innate immune danger signals following administration is a key strategy to overcome immunological barriers.

Keywords: AAV, gene therapy, immunogenicity, PAMPS, TLR9, CpG

OPEN ACCESS

Edited by:
Nicole K. Paulk, University of California, San Francisco, United States

Reviewed by:
David Markusic, Purdue University Indianapolis, United States
Annie Searls De Groot, EpiVax, United States
Longping Victor Tse, University of North Carolina at Chapel Hill, United States

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *04 March 2021*
Accepted: *22 April 2021*
Published: *17 May 2021*

Citation:
Hamilton BA and Wright JF (2021) Challenges Posed by Immune Responses to AAV Vectors: Addressing Root Causes. Front. Immunol. 12:675897. doi: 10.3389/fimmu.2021.675897

INTRODUCTION

Human gene therapies using recombinant adeno-associated virus (rAAV) vectors have demonstrated enormous promise. While rAAVs lack many characteristics of the wild-type viruses from which they are derived, they nevertheless retain legacy immunogenic features that affect their safety and efficacy. Marked inflammatory toxicities that have recently been observed as vector dosing has increased, including complement activation, cytopenias and severe hepatotoxicity (1), likely represent part of the continuum of diverse aspects of clinical immune responses that have emerged over the last two decades. Accurate identification and removal where possible of the immunogenic features in the vector design process, in conjunction with the optimization of immunomodulation protocols, will minimize rAAV-associated immunotoxicities and enhance therapeutic benefit. Process and product-related impurities are known to contribute to unwanted immune responses in biologics generally, and even highly purified rAAV vectors contain unique product-related impurities that present immunogenic risks (2). However, rAAV vectors comprising the viral capsid proteins (VP1, 2, 3), the vector DNA expression cassette, and the products generated by its transcription and translation are themselves immunogenic. Several comprehensive reviews of host immune responses to AAV gene therapy have been recently published (3–7). This article focuses on the inherent immunogenic features of rAAV vectors and provides a perspective that the removal of pathogen associated molecular patterns (PAMPs) from vector genomes is key to preventing pre-requisite innate immune signals that lead to deleterious adaptive immune responses.

Recombinant AAV capsids and their transgene products can provide targets for humoral and cellular immunity. Conceptually, immune responses to rAAV can be categorized into the quadrants shown in **Figure 1**, which summarizes innate immune triggers, adaptive effector functions, and clinical sequelae.

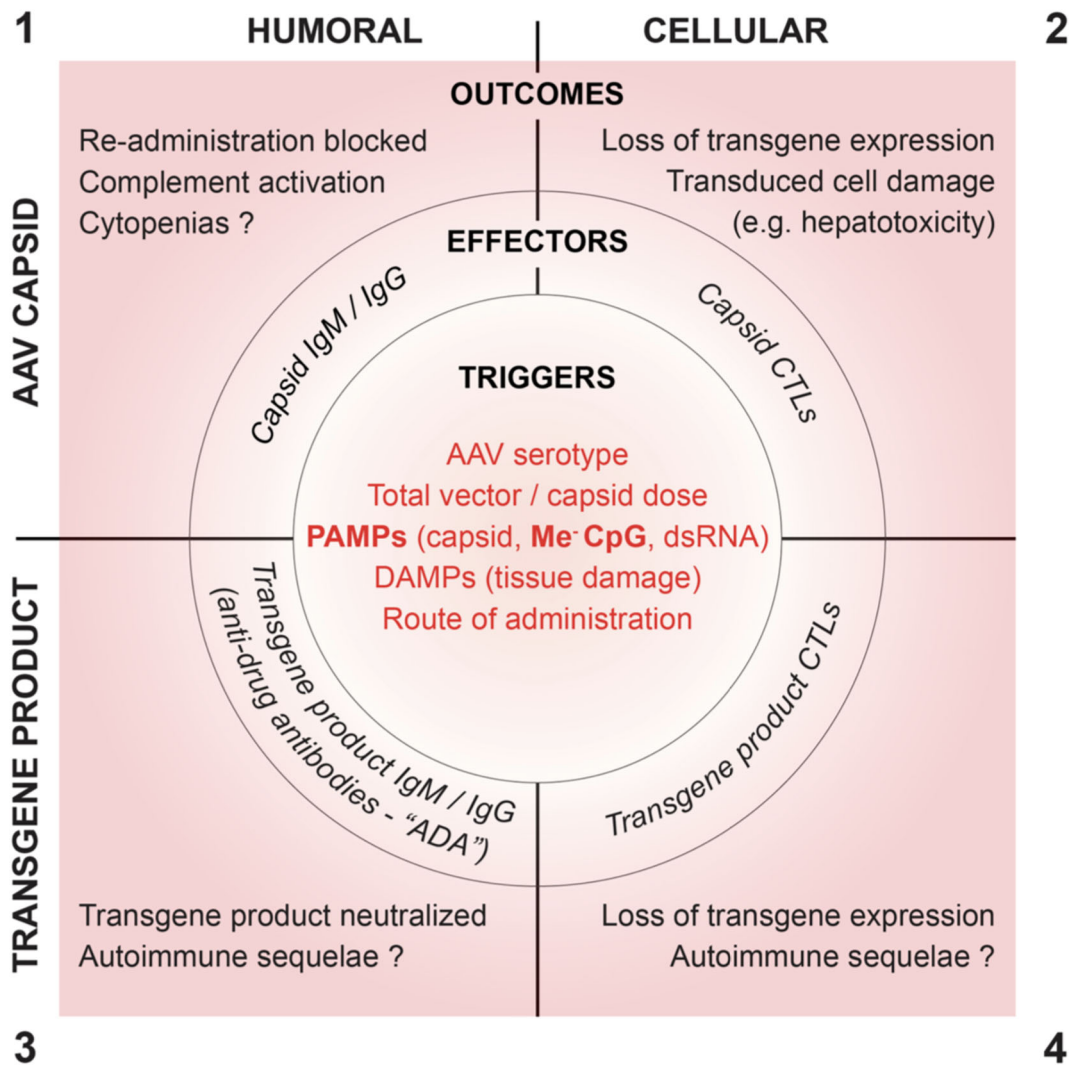

FIGURE 1 | Proposed root cause triggers, effector functions and clinical outcomes of humoral and cellular immune responses to AAV vectors.

Innate Immune Signals as Key Triggers for Deleterious Immune Responses

Robust immune responses require three signals (8). A pre-requisite innate immune danger signal (Signal 0) generated by binding of pathogen-associated and/or danger-associated molecular patterns (PAMPs and DAMPs) to host pattern recognition receptors (PRRs) results in pro-inflammatory cytokine release. An antigen-specific signal (Signal 1), generated by binding of non-self antigens or peptide/MHC complexes to antigen-specific receptors on B or T cells, respectively, then leads to clonal expansion of antigen-specific effector antibodies and cytotoxic T-lymphocytes (CTLs). Co-stimulatory signals (Signal 2), generated by additional receptor/ligand interactions between antigen-specific effector lymphocytes and helper T cells or professional antigen presenting cells, amplify effector functions and prevent anergy. Preventing generation of the initial danger signals by removal of PAMPs is a root cause-focused strategy to prevent immune responses to rAAV vectors. Three rAAV-associated PAMPs have been described: i) ligands present on rAAV viral capsids that bind toll-like receptor 2 (TLR2), a cell-surface PRR on non-parenchymal cells in the liver (9); ii) unmethylated CpG dinucleotides in viral DNA that bind TLR9, an endosomal PRR in plasmacytoid dendritic cells (pDCs) and B cells (10, 11); and iii) double-stranded RNA formed as a result of the bi-directional promoter activity associated with AAV inverted terminal repeats (ITRs), which binds melanoma differentiation-associated protein 5 (MDA5), a cytoplasmic PRR (12). Removal of these PAMPs by vector design optimization to avoid generation of the initial danger signal is predicted to markedly reduce immune responses to AAV.

HUMORAL IMMUNE RESPONSES TO AAV

AAV Capsid Antibodies

The generation of antibodies to the rAAV capsid is an expected outcome following challenge of the immune response with a non-self-i.e., viral protein, and generation of high titer capsid antibodies has been consistently reported following vector administration to human subjects. Tissue macrophages take up rAAV non-specifically by phagocytosis, process the capsid, and following migration to a draining lymph node, present capsid-derived peptides to effector lymphocytes. The presentation of AAV peptides on MHC class II molecules, with co-stimulatory signals, activates capsid-specific CD4+ helper T cells and B cells (10). T_h2 cells produce interleukins that stimulate B cell proliferation and antibody class switching, leading to increased neutralizing antibody production. A robust humoral immune response requires engagement of T_{FH} cell CD154 with CD40 on B cells. After germinal center formation, B cells differentiate into affinity matured plasma cells, antibody class switching from IgM to IgG occurs, and B memory cells are generated (13).

Prior exposure to wild-type AAV by natural infection or to rAAV by prior *in vivo* gene therapy results in neutralizing antibodies in serum that efficiently block target cell transduction and thereby therapeutic efficacy of systemically administered rAAV (14, 15). Potential participants are excluded from rAAV clinical trials if significant titers of neutralizing antibodies are measured in their sera during screening. Natural infections are common, and 70% of human sera contain antibodies against rAAV1 and rAAV2, 45% against rAAV6 and rAAV9, and 38% against rAAV8 (16). Children as young as two years of age are often seroconverted, with antibodies against multiple AAV serotypes (17), and antibodies have been shown to cross react between rAAV serotypes (18). Administration of rAAV to seronegative individuals resulting in high titer antibody formation will similarly block the option of systemic re-administration of AAV-based gene therapy vectors for many years. Capsid antibodies can also contribute to inflammation and cytotoxicity by activating the classical pathway of complement, and by mediating uptake by FcR-expressing immune cells such as dendritic cells and macrophages, potentiating AAV specific adaptive immune response and generation of proinflammatory cytokines (19).

Overcoming Challenges Posed by AAV Capsid Antibodies

To facilitate rAAV-mediated gene therapy in a broader patient population, several strategies are being developed to circumvent the barrier posed by capsid antibodies. Capsid modification by rational design or screening of capsid variant libraries to identify those that avoid recognition by AAV antibodies prevalent in human sera are two approaches (20). Novel capsids identified by modification of AAV2 following antibody epitope mapping and targeted disruption of epitopes by mutagenesis were reported to reduce neutralization by high titer individual human sera up to 42-fold and by pooled human serum IgG (IVIG) up to 10-fold (21). Developing AAV capsids in which the epitopes to prevalent human antibodies have been eliminated is an important strategy, but will not enable vector re-administration if needed due to waning or loss of expression following a first administration. If feasible, the development of stealth vectors with surface viral epitopes masked by non-antigenic features that do not interfere with target cell transduction (20) would be transformative to enable AAV administration in the presence of capsid antibodies. Alternative approaches to transiently remove antibodies in subjects prior to vector administration include *in vivo* enzymatic digestion to degrade total IgG (22) and plasmapheresis (23, 24) to remove total (23) or capsid-specific (24) IgG. Maneuvers to deplete antibodies present risks that need to be balanced with the potential benefits of gene transfer.

Complement Activation

Complement activation following high dose systemic administration is a recently described inflammatory toxicity associated with high dose rAAV administration (1). The complement system is comprised of over 30 proteins mainly synthesized in the liver and circulating in serum and interstitial fluid (25) that collectively play an important role in the recognition and elimination of pathogens (25). Complement acts by opsonizing pathogens and antigens for phagocytosis, co-stimulating B cell activation and antibody production, and by forming a membrane attack complex (MAC) for direct killing, among other functions. Complement can be triggered by the lectin pathway, the classical pathway, and the alternative pathway. The classical pathway is initiated when the antibody Fc regions of immune complexes composed of antigen-bound IgM or IgG bind complement component C1 causing confirmational changes activating C3 convertase, which cleaves C3 into fragments C3a and C3b. Soluble C3a recruits macrophages and neutrophils to the site of infection, and TNFα, IL-1, and IL-6 secretion by these leukocytes amplify the production of complement proteins (25). Membrane-bound C3b opsonizes antigens to facilitate their removal and propagates the formation of the MAC. Immune complexes composed of rAAV bound by IgM or IgG, formed when high doses of rAAV vector are administered to individuals with pre-existing or rapidly formed capsid antibodies, if deposited within recipient tissues would be predicted to cause complement activation-associated host cell damage. Covalent deposition of complement component C3b on AAV particles mediated uptake of the vector in a human monocyte cell line leading to production of pro-inflammatory cytokines (26). This highlights that AAV immune complexes involving pre-existing or newly formed capsid antibodies have the potential to activate complement and amplify capsid immune responses after AAV-mediated *in vivo* human gene transfer. Complement inhibitors are being investigated for their ability to modulate immune responses to AAV gene therapy vectors, including APL-9 as a C3 inhibitor, and Eculizumab as a C5 inhibitor (27).

CELLULAR IMMUNE RESPONSES TO AAV

Cytotoxic T Lymphocytes

As a central feature of immune responses to viruses, the generation of CD8+ capsid specific cytotoxic T lymphocytes

(CTLs) is an expected outcome following infection with wild type AAV. Capsid CTLs were not anticipated in early clinical trials using rAAV because vectors do not encode viral antigens in their genomes. However, capsid CTLs have been frequently reported following rAAV vector administration in humans and associated with the elimination of transduced cells. Both transduced target cells and professional antigen presenting cells (APCs) present capsid-derived peptide epitopes on MHC class 1 molecules to CD8+ T cells (28–30). After activation and expansion, the resulting CTLs have the potential to destroy rAAV-transduced cells, inducing inflammation in the target organ and adversely affecting the gene transfer outcome (14, 31). Herzog's group (29, 32), described the dendritic cell-dependent mechanism of CD8+T cell response against the AAV capsid. After endocytosis and partial degradation of rAAV particles in their lysosomal compartment, plasmacytoid dendritic cells (pDCs) present in lymph nodes or the spleen will generate type 1 interferons as a danger signal if CpG PAMP DNA is detected by TLR9. Conventional dendritic cells (cDCs) in the vicinity that have also taken up rAAV particles present capsid peptides on MHC class 2 molecules to CD4+ helper T cells, resulting in the generation of an antigen specific signal. These signals together promote maturation of the cDC, presentation of capsid peptides by MHC class 1 molecules to and activation of capsid-specific CD8+ CTLs that can then migrate to other tissues and eliminate transduced cells.

Systemic AAV Administration: Experience in Hemophilia Clinical Trials

In the first hemophilia B gene therapy clinical trial that reported therapeutic but transient levels of the FIX transgene product, elimination of vector-transduced hepatocytes by capsid-specific CTLs began four weeks after vector administration (14). Since that initial trial, several additional hemophilia B gene therapy trials have been reported, some achieving long-term efficacy, but others recapitulating the loss of transgene expression associated with immune-mediated loss of transduced cells (33). This failed outcome was recently reported in a trial using an AAV8 vector (34), demonstrating that consensus strategies to effectively minimize and manage deleterious capsid CTL response have not yet been fully established. The targeted nature of these efficacy-limiting responses when they are observed is evidenced by liver enzyme elevation concomitant with loss of therapeutic transgene expression. The timeframe of CTL effector manifestation after rAAV administration is delayed compared the kinetics following wild-type virus infection. One explanation is that, in contrast to the direct processing for MHC class I presentation of viral antigens following their transcription and translation during natural virus infections, the capsid protein component of the rAAV dose must be processed *via* the indirect pathway (14, 28), which is less efficient. Furthermore, the high stability of AAV capsids would be predicted to contribute to slow capsid processing by the indirect pathway (35). Alternatively, a delayed innate signal from dsRNA generated following vector transduction has been proposed to contribute to the delayed formation of capsid CTLs (12). In any case, the vector dose inoculum as the sole source of capsid antigen predicts that peptide presentation by MHC class I on transduced cells will be transient.

Intramuscular Administration: Experience in AAT-Deficiency Clinical Trials

Brantly and colleagues reported the occurrence of cellular immune responses in a clinical trial for alpha-1-antitrypsin (AAT) deficiency, including IFN-gamma ELISPOT responses to AAV capsid, that did not completely eliminate transduced cells (36). Capsid-specific T cells were detected at day 14 and remained present at day 90, yet subjects in the high dose cohort demonstrated sustained expression of AAT. Biopsy samples taken at three months post vector administration demonstrated inflammatory cells that were still present, although at a lower level at 1 year. Phenotyping revealed a substantial portion of the T cells present were regulatory T cells (Tregs), supporting that following intramuscular administration, rAAV induces Treg responses that allow ongoing transgene expression (37). The development of Tregs that provide immune tolerance to the therapeutic transgene product has also been shown to be induced by gene transfer to the liver by AAV (38), supporting that some level of liver transduction even when the primary target for gene expression is a different tissue may be beneficial (39).

Overcoming Immune Responses by Immune-Suppression

The inhibition of both capsid peptide presentation by MHC class I molecules and expansion of capsid-specific CTLs, each required for transduced cell lysis, have been proposed or used to prevent the elimination of vector-transduced cells and thereby enable long-term transgene expression. Proteosome inhibitors have been shown to prevent capsid processing by the indirect pathway (40, 41). Transient immune suppression with prednisone was used to block CTLs in subjects that experienced elevated liver enzymes in the first AAV gene therapy trial that reported therapeutic and durable expression of FIX (42). Prednisone initiated prior to vector administration and continued for at least 30 days is part of the administration protocol for Zolgensma, the first licensed AAV product approved for systemic administration, and similar protocols are used for many AAV products currently in clinical development. While prophylactic corticosteroids have improved outcomes in AAV clinical trials, they have failed to prevent loss of transgene expression in others (34), and present risks (34, 43). Additional novel immunomodulatory approaches have shown promise. The co-administration of polylactic acid nanoparticles containing rapamycin (sirolimus) can tolerize the non-human primate (NHP) immune system to the transgene product (44). However, blocking the interleukin-2 receptor with daclizumab to inhibit effector T cell activation in NHPs increased immunity against the transgene (45). Preclinical studies of rituximab-mediated B cell depletion (46), and mycophenolate mofetil depletion of guanosine nucleotides to arrest T cells and B cells (45), prevented anti-capsid humoral and cell-mediated responses. The standardization of

clinical immunomodulatory protocols has been complicated by the absence of an animal model in which vector immunogenicity can be reliably studied and predicted. The risks presented by immunomodulation, such as nosocomial and opportunistic infections, must be weighed against the potential benefits of gene therapy for each disease indication.

Overcoming Immune Responses by Improved Vector Design

Vector dose, serotype, genome configuration and method of manufacture have all been proposed as important factors contributing to prednisone-resistant loss of transgene expression. However, none of these factors consistently correlate with clinical observations, suggesting a different vector attribute(s) is key. Compelling evidence for a key role of unmethylated CpG motifs in efficacy-limiting immunotoxicity in AAV clinical trials has emerged. The recognition of unmethylated CpG motifs by TLR9 leads to its dimerization and downstream activation *via* MyD88 in professional APCs (11, 47, 48). The frequency of CpG dinucleotides in most microbial genomes approximates 6.25%, the frequency based on random nucleotide utilization, and most microorganisms do not methylate CpG dinucleotides. In contrast, the 0.97% frequency of CpGs in the human genome is markedly suppressed, and CpG dinucleotides in human DNA are predominantly methylated so that the frequency of unmethylated CpGs in human DNA is > 20-fold lower than in bacterial DNA. This difference provides the basis for discrimination between human and microbial DNA by TLR9. AAV expression cassettes that include components derived from viral or microbial sources such as AAV ITRs, viral promoters, and enhancers such as WPRE will therefore contain PAMP CpG motifs from these sources.

Increasing the number of CpG motifs in plasmid DNA vaccines is an effective way to increase both cellular and humoral immune responses (49–51), and plasmid methylation markedly reduces those responses (50, 51). These findings establish a key role for unmethylated CpG-triggered TLR9-MyD88 pathway activation in strong cellular and humoral immune responses. The well-established role of unmethylated CpG motifs as adjuvants in DNA vaccines further highlights the need, by vector genome and production process design, to reduce the frequency of unmethylated CpGs in AAV vector genomes to a level below the threshold that activates human TLR9.

Innate immunostimulatory CpG motifs (CpG PAMPs) are unexpectedly abundant in AAV vectors because of their hypomethylation. Using BrdU labeling to assess the origin of rAAV genomes, Hauck and colleagues reported that a large percentage of AAV2 vectors generated by transient transfection of HEK293 cells contain expression cassettes rescued directly from plasmid DNA (52). The authors proposed that the consequent high frequency of unmethylated CpG dinucleotides in AAV vector genomes contributed to clinical efficacy-limiting immune responses. The hypomethylation of CpGs in AAV vector genomes was further characterized by direct biochemical analysis (53, 54) supporting that, in addition to vector genome rescue from plasmid DNA, the kinetics of vector genome packaging leads to low methylation even in mammalian production cells that would normally achieve human levels of CpG methylation. CpG hypomethylation is a non-human epigenetic feature that explains why even wild-type human DNA sequences that do not bind TLR9 when they are predominantly methylated i.e., in the human genome, become TLR9-activating CpG PAMPs in AAV vector genomes.

Validation of the deleterious effect of CpG motifs in AAV vector genomes has been clearly demonstrated in animal models (10, 55–57), including demonstration that CpG-depleted AAV vectors evade immune responses (56), and human clinical trials (33, 34). The use of higher CpG content vectors correlated with hepatoxicity and absence of durable transgene expression in multiple hemophilia B gene therapy trials (33).

Synonymous codon substitutions to replace native CpG dinucleotides, and CpG methylation to 'humanize' the AAV genome are two approaches to remove TLR9-recognized PAMPs in AAV expression cassettes. The reduction of CpG dinucleotides within AAV vector genomes, especially those within motifs known to be strong activators of the TLR9 pathway, is supported as a key practical strategy to reduce adaptive immune response to AAV gene therapy vectors. Concerns with synonymous codon substitutions include deviations from naturally evolved codon usage patterns that may result in transgene product misfolding (58, 59). Components of the AAV genomes outside of the ORF, such as ITRs and promoters, are equally 'visible' to TLR9 receptors, and are often rich in CpG dinucleotides. **Table 1** lists calculated TLR9 activation risk factors for non-ORF components of AAV vector expression cassettes. If achievable, methylation of CpG motifs to 'humanize' this epigenic attribute is predicted to reduce TLR9 activation. Another approach is the incorporation of TLR9 inhibitory nucleotides sequences into the AAV expression cassette (60).

THE PATIENT PERSPECTIVE

The importance of effectively reducing the immunogenicity and potential for inflammatory toxicities during AAV-mediated gene therapies is emphasized by patient considerations. The weight participants bear in participating in rAAV clinical trials is compounded by the fact that failure to achieve efficacy after one administration is likely to preclude benefit from future improved AAV vectors. This long-term adverse outcome emphasizes the moral imperative towards scientific communication and data sharing to validate the vector attributes involved and to develop effective management of AAV immune responses towards the goals of low immunotoxicity and durable therapeutic transgene expression.

CONCLUSION

In order for potentially curative and definitive AAV-based gene therapies to be applicable to a broader range of indications and reach more patients, immune responses to rAAV must be reduced and better controlled. The formation of anti-capsid antibodies restricts rAAV gene therapy to a single administration at least for

TABLE 1 | TLR9 Activation Potential and Risk Factor (RF)* calculations for AAV genomes and components.

DNA test article	Size (nt)	RF_1	RF_3	Normalized RF_3	TLR9 Activation
Human genome					
complete genome *	3.21 x10^9	0.965	0.191	**1.00**	-
Bacterial genome					
Escherichia coli *	4.64 x10^6	7.471	4.683	24.5	+++
Clinical rAAV genomes					
low CpG AAV8-FIXsc (scAAV2/8-LP1-hFIXco[42]; clinicaltrials.gov NCT00979238)	4611	1.757	1.298	6.80	+
high CpG AAV8-FIXsc (BAX335[34]; clinicaltrials.gov NCT01687608)	4780	5.859	4.383	22.9	+++
rAAV genome components					
AAV2 ITRs	145	11.03	7.862	41.2	+++
ApoE HCR hAAT Promoter	828	3.019	2.754	14.4	+++
MHCK7 Promoter	770	2.078	1.851	9.69	++
CAG Promoter	584	8.562	2.603	13.6	+++
CMV Promoter	508	5.709	3.740	19.6	+++
WPRE	597	6.198	3.819	20.0	+++

Wright JF. Quantification of CpG motifs in rAAV genomes: avoiding the toll. Mol Ther 2020; **28(8):1756-58.*
RF_1 = f [CpG_T / nt] X 100%.
RF_3 = f [CpG_T + CpG_{S4} – 2CpG_{I4} / nt] X f [$CpGMe^{neg}$ / CpG_T] X 100%.
Normalized RF_3 = RF_3 (test article) / RF_3 (human genome).

the commonly used systemic route of administration. Therefore, trials that seroconvert human subjects without a reasonable expectation of clinical benefit, in particular those using vectors containing known significant innate immunogenic features, should not be performed. Furthermore, while capsid-specific CTL responses can sometimes be controlled by immune suppression, many diseases necessitate systemic administration of rAAV at high doses where immunomodulation is less effective, and inflammatory toxicities such as complement activation are likely to be worsened by strong innate immunogenic features of the vector. An effective strategy must combine immune-modulation with better design of vectors – 'humanized vectors' with reduced potential to trigger efficacy-limiting immune responses. The role of TLR9 activation as a seminal potentiator of cellular and humoral immune responses to rAAV is under-appreciated, and reducing TLR9 signaling by reducing the frequency of unmethylated CpG motifs in vector genomes is an important key to realizing the enormous promise of rAAV mediated gene therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by Stanford University. The authors thank John Day for critical review of the manuscript, and Cecile Martin for science graphics artistic support.

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Conflict of Interest: JFW is a co-founder of Spark Therapeutics and Kriya Therapeutics, consults to companies developing rAAV-based gene therapy products, and is inventor of patents relating to recombinant viral vector design and manufacture.

BAH declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 28 May 2021
doi: 10.3389/fimmu.2021.660302

Answered and Unanswered Questions in Early-Stage Viral Vector Transduction Biology and Innate Primary Cell Toxicity for *Ex-Vivo* Gene Editing

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OPEN ACCESS

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 29 January 2021
Accepted: 04 May 2021
Published: 28 May 2021

Citation:
Dudek AM and Porteus MH (2021) Answered and Unanswered Questions in Early-Stage Viral Vector Transduction Biology and Innate Primary Cell Toxicity for Ex-Vivo Gene Editing. Front. Immunol. 12:660302. doi: 10.3389/fimmu.2021.660302

Adeno-associated virus is a highly efficient DNA delivery vehicle for genome editing strategies that employ CRISPR/Cas9 and a DNA donor for homology-directed repair. Many groups have used this strategy in development of therapies for blood and immune disorders such as sickle-cell anemia and severe-combined immunodeficiency. However, recent events have called into question the immunogenicity of AAV as a gene therapy vector and the safety profile dictated by the immune response to this vector. The target cells dictating this response and the molecular mechanisms dictating cellular response to AAV are poorly understood. Here, we will investigate the current known AAV capsid and genome interactions with cellular proteins during early stage vector transduction and how these interactions may influence innate cellular responses. We will discuss the current understanding of innate immune activation and DNA damage response to AAV, and the limitations of what is currently known. In particular, we will focus on pathway differences in cell line verses primary cells, with a focus on hematopoietic stem and progenitor cells (HSPCs) in the context of *ex-vivo* gene editing, and what we can learn from HSPC infection by other parvoviruses. Finally, we will discuss how innate immune and DNA damage response pathway activation in these highly sensitive stem cell populations may impact long-term engraftment and clinical outcomes as these gene-editing strategies move towards the clinic, with the aim to propose pathways relevant for improved hematopoietic stem cell survival and long-term engraftment after AAV-mediated genome editing.

Keywords: AAV (adeno-associated virus), genome-edited cells, hematopoietic stem cell, toxicity, hematopoietic stem cell (HSC) transplantation, viral vector, DNA damage response (DDR), parvovirus

INTRODUCTION

The Efficacy of AAV in Genome Editing

Recombinant Adeno-associated virus (AAV) has been highly successful in gene-replacement therapy for monogenetic disorders. It has shown effectiveness in slowly or non-dividing cells such as the liver, retina or central nervous system because the extra-chromosomal non-replicative AAV genome is not diluted by cell division. However, for genetic diseases which manifest in cells that are highly proliferative such as the hematopoietic system, gene replacement therapy using AAV is not likely to be successful because of the dilutional effect. For highly replicative cells, such as hematopoietic stem and progenitor cells (HSPCs), long-term modification needs to occur by integrating into the genome. Permanent genetic modification of HSPCs then becomes a way to alter the function of the entire hematopoietic system because all cells of the blood and immune system are derived from HSPCs. While retroviral gene replacement has been curative in some gene-replacement strategies such as for treatment of X-linked SCID, malignant transformation due to the semi-random nature of retroviral integration (1–3) remains a concern. An alternative to semi-random integration by retroviral vectors is to use genome editing to integrate the transgene at a specific genomic location. The development of the highly-specific Cas9 endonuclease has catalyzed the development of highly efficient targeted integration strategies in HSPCs using genome editing and homology directed repair (HDR). Using Cas9 endonuclease to induce a double-strand break (DSB) by sequence-specific recognition through a guide RNA (4), we can force the cells to activate DSB repair, including by homologous recombination (an essential cellular repair pathway). In cases of homozygous genetic disorders, where both alleles of a gene contain a disease-causing mutation, by providing a DNA template to the cell which contains the corrective sequence flanked by regions of sequence homology in the presence of Cas9/guide RNA ribonucleoprotein complex (RNP) the cell will use the exogenous DNA donor as a template for homologous recombination, in a method termed HDR (5). The cell only uses the provided donor template ("donor") if sufficient quantities are provided, on the order of hundreds-thousands. While early studies showed successful HDR using plasmid or DNA fragments as a DNA donor template (6), the highly sensitive nature of many cell types, especially stem cells, to naked DNA in the cytoplasm causes significant Type I interferon toxicity and precludes using naked DNA plasmids from being an efficient option as a DNA donor for development of clinical therapies. AAV vectors however, as relatively simple replication-defective parvoviruses containing only capsid proteins VP1, VP2, VP3 and an ITR-flanked transgene have proved to be ideal DNA delivery vehicles because they do not significantly activate the anti-viral response like naked DNA does. The mechanism is from the non-pathogenic AAV evolving to shield its encapsidated DNA from detection such that it efficiently is delivered to the nucleus and can serve as the template for HDR.

Abbreviation: AAV, Adeno-associated virus; AAVR, AAV receptor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DAMP, damage-associated molecular pattern; DDR, DNA damage response; DSB, double-strand break; GMP, granulocyte monocyte progenitor; HDR, homology directed repair; HR, homologous recombination; HSPC, hematopoietic stem and progenitor cell; ITR, inverted terminal repeat; LT-HSC, long-term repopulating hematopoietic stem cell; MEP, myeloid erythroid progenitor; NHEJ, non-homologous end-joining; NPC, pnuclear pore complex; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RNP, ribonucleoprotein complex; TGN, Trans-golgi network.

What Cell Types Have Successfully Undergone HDR and What Are the Clinical Applications?

Using this method of AAV-mediated genome editing by AAV delivery of a DNA donor for HDR (**Figure 1**), multiple groups have demonstrated high levels of gene targeting (the targeted insertion of a gene into a specific location in the genome, including single nucleotide gene changes) in many clinically relevant cell types, including HSPCs (5, 7–9), T-cells (10, 11), IPSCs (12, 13), mesenchymal stromal cells (14), and airway basal cells (15). Highly efficient (often >60%) allele targeting occurs in CD34+ HSPCs for blood disorders such as sickle cell anemia and beta-thalassemia (16, 17), immune disorders such as X-linked severe-combined immunodeficiency and chronic granulomatous disease (18–20), and lysosomal storage disorders such as mucopolysaccharidoses and Gaucher disease (21, 22). The transition from using plasmid DNA to AAV as a DNA donor was crucial for these genome editing therapies to reach high efficiency allele correction, due to the high toxicity associated with plasmid DNA in these highly sensitive primary cell types.

What Are the Major Considerations for Continued Success of AAV-Mediated Gene Editing?

Successful development of AAV-mediated genome targeting is dependent on genome editing efficiency, genotoxicity, cell potency (including transplantability), and durability of the therapy. There have been many optimizations including cell culture conditions, nucleofection protocol, homology arm length in the DNA donor, guideRNA specificity, length of cell culture time, and the timing of the transduction of the AAV vector itself (23, 24). These optimizations have allowed highly efficient gene correction, demonstrated by as high as 90% allele targeting of the TRAC locus in human primary T cells (11). However, genotoxicity, transplantability, and durability of the gene-corrected cells after transplant are areas for continued improvement of these therapies to increase the probability of long-term safety and efficacy.

While AAV as a virus has evolved to avoid detection, data suggests that there can still be a residual detrimental effect in response to the AAV vector itself. Evidence that the AAV vector has toxicity is demonstrated by both measuring the response to the vector and by data showing that the toxicity increases as the amount of AAV used (multiplicity of infection or MOI) increases (24). While often genotoxicity is used to refer to aberrant DNA damage or mutations caused by the Cas9 endonuclease, the AAV genome burden within these cells can also be considered a type of genotoxicity. Single-stranded DNA (as in the AAV genome) normally only occurs in the cell in the context of viral infection

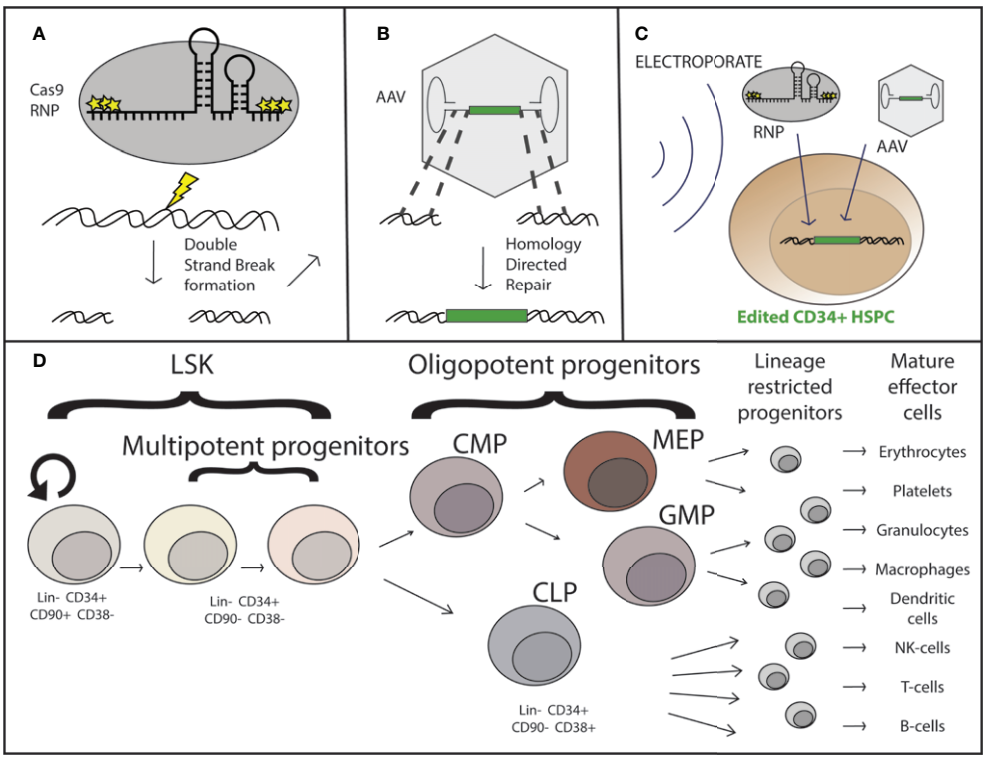

FIGURE 1 | Overview of AAV-based hematopoietic stem and progenitor cell gene editing. Purified Cas9 protein complexed with chemically modified guide RNA as a ribo-nucleoprotein complex (RNP) induces a double strand break at a specific locus **(A)**, at which site flanking regions of homology within the recombinant AAV genome serve as templates for homology directed repair and incorporation of the corrective sequence [green] after DNA delivery by the AAV vector **(B)**. The genome editing components are delivered simultaneously *ex-vivo via* nucleofection the CD34+ hematopoietic stem and progenitor cells which have been stimulated to enter the cell cycle through treatment with a cytokine cocktail including SCF, TPO, Ftl3, and IL-6 **(C)**. The CD34+ subset of cells is comprised of both long term hematopoietic stem cells (LT-HSCs) which can self-renew and maintain full differentiation potential to reconstitute the entire hematopoietic system, as well as multipotent and oligopotent progenitors which have limited or no self-renewal capacity and are lineage restricted **(D)**.

or DNA damage, as revealed during the DNA repair process. When using anywhere between 5,000 and as much as 100,000 AAV vector genomes per cell for gene-targeting, these genomes can persist long past when targeted integration takes place. Although cells that have undergone AAV-mediated HDR can maintain their transplantability as shown by serial HSPC engraftment into NSG mice (16), we and others have shown that there is a significant drop in the efficiency of engraftment of gene-targeted cells compared to mock treated or RNP-only treated cells. This deficiency can be overcome through transplantation of 4-5 times as many cells (23), yet this is not always feasible when considering both the number of cells available to undergo editing, as well as from a manufacturing perspective. Therefore increasing/restoring the potency of engraftment after gene targeting would allow improvement in the successful implementation of this class of gene and cell therapies. In addition to transplantability, the durability of engraftment is an important consideration. Though bone marrow and peripheral blood HSPC transplants have shown long-term (life-time) durability and graft maintenance in human patients, loss of the overall proportion of gene-corrected cells over the course of the transplant in NSG mice suggests that there may be some type of defect related to AAV-mediated genome editing that either prevents efficient correction of the "true" hematopoietic stem cells (LT-HSCs), causes a loss of fitness of gene targeted progenitors, a defect in self-renewal of LT-HSCs, or a combination of these effects (**Figure 2**). As these therapies progress from pre-clinical animal models to clinical trials, monitoring the durability of gene targeted cells over time is essential in patients even if the initial gene correction and transplantation are successful. Because AAV-mediated genome correction and transplantation are the most advanced in HSPCs as compared to other stem cell types such as airway and induced pluripotent stem cells, this review will primarily focus on what we have learned from HSPC gene targeting and transplantation, potential areas of improvement, and the unanswered questions related to how the AAV vector interacts with the HSPCs and influences the long term success of gene-targeting therapies.

What Are the Major Considerations for Successful Gene Editing Therapies in HSPCs?

When considering gene editing in HSPCs it is important to consider the functional and phenotypic differences in the HSPC

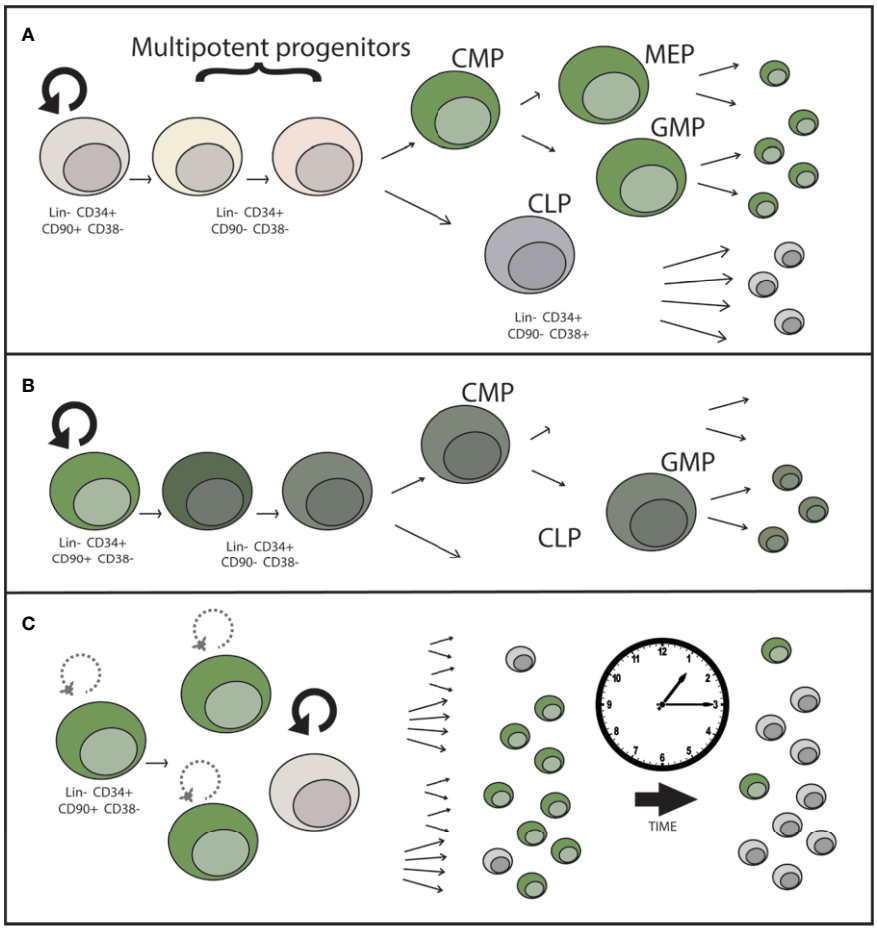

FIGURE 2 | Potential mechanisms for loss of gene-edited HSPCs over time after engraftment. **(A)** LT-HSCs may be less permissive to HSC transduction than committed progenitors due to differential expression of AAV attachment, entry, or restriction factors. This potential progenitor-biased transduction would lead to dilution of the proportion of edited hematopoietic cells over time over time, as shorter-lived progenitor cells reach the end of their lifespan. **(B)** Toxicity within the LT-HSC or progenitor cell population in response to AAV causes a loss of differentiation potential or cell death leading to incomplete reconstitution of gene edited cells over time. **(C)** Decrease in the self-renewal capacity of LT-HSCs over time causes a loss of gene-edited LT-HSCs over time, eventually causing loss or near complete loss of the HSC population contributing to hematopoiesis that contains the genetic correction.

populations. By definition, true long-term hematopoietic stem cells (LT-HSCs) have self-renewal capacity and have multi-lineage potential to reconstitute every cell-type of the hematopoietic system, including immune system cells (25). The LT-HSCs are primarily in a quiescent state *in vivo*, with the tremendous hematopoietic output (approximately 200 billion each of red blood cells, platelets, and neutrophils are made each day) deriving from progenitor cells. Progenitor cells have a limited self-renewal capacity, proliferate much more frequently, and become committed to a particular cell-type lineage within the hematopoietic system, the two predominant lineages being the myeloid and the lymphoid lineage. Immunophenotypically, LT-HSCs are currently defined by their cell surface markers (**Figure 1D**) as being Lin-, CD34+, CD90+, CD45RA+, and CD38-, while progenitors are defined as CD90-, CD34+, and CD38+ (26). Although immunophenotypic characterization is routinely done to define the HSPC subpopulations [for review see (27)], the current experimental gold-standard for determining LT-HSC function is through serial (2+) transplantation and multilineage hematopoietic reconstitution in immunodeficient mice with durability of engraftment shown for greater than 16-20 weeks and the true gold standard is engraftment and reconstitution in humans. For successful long term gene correction of cells in the hematopoietic system, correction of both LT-HSCs and progenitors is necessary as the progenitors are responsible for short-term blood and immune reconstitution early after transplantation, and correction of LT-HSCs is required for long-term correction as the shorter-lived progenitor cells are lost. While gene edited LT-HSCs have been maintained through sequential isolation and transplantation of human HSPCs and gene targeting of LT-HSCs has been confirmed through clonal tracking (28, 29), there is almost always a decrease in the proportion of corrected *vs* uncorrected alleles over course of the 16-20 week transplantation studies. This suggests that either true LT-HSCs are edited at a lower efficiency than progenitors, that edited cells are lost over time, or a combination of these possibilities.

This review will focus on what is currently known about the entry pathway and innate immune and DNA damage response activation in response to AAV, and what is known about how these effects may alter the viability of HSPCs in AAV-based ex-vivo gene editing therapies. We will focus on specific cellular mechanisms that have been implicated, and the perturbations thereof that have been shown to improve gene editing and/or cell survival outcomes with the hope of opening doors to decreasing cellular toxicity and improving clinical success in the stem cell gene editing field moving forward. This review complements the excellent review written by Kajaste-Rudnitski describing other aspects of the cellular response of HSPCs to genetic engineering (30).

BODY

Does Entry Mechanism Influence Toxicity in Gene Editing of HSPCs?

What Is the Canonical AAV Entry Pathway?

AAV first comes into contact with the cell through serotype-specific glycans that facilitate attachment of the external surface of the VP3 portion of capsid to the cell (31–33) (**Figure 3A**). After attachment, the vector undergoes endocytosis and trafficking within the endosome towards the perinuclear region *via* microtubules (34), although the exact endocytosis mechanism (eg: clathrin dependent, clathrin independent, micropinocytosis) is still debated and may be cell-type and vector dose dependent (35–38). In addition to glycan attachment factors, the external surface of most AAV capsids aside from the unique AAV4 and AAVrh32.3 serotypes (39) engage with the AAV receptor, AAVR, one of the only required AAV entry factors conserved across different capsid serotypes and shown to be required *in vivo* (40). While AAVR can be observed at the surface of cell lines in culture, steady state subcellular localization and endocytosis time-course experiments suggest that a major function of AAVR as a receptor is to traffic the endocytosed AAV particle to the appropriate cellular compartment. After attachment and receptor binding, the capsid is known to undergo a conformational change in acidified endosomes in which the internalized VP1unique portion of capsid is extruded through the intact capsid exposing the phospholipase domain (41, 42) within this region thought to facilitate endosomal escape through cleavage of the lipid membrane. Recent identification of GPR108 as the second cellular entry factor highly conserved across capsid serotypes

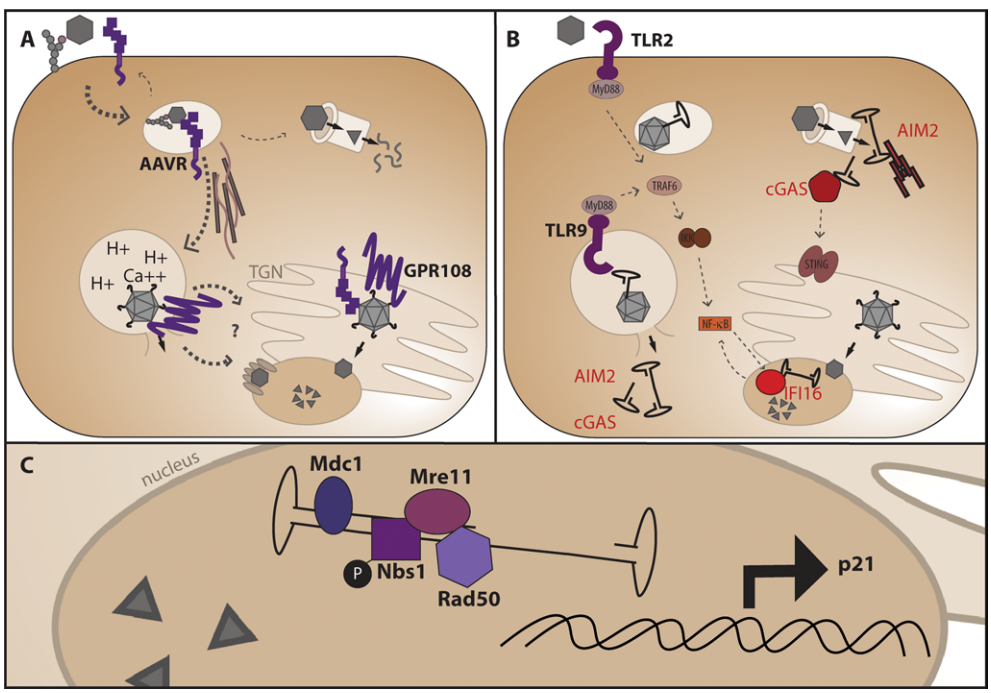

FIGURE 3 | AAV entry mechanism and points at which genome release may trigger DNA-specific pattern recognition receptors. **(A)** After glycan attachment, the AAV capsid is endocytosed and binds the conserved cellular receptor AAVR, is trafficked along microtubules for productive infection through the endosomal system and TGN (below), or non-productive infection and degradation through the proteasome. Endosomal acidification and potentially other ions such as calcium facilitate a conformational change in capsid which releases the VP1 portion of capsid for interaction with the conserved entry factor GPR108 prior to nuclear import. **(B)** Where the genome is first sensed in the cell may dictate the cellular response and alter viability of HSPCs. Non-productive infection and capsid degradation through the proteasome may leave cytoplasmic AAV genomes available for recognition by AIM2 leading to apoptosis, or cGAS leading to NF-κB signaling *via* the adaptor protein STING. Genome extrusion in endosomes may activate TLR2 and/or TLR9 causing NF-κB activation *via* MyD88 signaling, and aberrant endosomal escape may also allow sensing by AIM2 or cGAS. Nuclear AAV genomes may be sensed by the nuclear DNA sensor IFI16 causing subsequent NF-κB signaling. **(C)** Nuclear DNA damage response proteins known to interact with the AAV genome during second-strand synthesis or expressed in response to AAV transduction.

(43, 44) and required *in vivo* demonstrates an additional function for the VP1/2 unique region of the capsid in the entry pathway, as chimeric capsids and structural studies have demonstrated that while AAVR usage is dependent on the VP3 portion of capsid (45–49), GPR108 usage is dependent on the VP1/2 portion of capsid (44). Additional cellular factors may influence transduction efficiency either directly or indirectly such as the golgi-resident calcium transporter SPCA1, knockout of which demonstrates aberrant trafficking patterns within the cell (50). The recent identification and characterization of these golgi-resident factors over the past few years in addition to studies demonstrating that microinjection of AAV vectors into either the cytoplasm or nucleus does not facilitate transduction (51), highlight the importance of trafficking AAV through the endolysosomal system for productive and efficient transduction. While it is thought that AAV enters through the nuclear pore complex (52, 53), no nuclear pore proteins have been shown to interact directly with AAV capsid proteins as would be expected from a NPC-specific nuclear import mechanism and as seen with other NPC-using viruses, so questions remain about the exact nuclear import mechanism. Perhaps the most important and elusive question in the AAV entry pathway is at what point the AAV capsid disassembles and allows genome release. While we know the capsid must undergo conformational changes described above for transduction, and we know the capsid contains pores at the five-fold axes of symmetry large enough for the genome to fit through, it is unclear whether or not full capsid disassembly is required for genome release and if not where in the cell does the release occur.

What Is Special About AAV6?

Because AAV6 uses the same canonical factors AAVR and GPR108 as well as attachment factors shared by other AAV serotypes it is curious that AAV6 appears to transduce HSPCs for Cas9 mediated gene targeting so much more efficiently than other AAVs. Although there are reports that AAV capsid variants isolated from human HSPCs, termed AAVHSCs, can facilitate genome editing at higher frequencies than AAV6 in the absence of a specific DNA break (54, 55), this review is focused solely on AAV-based genome editing in the context of a Cas9-induced DSB so these capsids will not be reviewed at length there. Several groups have reported that AAV6 transduces HSPCs and other stem cell types higher than most other serotypes (56, 57), including the highly similar capsid AAV1 which differs by only 6 of the 737 capsid amino acids, for a sequence similarity of 99.2%. Indeed, we have reported that Cas9/AAV targeted integration at the *HBB* locus in HSPCs is 40% higher from AAV6 than from AAV1, while both episomal expression and targeted integration are 30-fold and 300-fold greater, respectively, from AAV6 than AAVHSC (58). The increased activity of AAV6 compared to AAVHSC in facilitating gene targeting in HSPCs following a DSB was also shown by the Cannon group (59). These 6 differential amino acids exist spread throughout the capsid as single amino acid substitutions, and these do not appear to come together to form any sort of unique domain in the fully assembled capsid (60). With the recent identification of the conserved AAVR and GPR108, it is possible that there are other entry or restriction factors specific to AAV6 in HSPCs that have yet to be discovered. It is also possible that in the absence of specific proteinaceous factors the capsid is inherently more efficient at uncoating or genome release in HSPCs therefore facilitating higher genome editing. In a sypro-orange thermostability assay, AAV6.2, the AAV6 capsid in which the unique amino acid in VP1 was reverted to the amino acid present in AAV1 (61), AAV6.2 demonstrates a significantly lower melting temperature than AAV1 suggesting it may be less stable than AAV1 (62). Interestingly, these capsids have the same melting temperature at very low pH of 3, yet the largest melting temperature difference is observed at neutral pH, so it is unclear how this melting data translates to the biological stability of these AAV capsids within the acidified endosome. While these details of AAV6 transduction in HSPCs may seem inconsequential as long as the genome reaches the nucleus, the process of how these AAV genomes reach the nucleus is of great interest both for improving genome editing efficiency, and in order to understand how and when the cell "see's" the AAV and alters the cellular function within this highly sensitive, highly specific cell type. During the next portion of this review we will discuss points at which the HSPCs may sense the AAV vector and potential impacts this may have on the HSPCs based on what is known about innate immune and DNA damage responses in HSPCs. Although there have been many studies and reviews aimed at addressing how transgene products can cause immune recognition in genetic diseases which cause a complete loss of protein and destruction of transduced cells through CD8+ T cell reactivity to the AAV capsid *in vivo* (63), this review is focused on aspects inherent to the AAV vector (capsid proteins and AAV genome ITRs) in an *ex-vivo* genome editing context.

How Might the AAV Genome Be Sensed as a PAMP in HSPCs?

Innate cellular responses are activated by pattern recognition receptors (PRRs) that sense cellular stress through pathogen-associated or damage-associated molecular patterns (PAMPS or DAMPS). For an extended review of PAMP and DAMP recognition by PRRs, we refer readers to (64, 65), and this review will only discuss the pathways relevant to cellular damage caused by intracellular sensing of AAV vector components, namely the AAV ITRs. Additionally, while vector quality and purity from production is of extreme importance to minimize cellular contaminants that could be recognized as pathogen or damage associated, we will only discuss aspects inherent to the vector itself and not the production process. AAV as a minimal vector comprised of three capsid proteins and only ~145 bases of inverted terminal repeat AAV genome on either side of the transgene for packaging leaves little to be recognized by innate cellular responses. However, it is known that TLR9 which exists in the endosome of antigen presenting cells and recognizes dsDNA can be activated by AAV, signaling through the MyD88 pathway and causing amplification of CD8 T cell responses (66, 67) (**Figure 3B**). TLR2 has also been implicated in innate activation in response to AAV vectors (68). While TLRs are classically thought of as PRRs specific to antigen presenting

cells such as macrophages and dendritic cells, several reports have demonstrated TLR expression on human HSPCs (69–71). In HSPCs, TLR-specific agonists induce cell cycle entry and lineage-specific differentiation. While several studies have demonstrated the importance of TLR signaling *in vivo* for hematopoiesis in response to microbial infection, ex vivo studies highlight the innate TLR functionality present in HSPCs. Ex vivo TLR9 activation by CpG DNA can induce mouse common lymphoid progenitors (CLPs) to differentiate to dendritic cells in response to herpesvirus infection (72). Additionally, TLR2 activation of human CD34+ cells *in vitro* with Pam_3CSK_4 pushes cells towards a myeloid-biased lineage, generating CD11c+ monocytes and dendritic cells (71, 73, 74). CpG depleted AAV vectors are in development to decrease TLR9 activation by vector genome CpG di-nucleotides *in vivo* (75–77). Recent incorporation of TLR9-inhibitory sequences derived from human telomeric DNA sequence have additionally been used to counteract TLR9 sensing *in vivo* (78). If AAV vectors are able to activate TLR9 in HSPCs *ex-vivo*, CpG depletion or incorporation of TLR9 inhibitory sequences may be a useful strategy for both *in vivo* AAV-mediated gene delivery as well as *ex-vivo* AAV-mediated gene editing.

In addition to TLR activation, the palindromic ITR hairpin DNA sequences could be sensed by cellular DNA sensors such as cGAS, IFI16, or AIM2. cGAS was originally described as a cytoplasmic DNA sensor which binds to short stretches of dsDNA and DNA/RNA hybrids and catalyzes the synthesis of cyclic GMP-AMP, a second messenger which activates STING to induce interferon through IRF3 dimerization and NF-κB signaling. However, recent immunofluorescent experiments have demonstrated that endogenous levels of cGAS can be found in the nucleus of LT-HSCs and it is kept silent through a small circular RNA called cia-cGAS to prevent activation in quiescent cells, yet this circular RNA does not exist in committed progenitors (79). In contrast, IFI16 is thought to be nuclear localized (80) and has multiple reported functions including regulation of transcription in hematopoiesis, as well as recruitment and activation of STING in the presence of viral infection (80). Expression of IFI16 has been shown in CD34+ cells from human bone marrow, which upon induction of differentiation was only preserved in cells of the monocytoid lineage as determined by expression of CD14 (81). Additionally, IFI16 expression has been shown to be 4-fold higher in quiescent HSCs compared to more proliferative progenitors (82). While cGAS and IFI16 are most commonly associated with induction of an interferon response, they have additionally been shown to induce non-canonical cellular responses such as cell cycle arrest (83, 84) and apoptosis or pyroptosis (85) in certain cell types. In contrast to cGAS and IFI16, the canonical activation of AIM2 causes induction of the AIM2-inflammasome (86) resulting in maturation of IL-1β and IL-18 as well as a type of programmed cell death called pyroptosis (87). Interestingly, mice deficient in AIM2 are protected from bone marrow failure after total body irradiation (88), demonstrating that AIM2 plays a role in apoptosis induction in the hematopoietic system. While some of these DNA sensing proteins have similar canonical downstream effector proteins involved in IFN activation, protein complex differences in unique cell types can greatly influence the cellular outcome from activation. There have been reports that activation of certain IFI16 isoforms can inhibit AIM2 inflammasome formation (89), and AIM2 can inhibit cGAS by cleavage through caspase-1 (90). DNA virus induced caspase-3 has also been shown to cleave cGAS, MAVS, and IRF3 to prevent overproduction of inflammatory cytokines (91), demonstrating that there is cross-talk between DNA sensing pathways and suggesting that aspects of the cellular response can be post-transcriptionally regulated. It is currently unclear if the AAV ITRs activate these cytoplasmic DNA sensors and how differential activation may influence the outcome of gene-editing therapies in HSPCs, yet observed toxicity from the vector *in vitro* suggests there is an inherent response which may influence cell survival after editing and transplant.

Where Is the Genome First Sensed in the Cell?

When considering potential cellular responses to the AAV vector it is important to consider where the genome is first sensed in the cell, as the most likely component to be sensed as a danger signal. Herein lies the important distinction of entry pathway usage, as endocytosis is not synonymous with true viral vector entry (ie: breach of a membrane barrier to the internal cytoplasm or nucleus) and may differ in cell types which have differential cellular responses to the vector. It has been demonstrated by other viral vectors such as adenovirus that capsids which traffic through and accumulate in the perinuclear region in late endosomes (as demonstrated through co-localization with LAMP1 and M6P) induce inflammatory cytokines while Ad5 which traffics directly to the nucleus does not (92), presumably due to increased recognition by intracellular viral DNA sensors. A variety of AAV serotypes have shown similar peri-nuclear accumulation during the entry pathway and co-localization with Rab7 and Rab11 suggest trafficking through late and/or recycling endosomes (93), as well as through the Trans-Golgi network (TGN) (94). The AAV receptor AAVR has demonstrated functional necessity for trafficking to these compartments late in the endolysosomal system, as domain swap chimeras containing the AAV binding and transmembrane domains with the cytoplasmic tails of well-defined trafficking domains demonstrates that trafficking to the TGN maintains full AAVR function yet chimeras containing c-tails that traffic to early or late endosomes but not TGN have drastically reduced functionality (40). In addition to trafficking considerations, the GPR108 protein has been implicated in regulation of NF-κB signaling, with knock-out mice demonstrating higher cytokine production (95), yet it is unclear whether AAV association with GPR108 triggers its functionality within the NF-κB pathway or if it solely takes advantage of the protein to gain access to the cell without triggering its activity. It is well-known that AAV can be degraded through the proteasome (96), and decreased efficiency of trafficking in some cell types may increase the amount of proteosomal degradation leading to increased availability of AAV genomes for intracellular recognition and a magnified cellular stress response. Additionally, when considering that

vector copy numbers of 5,000 to 100,000 genomes per cell are used for transduction and genome targeting, with hundreds or thousands of vector genomes per cell isolated from cytoplasmic extracts of transduced cells in order to achieve a functional multiplicity of infection (MOI) of 1 (meaning on average 1 functional transduction event per cell for all cells to have undergone successful gene replacement or gene targeting) a major question remains about what happens during non-productive infection and how are these extra hundreds/thousands of genomes being sensed and responded to within the cell. While there is great focus on genotoxicity caused by DNA break induction from Cas9 during genome editing, a source of genotoxicity especially in sensitive stem cell populations is likely caused by the genotoxicity of lingering AAV genomes from non-productive transduction events after uncoating or capsid degradation.

How Might the AAV Genome Be Sensed as a DAMP in HSPCs?

While lingering non-productive vector genomes may be detrimental the cell, an additional source of cellular stress may be during productive transduction when the genome is sensed in the nucleus. As a DNA hairpin with a free DNA end, the ITRs can also be sensed by DNA damage response (DDR) proteins in the nucleus (97). It has been demonstrated through co-localization experiments that a variety of DDR proteins co-localize with nascent vector genomes upon nuclear entry as they undergo second-strand synthesis in discrete nuclear foci. These proteins include Nbs1, phosphorylated NBS1 (p-S343-Nbs1), Mre11, Rad50, and Mdc1 (**Figure 3C**). The Mre11-Rad50-Nbs1 complex is responsible for recognizing dsDNA nicks near the 5'end of a DSB to facilitate end resection required for DSB repair through homologous recombination. The co-localization of these proteins within the nucleus is interesting, as it begs the question whether this recognition is harmful as the sensing of a DNA break, or helpful by recruitment of HR machinery during AAV-mediated HDR. Because the choice of HDR compared to the error prone non-homologous end joining (NHEJ) pathway is dictated by successful end resection (98) it is possible that presence of these proteins may be helpful for skewing the repair pathway post-break towards the HDR pathway in the presence of AAV. Exposure of cells to DNA-damaging agents which upregulate DDR proteins has been shown to increase recombinant AAV vector transduction in the absence of Adenovirus co-infection (99–101), yet the mechanism is not fully understood and it is unclear whether a similar effect would be observed in gene editing applications where transgene expression is driven after targeted integration rather than from the episomal genome. Although gene expression can be observed from episomal AAV through either inherent promoter activity in the ITRs or concatemeric AAV genomes (102–105), the highly proliferative nature of HSPCs causes a quick loss of this expression through a dilutional effect of unintegrated AAV genomes (5) and transcriptional activity may be regulated differently from episomal AAV compared to integrated DNA sequence after HDR. It has also been demonstrated that AAV transduction in HSPCs and other cell types upregulates p21 expression (24, 106, 107), and addition of the ATM-kinase inhibitor KU55933 has been shown to decrease the number of apoptotic HSPCs after AAV transduction (107). Addition of GSE56, a p53 dominant negative mRNA rescues engraftment defects after AAV/Cas9 gene editing when co-electroporated with the genome editing components (24), yet it is unclear if this inhibitor would counteract the loss of engraftment potential seen in HSPCs from AAV alone. Because the p53/p21 pathway influences many aspects of cell function including cell-cycle arrest, apoptosis, senescence, and suppression of HR, this activation could be affecting HSPC function in many different ways. Interestingly, it has been shown that p53-null cells undergo apoptosis after transduction with AAV (108), so it is clear there are aspects of the cellular response to AAV that are caused by p53 activation. While it is clear that there are many interactions of the AAV genome with cellular replication and DNA damage response proteins, it is unclear how AAV genome recognition dictates the cellular response after recognition.

While presented here as separate cellular responses, the innate immune and DNA-damage response pathways are inextricably linked. A major function of cellular DNA sensors is to prevent cellular replication in response to not only viral infection but to DNA damage as well and the cellular outcomes are determined by the mechanism and magnitude of the cellular response. The cGAS/STING pathway gets activated as a response to genotoxic stress due to DNA damage, and the magnitude determines whether cells will repair, go into senescence, or undergo cell death (109–111). In addition, IFI16 has been reported to negatively regulate p53 and p21 to influence p53-mediated cell cycle arrest (83, 112). In the following section we will discuss what can be learned from replication of other parvoviruses within the hematopoietic compartment, as well as what has been learned so far from initial investigations into pathways activated by AAV transduction in HSPCs

What Cell Intrinsic Effects Are Known to Occur in Response to Parvoviral Infection in HSPCs?

The HSPC population must undergo strict regulation to mobilize blood and immune cells in response to a variety of infectious agents, yet few viruses affect them directly. Parvoviruses, of which AAV is part of the dependoparvovirus subfamily, are one of the few types of viruses which can actually infect cells of the HSPC lineage. Human Parvovirus B19 has a selective preference for cells of the erythroid lineage within the bone marrow and can cause both transient and persistent erythroid aplasia, anemia, and bone marrow failure. B19 can cause apoptosis, G1 cell-cycle arrest, and G2 cell cycle arrest in target cells (**Figure 4**) and apoptosis and G1 arrest are a result of toxic genomic NS1 expression after entry (113). AAV Rep protein exerts analogous functions in virus replication to NS1, yet is provided in trans during vector production and is not present in the AAV vector after purification or encoded in the vector genome. Rare packaging of rep-containing DNA sequences have been observed in vector preparations (114, 115), yet packaging of undesirable genome elements such as rep-

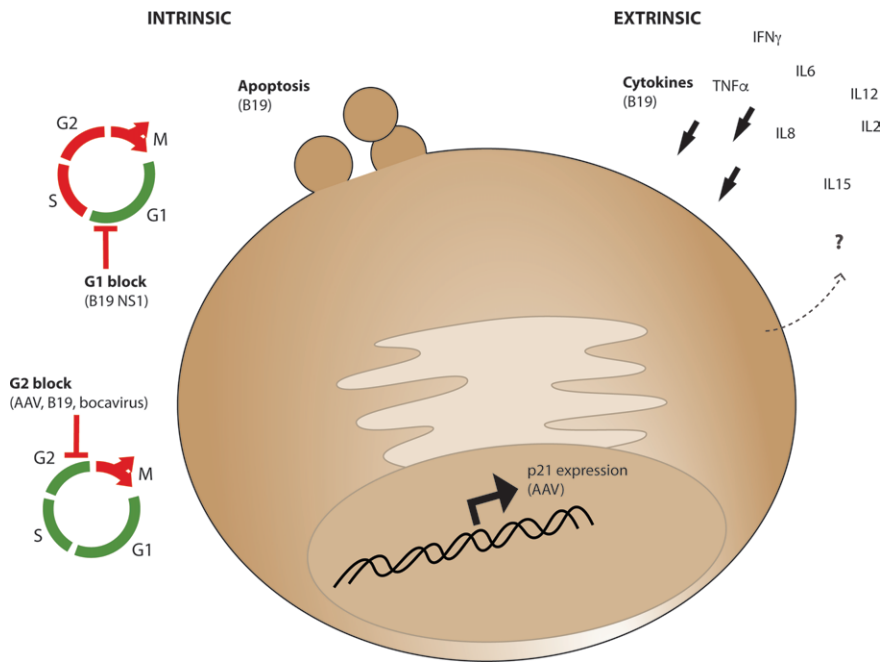

FIGURE 4 | Intrinsic and Extrinsic parvoviral effects which may influence CD34+ cell survival and functionality. Intrinsic cellular effects (left) observed from AAV or other parvoviruses include apoptosis, G1 cell cycle arrest through B19 parvovirus NS1 expression, or G2 cell cycle block from AAV, B19, and bocavirus genomes. Extrinsic effects (right) observed from parvovirus B19 infection include expression of cytokines which promote CD34+ cell differentiation including IL6, which are expressed from various cell types *in vivo* during acute and chronic infection.

containing plasmid sequence or human sequence are rare (116) and prevalence of aberrant genome packaging likely is influenced by transgene sequence (117). However, it has been demonstrated that G2 cell-cycle arrest can be induced by both live and UV-inactivated B19, AAV, and bocavirus (118) suggesting that a major cause of cellular toxicity is simply the presence of parvovirus genome even in the absence of replication. This toxicity was shown to be caused by the bocavirus terminal repeats (119), and the AAV ITRs through nuclear injection of the AAV ITR sequence in human embryonic stem cells which causes apoptosis (120). Importantly for AAV vector considerations, this toxicity was observed in response to purified full but not empty capsids, demonstrating that toxicity in stem cells is due to the presence of encapsidated vector genomes, not cellular contaminants from the production process or from the capsid itself. While the replication competent B19 causes extreme hematopoietic toxicity and WT AAV infection has no known human pathology despite anti-capsid antibodies demonstrating common infection in humans (121–123), by investigating commonalities within this virus family we may identify points of intervention at which the health and survival of HSPCs after AAV-mediated gene editing can be improved.

Preliminary studies have aimed to identify transcriptional changes induced in HSPCs after editing by microarray gene expression profiles after treatment of cells with the individual gene editing components including AAV treatment (124). 24 hours after electroporation and AAV transduction HSPCs upregulate apoptosis factors DHRS2, GZMB, GDF15, and CCL2, as well as stress response genes RAP1GAP and BICC1. Transduction alone specifically upregulates DDR proteins RPA4 and PHLDA3, stress response proteins RAP1GAP and BICC1, and the immune molecule CD86. Of note, RPA has previously been implicated in AAV genome replication (125) and PHLDA3 is a p53-regulated Akt suppressor, deletion of which decreases p53-dependent apoptosis (126). These data are suggestive that AAV transduction of HSPCs induces an apoptosis program in some cells. Additionally, upregulation of CD86 which is normally expressed on antigen presenting cells such as dendritic cells and monocytes but not HSPCs suggests that AAV transduction pushes cells toward a lineage committed state and decreases regenerative potential. While it remains to be determined the mechanism by which AAV is altering hematopoietic stem cell fate, it is clear that there is a transcriptional response occurring that may alter HSPC function.

What Cell Extrinsic Effects Are Known to Occur in Response to Parvoviral Infection in HSPCs?

The tight regulation dictating hematopoiesis is determined by cytokines in the blood and bone marrow niche which determine the proliferative, regenerative, and differentiation fate of the stem and progenitor cells in response to a variety of stimuli including infection. The bone marrow niche plays a huge role in this regulation, but cytokines in ex vivo culture play a major role in the survival and function of HSPCs as well. In culture, a combination of cytokines usually including stem cell factor (SCF), thrombopoietin (TPO), Fms-related tyrosine kinase 3 ligand

(Flt-3 ligand), and interleukin-6 (IL-6) are used to both maintain HSPC survival as well as push quiescent HSCs into the cell cycle in order to express the genes required for successful homologous recombination. Studies of parvovirus B19 infection *in vivo* have demonstrated increased levels of cytokines known to influence HSPC survival and differentiation including interferon-γ, tumor necrosis factor-α, IL-6, and IL-8 (127). Early infection at the initial peak viral load during acute infection has also demonstrated elevated IL-2, IL12, IL-15 levels in the absence of IFN-γ, while persistent infection was associated with elevated IFN-γ (128). Because these data were collected from patients it is presumed these cytokines were produced from B19 responsive CD8+ T cells (129, 130). However, it has been shown that LSK cells have the ability to produce a variety of cytokines including IFN-γ, IL-6, IL-2, IL-12 at high levels in response to danger signals (131). Single-cell analysis of multipotent progenitors (MPPs) demonstrated that 69% of cells produced at least one and up to 12 different cytokines in response to TLR stimulation by LPS and Pam_3CSK_4 (131), a TLR-2 ligand previously shown to activate TLR-2 in human CD34+ cells (71, 74). It is unclear if HSPCs directly express cytokines in response to parvoviral infection with B19 or AAV transduction during gene editing, but if so then paracrine functions on neighboring cells in culture may influence the engraftability of cells after editing.

Inflammatory cytokines are well known to modulate the hematopoietic compartment, and play important roles in mobilization from the bone marrow (132–134). Tight regulation of HSC response to inflammatory cytokines through cellular factors such as interferon regulatory factor-2 (IRF2) and ADAR1 are essential for the suppression of HSC exhaustion due to interferon signaling (135, 136). Factors such as TGF-β differentially regulate distinct hematopoietic stem cell subtypes; even true LT-HSCs are thought to be a somewhat diverse cellular population with individual cells being biased towards the myeloid or lymphoid lineage, and this differentiation is thought to be regulated through TGF-β (137). Conflicting reports on the effect of IFN-γ demonstrate an obvious but poorly understood effect of IFN-γ on hematopoiesis (138). Some reports have suggested that treatment of HSPCs with IFN-γ in culture causes expression of pro-apoptotic genes and reduced progenitor survival in a colony forming assay, yet a conflicting report suggests that IFN-γ exposure increases cell viability and colony formation. In vivo, IFN-γ injection has been shown to increase progenitor proliferation and activate dormant hematopoietic cells. Based on these experiments, it is suggestive that low cytokine levels leading to low levels of immune activation may increase survival of HSPCs, while high levels of activation are detrimental and lead to exhaustion of the hematopoietic compartment.

DISCUSSION

How Do These Cellular Responses to AAV Alter the Long Term Potential and Engraftment of HSPCs?

The DNA damage and innate immune response pathways have integral interactions within them that influence cellular responses (139). This leads us to a chicken and egg problem about what cellular response comes first in response to AAV transduction of HSPCs and what is the rotten egg that is detrimental to HSPC engraftment after AAV-mediated gene editing. A variety of advances have been made to improve gene-editing efficiency and specificity in HSPCs including transition from using Cas9 mRNA to purified Cas9 RNP complex, chemical modification of gRNAs to decrease immune sensing (140), optimization of homology arm and guide RNA specificity, engineering of high fidelity Cas9 nucleases (141), increasing the purity of AAV vector preps, and decreasing the amount of AAV used. In addition, a variety of small molecules have been used to try to boost HDR efficiency during genome editing (142). These advances have led to extremely specific and efficient AAV-mediated genome targeting in many primary cells, and now the major area for improvement is increasing cell potency and viability during editing and engraftment after AAV transduction.

How Do DDR and Innate Immune Pathway Activation Alter Engraftment Efficiency of HSPCs?

A lot can be learned about HSPC function from examining genetic diseases which cause a defect in components of the DDR pathway. Patients with defects in DNA damage signaling and repair [such as in ataxia-telangiectasia (A-T) and Fanconi-anemia (FA), respectively] highlight the importance of these pathways in HSCs as these patients have a high incidence of bone marrow failure and hematological malignancies. Competitive reconstitution experiments in mice demonstrate that deletion of a variety of different DDR pathway proteins cause a defect in hematopoietic reconstitution. Cells transplanted from knock-out mice demonstrate a decrease in progenitor cell reconstitution relative to WT cells, while young mice have no defect in overall HSC number suggesting this defect is a proliferative or regenerative defect, not a HSC developmental defect. Additionally, macrophages deficient in FA proteins have an increased production of inflammatory cytokines in response to TLR ligands, while the progenitor cells are hypersensitive to these cytokines which include IFN-γ, TGF-β, and IL-1. Though the mechanisms are still debated it is clear that these pathways are intricately related and greatly influence HSPC function and survival *in vivo*. There appears to be a balancing act for the level of innate immune activation to promote HSC quiescence and self-renewal compared to activation and differentiation (143). The proposed mechanism of the self-renewal improving small-molecule UM171 is through low level activation of inflammation but high concentrations of UM171 are detrimental to cell survival (144, 145), supporting this balancing act model. Several reports have demonstrated that low-level NF-κB activation promotes quiescence and self-renewal of LT-HSCs, suggesting that some level of innate response is beneficial to the survival and function of these cells.

What Methods Have Been Used to Improve Engraftment After AAV-Mediated Genome Editing, and Where Do We Go From Here?

There are currently no specific targets known to increase efficiency of engraftment specifically after AAV transduction. During *in vivo*

engraftment experiments, the main method to increase engraftment is by transplanting more cells but as these studies move from small animal (NSG mouse) to human studies the feasibility of transplanting higher numbers of edited cells becomes limiting. In addition to transplanting more cells, inclusion of several cytokines and small molecules such as UM171 in culture throughout the course of editing are known to improve engraftment (23, 144, 146). Factors which support stem cell survival such as stem cell factor (SCF) and thrombopoietin (TPO) greatly enhance the engraftment potential of HSPCs when maintained in culture. However, the benefit of including these cytokines occurs in all transplant groups, not just when transplanting AAV-transduced cells. While the incorporation of GSE56 during AAV-mediated genome-editing has been shown to preserve engraftment efficiency and increase clonality of transplanted cells (24), it is of great importance to further understand specific pathways which can be safely manipulated in the absence of potential unwanted cellular responses. Over 50 years of hematopoietic stem cell research has taught us much about HSPC biology, yet we have little understanding of how AAV transduction alters the function of HSPCs and their health and survival during *ex-vivo* AAV-mediated genome editing. It is likely that the first-in-human clinical trials using AAV6 as a donor for gene correction, will teach us even more about the potency of gene-targeted HSCs and serve as the basis for further optimization. Nonetheless, as this novel class of genetic medicines moves towards the clinic, continued rigorous study of the basic biology in primary cells such as HSPCs to determine pathways activated and altered by AAV transduction will allow for the improved success of AAV-mediated gene editing in the future.

AUTHOR CONTRIBUTIONS

AMD and MHP conceived and assembled information for this review. AMD wrote and generated figures, and MHP contributed significant edits and intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

Grant support for AMD is through NIH F32 grant number 1F32HL154667-01. MHP thanks the Doris Duke Charitable Trust (#22399) and the Sutardja Chuk Professorship for support of this work.

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Conflict of Interest: MHP holds equity in CRISPR Tx, Graphite Bio, and Allogene Tx; serves on the Board of Directors of Graphite Bio and SAB's of Allogene Tx and Ziopharm Oncology Inc. and receives compensation for those activities.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 31 May 2021
doi: 10.3389/fimmu.2021.672449

Effect of CpG Depletion of Vector Genome on CD8$^+$ T Cell Responses in AAV Gene Therapy

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Adeno associated viral (AAV) vectors have emerged as a preferred platform for *in vivo* gene replacement therapy and represent one of the most promising strategies to treat monogenetic disorders such as hemophilia. However, immune responses to gene transfer have hampered human gene therapy in clinical trials. Over the past decade, it has become clear that innate immune recognition provides signals for the induction of antigen-specific responses against vector or transgene product. In particular, TLR9 recognition of the vector's DNA genome in plasmacytoid dendritic cells (pDCs) has been identified as a key factor. Data from clinical trials and pre-clinical studies implement CpG motifs in the vector genome as drivers of immune responses, especially of CD8$^+$ T cell activation. Here, we demonstrate that cross-priming of AAV capsid-specific CD8$^+$ T cells depends on XCR1$^+$ dendritic cells (which are likely the main cross-presenting cell that cooperates with pDCs to activate CD8$^+$ T cells) and can be minimized by the elimination of CpG motifs in the vector genome. Further, a CpG-depleted vector expressing human coagulation factor IX showed markedly reduced (albeit not entirely eliminated) CD8$^+$ T cell infiltration upon intramuscular gene transfer in hemophilia B mice when compared to conventional CpG$^+$ vector (comprised of native sequences), resulting in better preservation of transduced muscle fibers. Therefore, this deimmunization strategy is helpful in reducing the potential for CD8$^+$ T cell responses to capsid or transgene product. However, CpG depletion had minimal effects on antibody responses against capsid or transgene product, which appear to be largely independent of CpG motifs.

Keywords: CD8$^+$ T cell, dendritic cells, CpG, adeno-associated virus, TLR9, gene therapy, hemophilia

OPEN ACCESS

Edited by:
Jose Martinez-Navio, University of Miami Health System, United States

Reviewed by:
Denise Sabatino, University of Pennsylvania, United States
Gloria Gonzalez-Aseguinolaza, University of Navarra, Spain

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *25 February 2021*
Accepted: *20 April 2021*
Published: *31 May 2021*

Citation:
Bertolini TB, Shirley JL, Zolotukhin I, Li X, Kaisho T, Xiao W, Kumar SRP and Herzog RW (2021) Effect of CpG Depletion of Vector Genome on CD8$^+$ T Cell Responses in AAV Gene Therapy. Front. Immunol. 12:672449. doi: 10.3389/fimmu.2021.672449

INTRODUCTION

Adeno associated virus (AAV) has emerged as the preferred platform for *in vivo* gene replacement therapy and represents one of the most promising strategies to treat monogenetic disorders such as hemophilia. Two AAV-based gene therapies have received regulatory approval and many more are currently under investigation in late-stage clinical trials (1, 2). However, adaptive immune responses

directed against the AAV capsid and the transgene product continue to limit long-term clinical success (3–6). These include cytotoxic T lymphocytes (CTLs) that eliminate transduced cells by recognizing peptides derived from the AAV capsid and activated B cells that produce antibodies capable of neutralizing the therapeutic transgene product. In clinical trials, immunosuppressive drugs are frequently administered to mitigate immune mediated rejection of AAV and preserve therapeutic gene expression. However, these drugs come with significant safety concerns and inadequately address AAV-associated immune responses and immunotoxicities. Thus, there is a growing interest in developing AAV vectors that are devoid of immunogenic features and can escape immune detection all together.

Innate immune sensing is critical for the initial detection of microbes and conditions downstream adaptive immune responses. Antigen presenting cells like macrophages and dendritic cells (DCs), express a diverse repertoire of pattern recognition receptors such as toll-like receptors (TLRs) that each recognize distinct, evolutionarily conserved, pathogen associated molecular patterns (PAMPs). Signaling through these receptors triggers a cascade of immunoregulatory events that balance the decision to respond with tolerance or immunity. Initial innate immune detection of AAV vectors occurs *via* toll-like receptor 9 (TLR9) (7, 8). TLR9 is an endosomal DNA sensor that recognizes unmethylated cytosine-phosphate-guanine (CpG) motifs commonly present in viral and bacterial DNA genomes. Importantly, TLR9 sensing of AAV vector genomes has been shown to be required for anti-capsid $CD8^+$ T cell responses (9–14). Specifically, TLR9 signaling in plasmacytoid dendritic cells (pDCs) is required for adaptive immune responses to AAV. Type I interferons (T1 IFN) produced downstream of TLR9 in pDCs are necessary to activate conventional DCs (cDCs) which process and present AAV capsid derived antigens on MHC class I (MHC I) to prime anti-capsid $CD8^+$ T cells. Indeed, both TLR9 and T1 IFNs (and also $CD4^+$ T help) are requisite for anti-capsid $CD8^+$ T cell priming (9, 15). Thus, CpG motifs present in the DNA genome of AAV gene therapy vectors are key determinants of vector immunogenicity.

Evidence from early clinical trials, along with subsequent laboratory studies, suggest that $CD8^+$ T cell responses against AAV capsid target transduced hepatocytes (16–18). Moreover, a recent meta-analysis of clinical trials using hepatic AAV gene transfer concluded that vectors rich in CpG motifs results in failure to achieve sustained expression, owing to activation of capsid specific $CD8^+$ T cells (19–22). Therefore, elimination of CpG motifs has become a feature of more recent vector designs. Additionally, Faust et al. demonstrated that use of a CpG depleted expression cassette ablated CTL responses to the β-galactosidase transgene upon AAV gene transfer in mice (23), suggesting this strategy may reduce the risk of immune response directed against the transgene product.

Here, we demonstrate that depletion of CpG motifs from the AAV expression cassette substantially reduces cross-priming of capsid specific $CD8^+$ T cells in mice. We further show in hemophilia B mice that $CD8^+$ T cell responses against the therapeutic transgene product, coagulation factor IX (FIX), are dramatically reduced using the CpG-depleted vector construct compared to native sequences. Interestingly, this approach was not useful to prevent antibody formation against capsid or transgene product.

MATERIALS AND METHODS

Mice

Wild type (WT) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). $XCR1^{+/DTRvenus}$ mice were obtained from RIKEN Center for Integrative Medical Sciences. In $XCR1^{+/DTRvenus}$ mice, coding region of the *Xcr1* gene was replaced with a genetic cassette encoding diphtheria toxin receptor (DTR) and reporter protein "Venus" (a yellow fluorescent protein) (24, 25). Expression of DTR under XCR1 promoter allowed specific depletion of $XCR1^+$ DCs by administration of diphtheria toxin (DT) whereas expression of fluorescent protein allowed their easy detection and tracking. Hemophilia B (C3H/HeJ-F9-/Y) mice with a targeted deletion of murine *F9* gene had been bred on the C3H/HeJ background as published (24–26). All animals were maintained at laboratory animal resource center facility at Indiana University–Purdue University, Indianapolis (IUPUI). All animal experiments were performed as per the guidelines of Institutional Animal Care and Use Committee (IACUC). Male mice, 6 to 8 weeks of age were used. The specific number of mice in each cohort is indicated in each figure with a minimum of four mice per group.

AAV Vector

AAV1 and AAV2 serotype were used to perform these studies. To assess capsid specific $CD8^+$ T cells a surrogate epitope SIINFEKL (a dominant $CD8^+$ T cell epitope derived from ovalbumin) was cloned into the AAV2 capsid HI loop (9). Two gene cassettes [one with native CpG sequence (27) and other with CpG-depleted sequence] of human coagulation factor IX (hFIX) containing Padua mutation were used in these studies. CpG depleted sequence of hFIX with Padua mutation was custom synthesized by Invivogen (San Diego, CA, USA) and is provided in **Supplementary Figure 1**. Both cassettes were under the transcriptional control of a cytomegalovirus (CMV) immediate early gene 1 enhancer/human elongation factor-1α (EF1α) promoter combination and had SV40 polyadenylation sequence (Invivogen). The naturally occurring Padua mutation represents a single amino acid change (R338L) in human FIX that results in a ~1 log increased specific activity, resulting for higher efficacy in gene therapy per FIX antigen level (28). All AAV vectors used in this study were single stranded (ss) and were produced by triple transfection of HEK-293 cells. All AAV vectors were purified by double iodixanol gradient centrifugation and titers determined by quantitative PCR (29).

Animal Procedures

$XCR1^+$ DCs were depleted by administering 1 μg of DT (Millipore, Massachusetts, USA) to $XCR1^{+/DTRvenus}$ mice.

$CD103^+$ DCs were neutralized by administration of 150 μg of anti-CD103 antibody (BioXCell, Lebanon, NH) to C57BL6 wild type (WT) mice. In order to maintain the depletion of $XCR1^+$ DCs and neutralization of $CD103^+$ DCs, DT was administered on day −1, 3, and 7, and anti-CD103 antibody was administered on day −1, 3, 7, and 10, both *via* intraperitoneal (i.p) route. For all experiments, mice were injected intramuscularly (i.m) into the quadricep muscle with 50 μL of AAV containing 1×10^{11} vg. Post AAV administration mice were bled at different time points *via* retro-orbital plexus using heparinized capillaries. Blood from hemophilia B mice was collected using untreated capillaries in to 0.38% sodium citrate buffer. Hemophilia B mice were euthanized at the end of experiment (28 days post AAV administration) and quadricep anterior muscle was harvested. Excised muscles were cryo-protected in optimal cutting temperature media using liquid N_2-cooled 2-methylbutane (30).

Flow Cytometry

To quantify capsid specific $CD8^+$ T cells, flow cytometry was performed on peripheral blood mononuclear cells (PBMC) using antibodies to CD3 (17A2), CD8a (53–6.7) and MHC I tetramer (H-2Kb-SIINFEKL, MBL International, Woburn, MA, USA) (15). To assess depletion of $XCR1^+$ DCs, spleens were pretreated with collagenase D (Roche, Basel, Switzerland) at 2 mg/mL for 20 min at 37°C. Single cell suspension of splenocytes were prepared and stained with CD11c (N418), XCR1 (ZET), and CD8a (53–6.7) antibodies (BioLegend, San Diego, CA). Flow cytometry data was acquired on the Fortessa flow cytometer (BD Biosciences) and analyzed using FCS express 7 (DeNovo Software, Los Angeles, CA).

Immunohistochemistry

Immunohistochemistry was performed on cryo-sections of quadricep muscle, as previously described (30). Briefly, cryosections (10 μm) of muscle were fixed in pre-cooled acetone at −20°C, blocked with 5% donkey serum (Sigma, St. Louis, MO) and stained with rat anti-CD8α (eBioscience) and goat anti-hFIX (Affinity Biologicals, Ontario, CA) antibodies at room temperature. Secondary antibodies, donkey anti-rat conjugated to Alexa Fluor 488 and donkey anti-goat conjugated to Alexa Fluor 568 (Life Technologies, Carlsbad, CA, USA) were used for detection. Sections were mounted using Prolong Diamond antifade with DAPI mounting media (Invitrogen, Carlsbad, CA). Mounted sections were stored protected from light at 4°C until visualization and image acquisition.

Image Acquisition and Analyses

Slides were scanned and digitized using an Axio observer 7 Zeiss microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY). Whole muscle sections were captured with a 40× objective, using the tiles option. Infiltrating $CD8^+$ T cell were quantified on whole muscle sections (area average 612.2519) using Fiji-ImageJ software after generating a grid (area per point, 1200 square pixels). Fluorescent signal for hFIX was quantified as mean gray value with Fiji-ImageJ software.

Analyses of Plasma Samples

Plasma samples from hemophilia B mice were collected by retro-orbital bleed into 0.38% sodium citrate buffer. Inhibitory antibodies to hFIX were measured by Bethesda assay as described (26). One Bethesda Unit (BU) is defined as the reciprocal of the dilution of test plasma at which 50% of FIX activity is inhibited. Measurements were carried out in a Diagnostica Stago STart Hemostasis Analyzer (Parsippany, NJ, USA). The activity of the expressed hFIX was assessed by ROX FIX chromogenic assay (Diapharma, Louisville, KY) following the manufacturer's protocol. Enzyme-linked immunosorbent assay (ELISA)-based measurements of anti-AAV2 IgG2c, AAV1 IgG2a, and anti-FIX IgG1 antibodies were carried out as described (12, 26).

Statistical Analysis

Results are reported as means ± standard error of the mean (SEM). Statistical significance between groups was determined by either unpaired Student's t test or two-way ANOVA using GraphPad Prism 7 software (San Diego, CA, USA). *P* value of <0.05 was considered significant. Differences are indicated as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RESULTS

Capsid-Specific $CD8^+$ T Cell Responses Depend on $XCR1^+$ Dendritic Cells

We have previously shown that cross-priming of AAV capsid specific $CD8^+$ T cells requires cooperation of pDCs, which sense the AAV genome *via* TLR9 and produce T1 IFN, and cDCs, which sense T1 IFN and present antigen (9, 15). The cDC compartment can be divided into two main subsets, cDC1s and cDC2s which are $XCR1^+$ and $CD11b^+$, respectively. The cDC1 compartment can be further broken down into tissue resident $CD8\alpha^+$ DCs and migratory $CD103^+$ DCs (31–35). To determine whether $XCR1^+$ DCs are required for $CD8^+$ T cell responses to capsid, we used $XCR1^{+/DTRvenus}$ mice, in which $XCR1^+$ resident DC can be depleted upon administration of diphtheria toxin (DT) (36). We also evaluated whether $CD103^+$ DCs are necessary for anti-capsid $CD8^+$ T cell responses by neutralizing $CD103^+$ DCs in WT C57BL/6 mice using an anti-CD103 antibody (37). To quantify capsid specific $CD8^+$ T cells, we used an AAV2 capsid containing SIINFEKL (AAV2-SIINFEKL), the immunodominant epitope of ovalbumin recognized by $CD8^+$ T cells (9). AAV-capsid specific $CD8^+$ T cells in peripheral blood were quantitated over time by flow cytometry using an H-2Kb SIINFEKL tetramer (9). $XCR1^{+/DTRvenus}$ mice (on C57BL/6 genetic background) were treated with 1 μg of DT i.p. on day −1, 3, and 7. Mice not receiving DT served as positive control for the T cell response. On days 0, animals received an i.m. injection into the quadriceps with 1×10^{11} viral genomes (vg) of AAV2-SIINFEKL (**Figure 1A**). Upon DT injection to $XCR1^{+/DTRvenus}$ mice, $XCR1^+$ DCs (expressing the YFP "venus" reporter) and $CD8\alpha^+CD11c^{hi}$ DCs (which represent the majority of $XCR1^+$ DCs) were efficiently

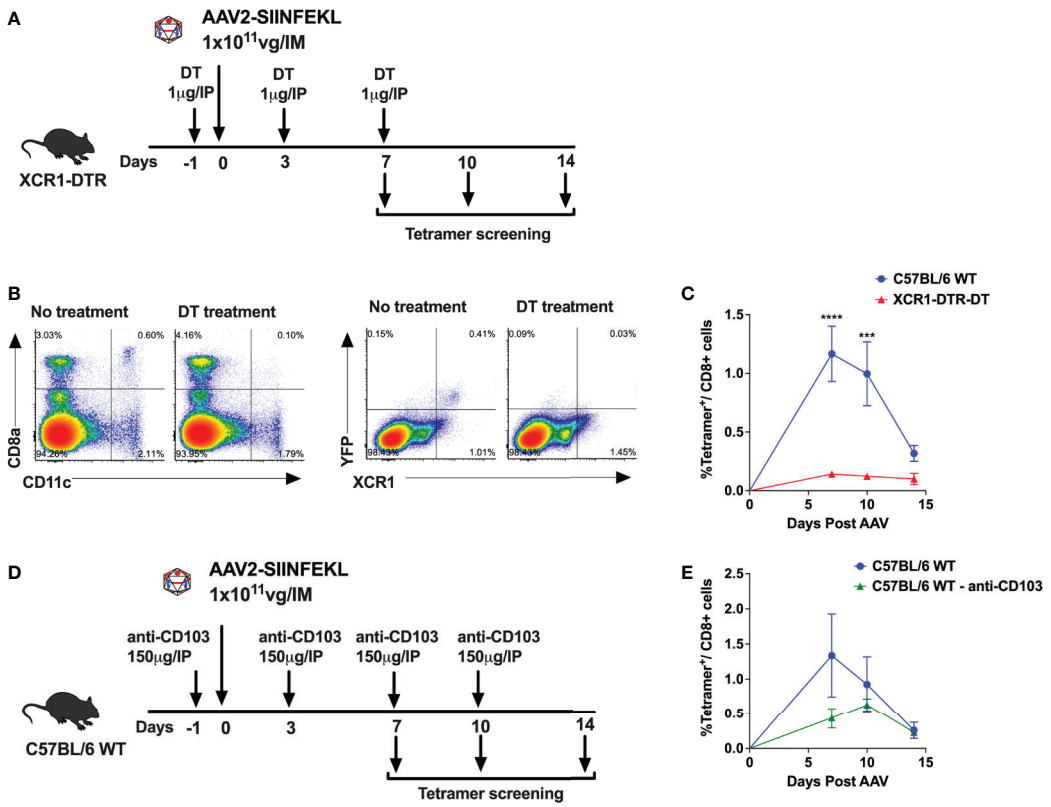

FIGURE 1 | XCR1$^+$ DCs required for cross-priming of capsid specific CD8$^+$ T cells. **(A)** Experimental timeline showing treatment of XCR1$^{+/DTRvenus}$ mice with DT to ablate XCR1$^+$ DCs. **(B)** Representative flow cytometry plots showing CD8α$^+$CD11c^{hi} DCs and XCR1$^+$YFP$^+$ DCs ("venus" reporter) from DT treated and untreated mice. **(C)** Anti-capsid CD8$^+$ T cell response reported as percent tetramer$^+$CD8$^+$ T cells at 7-, 10- and 14-day time points. **(D)** Experimental timeline showing treatment of C57BL/6 WT mice with anti-CD103 antibody to neutralize CD103$^+$ DCs. **(E)** Percentage of tetramer$^+$CD8$^+$ T cells over time in anti-CD103 antibody treated and untreated mice. Data are average ± SEM of at least five animals per group. Each circle represents an individual animal. Statistically significant differences are indicated. ***P < 0.001, ****P < 0.0001.

ablated as shown in **Figure 1B**. Importantly, XCR1$^+$ cell depletion significantly hampered development of capsid specific CD8$^+$ T cells (**Figure 1C**). In another experiment, WT C57BL/6 mice were treated with 150 μg of α-CD103 i.p. on days −1, 3, 7, and 10 to neutralize CD103$^+$ DCs (**Figure 1D**). Mice treated with anti-CD103 exhibited a significantly lower frequency of tetramer$^+$ CD8$^+$ T cells compared with control mice (**Figure 1E**). Thus, while CD103$^+$ DC neutralization produced a partial decrease of CD8$^+$ T cell responses, depletion of XCR1$^+$ DCs led to a complete abolishment of capsid-specific CD8$^+$ T cell responses. Therefore, cross-priming of capsid-specific CD8$^+$ T cells strictly relies on XCR1$^+$ DCs, with the CD103$^+$XCR1$^+$ DC subset partially contributing.

Depletion of Immune Stimulatory CpG Motifs Prevent AAV Capsid-Specific CD8$^+$ T Cells

Because depleting CpG motifs from the transgene gene has been shown to mitigate AAV-immune responses (23), we hypothesized that depletion of CpG motifs from the vector genome may "deimmunize" AAV vectors and reduce the CD8$^+$ T cell response. To test our hypothesis, we constructed a vector using a CpG-free expression cassette. This cassette contains a CpG-free edited sequence of the coding region for human coagulation factor IX (hFIX) and the following CpG-free elements: a CMV enhancer/EF1α promoter combination, a synthetic intron, and an SV40 polyA signal. The assembled cassette was inserted in between AAV2 ITRs and packaged into AAV2-SIINFEKL (**Figure 2A**). While this cassette contains 0 CpG motifs, the control vector with native sequences contains 41 CpG motifs (while the ITRs were unaltered in both vectors, each containing 16 CpG motifs per ITR). On day 0, WT C57BL/6 mice received 1 × 10^{11} vg of AAV2-SIINFEKL vector containing either hFIX cassette depleted of CpG motifs (AAV2-SIINFEKL-CpG$^-$) or hFIX cassette containing CpG motifs (AAV2-SIINFEKL-CpG$^+$; containing native sequence) *via* i.m. injection into the quadriceps muscle (**Figure 2B**). Anti-capsid CD8$^+$ T cells were quantified in peripheral blood on days 7, 10 and 14. Mice injected with AAV-SIINFEKL-CpG$^-$ vector had a substantially reduced frequency of tetramer$^+$ CD8$^+$ T cells compared to mice injected with AAV2-SIINFEKL-CpG at 7 and 10 days after vector injection (**Figures 2C, D**). Low induction of anti-capsid CD8$^+$ T cells by the CpG$^-$ vector was also observed in a similar second

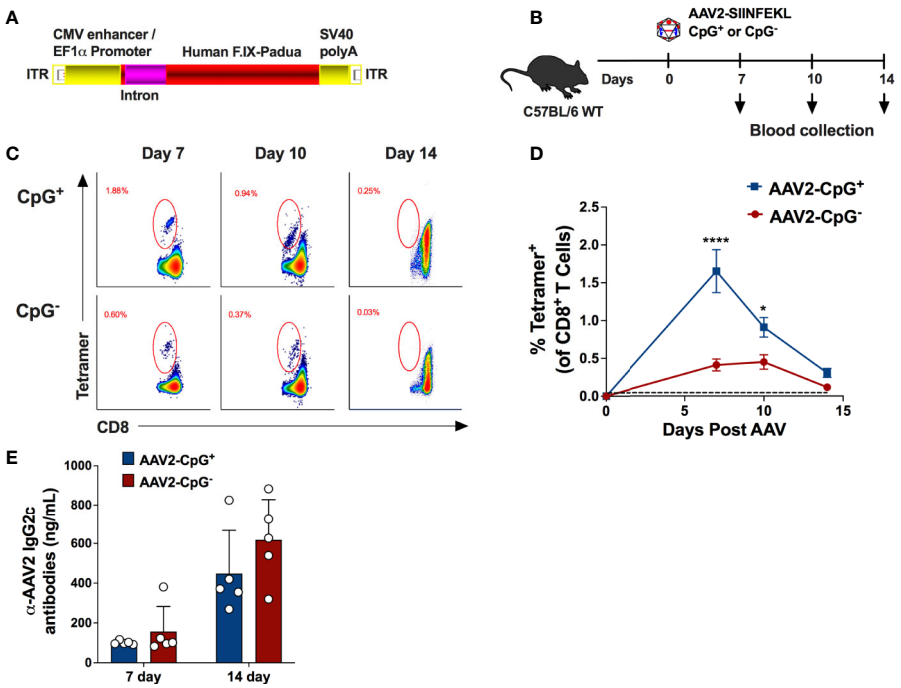

FIGURE 2 | Absence of CpG motifs significantly reduce the percentage of AAV capsid specific CD8$^+$ T cells but does not impact capsid-specific antibody formation. **(A)** Schematic representation of CpG-free expression cassette encoding hFIX with padua mutation. This cassette was packaged into AAV2-SIINFEKL capsid. **(B)** Experimental timeline of WT C57BL/6 mice injected with either AAV2-SIINFEKL-CpG$^+$ or AAV2-SIINFEKL-CpG$^-$ vector. **(C)** Representative flow plots showing AAV capsid-specific CD8$^+$ T cells at different time points in mice injected with AAV2-SIINFEKL-CpG$^+$ and AAV2-SIINFEKL-CpG$^-$ vector. **(D)** Percentages of capsid specific CD8$^+$ T cell in mice injected with AAV2-SIINFEKL-CpG$^+$ and AAV2-SIINFEKL-CpG$^-$ vector. The dotted line at 0.045% represents the limit of detection of capsid specific CD8$^+$ T cells using the tetramer. **(E)** Anti-AAV2 IgG2c antibody titers in plasma samples from mice injected with AAV2-SIINFEKL-CpG$^+$ or AAV2-SIINFEKL-CpG$^-$ vector. Samples were collected at days 14 and 28 post vector injection. Data are average ± SEM of at least five animals per group. Each circle represents an individual animal. Statistically significant differences are indicated. *P < 0.05, ****P < 0.0001.

experiment (**Supplementary Figure 2**). However, CpG depletion failed to prevent capsid-specific antibody formation as there was no difference in anti-AAV2 IgG2c titers between mice injected with AAV2-SIINFEKL-CpG$^-$ and AAV2-SIINFEKL-CpG$^+$ vector (**Figure 2E**). Thus, CpG motifs represent a critical activation signal for the generation of a CD8$^+$ T cell response but not an antibody response against capsid.

The Effect of CpG Motifs on Immune Responses to Gene Transfer in Hemophilia B Mice

After confirming that CpG depletion reduces AAV capsid specific CD8$^+$ T cell responses, we asked whether depleting CpG motifs from hFIX expression cassette improves the outcome of gene transfer in hemophilia B mice. For that, we evaluated adaptive immune responses to hFIX in male hemophilia B (C3H/HeJ F9$^{-/Y}$) mice injected with 1×10^{11} vg of AAV1-CpG$^-$ or AAV1-CpG$^+$ into the quadriceps (**Figure 3A**). These hemophilia B mice have a deletion of endogenous *F9* gene and therefore lack tolerance to FIX antigen (26). The immunogenic i.m. route was chosen for these experiments, as hepatic gene transfer typically results in tolerance induction to FIX (24, 38). Because the serotype AAV1, shows a superior efficiency for muscles gene transfer, AAV1 capsid was used (39). Blood samples were collected at day 14 and 28 after vector injection and antibody titers measured by ELISA. IgG2a titers (the dominant anti-capsid immunoglobulin) against AAV1 capsid were similar between groups at both time points suggesting that CpG content is less critical for anti-capsid antibody formation (**Figure 3B**). On the other hand, AAV1-CpG$^-$ group showed >10-fold lower IgG1 formation against hFIX (the main IgG subclass against FIX as we had identified in our published studies) at day 14 but only ~2.5-fold lower at day 28 compared to AAV1-CpG$^+$ (**Figure 3C**). Thus, CpG depletion did not impact capsid specific antibody formation, but initially reduced IgG1 formation against hFIX.

To further characterize the antibodies against hFIX, inhibitor titers were measured by Bethesda assay. At day 14, AAV1-CpG$^-$ treated mice showed modestly higher incidence of inhibitor development (antibodies that inhibit FIX coagulation activity as determined by Bethesda assay); 58.3% (7 of 12) of AAV1-CpG$^-$ treated mice, compared to 33.3% (3 of 9) in the AAV1-CpG$^+$ injected mice (**Figure 3D**). In contrast, AAV1-CpG$^+$ treated mice had increased inhibitor titers by day 28, which now were somewhat higher compared to the AAV1-CpG$^-$ group (**Figure 3D**). Interestingly, when comparing the BU titers for different time points within the same group, we observed that at day 28, AAV1-CpG$^-$ treated mice showed a decrease in the inhibitor formation compared to earlier time point. In contrast, AAV1-CpG$^+$ mice showed an increase of inhibitor formation

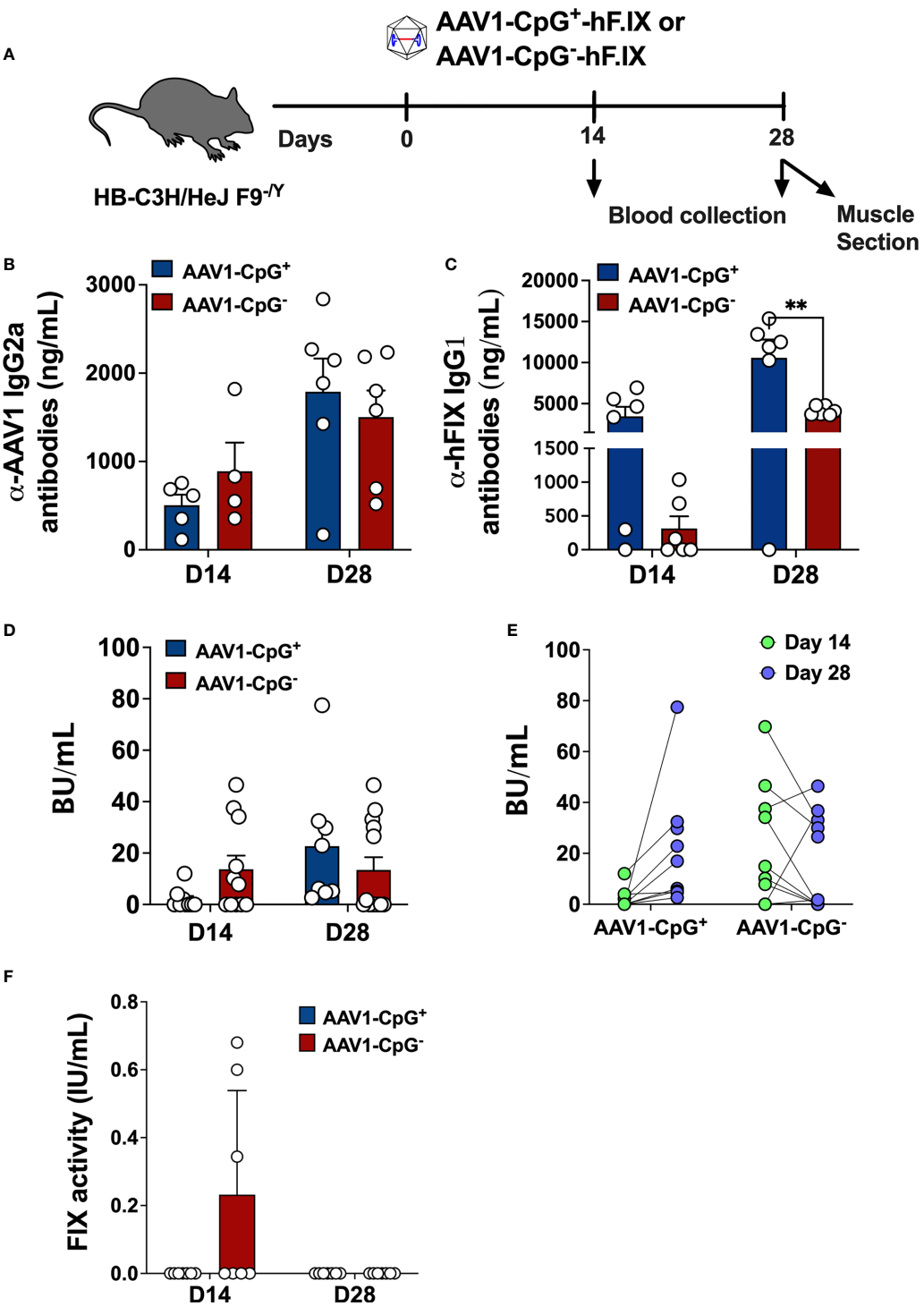

FIGURE 3 | CpG depletion has no effect on antibody formation against capsid but changes the dynamics of the anti-hFIX response in hemophilia B mice. **(A)** Experimental timeline of hemophilia B (C3H/HeJ $F9^{-/Y}$) mice injected with AAV1-CpG^+ or AAV1-CpG^- vector. **(B)** Anti-AAV1 IgG2a antibody titers in plasma samples of hemophilia B mice injected with AAV1-CpG^+ or AAV1-CpG^- vector. Samples were collected at day 14 and 28 post vector injection. **(C)** anti-hFIX IgG1 antibody titers anti-AAV1 IgG2a antibody titers in plasma samples of hemophilia B mice injected with AAV1-CpG^+ or AAV1-CpG^- vector. **(D)** Bethesda inhibitor titers (BU/ml) in plasma samples of hemophilia B mice injected with AAV1-CpG^+ or AAV1-CpG^- vector. **(E)** Correlation between inhibitor titer at different time points within the same group. **(F)** Percent hFIX coagulation activity as assessed by Rossix ROX Factor IX chromogenic assay. Data are average ± SEM of at least four animals per group and is representative of two independent experiments. Each circle represents an individual animal. Statistically significant differences are indicated. **$P < 0.01$.

over time (**Figure 3E**). Although no FIX antigen was detectable in circulation (likely owing to the antibody formation), low hFIX activity of 0.3% of normal was initially detected in mice treated with AAV1-CpG$^-$ (**Figure 3F**). In order to rule out that CpG depletion affected transgene expression, we also compared both vectors in a setting of immune tolerance. Hepatic gene transfer in C57BL/6 mice resulted in nearly identical levels of hFIX expression from CpG$^+$ and CpG$^-$ vectors, indicating that these were equally potent in hFIX transgene expression (**Supplementary Figure 3**). In conclusion, CpG motifs merely modify the time course and potency of antibody formation against the transgene product but are ultimately not required. Therefore, CpG depletion has little utility in prevention of antibody formation.

CpG Depletion Substantially Decreased But Did Not Entirely Eliminate CD8$^+$ T Cells Infiltration in Skeletal Muscle

Next, we studied the effect of CpG depletion on CD8$^+$ T cell infiltration in injected muscle. We know from prior experiments, that CD8$^+$ T cell infiltrating AAV-hFIX transduced muscle are directed against hFIX in this strain of mouse, since hemophilic C3H/HeJ mice transgenically expressing non-functional forms of hFIX do not show this response (13, 26). We evaluated CD8$^+$ T cells infiltration and hFIX expression in skeletal muscles tissue using immunofluorescence staining. Transduced quadriceps muscles of hemophilia B were harvested 28 days after gene transfer, cryosectioned, and immunostained for CD8 and hFIX. CD8$^+$ T cell infiltration was consistently low in skeletal muscle of all 5 AAV1-CpG$^-$ injected mice (**Figures 4A, B)**. In contrast, 3 of 5 AAV1-CpG$^+$ injected mice showed robust CD8$^+$ T cell infiltration and substantial tissue damage (**Figure 4A** and **Supplementary Figure 4**). CD8$^+$ T cell infiltration in AAV1-CpG$^+$ transduced muscle was on average 8-fold higher compared to AAV1-CpG$^-$. This difference did not reach statistical significance because of variability in the AAV1-CpG$^+$ group, where 2 of 5 animals had low levels of infiltrates. In agreement with the increased tissue damage caused by the AAV1-CpG$^+$ vector, the areas of hFIX expression in cross sections of skeletal muscles was lower in AAV1-CpG$^+$ injected compared to AAV1-CpG$^-$ injected mice (**Figure 4C**). To confirm that CpG depletion reduced rather than merely delayed a CD8$^+$ T cell response, we performed gene transfer in an additional cohort of hemophilia B mice and analyzed transduced muscles 8 weeks later. For both vectors we observed minimal CD8$^+$ T cell infiltration in muscles at 8 weeks (**Figures 4A, B** and **Supplementary Figure 4**) and a reduction in hFIX expression compared to the 4-week time point (**Figure 4C**). However, AAV1-CpG$^-$ vector had a significantly greater FIX expression in the muscle at 8 weeks time point compared with AAV1-CpG$^-$ vector (**Figure 4C**). In summary, CpG-rich vectors were prone to causing inflammatory CD8$^+$ T cell responses during the first month in transduced muscle, while CpG-depleted vectors consistently caused only mild responses. As a result, muscles transduced with CpG$^-$ vector showed less tissue destruction and better preservation of transgene expression.

DISCUSSION

Development of immune response to the viral vector or the transgene product, particularly CD8$^+$ T cell responses, represents a significant challenge for long-term success of gene therapy. DCs are responsible for the crosstalk between innate and adaptative immune systems. In case of AAV vectors, the immune response initiates with pDC sensing of hypomethylated CpG motifs in the AAV genome by TLR9. This sensing leads to T1 IFN production by pDCs. T1 IFN along with CD4$^+$ T cell help contributes to licensing of cDCs, which then prime capsid specific CD8$^+$ T cells (9, 15). Our new data strongly suggest that among cDCs, the XCR1$^+$ subset is primarily responsible for the priming of CD8$^+$ T cells. This result is consistent with the known biology of XCR1$^+$ DCs, which are specialized in cross-presentation of antigen. XCR1$^+$ DCs consist of CD8α^+ and CD103$^+$ DCs; and it is likely that both subsets contribute, as neutralization of the CD103$^+$ subset only partially blocked the response (albeit that we cannot rule out that the antibody against CD103 was only partially effective). Others have shown that pDC-derived T1 IFN induces expression of XCR1$^+$ in cDCs, optimizing their maturation, costimulatory capacity, and ability to cross-present (40). XCR1$^+$ cDCs are able to present antigen on both class I and II MHC molecules, serving as a platform for simultaneous interactions between CD4$^+$ and CD8$^+$ T cell (41). CD4$^+$ T help *via* CD40L/CD40 co-stimulation is also required for an effective CD8$^+$ T cell response against AAV capsid (15). Thus, XCR1$^+$ DCs, besides cross-presenting antigen to CD8$^+$ T cells, establish a platform to orchestrate the cooperation between CD8$^+$ T cells and CD4$^+$ T help, resulting in optimal CD8$^+$ T cell activation, as summarized in the proposed model in **Supplementary Figure 5**.

Pre-clinical studies have applied immunosuppression as an alternative to control anti-capsid cellular immune response (42–44). However, this may not be sufficient to prevent the loss of transgene expression, in particular if the vector is too immunogenic. *In vitro* studies showed TLR9-dependent induction of IFN I production in human pDCs pulsed with AAV vectors (8). Clinical experience with hepatic AAV gene transfer for hemophilia suggests that CpG motifs contribute to the loss of therapeutic expression and that immune suppression, when still needed, is more effective when using CpG depleted vectors (45–47). Low CpG level correlates strongly with long-term transgene expression (20). Conversely, CpG enrichment negatively affected the outcome of gene therapy for hemophilia (47, 48). Therefore, genome editing to eliminate CpG motifs and thus decrease immunogenicity of AAV vectors is a promising approach. In a proof-of-principle study, others have shown that absence of CpG sequences in the lacZ reporter transgene minimized CD8$^+$ T cell infiltration and prolonged expression of a reporter transgene upon AAV gene transfer in mice (23). Curiously, Xiang et al. found that capsid-specific memory CD8$^+$ T cells showed strong *in vivo* proliferative responses to AAV vectors with CpG-depleted genomes, while naive CD8$^+$ T cells responded much more vigorously to CpG$^+$ vectors, which provide stronger TLR9 stimulation (49). Given the above-mentioned clinical experience with these vectors, the authors

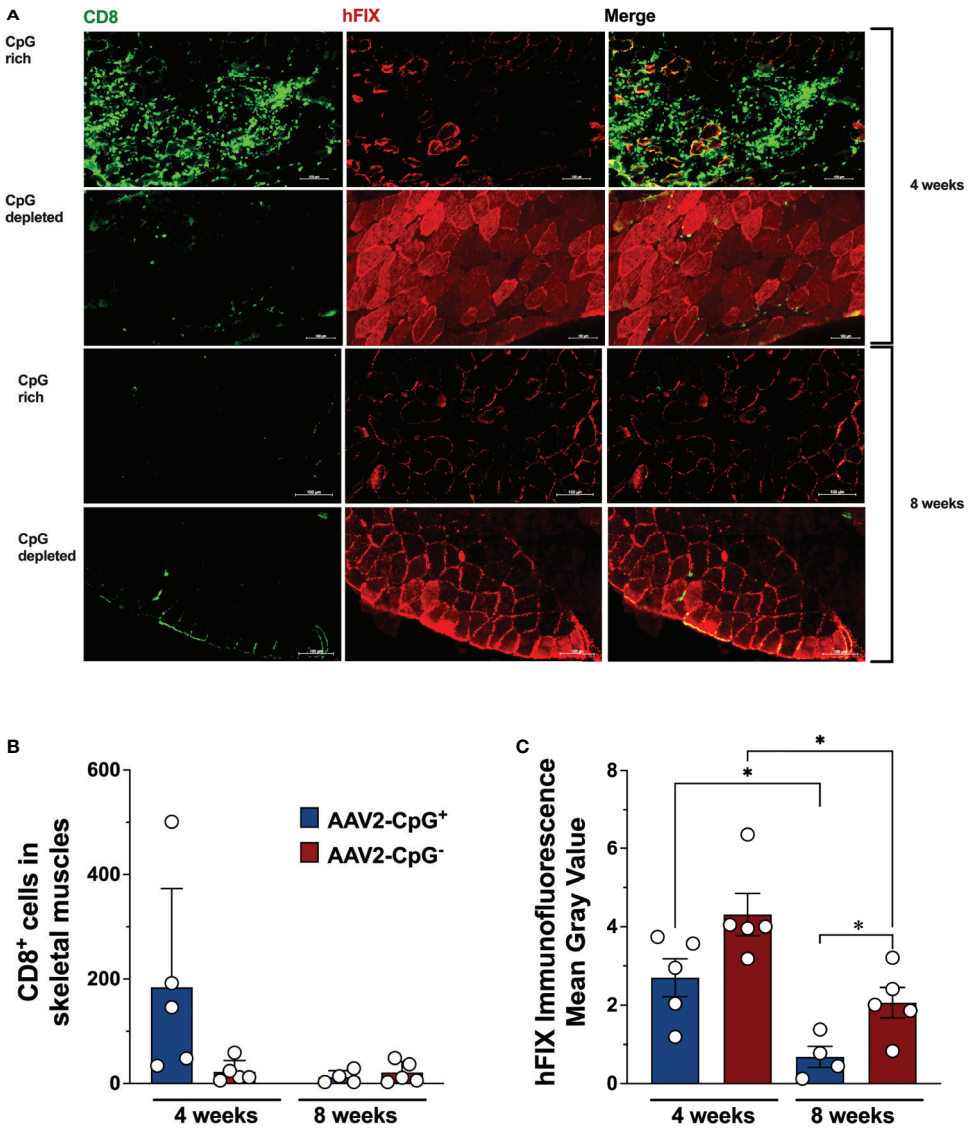

FIGURE 4 | hFIX expression and CD8$^+$ T cell infiltration in transduced muscles. Skeletal muscles from hemophilia B (C3H/HeJ F9$^{-/Y}$) mice were harvested, cryosectioned, and stained for hFIX (red) and CD8 (green) 4 weeks after AAV1-CpG$^-$ or AAV1-CpG$^+$ injection, as described in Figure 3A. Muscles section was entirely scan and images were taken for both channels using a 40× objective with ZEISS Microscopy. **(A)** Sequential scan image of transduced muscle from AAV1-CpG$^+$ (left) or AAV1-CpG$^-$ (right) injected mice shown for individual channel or merge image. **(B)** Numbers of infiltrating CD8$^+$ T cells in transduced muscle as quantified by Fiji-ImageJ software after generating a grid (area per point, 1200 square pixels). Whole muscle sections were quantified. **(C)** Fluorescent signal for hFIX quantified as mean gray value with Fiji-ImageJ software. Representative images from five mice are shown for each condition. The scale bar represents 100 μm. Data are average ± SEM of at least five animals per group. Each circle represents an individual animal. Statistically significant differences are indicated. *P < 0.05.

concluded that responses seen in humans may mostly reflect primary responses.

Both pre-clinical studies in animal models and clinical trial data support that CD8$^+$ T cell activation is linked to innate immune sensing, which serve to provide activation or "danger" signals. Here, we have refined our model for cross-priming of capsid specific CD8$^+$ T cells. Sensing of CpG motifs by TLR9 in pDCs leads to T1 IFN production and activation of cross-presenting XCR1$^+$ cDCs. Thus, depletion of CpG motifs lead to substantially reduced CD8$^+$ T cell responses against the viral capsid and against a therapeutic transgene product. For instance, CD8$^+$ T cell responses were significantly reduced in CpG$^-$ transduced muscle of hemophilia B mice, thereby avoiding muscle damage and better preserving hFIX transgene expression. CpG depletion did not much affect the kinetics of the CD8$^+$ T cell response but rather substantially reduced the magnitude. The reason why hFIX expression was not entirely lost in muscle of CpG$^+$ treated mice may have related to the use of

single-stranded AAV (ssAAV) vector. These often induce functionally impaired $CD8^+$ T cells against the transgene product with a reduced cytotoxic and proliferative capacity (50), ultimately undergoing apoptosis (51). This is also the case in AAV-hFIX gene transfer to hemophilia B mice, while use of self-complementary AAV (scAAV) results in a more destructive $CD8^+$ T cell response that rapidly eliminates of hFIX expressing muscles fibers (13). While we have not tested CpG depletion in scAAV vectors in this study, clinical trial results support that such a vector is also less prone to $CD8^+$ T cell activation, at least against capsid (47, 52).

Our prior study in mice showed a substantial reduction in $CD8^+$ T cell responses against a transgene product encoded by scAAV vector in TLR9-deficient mice (13). A quantitative method to evaluate TLR9 activation risk factors in candidate expression cassettes may be helpful to reduce AAV vector immunogenicity (22). This method has predictive potential for selected DNA sequences, thus increasing the chances for long-term clinical benefits.

However, the approach also has limitations. We still detected a residual $CD8^+$ T cell response and partial loss of transduced cells. Since we only eliminated CpG motifs in the expression cassette, it is possible that CpG motifs in the inverted terminal repeats (ITRs) contributed. Also, TLR9 signaling is not entirely CpG dependent (53). For other expression cassettes, it may not be possible to remove all CpG motifs. In case of editing the promoter, one would have to empirically determine which sequences can be changed and what to change them to without altering promoter strength or specificity. An alternative approach to eliminate TLR9 signaling is to include TLR9 inhibitory DNA sequences in the vector construct, as recently been shown by Chan et al. (54). However, it should be pointed out that even TLR9 deficient mice do not show complete abolishment of $CD8^+$ T cell responses, as our current and published studies have shown (7–9). Therefore, additional pathways may exist that result in $CD8^+$ T cell activation. In clinical trials, $CD8^+$ T cell responses have been observed against dystrophin and α1-antitrypsin in treatment of Duchenne's muscular dystrophy and α1-antitrypsin (AAT) deficiency, respectively (55, 56). Interestingly, pre-existing cellular immunity to dystrophin may occur in some patients with Duchene's muscular dystrophy, a disease characterized by muscle regeneration and some level inflammation (55). One should therefore caution that the underlying disease may contribute additional inflammatory signals in the target tissue of gene transfer.

Another limitation is that CpG depletion does not eliminate antibody formation against the capsid or transgene product, which can be modified by but does not depend on the TLR9-MyD88 pathway (9, 12, 15). In this new study, CpG depletion had no effect on antibody formation against capsid but changed the dynamics of the anti-hFIX response. CpG^+ vector rapidly induced high-titer mostly non-inhibitory antibodies, which evolved into a more neutralizing response over time. CpG^- vector showed a lower-titer IgG1 but more focused response by day 14, with higher inhibitory titers that modestly decreased afterwards. Antibody formation against capsid was also not impacted by CpG depletion in clinical trials (19, 57).

In conclusion, our results support CpG depletion as a strategy to limit $CD8^+$ T cell activation against capsid and transgene product while also pointing to limitations such as minimal impact on antibody formation and a minimized but still detectable $CD8^+$ T cell response.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC, Indiana University.

AUTHOR CONTRIBUTIONS

TB, JS, SK, IZ, and XL performed experiments. TB, JS, SK, TK, WX, and RH designed, analyzed, and interpreted experiments. TB, JS, SK, and RH wrote the manuscript. RH supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institutes of Health, National Institute of Allergy and Infectious Diseases Grant R01 AI51390, National Institutes of Health, National Heart, Lung, and Blood Institute grants R01 HL131093, R01 HL097088 to RH, U54 HL142012 to RH, and HL142019 WX, and by Indiana Collaborative Initiative for Talent Enrichment (INCITE) funds provided by Lilly Endowment to RH and WX.

ACKNOWLEDGMENTS

The authors would like to thank Flow Cytometry Core at IU Simon Comprehensive Cancer Center, IUPUI, Indianapolis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.672449/full#supplementary-material

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Conflict of Interest: RH served on the scientific advisory board or Ally Therapeutics and has been receiving grant funding from Luye R&D, Boston, and Spark Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 June 2021
doi: 10.3389/fimmu.2021.612910

Liver-Directed AAV8 Booster Vaccine Expressing *Plasmodium falciparum* Antigen Following Adenovirus Vaccine Priming Elicits Sterile Protection in a Murine Model

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OPEN ACCESS

Edited by:
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Katsuyuki Yui,
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Specialty section:
This article was submitted to
Vaccines and Molecular
Therapeutics,
a section of the journal
Frontiers in Immunology

Received: 01 October 2020
Accepted: 04 June 2021
Published: 23 June 2021

Citation:
Shahnaij M, Iyori M, Mizukami H,
Kajino M, Yamagoshi I, Syafira I,
Yusuf Y, Fujiwara K, Yamamoto DS,
Kato H, Ohno N and Yoshida S (2021)
Liver-Directed AAV8 Booster Vaccine
Expressing Plasmodium falciparum
antigen Following Adenovirus
Vaccine Priming Elicits Sterile
Protection in a Murine Model.
Front. Immunol. 12:612910.
doi: 10.3389/fimmu.2021.612910

Hepatocyte infection by malaria sporozoites is a bottleneck in the life-cycle of *Plasmodium* spp. including *P. falciparum*, which causes the most lethal form of malaria. Therefore, developing an effective vaccine capable of inducing the strong humoral and cellular immune responses necessary to block the pre-erythrocytic stage has potential to overcome the spatiotemporal hindrances pertaining to parasite biology and hepatic microanatomy. We recently showed that when combined with a human adenovirus type 5 (AdHu5)-priming vaccine, adeno-associated virus serotype 1 (AAV1) is a potent booster malaria vaccine vector capable of inducing strong and long-lasting protective immune responses in a rodent malaria model. Here, we evaluated the protective efficacy of a hepatotropic virus, adeno-associated virus serotype 8 (AAV8), as a booster vector because it can deliver a transgene potently and rapidly to the liver, the organ malaria sporozoites initially infect and multiply in following sporozoite injection by the bite of an infected mosquito. We first generated an AAV8-vectored vaccine expressing *P. falciparum* circumsporozoite protein (PfCSP). Intravenous (i.v.) administration of AAV8-PfCSP to mice initially primed with AdHu5-PfCSP resulted in a hepatocyte transduction rate ~2.5 times above that seen with intramuscular (i.m.) administration. This immunization regimen provided a better protection rate (100% sterile protection) than that of the i.m. AdHu5-prime/i.m. AAV8-boost regimen (60%, $p < 0.05$), i.m. AdHu5-prime/i.v. AAV1-boost (78%), or i.m. AdHu5-prime/i.m. AAV1-boost (80%) against challenge with transgenic PfCSP-expressing *P. berghei* sporozoites. Compared with the i.m. AdHu5-prime/i.v. AAV1-boost regimen, three other regimens induced higher levels of PfCSP-specific humoral immune responses. Importantly, a single i.v. dose of AAV8-PfCSP recruited $CD8^+$ T cells, especially resident memory $CD8^+$ T cells, in the liver. These data

suggest that boost with i.v. AAV8-PfCSP can improve humoral and cellular immune responses in BALB/c mice. Therefore, this regimen holds great promise as a next-generation platform for the development of an effective malaria vaccine.

Keywords: human adenovirus serotype 5, adeno-assoicated virus, AAV8, *Plasmodium falciparum* circumsporozoite protein, malaria, vaccine

INTRODUCTION

Malaria remains an important cause of global morbidity and mortality, predominantly in infants and young children in sub-Saharan Africa. Numerous efforts have been made to develop an effective malaria vaccine. The most clinically advanced malaria vaccine to date, RTS,S/AS01 (also known as Mosquirix™), is a protein-in-adjuvant component vaccine based on the *Plasmodium falciparum* circumsporozoite protein (PfCSP), which targets the pre-erythrocytic parasite stage and has been partially successful in human clinical trials. However, a phase III clinical trial in sub-Saharan Africa found that RTS,S/AS01 has limited efficacy (18%–26% in infants) in the first year after vaccination, and that its protection level wanes rapidly, dropping to almost zero in the fourth year after vaccination (1, 2).

The development of an effective malaria vaccine has been impeded by two major spatiotemporal factors: liver microanatomy and parasite biology. Within 30 minutes of *Plasmodium* sporozoites entering a new host after it has been bitten by a *Plasmodium*-infected female mosquito, the parasites reaching the liver invade hepatocytes. Later it undergo exoerythrocytic schizogony, and are subsequently released into the circulation as thousands of blood-stage merozoites. Therefore, to be effective, a pre-erythrocytic vaccine should induce a robust cell-mediated immune response in the liver, and this response must clear the parasites or infected hepatocytes within a narrow window of 5 to 7 days after liver infection (3). $CD8^+$ T cells are necessary for protection against intrahepatic malaria parasites, and viral-vectored vaccines are better at inducing $CD8^+$ T cells than protein-in-adjuvant regimens (4). Recombinant adeno-associated virus (AAV) has emerged as a promising viral vector for use in the development of effective and safe vaccines due to its broad tissue tropism, non-pathogenicity, and ability to induce efficient and long-term gene expression without causing toxicity *in vivo* (5). Moreover, the low prevalence of neutralizing antibodies against the viral capsid in human sera, rapid viral uncoating, and excellent safety profile in human clinical trials underpins the position of AAVs as viral vector-based vaccination tools (6).

We recently reported that AAV1 has the potential to induce specific antibodies targeting malaria vaccine candidate antigens (e.g., PfCSP and Pfs25). It also affords durable protection in a rodent model when administered following intramuscular (i.m.) injection of an adenovirus-vectored vaccine, but only when AAV1 is administered as the booster, not as the prime (7, 8). Differences in cell entry, tissue tropism, and/or interactions with host innate immune factors among the AAV serotypes can dictate the adaptive immune responses in the host following administration of recombinant AAV (rAAV) (9). Consequently, the administration of rAAV encoding different pathogenic antigens by various delivery methods can induce immunized animals to produce varying immune responses (10–12). The strategy behind the immunization regimen tested in the present study was to generate and maintain the level of T cell-mediated immune responses in the liver to confer adequate protection by efficiently delivering the *pfcsp* gene into hepatocytes using a liver-directed AAV serotype 8 (AAV8) vaccine construct.

Thus, we performed a comparative study in BALB/c mice to evaluate the immune responses and protective efficacy induced by immunization regimens each consisting of a prime with the i.m.-delivered human adenovirus type 5 (AdHu5) vaccine and an AAV1 or AAV8 booster vaccine delivered by i.m. or intravenous (i.v.) injection. Together with the induction of strong humoral and cellular immune responses from the AAV-based booster vaccine, the introduction of a pre-erythrocytic antigen into the liver by a hepatotropic AAV8 vaccine may result in the direct induction of liver-specific cellular immune responses capable of killing malaria parasites in the liver, the organ where they develop into their exoerythrocytic form and multiply by schizogony.

MATERIALS AND METHODS

Ethics Statement

All animal care and handling procedures were performed under the approved guidelines of the Animal Care and Ethical Review Committee of Kanazawa University (No. 22118–1) and Jichi Medical University (No. 17086-01), Japan. All efforts were made to minimize animal suffering during the experiments.

Parasites and Animals

Female inbred BALB/c and ddY mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). BALB/c mice were used to assess tissue tropism, cellular immune responses, and protection from challenge infections. A transgenic *P. berghei* parasite expressing full-length PfCSP in place of PbCSP (PfCSP-Tc/Pb) was used for the protective efficacy experiments as described previously (13–15). This transgenic parasite was maintained in the Laboratory of Vaccinology and Applied Immunology, Kanazawa University. *Anopheles stephensi* mosquitoes (SDA 500 strain) were infected with the parasites by allowing them to feed on parasitized 6-week-old ddY mice.

Recombinant Viral-Vectored Vaccines

AAV1 and AAV8 expressing luciferase (AAV1-Luc or AAV8-Luc, respectively) were generated as described previously (16, 17). AdHu5-PfCSP-G, AAV1-PfCSP-G, and AAV8-PfCSP-G were

also generated as described previously (7, 16). Briefly, the codon-optimized gene cassette encoding a GPI anchor-lacking full-length PfCSP (Leu19-Val377) gene from *P. falciparum* 3D7 strain was fused between the mouse IgGκ signal peptide and the membrane anchor sequence of the vesicular stomatitis virus (VSV)-G protein to direct localization and efficient translocation of the antigen to the cell membrane, respectively. The posttranscriptional regulatory element from woodchuck hepatitis virus (*wpre*) was then inserted into the gene cassette to enhance the expression of PfCSP protein in mammalian cells. The resulting gene construct was inserted into pAd/PL-DEST (Invitrogen, Carlsbad, CA, USA) under the control of a strong synthetic CAG promoter sequence. The adenovirus was produced (15), purified, and titrated using the Fast-Trap Adenovirus Purification and Concentration Kit (Millipore, Temecula, CA, USA) and the Adeno-X™ Rapid Titer Kit (Clontech, Palo Alto, CA, USA) according to the manufacturers' protocols.

In the case of the AAV vectors, the gene construct was introduced into pAAV-MCS under the control of a universal cytomegalovirus (CMV) promoter sequence to construct pAAV-CMV-sPfCSP2-G(+). These plasmids were used to produce the AAV1-PfCSP-G and AAV8-PfCSP-G constructs by transfecting HEK293 cells, as described elsewhere (11, 12). AdHu5-PfCSP-G, AAV1-PfCSP-G, and AAV8-PfCSP-G were re-named AdHu5-PfCSP, AAV1-PfCSP, and AAV8-PfCSP, respectively, in the present study.

In Vivo Bioluminescent Imaging

AAV1-Luc and AAV8-Luc were injected into the right tibialis anterior muscles or tail veins of the BALB/c mice ($n = 3$; 1.0×10^{11} plaque-forming units (pfu)/mouse) on day 0. The animals were anesthetized with a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture and, after 10 minutes, D-Luciferin (15 mg/ml; OZ Biosciences, Marseille, France) was administered intraperitoneally (i.p.) (150 µl/mouse) at the appropriate time points. On days 8 or 224, the sacrificed mice from this experiment were dissected to measure liver bioluminescence. Luciferase expression in their livers (and whole bodies) was detected using the IVIS® Lumina LT *in vivo* imaging system (PerkinElmer, Waltham, MA, USA) as described previously (15, 16). The accumulated emissions were calculated, and a color heatmap was used to show the expression intensities. The visible *in vivo* luminescent images in the mice represent the total flux of photons in photons/second/cm^2 (p/s/cm^2) in the region of interest (ROI). The measured signal intensities are represented by radiance (p/s/cm^2/sr), a value that refers to the number of photons per second that leave a square centimeter of tissue and radiate into a solid angle of one steradian (sr).

Immunoblotting

HEK293T or Hepa1-6 cells (4.0×10^4 cells/well) were transduced with AdHu5-PfCSP at a multiplicity of infection (MOI) of 3, with AAV1-PfCSP (MOI = 10^5), or with AAV8-PfCSP (MOI = 10^5) after seeding onto 48-well plates. The cell lysates collected using Laemmli buffer at 48 h post-infection were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels under reducing conditions. Proteins were transferred onto Immobilon FL® PVDF membranes (Merck Millipore, Tokyo, Japan). The resulting blots were blocked in 5% skim milk (Wako Chemical Inc., Tokyo, Japan) in PBS containing 0.1% Tween 20 (PBS-T). Blots were probed with the anti-PfCSP 2A10 monoclonal antibody (mAb) in 5% skim milk/PBS-T. After PBS-T washing, each membrane was probed with goat anti-mouse IgG conjugated to IRDye 800cw (Rockland Immunochemicals, Limerick, PA, USA) diluted in 5% skim milk. The membrane was visualized using an Odyssey infrared imager (LI-COR, Lincoln, NE, USA).

Immunofluorescence Assay

HEK293T cells were transduced with AdHu5-PfCSP (MOI = 3), AAV1-PfCSP (MOI = 10^5), or AAV8-PfCSP (MOI = 10^5) on an 8-well chamber slide. At 48 h post-infection, the samples were treated with ice-cold 100% methanol (permeabilized) or 4% paraformaldehyde (non-permeabilized) for 15 min. The samples were then blocked with 10% normal goat serum (NGS) in PBS and incubated with 2A10 mAb in 10% NGS/PBS at room temperature. After three PBS washes, the samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). After PBS washing, the slide was mounted with a drop of VECTASHIELD™ containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). All micrographs were acquired by BZ-X710 fluorescence microscopy (Keyence Corp., Tokyo, Japan).

Immunization and Parasite Challenge

Mice were immunized with an i.m. injection of 5×10^7 pfu AdHu5-PfCSP into the musculus tibialis followed by a booster with 1×10^{11} vector genome (vg) per mouse of either AAV1- or AAV8-PfCSP administered i.m. or i.v. into the tail vein with a 6-week interval. Control mice received 100 µl of endotoxin-free PBS. Six weeks after the last immunization, the mice were challenged with an i.v. dose of 500 PfCSP-Tc/Pb sporozoites per mouse suspended in RPMI-1640 media (Gibco, Life Technologies, Tokyo, Japan). Infections were monitored (on days 4 to 14) using the Giemsa-stained thin blood smears obtained from the tail. At least 20 fields (magnification: ×1,000) were examined before a mouse was deemed to be malaria-infection negative. Protection was defined as the complete absence of blood-stage parasitemia on day 14 after challenge.

Enzyme-Linked Immunosorbent Assay

The PfCSP-specific antibody titers in the sera collected from the tail vein blood samples of the immunized mice, which were taken 1 day before the booster dose and sporozoite challenge, were measured by ELISA as previously described (7). Briefly, Costar® EIA/RIA polystyrene plates (Corning Inc., NY, USA) pre-coated with *Escherichia coli*-produced recombinant PfCSP (400 ng/well) were blocked with 1% bovine serum albumin (BSA) in PBS and then incubated with the serially diluted serum samples or with the negative or the positive controls (2A10 mAb). An anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad Lab Inc., Tokyo, Japan) was used as the secondary antibody. The endpoint titer was expressed as the reciprocal of the last dilution that produced an optical density (O.D.) at 414 nm of 0.15 U

above the values of the negative controls (<0.1). All mice used in the experiments were seronegative before immunization.

Liver DNA Isolation and Quantitation

Whole livers were obtained from the vaccine-protected mice in the challenge study promptly after the animals were sacrificed. Each liver was placed in a 5-ml plastic tube containing 4.0 ml of ALT buffer (Qiagen, Valencia, CA, USA), and then homogenized at 2,500 rpm for 3.5 min using a μT-12 bead crusher (Tatitec, Saitama, Japan). Total DNA was isolated from 100-μl aliquots of the resulting homogenates using a DNA isolation kit (Qiagen) in accordance with the manufacturer's instructions. A quantitative analysis of the DNA was performed by qPCR with SYBR Green Premix Ex Taq (Takara, Tokyo, Japan). The oligonucleotide primers used for qPCR are shown in **Supplemental Table 1**. pAAV-CMV-sPfCSP2-G(+) plasmid DNA was used to generate a standard curve for the qPCR assays targeting the *pfcsp* gene sequences. A C_t cutoff was determined for each assay, whereby any well with a C_t value ≥ the mean C_q of the 10^3 standard was omitted from the analysis because it would lie outside of the linear range of the assay. The fit-points method for absolute quantification was used for the analysis, and the noise band and threshold were set to Auto (18).

Immunohistochemistry on the Liver Sections

Whole livers from the vaccine-protected mice in the challenge experiments were obtained after perfusion with 4% paraformaldehyde in 0.1M phosphate buffer under anesthesia. The liver tissues were subsequently immersed in the same fixatives, and 8-μm-thick cryosections were obtained following infiltration with 30% sucrose in PBS. The sections were heated at 95°C for 10 min in 0.5% Immunosaver (Nisshin EM, Tokyo, Japan), treated with 100% methanol for 10 min, and incubated overnight at 4°C in PBS containing 2.5% BSA and the 2A10 mAb. Some sections from the same mice were incubated in 2.5% BSA in PBS without 2A10 mAb for use as negative controls. After washing, all the sections were incubated in PBS containing Alexa 488-conjugated donkey anti-mouse IgG and 2.5% BSA for 3 h at room temperature. Sections were observed by light microscopy after coverslipping with VECTASHIELD™ mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI).

Liver-Resident Memory CD8$^+$ T Cells

Mice were sacrificed 2 weeks after the i.v. administration of AAV8-PfCSP (1.0×10^9, 1.0×10^{10}, or 1.0×10^{11} vg) or PBS into their tail veins. After being perfused with buffer 2 (66.74 mM NaCl, 6.71 mM KCl, 6.31 mM $CaCl_2$, 100 mM HEPES, 0.226 mM BSA) containing collagenase type IV (0.53 mg/ml, Sigma-Aldrich, St. Louis, UK), the livers were harvested and homogenized using frosted glass to generate single-cell suspensions. The cells were passed through a 100-μm mesh, resuspended in 35% Percoll/PBS, and centrifuged at 500 ×*g* for 20 min at room temperature. The red blood cells were subsequently lysed. Spleen cells were filtered through a 40-μm mesh and the buffy coat layer resulting from density gradient centrifugation with Histopaque®-1083 (Sigma-Aldrich) was collected. After the number of cells in the pellets and supernatants, excluding debris, was counted, the cells were antibody-stained in the presence of TruStain FcX™ Ab. The following antibodies and tetramer were used to stain the liver T_{RM} cells: CD45-FITC, CD8β-PerCP-Cy5.5, CD44-Brilliant Violet 510™, CD62L-APC, CD69-Brilliant Violet 421™, CXCR6 (CD186)-PE-Cy7, KLRG1 (MAFA)-APC-Cy7, and PE-conjugated PfCSP tetramer (the H-2K^d-restricted PfCSP NYDNAGTNL epitope was provided by the National Institutes of Health Tetramer Core). The cells were washed and flow cytometrically analyzed using BD FACSVerse (the gating strategy is shown in **Figure S1**). The number of leukocytes per gram of tissue was calculated based on the percentage of CD45$^+$ cells.

Statistical Analyses

Statistical analyses were performed in Prism version 7.0a (GraphPad Software Inc., La Jolla, CA, USA) and RStudio. Depending on the data distribution, a Student's t-test or Mann–Whitney rank test was used for comparing two groups. To assess the differences among the immunization groups, a Kruskal–Wallis test with Dunn's correction for multiple comparisons or Tukey's multiple comparison was used. All ELISA end-point titers were $\log_{10}$-transformed before analysis. The infection protection level was analyzed using Fisher's exact test. A p-value of <0.05 was considered statistically significant.

RESULTS

AAV8 Exhibits a Stronger Luciferase Expression Profile Than AAV1

Among the wide range of AAV serotypes, AAV1 and AAV8 are the two best options for producing an efficient, durable transfer and expression of the transgene in the desired tissue (19, 20). Previous *in vivo* bioluminescence imaging system (IVIS) studies have revealed that the AAV8 vector is highly hepatotropic after i.v. administration, whereas AAV1 produces an intense expression in skeletal muscle after its i.m. administration (21, 22). Therefore, we compared the expression profiles of these two promising serotypes following either i.m. or i.v. administration of a standard dose of 10^{11} vg/mouse. The resulting luminescence was robust; it gradually increased from day 0 to day 7, plateaued within 10 days, and persisted for over 224 days. The i.m. administration of AAV1-Luc or AAV8-Luc induced extensive luciferase expression (10^{11} p/s/cm^2/sr) in the right medial thigh muscles (**Figure 1A**). When AAV1 or AAV8 were i.m. administered, the luciferase activity levels did not significantly differ between them. Conversely, following i.v. administration of these vectors, the luciferase expression level transduced by AAV8 was ~100 times higher than that transduced by AAV1 (**Figures 1B, C**). Consistent with the results from a previous study, luciferase expression (10^8 p/s/cm^2/sr) was also observed in the liver after i.m. administration of AAV8-Luc although the intensity was not as higher as i.v. route of delivery. Unlike in C57BL/6 mice, where it was reported that after luciferase was initially expressed in the livers of mice receiving i.m. AAV8-Luc,

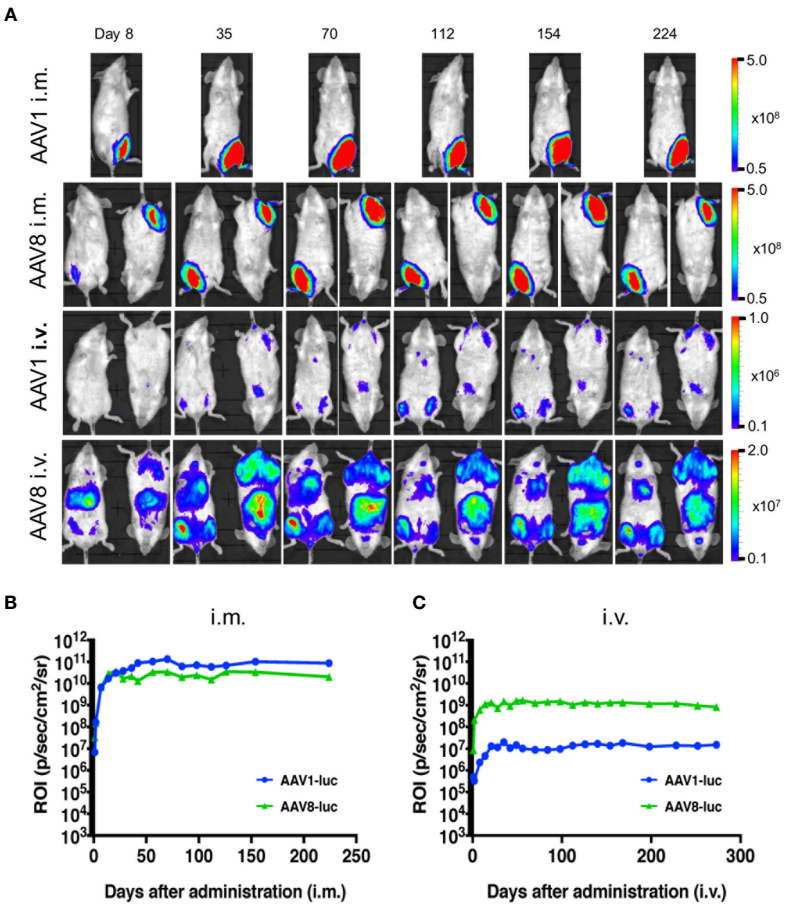

FIGURE 1 | Comparison of long-term transgene expression between AAV1-Luc and AAV8-Luc. **(A)** AAV1-Luc or AAV8-Luc was injected into the right medial thigh muscles or tail veins of mice (n = 2; 1.0×10^{11} vg/mouse) on day 0. Luciferase expression at different time points was detected using the IVIS Lumina LT Series III *in vivo* imaging system. The heatmap images visible in the mice represent the total flux of photons (p/s/cm^2) in the area of interest. Rainbow scale ranges are expressed in radiance (p/s/cm^2/sr. **(B, C)** The mean total flux of photons is shown as the region of interest (ROI) from day 0 to day 224 after i.m. **(B)** or i.v. administration **(C)** AAV-Luc administration.

the luciferase levels gradually became undetectable (23), we found that luciferase expression in our BALB/c mice persisted for more than 224 days post-administration (**Figures 1**, **2**).

Moreover, the IVIS imaging results for the livers collected from mice on day 8 or day 224 post-administration of AAV-Luc showed that systemic administration of AAV8 induced ~24–43 times better liver luciferase enzyme activity when compared with i.m. administration (**Figure 2**). In the cases of i.v. or i.m. administration of AAV1, no trace of luciferase activity (detection limit ~6.0×10^3 p/s/cm^2/sr) was observed in the liver. The high levels of hepatic- and muscular-directed transgene transduction and expression we observed after a single dose of the AAV vector was administered support the superiority of AAV8 over AAV1.

AAV Construction and *In Vitro* Transduction Efficiency in Mammalian Cell Lines

In the liver-directed vaccine delivery experiments, we constructed an AAV8 similar to that of the AAV1-PfCSP vaccine described in our previous study (7). This construct expresses the full-length PfCSP, whereby PfCSP is anchored on the surfaces of the infected cells *via* the VSV-G protein membrane anchor, followed by the *wpre* sequence, under the control of the CMV promoter. While the CMV promoter-controlled transgene construct ensures the strong and constitutive expression of PfCSP, *wpre* enhances PfCSP gene expression. We also included VSV glycoprotein G because antigen display on the transduced cells will be more efficient in its presence. The immunofluorescence analysis of HEK293T cells transduced with AdHu5-PfCSP, AAV1-PfCSP or AAV8-PfCSP vectors revealed that PfCSP had accumulated in both the cell cytoplasmic regions and on the cell surfaces (**Figure 3A**). We next evaluated the transduction efficacy and resulting transgene expression of the virus-vectored vaccines in immortalized cell lines (HEK293T or Hepa1-6) using immunoblotting assays. Compared with AAV8-PfCSP, an identical infection dose (MOI = 10^5) of AAV1 transduced more efficient expression in the HEK293T cell line (**Figure 3B**). This IVIS result led us to expect greater transduction and expression efficiency with AAV8-PfCSP

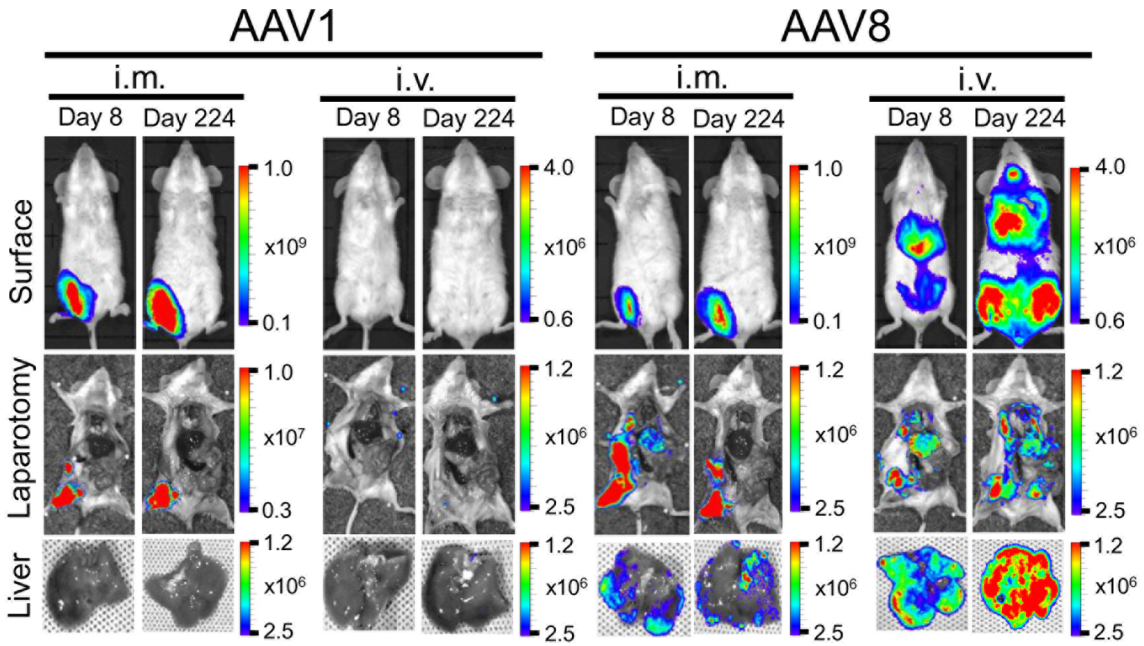

FIGURE 2 | Comparison of luciferase expression in the liver between AAV1-Luc and AAV8-Luc. Luciferase expression in the liver on day 8 or day 224 was detected using the IVIS Lumina LT Series III *in vivo* imaging system. AAV1-Luc or AAV8-Luc was injected into the right medial thigh muscles or tail veins of mice (n = 2; 1.0×10^{11} vg/mouse) on day 0. On days 8 and 224, mice were dissected 15 min after D-luciferin was administered i.p. and luciferase expression in the liver was measured. The heatmap images in the mice represent the total flux of photons (p/s/cm^2) in the area of interest. Rainbow scale ranges are expressed in radiance (p/s/cm^2/sr). The mean total flux of photons is shown as the region of interest on days 8 and 224 after AAV-Luc administration with the indicated serotype and route.

than with the AAV1 vector in the Hepa1-6 immortalized mouse hepatic cell line. However, similar to that observed with the HEK293T cell results, a lower level of PfCSP expression was observed following AAV8-PfCSP transduction in the Hepa1-6 cell line (**Figure 3C**). This result may be explained by an improved ability of the liver tissue to take up AAV8 virus in a living organism unlike that which occurs in hepatic cell cultures.

Administering AAV8-PfCSP by the I.V. Route After I.M. Administration of AdHu5-PfCSP Confers Complete Protection Against Transgenic *P. berghei* Expressing PfCSP

To investigate the protective efficacy of the prime-boost regimens, mice primed by i.m. injection with the AdHu5-PfCSP vaccine followed by an i.m. or i.v. booster vaccine dose of AAV1 or AAV8 were challenged with i.v. administration of 500 transgenic PfCSP-Tc/Pb sporozoites. Consistent with our previous results (7), the i.m. AdHu5-PfCSP/i.m. AAV1-PfCSP regimen elicited an 80% protection rate. A similar level of protection (78%) was observed in mice receiving the i.m. AdHu5-PfCSP/i.v. AAV1-PfCSP regimen (**Table 1**). The immunization regimen of i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP induced significantly better protection against challenge than did the immunization regimen of i.m. AdHu5-PfCSP/i.m. AAV8-PfCSP (100% vs 60%, $p < 0.05$) (**Figure 4**). The strong protective efficacy of i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP against sporozoite challenge indicates that the successful elimination of sporozoites by PfCSP-specific immune responses was not only dependent on the AAV serotype but also on the administration route.

Induction of a Potent Humoral Immune Response in Mice Immunized With AdHu5-PfCSP/AAV8-PfCSP

To compare the immunogenicity of the different vaccine regimens, we first assessed the PfCSP-specific humoral immune responses among the mouse groups receiving the various booster immunization regimens and administration routes (i.e., the PBS-treated control mice and the four groups of mice that received a booster dose with i.m. or i.v. AAV1-PfCSP or AAV8-PfCSP. All booster doses were administered 6 weeks after the mice were primed with i.m. AdHu5-PfCSP. We collected sera from tail veins 1 day before the booster injections were given and the sporozoite challenge infections commenced, and the samples were assessed by ELISA to measure the induction of PfCSP-specific humoral immune responses. All four groups of immunized mice had similar anti-PfCSP antibody levels after i.m. priming with AdHu5-PfCSP (**Figure 5**). Six weeks after the booster immunization, significant increases in the anti-PfCSP antibody titers were observed in all the test groups. Overall, the results indicate that after transduction with the booster dose, all the regimens induced high-level, PfCSP-specific humoral immune

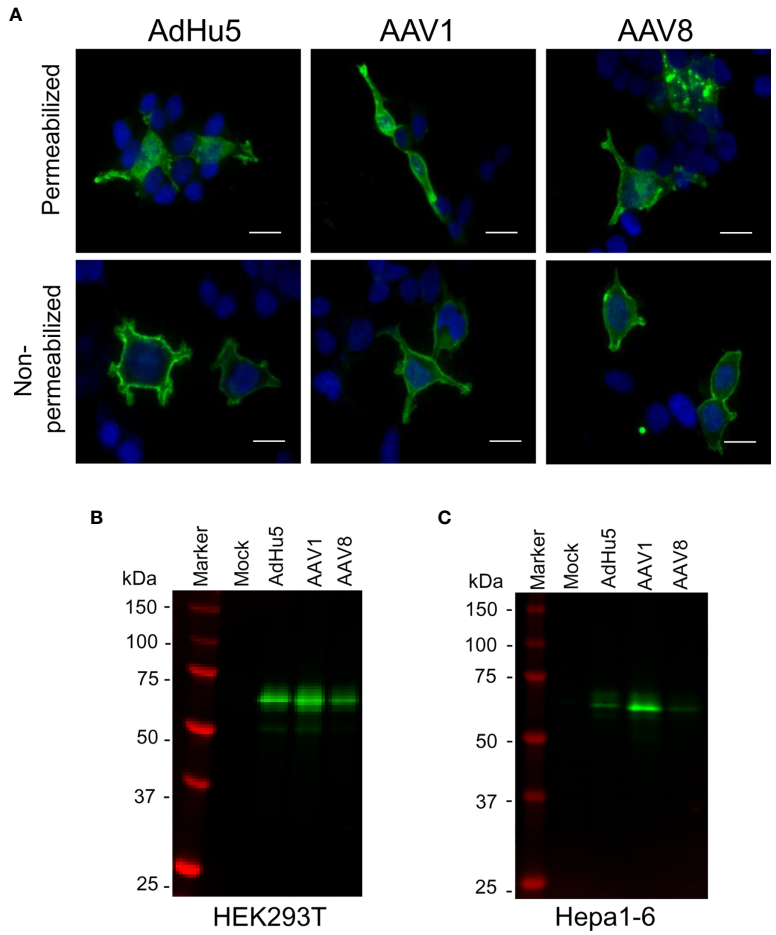

FIGURE 3 | PfCSP expression in cells transduced with AAV-PfCSP. **(A)** Cellular localization of PfCSP expression in transduced cells as assessed by an immunofluorescence assay. HEK293T cells were transduced with AdHu5-PfCSP (MOI = 3), AAV1-PfCSP (MOI = 10^5), or AAV8-PfCSP (MOI = 10^5). After 48 h, the cells were fixed with methanol (permeabilized) or paraformaldehyde (non-permeabilized), and then treated with 2A10 mAb followed by FITC-conjugated anti-mouse secondary antibody and simultaneous staining with 4′, 6-diamidino-2-phenylindole (DAPI; blue). Original magnification, 1,000. Bars = 20 μm. **(B, C)** Detection of PfCSP in HEK293T **(B)** or Hepa1-6 **(C)** cells transduced with AdHu5-PfCSP (MOI = 3, lane 3), AAV1-PfCSP (MOI = 10^5, lane 4), or AAV8-PfCSP (MOI = 10^5, lane 5). Cell lysate proteins were separated by 10% SDS-PAGE and immunoblotted with 2A10 mAb.

TABLE 1 | Protective efficacies against sporozoite challenge by immunization with heterologous prime-boost regimens in mice[a].

Prime (route)	Boost (route)	Protected/challenged (% protection)[b,c]
PBS	PBS	0/10 (0)
AdHu5-PfCSP (i.m.)	AAV1-PfCSP (i.m.)	7/9 (78)****
AdHu5-PfCSP (i.m.)	AAV1-PfCSP (i.v.)	8/10 (80)****
AdHu5-PfCSP (i.m.)	AAV8-PfCSP (i.m.)	6/10 (60)****
AdHu5-PfCSP (i.m.)	AAV8-PfCSP (i.v.)	10/10 (100)****

[a]*Mice were immunized with an i.m. injection of 5×10^7 pfu AdHu5-PfCSP followed by a booster with 1×10^{11} vg per mouse of either AAV1- or AAV8-PfCSP administered i.m. or i.v. into the tail vein at a 6-week interval. The immunized mice were challenged by i.v. administration of 500 PfCSP-Tc/Pb sporozoites. They were then screened for blood-stage infections by microscopic examination of Giemsa-stained thin smears of tail blood. Protection was defined as the complete absence of blood-stage parasitemia on day 14 post-challenge.*

[b]*Protective efficacy was calculated as described in the Materials and Methods.*

[c]*Significant difference from the control/PBS group as determined using Fisher's exact probability test (****$p < 0.0001$).*

responses. A similar high-level antibody response was observed with the i.m. AAV1-PfCSP booster, a finding consistent with that reported in our previous study (7), but the immunity induced with the i.v. AAV1-PfCSP booster was significantly lower ($p<0.01$). However, the reduced humoral immune responses did not interfere with the vaccine's protective efficacy (80% i.v. vs 78% i.m.). This finding is similar to that of the i.v. AAV8-boosted mouse group in that although a high anti-PfCSP response was

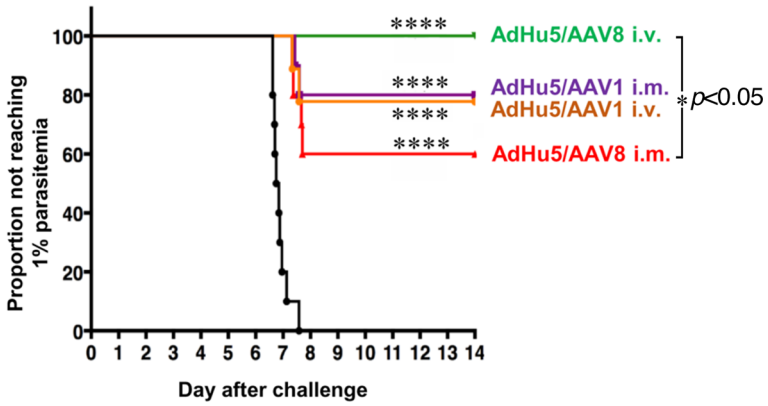

FIGURE 4 | Protective efficacy of AdHu5-PfCSP prime/AAV-PfCSP boost in immunized mice. Mice (n = 9–10 per group) were immunized with an AdHu5-PfCSP prime (5×10^7 pfu/mouse)/AAV-PfCSP boost (1×10^{11} vg/mouse) administered with a 6-week interval *via* the indicated administration routes. Six weeks after receiving booster immunizations, the mice that were challenged with 500 intravenously administered PfCSP-Tc/Pb sporozoites were checked for blood-stage infections by microscopic examination of Giemsa-stained thin smears of tail blood. Protection was defined as the complete absence of blood-stage parasitemia on day 14 post-challenge. The resulting data were statistically analyzed using the Kaplan–Meier log-rank (Mantel–Cox) test. Differences from the PBS group was assessed by a two-way ANOVA. ****$p < 0.0001$, and *$p < 0.05$.

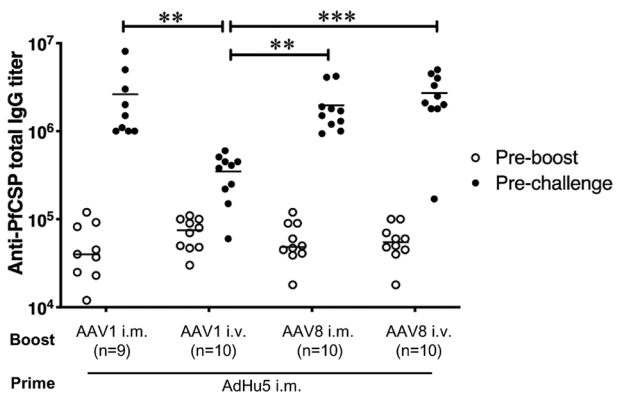

FIGURE 5 | Anti-PfCSP antibody levels in mice immunized with heterologous prime-boost regimens. After immunization with i.m. AdHu5-PfCSP prime (5.0×10^7 pfu/mouse)/AAV-PfCSP boost (1.0×10^{11} vg/mouse), mice were challenged with 500 PfCSP-Tc/Pb sporozoites. Sera were collected one day before the boost and before the sporozoite challenge from the mice used in the challenge study. Anti-PfCSP antibody titers were determined by ELISA. Bars and error bars indicate the means and SDs of the values, respectively. Between-group differences were assessed with the Kruskal–Wallis test and Dunn's correction for multiple comparisons. ***$p < 0.001$ and **$p < 0.01$.

observed (i.v. AAV1 vs i.v. AAV8, $p<0.01$), the AAV1 vaccines failed to elicit complete protection. Moreover, no significant difference in the total IgG level was observed between the protected and non-protected subgroups of the vaccinated mouse groups (**Figure S2**). Interestingly, i.m. AdHu5-PfCSP/i.m. AAV8-PfCSP and i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP regimens produced similar total IgG levels against PfCSP with varying protective efficacies (60% i.v. vs 100% i.m., $p<0.05$).

AAV8 Induces Superior Liver-Directed Gene Transfer Compared With AAV1

The ability of i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP immunization to transduce *pfcsp* gene expression in the liver was confirmed by immunohistochemistry (**Figure 6A**). Therefore, we performed qPCR to quantify the DNA transduction levels in the livers dissected from the mice that were protected against sporozoite challenge (**Figure 6B**). The qPCR results indicated that the i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP regimen was ~2.5 times better at transducing hepatocytes in the liver compared with the i.m. AdHu5-PfCSP/i.m. AAV8-PfCSP regimen. No traceable signal was detected in the liver DNA from the AAV1-immunized mouse groups.

AAV8-PfCSP I.V. Administration Induces Liver-Resident Memory $CD8^+$ T Cells

Antigen-expressing hepatocytes can promote the development of cytotoxic T cells in the liver (24). $CD8^+$ T cell populations and their subsets were quantified to examine an immunological indicator of protection after i.v. administration of AAV8-

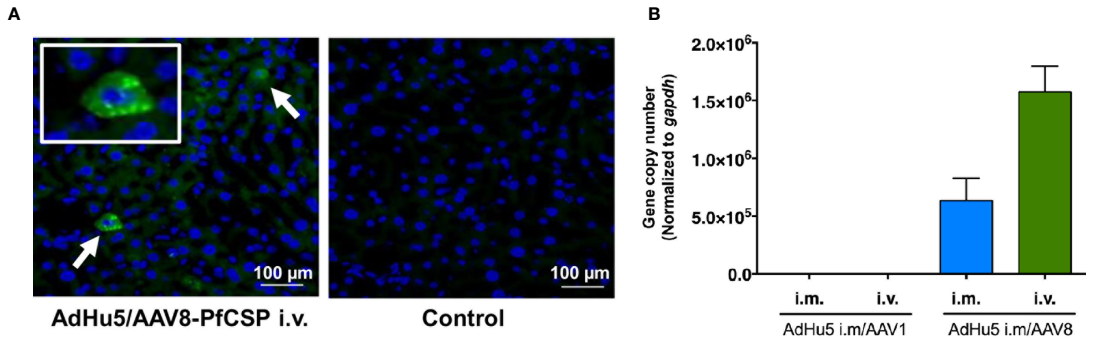

FIGURE 6 | PfCSP expression profiles in the livers of the protected mice. Mice were primed with i.m. AdHu5-PfCSP (5.0×10^7 pfu/mouse) and boosted with AAV-PfCSP (1.0×10^{11} vg/mouse) followed by a challenge with 500 PfCSP-Tc/Pb sporozoites. Whole livers were obtained from the protected mice in the challenge study. **(A)** Immunohistochemistry was performed on the liver microsections with 2A10 mAb followed by Alexa 488-conjugated anti-mouse secondary antibody (green). (**A**, left panel) Arrows show PfCSP-expressing hepatocytes. (**A**, right panel) As a negative control, a liver section from the same mouse was incubated with the solution without the mAb. Nuclei were simultaneously stained with DAPI (blue). **(B)** Quantitation of the *pfcsp* gene in the livers collected from the protected mice in the challenge experiments. The pAAV-CMV-sPfCSP2-(G+) plasmid was used to generate a standard curve with which to determine the *pfcsp* copy number in the liver. The mouse *gapdh* gene was used for normalization. 'i.m.' and 'i.v.' indicate the route of booster immunization with AAV1 or AAV8 following AdHu5 i.m. prime.

PfCSP. Liver-resident memory T (T_{RM}) cells were extensively investigated because these cells may act as sentinels against invading pathogens (25, 26). T_{RM} cells are defined as $CD69^+$/$KLRG1^{lo}$ or $CD69^+$/$CXCR6^+$ effector memory T (T_{EM}) cells ($CD8^+CD62L^{lo}CD44^{hi}$ cells). Two weeks after administering i.v. AAV8 to each mouse in a dose-dependent manner, the number of liver $CD8^+$ T cells, especially those belonging to the T_{EM} cell population, was increased at a dose of 10^{11} vg per mouse, whereas these cells were gradually decreased in the spleen (**Figure 7A**). PfCSP-specific $CD8^+$ liver T_{RM} cells (both $KLRG1^{lo}$ and highly efficient $CXCR6^+$ T_{RM} cells) were significantly induced by immunization with the standard dose of 10^{11} vg compared with the non-immunized group (**Figure 7B**). These results suggest that vaccine doses below 1.0×10^{11} vg/mouse are unable to efficiently recruit T cells to the liver, particularly antigen-specific $CD8^+$ T_{RM} cells, 2 weeks after immunization with AAV8. This result is noteworthy because such cells may play an important role in eliminating sporozoite and/or infected hepatic cells upon parasite challenge.

DISCUSSION

Our recent study showed that a heterologous AdHu5-PfCSP-prime and AAV1-PfCSP-boost immunization regimen administered by the i.m. route in a murine model can elicit 80% protection against sporozoite challenge with a strong and durable PfCSP-specific humoral and cellular immune response (7). We hypothesized that the induction of local immunity in the liver could be important for preventing the exoerythrocytic stages of malaria and subsequent blood infection. Therefore, we investigated whether hepatotropic AAV8 was capable of stimulating intense transgene expression and effective T cell recruitment in the liver. We also compared the protective efficacies of i.m. or i.v. AAV1-PfCSP and AAV8-PfCSP boost regimens administered 6 weeks after i.m. AdHu5-PfCSP priming. Notably, immunization with the i.m. AdHu5-PfCSP-prime/i.v. AAV8-PfCSP-boost regimen elicited 100% protection against sporozoite challenge.

Vaccines aimed at inducing humoral and cell-mediated immunity against malaria parasites have been hindered by parasite biology and liver microanatomy (3). Successful parasite clearance in the short window of time (5 to 7 days) during the asymptomatic pre-erythrocytic malarial infection stage requires the generation and maintenance of an adequate amount of *Plasmodium*-specific humoral and cellular immune responses to confer protection (24, 27, 28). Our heterologous prime/boost immunization regimens generated a high level of PfCSP-specific antibodies in our mouse model (**Figure 5**), although the anti-PfCSP antibody titers were not correlated with protective efficacy against sporozoites (**Figure S2**). However, it has been suggested that anti-CSP antibody titers can be used as surrogate markers of protection. Therefore, such titers were previously employed to study the magnitude and duration of RTS,S/AS01 efficacy (29). Thus, the strong PfCSP-specific antibody titer induced by our two-dose AdHu5/AAV immunization strategy, especially by the i.m. AdHu5-PfCSP-prime/i.v. AAV8-PfCSP-boost regimen, is a crucial feature of translatable vaccines. Similarly, we investigated the recruitment and proliferation of $CD8^+$ T cells in the liver following i.v. AAV8 administration because induced $CD8^+$ T cells are reportedly required for sterile protection and are correlated with efficacy against challenge with malaria parasites (3, 30, 31). However, our current evaluation system did not address whether the protection rate was correlated with the anti-PfCSP IgG level and/or with liver-resident T cells. To elucidate the mechanism(s) underlying the protective efficacy of the i.m. AdHu5-PfCSP-prime/i.v. AAV8-PfCSP-boost regimen will require further experiments to clarify how immune responses operate in the protected and non-protected mice.

Because of the high abundance of a laminin co-receptor and a possible unknown receptor in the liver, AAV8-based vectors are very efficiently taken up by the parenchymal cells in the liver (32). Consistent with other reports, our results confirm the rapid and

efficient *in vivo* transgene expression transduced by AAV8; thus, an AAV8-based vector is an attractive vector to choose for transgene delivery to the liver (3, 6, 22, 33). We found that, depending on the dose used, AAV8 could transduce as much as 90%–95% of hepatocytes in the mouse liver following its i.v. administration (34), which delivered ~2.5 times more DNA into the liver compared with i.m. administration (**Figure 6B**). Antigen expression by hepatocytes may modulate the T-cell immune response, thereby improving the clinical efficacy of rAAV (3). Our flow cytometric experiment revealed that, in a dose-dependent manner, $CD8^+$ T cells, particularly T_{EM} cells and T_{RM} cells, were generated and recruited to the liver following a single dose of i.v. AAV8 (**Figure 7**). Higher efficacy against *P. falciparum* malaria is correlated with higher numbers of CXC6 T_{RM} cells in an animal model and circulating IFN-γ secreting T_{EM} cells in human recipients after immunization with attenuated *P. falciparum* sporozoites (35, 36).

We investigated the capability of i.v. AAV8-PfCSP to recruit and induce PfCSP-specific T_{RM} cells in the liver. Because liver-resident T_{RM} cells are capable of providing protection against a malaria sporozoite challenge (3, 33, 37), the observed significant increase in the number of T_{RM} cells may act to improve the protection rate. T_{RM} cells migrate to and patrol the liver sinusoids using a crawling motion and by scanning for the cognate antigen on the cell surfaces of the hepatocytes and/or the invading malaria sporozoites (37). Cytokine production (e.g., interferon-γ, IFN-γ; tumor necrosis factor α, TNF-α) and cytotoxicity marker production [e.g., granzyme B and CD107a (33)], are associated with T_{RM} cell-mediated protection against malaria, indicating that these cells may be poised to respond to immediate threats (37).

The use of both adenovirus and AAV as vaccine vehicles has been shown to be safe in human trials (38, 39). However, capsid-specific T-cell responses and pre-existing natural antibodies against these vectors from previous exposure to natural infections with them, particularly adenovirus transduction, are important considerations for their clinical application. AAV8, a relatively new isolate, has been cloned from nonhuman primate tissues, and experience with vectors based on this serotype is limited (40). However, the seroprevalence of anti-AAV8 capsid protein antibodies and cross-reactions with natural antibodies against other AAV serotypes were found to be low in human sera and, when present, they were found to have low activities (41, 42). Because a high prevalence of AdHu5 viral capsid-specific

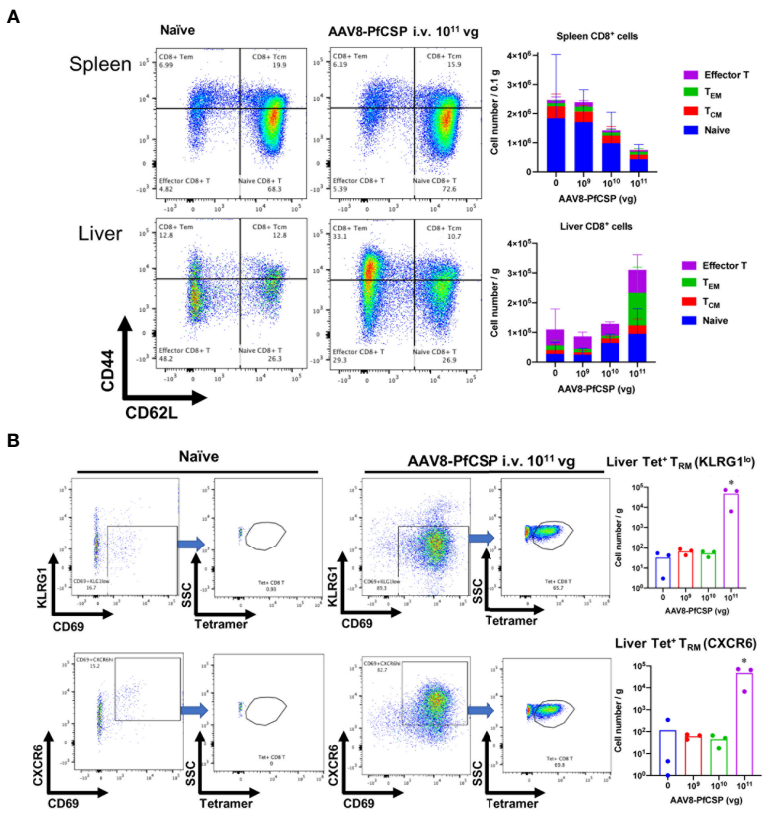

FIGURE 7 | T-cell responses in the liver and spleen after i.v. immunization with AAV8. Mice were immunized with AAV8-PfCSP (n = 3 per group; 1.0×10^9, 1.0×10^{10}, or 1.0×10^{11} vg) or PBS *via* i.v. administration into their tail veins. After 2 weeks, the $CD8^+$ T cells collected from the livers and spleens were stained as described in the Materials and Methods. Gated $CD45^+CD8^+$T-cells in livers (**A**, lower panels) or spleens (**A**, upper panels) were analyzed for CD62L and CD44 expression. The mean absolute numbers of the spleen or liver $CD8^+$T cells are shown in the right-hand graphs. **(B)** Flow cytometric measurement of $CD69^+$ and $KLRG1^{lo}$ (**B**, upper panels) or $CXCR6^+$ (**B**, lower panels) tetramer-stained, CSP-specific liver T_{RM} cells in the $CD45^+CD8^+CD62L^{lo}CD44^{hi}$ T_{EM} population. Mean absolute numbers of the spleen or liver PfCSP-specific $CD8^+T_{RM}$ cells are shown in the right-hand graphs. *$p < 0.05$ by one-way nonparametric ANOVA (Kruskal–Wallis) followed by the Dunn multiple comparisons test.

antibody was detected in African countries where malaria prevalence is high, administrating an AAV8 booster may help with maintaining the magnitude of the humoral immune response against the encoded antigen. A better priming vaccine might be achieved by the application of a different clinically tested non-immunogenic human or chimpanzee adenoviral vector [i.e., serotype 26 or serotype 63 (16)].

In summary, we exploited hepatotropic AAV8 as the booster component in a promising immunization regimen beginning with an AdHu5 priming vaccine targeting the PfCSP antigen against the malaria parasite's liver stage in preclinical settings. AAV8 is intensely hepatotropic, and its i.v. administration induces robust expression of the *pfcsp* gene after its liver-directed delivery. Consequently, strong PfCSP-specific humoral immune responses along with high levels of $CD8^+$ T cells, particularly T_{RM} cells, were generated and recruited in adequate amounts to the liver through its use. Although further studies are required to assess the involvement and durability of the T cells conferring the type of sterile protection we observed, the i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP immunization regime successfully eliminated transgenic *P. berghei* parasites in sporozoite challenge infections and achieved 100% sterile protection in our BALB/c mouse model. Therefore, we propose that the i.m. AdHu5-PfCSP/i.v. AAV8-PfCSP immunization regimen has great potential for use in the development of an effective malaria vaccine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Ethical Review Committee of Kanazawa University (No. 22118–1) and Jichi Medical University (No. 17086-01).

AUTHOR CONTRIBUTIONS

Study concept and design: MS, MI, and SY. Acquisition of data: MS, MI, HM, MK, IY, IS, YY, KF, DY, HK, NO, and SY. Analyses and interpretation of data: MS, MI, NO, and SY. Drafting the manuscript: MS, MI, and SY. Critical revision of the manuscript for important intellectual content: MS, MI, YY, and SY. Statistical analyses: MS, and MI. Technical or material support: MS, MI, HM, DY, HK, NO, and SY. Study supervision: SY. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a Grant-in-Aid for Scientific Research (B) (JSPS KAKENHI grant number 19H03458) and Grant-in-Aid for Scientific Research (JSPS KAKENHI grant number 18K19394) to SY, by a Grant-in-Aid for Scientific Research (C) (JSPS KAKENHI grant number 18K06655) to MI, and by a MEXT fellowship (183193) to MS.

ACKNOWLEDGMENTS

The authors are grateful for the assistance of Chisato Seki and Ashekul Islam as well as all the members of the Laboratory of Vaccinology and Applied Immunology, Kanazawa University. The authors also thank Katie Oakley, and Sandra Cheesman, PhD, from Edanz (https://jp.edanz.com/ac), for editing a draft of this manuscript, and Megumi Yatabe (Jichi Medical University) for her technical assistance in immunohistochemistry.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.612910/full#supplementary-material

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Conflict of Interest: SY, MI, and HM are named inventors on filed patents related to immunization with the AAV anti-malarial vaccines. These products have not been commercialized.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 October 2021
doi: 10.3389/fimmu.2021.730825

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 25 June 2021
Accepted: 17 September 2021
Published: 21 October 2021

Citation:
Brown D, Altermatt M, Dobreva T, Chen S, Wang A, Thomson M and Gradinaru V (2021) Deep Parallel Characterization of AAV Tropism and AAV-Mediated Transcriptional Changes via Single-Cell RNA Sequencing. Front. Immunol. 12:730825. doi: 10.3389/fimmu.2021.730825

Deep Parallel Characterization of AAV Tropism and AAV-Mediated Transcriptional Changes *via* Single-Cell RNA Sequencing

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Engineered variants of recombinant adeno-associated viruses (rAAVs) are being developed rapidly to meet the need for gene-therapy delivery vehicles with particular cell-type and tissue tropisms. While high-throughput AAV engineering and selection methods have generated numerous variants, subsequent tropism and response characterization have remained low throughput and lack resolution across the many relevant cell and tissue types. To fully leverage the output of these large screening paradigms across multiple targets, we have developed an experimental and computational single-cell RNA sequencing (scRNA-seq) pipeline for *in vivo* characterization of barcoded rAAV pools at high resolution. Using this platform, we have both corroborated previously reported viral tropisms and discovered unidentified AAV capsid targeting biases. As expected, we observed that the tropism profile of AAV.CAP-B10 in mice was shifted toward neurons and away from astrocytes when compared with AAV-PHP.eB. Transcriptomic analysis revealed that this neuronal bias is due mainly to increased targeting efficiency for glutamatergic neurons, which we confirmed by RNA fluorescence *in situ* hybridization. We further uncovered cell subtype tropisms of AAV variants in vascular and glial cells, such as low transduction of pericytes and Myoc+ astrocytes. Additionally, we have observed cell-type-specific transitory responses to systemic AAV-PHP.eB administration, such as upregulation of genes involved in p53 signaling in endothelial cells three days post-injection, which return to control levels by day twenty-five. The presented experimental and computational approaches for parallel characterization of AAV tropism will facilitate the advancement of safe and precise gene delivery vehicles, and showcase the power of understanding responses to gene therapies at the single-cell level.

Keywords: gene therapy, next generation sequencing, computational biology, molecular biology, neuroscience

1 INTRODUCTION

Recombinant AAVs (rAAVs) have become the preferred gene delivery vehicles for many clinical and research applications (1, 2) owing to their broad viral tropism, ability to transduce dividing and non-dividing cells, low immunogenicity, and stable persistence as episomal DNA ensuring long-term transgene expression (3–8). However, current systemic gene therapies using AAVs have a relatively low therapeutic index (9). High doses are necessary to achieve sufficient transgene expression in target cell populations, which can lead to severe adverse effects from off-target expression (10–12). Increased target specificity of rAAVs would reduce both the necessary viral dose and off-target effects; thus, there is an urgent need for AAV gene delivery vectors that are optimized for cell-type-specific delivery (13). Lower viral doses would also alleviate demands on vector manufacturing and minimize the chances of undesirable immunological responses (14–16). Capsid-specific T-cell activation was reported to be dose-dependent *in vitro* (17, 18) and in humans (19, 20). Shaping the tropism of existing AAVs to the needs of a specific disease has the potential to reduce activation of the immune system by detargeting cell types, such as dendritic cells, that have an increased ability to activate T-cells (21–26).

Several studies have demonstrated that the transduction efficiency and specificity of natural AAVs can be improved by engineering their capsids using rational design (27–31) or directed evolution (32–47). These engineering methods yield diverse candidates that require thorough, preferably high-throughput, *in vivo* vector characterization to identify optimal candidates for a particular clinical or research application. Toward this end, conventional immunohistochemistry (IHC) and various *in situ* hybridization (ISH) techniques are commonly employed to profile viral tropism by labeling proteins expressed by the viral transgene or viral nucleic acids, respectively (10, 32, 34, 45, 48–59).

Although these histological approaches preserve spatial information, current technical challenges limit their application to profiling the viral tropism of just one or two AAV variants across a few gene markers, thus falling short of efficiently characterizing multiple AAVs across many complex cell types characteristic of tissues in the central nervous system (CNS). The reliance on known marker genes also prevents the unbiased discovery of tropisms since such marker genes need to be chosen *a priori*. Choosing marker genes is particularly challenging for supporting cell types, such as pericytes in the CNS microvasculature and oligodendrocytes, which often have less established cell type identification strategies (60, 61). The advent of single-cell RNA sequencing (scRNA-seq) has enabled comprehensive transcriptomic analysis of entire cell-type hierarchies, and brought new appreciation to the role of cell subtypes in disease (62–66). However, experimental and computational challenges, such as the sparsity of RNA capture and detection, strong batch effects between samples, and the presence of ambient RNA in droplets, reduce the statistical confidence of claims about individual gene expression (67–69). Computational methods have been developed to address some of these challenges, such as identifying contaminating RNA (68), accounting for or removing batch effects (70–72), and distinguishing intact cells from empty droplets (69, 73, 74). However, strategies for simultaneously processing transcripts from multiple delivery vehicles and overcoming the computational challenges of confidently detecting individual transcripts have not yet been developed for probing the tropism of AAVs in complex, heterogeneous cell populations.

Collecting the entire transcriptome of injected and non-injected animals also offers an opportunity to study the effects of AAV transduction on the host cell transcriptome. A similar investigation has been conducted with G-deleted rabies virus (75). This study demonstrated that virus infection led to the downregulation of genes involved in metabolic processes and neurotransmission in host cells, whereas genes related to cytokine signaling and the adaptive immune system were upregulated. At present, no such detailed examination of transcriptome changes upon systemic AAV injection has been conducted. High-throughput single-cell transcriptomic analysis could provide further insight into the ramifications of AAV capsid and transgene modifications with regard to innate (76–80) and adaptive immune recognition (20, 81–84). Innate and adaptive immune responses to AAV gene delivery vectors and transgene products constitute substantial hurdles to their clinical development (85, 86). The study of brain immune response to viral gene therapy has been limited to antibody staining and observation of brain tissue slices post direct injection. In particular, prior studies have shown that intracerebral injection of rAAV vectors in rat brains does not induce leukocytic infiltration or gliosis (87, 88); however, innate inflammatory responses were observed (89). Results reported by these methods are rooted in single-marker staining and thus prevent the discovery of unexpected cell-type-specific responses. A comprehensive understanding of the processes underlying viral vector or transgene-mediated responses is critical for further optimizing AAV gene delivery vectors and treatment modalities that mitigate such immune responses.

Here, we introduce an experimental and bioinformatics workflow capable of profiling the viral tropism and response of multiple barcoded AAV variants in a single animal across numerous complex cell types by taking advantage of the transcriptomic resolution of scRNA-seq techniques (**Figure 1A**). For this proof-of-concept study, we profile the tropism of previously-characterized AAV variants that emerged from directed evolution with the CREATE (AAV-PHP.B, AAV-PHP.eB) (32, 34) or M-CREATE (AAV-PHP.C1, AAV-PHP.C2, AAV-PHP.V1, AAV.CAP-B10) (45, 90) platforms. We selected the AAV variants based on their unique CNS tropism following intravenous injection. AAV-PHP.B and AAV-PHP.eB are known to exhibit overall increased targeting of the CNS compared with AAV9 and preferential targeting of neurons and astrocytes. Despite its sequence similarity to AAV-PHP.B, the tropism of AAV-PHP.V1 is known to be biased toward transducing brain vascular cells. AAV-PHP.C1 and AAV-PHP.C2 have both demonstrated enhanced blood–brain barrier (BBB) crossing relative to AAV9 across two mouse strains (C57BL/6J and

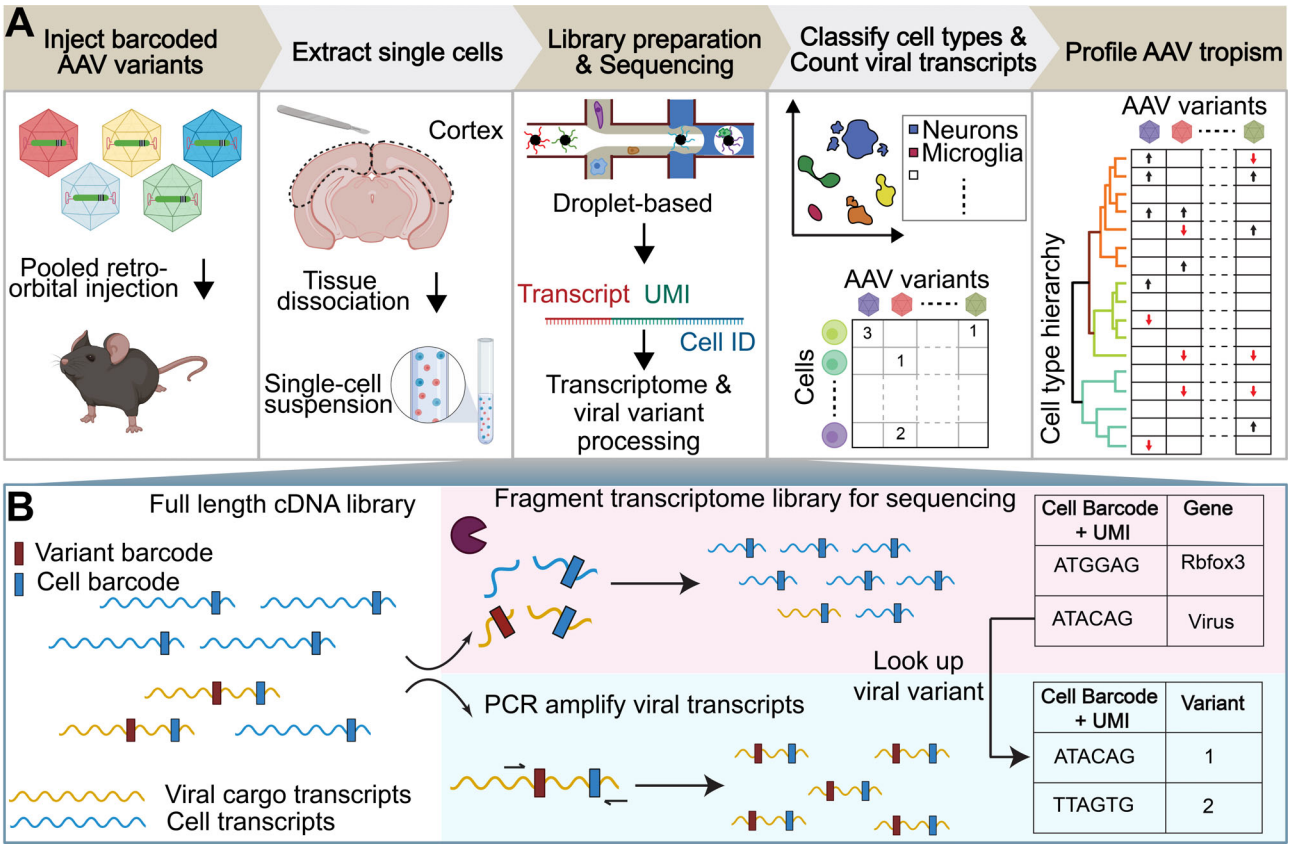

FIGURE 1 | Workflow of AAV tropism characterization by scRNA-seq. **(A)** (I) Injection of a single AAV variant or multiple barcoded AAV variants into the retro-orbital sinus. (II) After 3–4 weeks post-injection, the brain region of interest is extracted and the tissue is dissociated into a single-cell suspension. (III) The droplet-based 10x Genomics Chromium system is used to isolate cells and build transcriptomic libraries [see **(B)**]. (IV) Cells are assigned a cell-type annotation and a viral transcript count. (V) AAV tropism profiling across numerous cell types. **(B)** The full length cDNA library is fragmented for sequencing as part of the single-cell sequencing protocol (top). To enable viral tropism characterization of multiple rAAVs in parallel, an aliquot of the intact cDNA library undergoes further PCR amplification of viral transcripts (bottom). During cDNA amplification, Illumina sequencing primer targets are added to the viral transcripts such that the sequence in between the Illumina primer targets contains the AAV capsid barcode sequence. Viral cargo in the cell transcriptome is converted to variant barcodes by matching the corresponding cell barcode + UMI in the amplified viral transcript library (right).

BALB/cJ). Finally, AAV.CAP-B10 is a recently-developed variant with a bias toward neurons compared to AAV-PHP.eB (90).

In the initial validation experiment, we quantify the transduction biases of AAV-PHP.eB and AAV.CAP-B10 across major cell types using scRNA-seq, and demonstrate results which correlate well with both published results and conventional IHC-based quantification. We then demonstrate the power of the transcriptomic approach by going beyond the major cell types to reveal significant differences in cell-subtype transduction specificity. Compared with AAV.CAP-B10, AAV-PHP.eB displays biased targeting of inhibitory neurons, and both variants transduce Sst+ or Pvalb+ inhibitory neurons more efficiently than Vip+ inhibitory neurons. We validate these results with fluorescent *in situ* hybridization – hybridization chain reaction (FISH-HCR). We then develop and validate a barcoding strategy to investigate the tropism of AAV-PHP.V1 relative to AAV-PHP.eB in non-neuronal cells and reveal that pericytes, a subclass of vascular cells, evade transduction by this and other variants. We further use scRNA-seq to profile cell-type-specific responses to AAV-PHP.eB at 3 and 25 days post-injection (DPI), finding numerous genes implicated in the p53 pathway in endothelial cells to be upregulated at 3 DPI and returning back to control levels at 25 DPI. Finally, we showcase the capabilities of parallel characterization by verifying the preceding findings in a single animal with seven co-injected AAV variants and revealing their respective cell-type biases.

2 RESULTS

2.1 Multiplexed Single-Cell RNA Sequencing-Based AAV Profiling Pipeline

To address the current bottleneck in AAV tropism profiling, we devised an experimental and computational workflow (**Figure 1A**) that exploits the transcriptomic resolution of scRNA-seq to profile the tropism of multiple AAV variants

across complex cell-type hierarchies. In this workflow, single or multiple barcoded rAAVs are injected into the retro-orbital sinus of mice followed by tissue dissociation, single-cell library construction using the 10X Genomics Chromium system, and sequencing with multiplexed Illumina next-generation sequencing (NGS) (69). The standard mRNA library construction procedure includes an enzymatic fragmentation step that truncates the cDNA amplicon such that its final size falls within the bounds of NGS platforms (**Figure 1B**). These cDNA fragments are only approximately 450 bp in length and, due to the stochastic nature of the fragmentation, sequencing from their 5' end does not consistently capture any particular region. The fragment length limit and heterogeneity pose a problem for parallelizing AAV tropism profiling, which requires reliable recovery of regions of the transgene that identify the originating AAV capsid. For example, posttranscriptional regulatory elements, such as the 600 bp Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), are commonly placed at the 3' end of viral transgenes to modulate transgene expression. The insertion of such elements pushes any uniquely identifying cargo outside the 450 bp capture range, making them indistinguishable based on the cDNA library alone (**Supplemental Figure 1A**). An alternative strategy of adding barcodes in the 3' polyadenylation site also places the barcode too distant for a 5' sequencing read, and reading from the 3' end would require sequencing through the homopolymeric polyA tail, which is believed to be unreliable in NGS platforms (91, 92).

We circumvented these limitations in viral cargo identification by taking an aliquot of the intact cDNA library and adding standard Illumina sequencing primer recognition sites to the viral transcripts using PCR amplification such that the identifying region is within the two Illumina primer target sequences (e.g. **Figure 2B**). The cell transcriptome aliquots undergoing the standard library construction protocol and the amplified viral transcripts are then sequenced as separate NGS libraries. We sequence shorter viral transcripts in the same flow cell as the cell transcriptomes and longer viral transcripts on the Illumina MiSeq, which we found to be successful at sequencing cDNAs up to 890 bp long. The sequencing data undergoes a comprehensive data processing pipeline (see *Methods*). Using a custom genome reference, reads from the cell transcriptome that

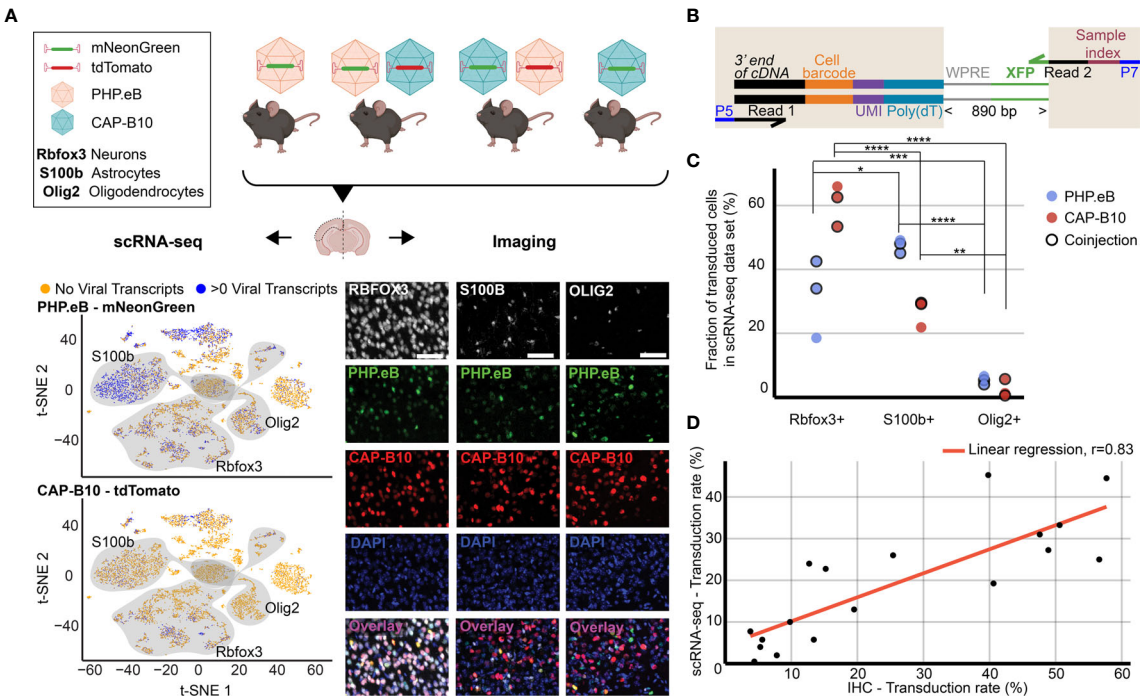

FIGURE 2 | Comparison of viral tropism profiling with traditional IHC and scRNA-seq. **(A)** Overview of the experiment. Four animals were injected with $1.5 \times 10_{11}$ viral genomes (vg) packaged in AAV-PHP.eB and/or AAV.CAP-B10. The bottom panels show a representative dataset collected from an animal that was co-injected with AAV-PHP.eB and AAV.CAP-B10. The left side displays the scRNA-seq dataset in the lower dimensional t-SNE space, with cells colored according to presence of viral transcripts. The shaded areas indicate clusters with high expression of the corresponding gene marker. The right side shows representative confocal images of cortical tissue labeled with IHC. Scale bar, 50 µm. **(B)** Viral transcript recovery strategy. The shaded areas highlight sequences added during library construction. **(C)** The fraction of the total number of transduced cells labeled as expressing the corresponding marker gene. For each AAV variant, the results of a two-way ANOVA with correction for multiple comparisons using Sidak's test are reported with adjusted P-values (****P ≤ 0.0001, ***P ≤ 0.001, and **P ≤ 0.01, and *P ≤ 0.05 are shown; P > 0.05 is not shown). **(D)** Comparison of transduction rates based on quantification *via* scRNA-seq or IHC. Transduction rate in IHC was calculated as (number of transduced cells in the group)/(total number of cells in the group). Transduction rate in scRNA-seq is based on an estimate of the fraaction of cells containing transcripts above background (see *Methods*). Each dot represents the transduction rate of neurons/Rbfox3+, astrocytes/S100b+, or oligodendrocytes/Olig2+ by AAV-PHP.eB or AAV.CAP-B10 in one animal. Histology data are averages across three brain slices per gene marker and animal. r indicates the Pearson correlation coefficient.

align to the viral cargo plasmid sequences are counted as part of the standard 10X Cell Ranger count pipeline (see *Methods* and **Supplemental Figure 1C**). In parallel, reads from the amplified viral transcripts are used to count the abundance of each viral barcode associated with each cell barcode and unique molecular identifier (UMI). The most abundant viral barcode for each cell barcode and UMI is assumed to be the correct viral barcode, and is used to construct a variant lookup table. This lookup table approach identifies an originating capsid in 65.7 ± 2.3% of viral transcripts detected in the cell transcriptome aliquots (**Supplemental Table 4**).

For determining viral cell-type tropism, we developed a method to estimate the fraction of cells within a cell type that express viral transcripts. Viral RNA expression levels depend on both the multiplicity of infection and the transcription rate of the delivered cargo. Thus, directly using viral RNA counts to determine tropism is confounded by differences in transcription rate between cell types, limiting comparison with imaging-based tropism quantification methods. As evidence of this, we detected that viral RNA expression levels can vary by cell type but are not perfectly rank correlated with the percent of cells detected as expressing that transcript (**Supplemental Figure 2B**). An additional confound arises from the ambient RNA from cellular debris co-encapsulated with cell-containing droplets, which can lead to false positives, i.e., detecting viral RNA in droplets containing a cell that was not expressing viral RNA. For example, we detected low levels of viral transcripts in large percentages of cells, even in cell types suspected to evade transduction, such as immune cells (**Supplemental Figure 2A**). To reduce the effect of both variability in expression and ambient RNA, we developed an empirical method to estimate the percentage of cells expressing transcripts above the noise, wherein the distribution of viral transcript counts in a set of cells of interest is compared to a background distribution of cell-free (empty) droplets (see *Methods*, **Supplemental Figure 2C**). In simulation, this method accurately recovers the estimated number of cells expressing transcripts above background across a wide range of parameterizations of negative binomial distributions (see *Methods*, **Supplemental Figure 2D**). Importantly, this method can yield a different ranking of viral tropism as compared to mean transcript expression rate (**Supplemental Figure 2E**).

To address several additional technical problems in default single-cell pipelines, we developed a simultaneous quality control (QC) and droplet identification pipeline. Our viral transduction rate estimation method described above relies on having an empirical background distribution of viral transcript counts in empty droplets to compare against the cell type of interest. However, the default cell *vs.* empty droplet identification method provided by the 10X Cell Ranger software, which is based on the EmptyDrops method (73), yielded unexpectedly high numbers of cells and clusters with no recognizable marker genes, suggesting they may consist of empty droplets of ambient RNA or cellular debris (**Supplemental Figures 3A, B**). Additionally, we sought to remove droplets containing multiple cells (multiplets) from our data due to the risk of falsely attributing viral tropism of one cell type to another. However, using Scrublet (93), an established method for identifying droplets containing multiplets, failed to identify multiplets in some of our samples and only identified small proportions of clusters positive for known non-overlapping marker genes, such as Cldn5 and Cx3cr1 (**Supplemental Figure 3C**). To address both the empty droplet and multiplet detection issues, we built a droplet classification pipeline based on scANVI, a framework for classifying single-cell data *via* neural-network-based generative models (94). Using clusters with a high percentage of predicted multiplets from Scrublet as training examples of multiplets, and clusters positive for known neuronal and non-neuronal marker genes as training examples of neurons and non-neuronal cells, we trained a predictive model to classify each droplet as a neuron, non-neuron, multiplet, or empty droplet (see *Methods*, **Supplemental Figure 4A**). This model performed with 97.4% accuracy on 10% of cells held out for testing, and yielded a database of 334,151 cortical cells (**Supplemental Figure 4B**). Inspection of the cells classified as empty droplets reveals that these droplets have lower transcript counts and higher mitochondrial gene ratios, consistent with other single-cell quality control pipelines (**Supplemental Figure 4D**). Critically, we discovered that non-neuronal clusters contained significantly more cells that had been previously removed by the Cell Ranger filtering method as compared to neuronal clusters ($P = 0.025$, 2-sided student t-test). In some clusters within cell subtypes, such as mature oligodendrocytes and endothelial cells, we identified up to 67% more cells than what were recovered *via* Cell Ranger.

Using our combined experimental and computational pipeline for viral transcript recovery and droplet identification, we can recover a lower bound on the expected number of cells expressing each unique viral cargo within groups of cells in heterogeneous samples.

2.2 Single-Cell RNA Sequencing Recapitulates AAV Capsid Cell-Type-Specific Tropisms

As a first step, we validated our method by comparing the quantification of AAV transduction of major cell types *via* scRNA-seq to conventional IHC. For this purpose, we characterized the tropism of two previously reported AAV variants, AAV-PHP.eB (32) and AAV.CAP-B10 (90) (**Figure 2A**). In total, four animals received single or dual retro-orbital injections of AAV-PHP.eB and/or AAV.CAP-B10 with 1.5×10^{11} viral genomes (vg) per variant. Co-injection of both variants served to test the ability of our approach to parallelize tropism profiling. By having each variant package a distinct fluorophore, tropism could be simultaneously assessed *via* multi-channel fluorescence and mRNA expression of the distinct transgene. After 3–4 weeks of expression, we harvested the brains and used one hemisphere for IHC and one hemisphere for scRNA-seq. To recover viral transcripts, we chose primers such that enough of the XFP sequence was contained within the Illumina primer target sequences to differentiate the two variants (**Figure 2B**, **Supplemental Table 1**). For this comparison, we

focused on the transduction rate for neurons (Rbfox3), astrocytes (S100b), and oligodendrocytes (Olig2). For IHC, a cell was classified as positive for the marker gene on the basis of antibody staining, and was classified as transduced on the basis of expression of the delivered fluorophore. For scRNA-seq, all cells that passed our QC pipeline were projected into a joint scVI latent space and clustered. To most closely match our imaging quantification, we considered all clusters that were determined to be positive for the respective marker gene as belonging to the corresponding cell type (see *Methods*). All clusters of the same marker gene were grouped together, and the transduction rate of the combined group of cells was determined using our viral transduction rate estimation method.

Our analysis of the scRNA-seq data demonstrates that the viral tropism biases across the three canonical marker genes are consistent with previous reports (**Figure 2C**) (32, 90). In contrast to AAV-PHP.eB, AAV.CAP-B10 preferentially targets neurons over astrocytes and oligodendrocytes. No marked discrepancies in viral tropism characterization were observed with single *versus* dual injections.

To quantify the similarity of the AAV tropism characterizations obtained with IHC and scRNA-seq, we directly compared the transduction rate of each AAV variant for every cell type and its corresponding marker gene (i.e., Rbfox3, S100b, or Olig2) as determined by each technique and noticed a good correlation (**Figure 2D**). Despite the different underlying biological readouts–protein expression in IHC and RNA molecules in labeled cell types for scRNA-seq–the two techniques reveal similar viral tropisms.

2.3 Tropism Profiling at Transcriptomic Resolution Reveals AAV Variant Biases for Neuronal Subtypes

After validating our approach against the current standard of AAV tropism characterization (IHC imaging), we scrutinized the tropism of AAV-PHP.eB and AAV.CAP-B10 beyond the major cell types (**Figure 3**). Since AAV.CAP-B10 has increased neuronal bias relative to AAV-PHP.eB, we first sought to understand if there were neuronal subtypes that were differentially responsible for this bias. However, in-depth cell typing of transcriptomes collected from tissues with numerous and complex cell types, such as neurons in the brain, requires expert knowledge of the tissue composition, time to manually curate the data, and the availability of large datasets (66). To minimize the burden of manual annotation, computational tools have been developed that use previously-annotated single-cell databases to predict the cell type of cells in new, unannotated single-cell experiments, even across single-cell platforms (94, 96, 97). We decided to leverage these tools and expanded our marker gene-based cell typing approach by having more complicated or well-established cell types be assigned based on annotations in a reference dataset (**Supplemental Figure 4A**). To this end, we again employed scANVI to construct a joint model of cells from our samples and cells from an annotated reference database. For this model, we used the Mouse Whole Cortex and Hippocampus 10x v2 dataset available from the Allen Brain Institute (95). Since this is a neuron-enriched dataset, we constructed the model using only the 125,341 cells in our dataset classified as neurons from our marker-based QC pipeline combined with the 561,543 neuronal cells from cortical regions from the reference database. We trained this model to predict to which of 14 neuron subtype groupings each cell belonged. We held out 10% of the data for testing: the model performed with 97.9% classification accuracy on the held-out data. We then applied the model to predict the neuron subtypes of our cells.

During our in-depth characterization, we discovered several previously unnoticed cell-subtype biases for AAV-PHP.eB and AAV.CAP-B10 (**Figure 3A**). Starting at the top of our neuronal hierarchy, the fraction of transduced cells that were glutamatergic neurons was markedly reduced for AAV-PHP.eB compared with AAV.CAP-B10 ($P = 0.03$, 2-sided student t-test, corrected for 2 neuron subtype comparisons). Furthermore, Pvalb+ and Sst+ inhibitory neurons both represented a larger fraction of transduced cells than Vip+ inhibitory neurons (adjusted $P = 0.0009$, $P = 0.045$, respectively, two-way ANOVA with multiple comparison correction for inhibitory neuron subtypes using Tukey's method).

To confirm these tropism biases in neuronal subtypes with a traditional technique, we performed FISH-HCR for glutamatergic and GABAergic gene markers (**Figure 3B**) (98, 99). As indicated by our scRNA-seq data, AAV.CAP-B10, when compared with AAV-PHP.eB, has increased transduction efficiency of glutamatergic neurons (SLC17A7). Furthermore, FISH-HCR verified the downward trend in transduction efficiency from Pvalb+, to Sst+, to Vip+ neurons in both AAV variants (**Figure 3C**).

2.4 Pooled AAVs Packaging Barcoded Cargo Recapitulate the Non-Neuronal Tropism Bias of PHP.V1

To enable profiling viral variants in parallel without needing distinct transgenes per variant, we established a barcoding strategy whereby we package AAV variants with the same transgene and regulatory elements but with short, distinguishing nucleotide sequences within the 3' UTR (**Figure 4A**). To verify that this barcoding strategy can recover tropisms consistent with our previous transgene-based capsid-identification strategy, we performed a set of experiments to re-characterize the tropism of AAV-PHP.eB in parallel with that of the recently developed AAV-PHP.V1, which has increased specificity for vascular cells over AAV-PHP.eB (45).

We produced AAV-PHP.eB carrying CAG-mNeonGreen and AAV-PHP.V1 carrying either CAG-mRuby2 or CAG-tdTomato. Additionally, we produced AAV-PHP.eB and AAV-PHP.V1 both carrying CAG-mNeonGreen with 7-nucleotide barcodes 89 bp upstream of the polyadenylation start site such that they did not interfere with the WPRE. We ensured each barcode had equal G/C content, and that all barcodes were Hamming distance 3 from each other (**Supplemental Table 5**). Each of the barcoded variants was packaged with multiple barcodes that were pooled together during virus production. Four animals received a retro-orbital co-injection of 1.5×10^{11} vg/each of AAV-PHP.V1 and AAV-PHP.eB. Two animals received viruses carrying separate fluorophores (cargo-based), and two animals received viruses

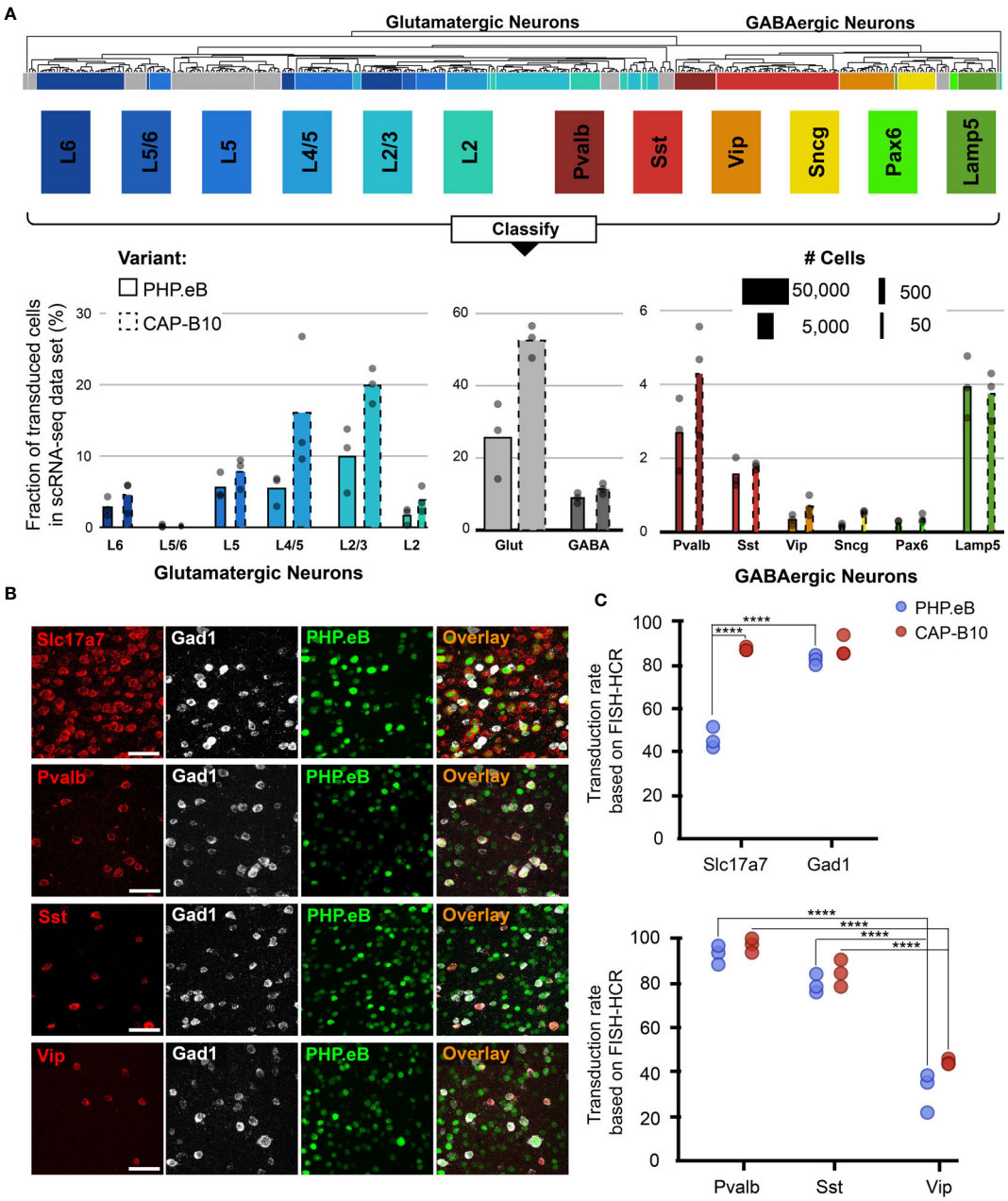

FIGURE 3 | In-depth AAV tropism characterization of neuronal subtypes at transcriptomic resolution. **(A)** Viral tropism profiling across neuronal subtypes. Neuronal subtype annotations are predicted by a model learned from the Allen Institute reference dataset using scANVI (94, 95). Each dot represents data from one animal injected with AAV-PHP.eB and/or AAV.CAP-B10. Bar width indicates the total number of cells of a particular cell type present in our dataset. **(B)** Representative confocal images of cortical tissue from an animal injected with 1.5×10^{11} vg of AAV-PHP.eB. Tissue was labeled with FISH-HCR for gene markers of glutamatergic neurons (Slc17a7) and GABAergic neurons (Gad1, Pvalb, Sst, Vip). AAV-PHP.eB shows the endogenous fluorescence of mNeonGreen. Scale bar, 50 µm. **(C)** Confirmation of viral tropism biases across neuronal subtypes using FISH-HCR (3 mice per AAV variant, 1.5×10^{11} vg dose). Dots represent the average values across three brain slices from one animal. Results from a two-way ANOVA with correction for multiple comparisons using Tukey's test is reported with adjusted P-values (****$P \leq 0.0001$; and $P > 0.05$ is not shown on the plot).

carrying the barcoded cargo (barcode-based). For amplification of the viral cDNA in the animals receiving the barcoded cargo, we used primers closer to the polyA region such that the sequencing read covered the barcoded region (**Supplemental Table 1**). During the single-cell sequencing dissociation and recovery, one of our dissociations resulted in low recovery of neurons (**Supplemental Figure 4C**); thus, we investigated only non-neuronal cells for this experiment.

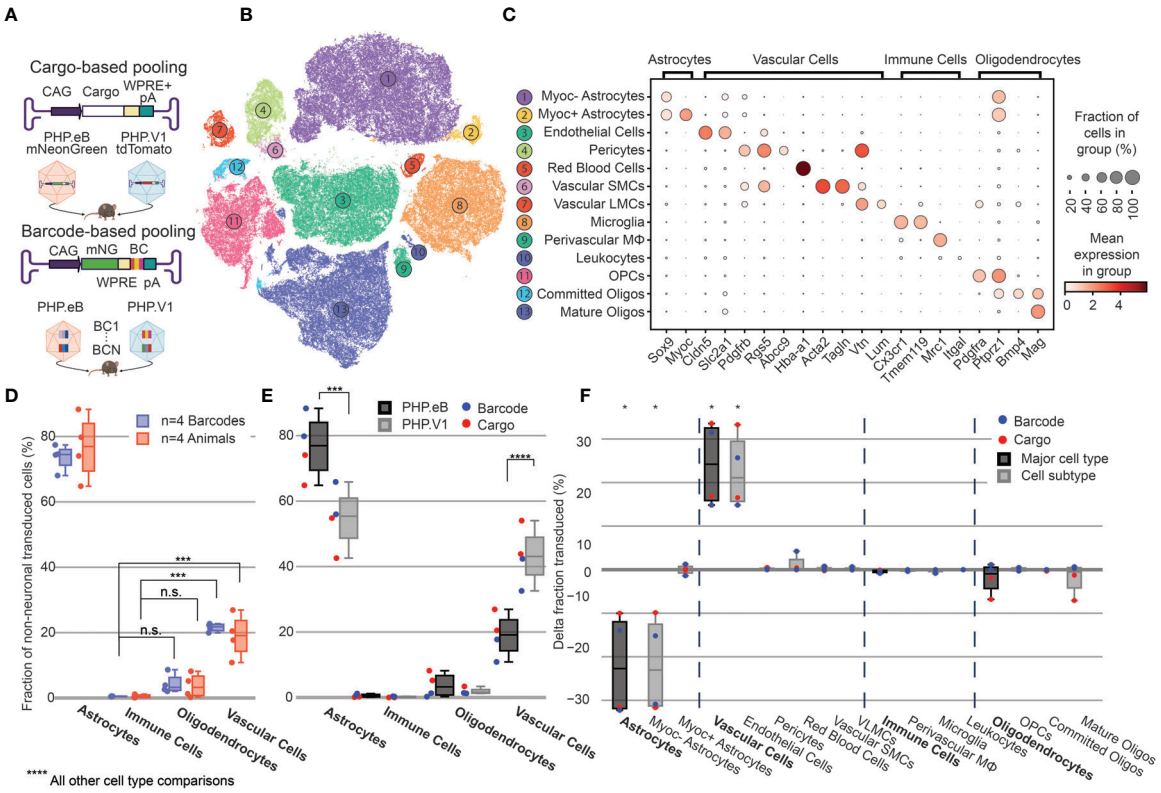

FIGURE 4 | Barcoded co-injected rAAVs reveal the non-neuronal tropism bias of AAV-PHP.V1. **(A)** Experimental design for comparing barcode *vs* cargo-based tropism profiling. Animals received dual injections of AAV-PHP.eB and AAV-PHP.V1, carrying either distinct fluorophores (cargo) or the same fluorophore with distinct barcodes. **(B)** t-SNE projection of the single-cell Variational Inference (scVI) latent space of cells and their cell type classification of the 176,724 labeled non-neuronal cells across all our samples. Each number corresponds to the cell type labeled in **(C)**. **(C)** Marker genes used to identify non-neuronal cell types. Darker colors indicate higher mean expression, and dot size correlates with the abundance of the gene in that cell type. **(D)** The distribution of non-neuronal cells expressing transcripts from AAV-PHP.eB across 4 barcodes within one animal (blue) and across 5 animals (red). All animals received dual injections, with one of the vectors being 1.5 x 10^{11} vg of PHP.eB carrying CAG-mNeonGreen. The y-axis represents the fraction of transduced non-neuronal cells that are of the specified cell type. (***P≤ 0.001 and P > 0.05 (n.s.) is shown; all other cell-type comparisons within a paradigm were significant at P ≤ 0.0001). **(E)** The distribution of non-neuronal cells expressing transcripts from AAV-PHP.eB (black) and AAV-PHP.V1 (gray). Results from the different experimental paradigms are combined. Results shown are from a two-way ANOVA with correction for multiple comparisons using Sidak's test comparing transduction by AAV-PHP.eB to AAV-PHP.V1 for each cell type, with adjusted P-values (****P ≤ 0.0001, ***P ≤ 0.001 is shown; P > 0.05 is not shown). **(F)** Within-animal difference in the fraction of cells transduced with AAV-PHP.V1 relative to AAV-PHP.eB across four animals, two from each experimental paradigm. For each cell type in each sample, the combined 2-proportion z score for the proportion of that cell type transduced by AAV-PHP.V1 *vs* AAV-PHP.eB is reported. Cell types with fewer than 2 cells transduced by both variants were discarded. Z scores were combined across multiple animals using Stouffer's method and corrected for multiple comparisons. Cell-type differences with an adjusted P-value below 0.05 are indicated with *.

Despite variability in the total transgene RNA content between barcodes of the same variant (**Supplemental Figure 5A**), the estimated percent of cells expressing the transgene within each cell type was consistent between barcodes within a single animal, with standard deviations ranging from 0.002 to 0.056 (**Supplemental Figure 6A**). Our analysis of both the barcode-based animals and cargo-based animals shows the same bias in non-neuronal tropism, with AAV-PHP.eB significantly preferring astrocytes over oligodendrocytes, vascular cells, and immune cells (**Figure 4D**). Interestingly, our analysis also revealed that the variance between barcodes within an animal was less than the variance between animals, even when controlling for cargo and dosage (P = 0.030, Bartlett's test, P-values combined across all variants and cell types using Stouffer's method, weighted by transduced cell type distribution). This is not surprising, since we found differences in cell type distribution alone can account for up to 58% of the perceived variability in tropism bias (**Supplemental Figure 6B**).

Next, we investigated the distribution of cells transduced by AAV-PHP.eB *vs* AAV-PHP.V1 in the major non-neuronal cell types across both barcode-based and cargo-based paradigms (**Figure 4E**). The single-cell tropism data confirms the previously-established finding that AAV-PHP.V1 has a bias toward vascular cells relative to AAV-PHP.eB. Additionally, we uncovered that this is coupled with a bias away from astrocytes relative to AAV-PHP.eB, but that transduction bias of oligodendrocytes and immune cells did not differ between the variants. To investigate for a specific effect of the barcoding strategy, we performed a three-way ANOVA across the variant,

cell type, and experimental paradigm factors. We found that the cell type factor accounted for 87.80% of the total variation, the combined cell type + variant factor accounted for 8.39% of the total variation, and the combined cell type + experimental paradigm factor accounted for only 2.36% of the total variation, confirming our hypothesis that barcoded pools can recover tropism with minimal effect.

2.5 Relative Tropism Biases Reveal Non-Neuronal Subtypes With Reduced AAV Transduction

To further characterize the tropism biases of AAV-PHP.V1 and expand our method to less well-established cell hierarchies, we explored the non-neuronal cell types in our dataset. Since the Allen Brain Institute reference database that we used to investigate neuronal tropism was enriched for neurons, it does not contain enough non-neuronal cells to form a robust non-neuronal cell atlas. Our combined dataset consists of 203,661 non-neuronal cells, making it large enough to establish our own non-neuronal cell clustering. Thus, we performed an additional round of automatic clustering on the cells classified as non-neuronal in our combined dataset, and identified 13 non-neuronal cell subtypes based on previously established marker genes (**Figures 4B, C** and **Supplemental Table 2**).

Most cell subtypes had multiple clusters assigned to them, which suggested there may be additional subtypes of cells for which we did not find established marker genes. To determine whether any of these clusters delineated cell types with distinct transcriptional profiles, we investigated the probability of gene expression in each cluster compared to the other clusters of the same cell subtype (see *Methods*). Our approach determined two subclusters of pericytes and astrocytes. Both clusters of pericytes had strong expression of canonical pericytes marker genes Rgs5, Abcc9, and Higd1b. However, one of the clusters had no marker genes that made it distinct from the other pericyte cluster, nor from endothelial cells. Consistent with previous reports, this suggests that this cluster could be pericytes contaminated with endothelial cell fragments, and thus was not considered for further analysis (100–102). Two distinct groups of astrocytes were detected, one of which had unique expression of Myoc and Fxyd6. Using these new marker genes, we expanded our non-neuronal cell taxonomy to 13 cell types, now including Myoc+ and Myoc- astrocytes.

Given our finding that inter-sample variability exceeds intra-sample variability, we established a normalization method for comparing transduction biases between variants co-injected into the same animal. This normalization–calculating the difference in the fraction of transduced cells between variants–captures the relative bias between variants, instead of the absolute tropism of a single variant (see *Methods*). By considering the relative bias between variants, we are able to interrogate tropism in a way that is more robust to inter-sample variability that arises from different distributions of recovered cells, expression rate of delivered cargo, and success of the injection. Using this normalization method, we evaluated the non-neuronal cell type bias of AAV-PHP.V1 relative to AAV-PHP.eB in both the cargo-based animals and the barcode-based animals across our non-neuronal cell-type taxonomy (**Figure 4F**). We discovered that the bias of AAV-PHP.V1 for vascular cells is driven by an increase in transduction of endothelial cells, but not pericytes. Similarly, AAV-PHP.V1's bias away from astrocytes is driven by a decrease in transduction of Myoc- astrocytes, but not Myoc+ astrocytes. Further inspection of the transduction of pericytes and Myoc+ astrocytes revealed that pericytes are not highly transduced by any of the AAVs tested in this work, and that Myoc+ astrocytes have both lower viral transcript expression and lower abundance than Myoc- astrocytes, and thus do not contribute significantly to tropism (**Supplemental Figures 4, 7A, B**).

2.6 Single-Cell RNA Sequencing Reveals Early Cell-Type-Specific Responses to IV Administration of AAV-PHP.eB That Return to Baseline by 3.5 Weeks

To investigate the temporal cell-type-specific transcriptional effects of systemic AAV delivery and cargo expression, we performed a single-cell profiling experiment comparing animals injected with AAV to saline controls. We injected six male mice with AAV-PHP.eB (1.5×10^{11} vg) carrying mNeonGreen, and performed single-cell sequencing on three mice three days post-injection (3 DPI) and three mice twenty-five days post-injection (25 DPI). These time points were chosen based on previous work showing MHC presentation response peaking around day seven and transgene response peaking around day 30 (89). Three saline control mice were processed 3 DPI. We then analyzed differential gene expression for each cell type between injected animals and controls using DESeq2 (**Supplemental Table 8**). Of note, we excluded cell types with less than 50 cells in each sample, and excluded leukocytes and red blood cells given the risk of their presence due to dissociation rather than chemokine mediated infiltration. Additionally, we collapsed subtypes of excitatory neurons, inhibitory neurons, and OPCs to have greater than 50 cells for differential expression analysis. We estimated viral transduction rate of AAV-PHP.eB using its delivered cargo, mNeonGreen, across cell types and time points. We identified that Myoc- astrocytes have significantly higher estimated transduction rate at 25 DPI compared to 3 DPI (adjusted P-value = 0.0438, two-way ANOVA with multiple comparison correction using Sidak's method). It is also worth noting that endothelial cells have a similar transduction rate between the time points in all animals, while one of the animals at 25 DPI exhibited higher transduction in neurons (**Figure 5A**). The number of statistically relevant genes between the injected and control group (adjusted P-value < 0.05, DESeq2) were highest in endothelial cells (41 genes) at 3 DPI, followed by inhibitory neurons (9 genes) at 25 DPI (**Figure 5B**) (adjusted P-value < 0.05, DESeq2).

We found that endothelial cells had the most acute response at 3 DPI with p53 signaling pathway notably impacted. A significant upregulation of Phlda3 and its effectors Bax, Aen, Mdm2, and Cdkn1a, all involved in the p53/Akt signaling pathway, was present (**Figures 5C, E**) (103, 104). Of relevance, we also detected Trp53cor1/LincRNA-p21, responsible for negative regulation of gene expression (105), upregulated in

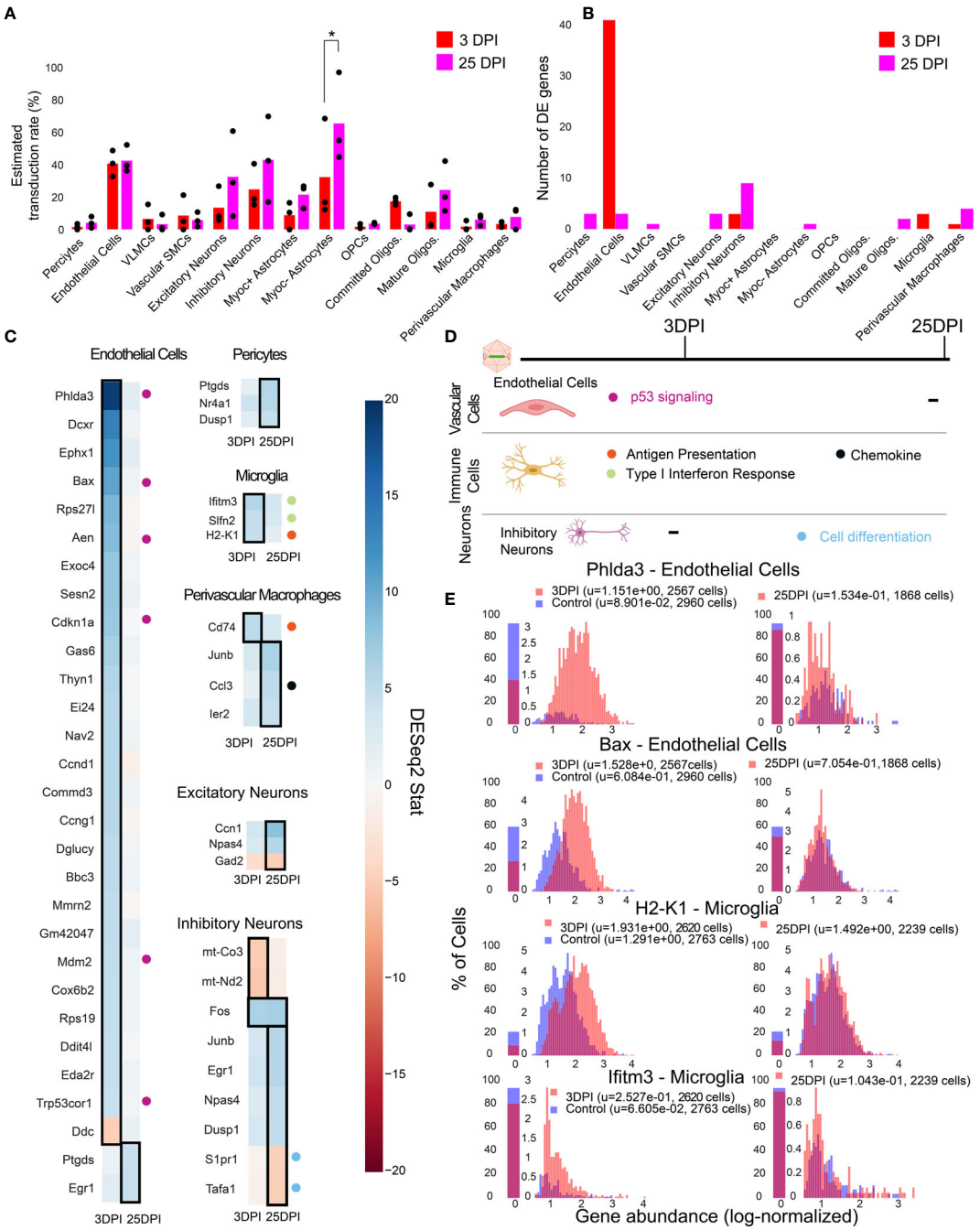

FIGURE 5 | Single-cell gene expression profiling finds cell-type-specific responses to AAV transduction in endothelial cells. **(A)** Estimated transduction rate (%) of mNeonGreen cargo at three and twenty-five days post-injection (DPI). Results from a two-way ANOVA with correction for multiple comparisons using Sidak's method is reported with adjusted P-values (*P ≤ 0.05 is shown; and P > 0.05 is not shown on the plot). **(B)** Number of differentially expressed genes (adjusted P-value < 0.05, DESeq2) at 3 DPI and 25 DPI across 3 animals. **(C)** Differentially expressed genes across the two time points in endothelial cells, pericytes, microglia, perivascular macrophages, inhibitory neurons, and excitatory neurons. Color indicates DESeq2 test statistic with red representing downregulation and blue representing upregulation. Genes outlined by a black rectangle are determined to have statistically significant differential expression compared to controls (adjusted P-value < 0.05, DESeq2, after Benjamini/Hochberg multiple comparison correction across all cell types and conditions). Colored circles adjacent to each gene indicate the corresponding pathway presented in **(D)**. **(D)** A summary of corresponding pathways in which the differentially regulated genes in **(C)** are involved across the time points. **(E)** Distribution of p53 signaling transcripts in endothelial cells (3 animals are combined) and an example of an MHC-I gene upregulated in microglia at 3 DPI.

endothelial cells at 3 DPI. Another example of an upregulated gene relevant to inflammation and stress response in endothelial cells is Mmrn2, responsible for regulating angiogenesis in endothelial cells (106).

In brain immune cells, we observe a few substantial changes in genes pertaining to immune regulation at 3 DPI for microglia and at 25 DPI for perivascular macrophages. For example, we observe an upregulation of MHC-I gene H2-K1 at 3 DPI in microglia, which then stabilizes back to control levels at 25 DPI (**Figure 5C**). Ifitm3 and Slfn2, genes implicated in type I interferon response (107, 108), also show upregulation at 3 DPI in microglia. Cd74, a chaperon responsible for regulating antigen presentation during immune response, was upregulated in perivascular macrophages at 3 DPI (109). We did not observe significant differences in pro-inflammatory chemokines, Ccl2 and Ccl5, which are related to breakdown of the blood-brain barrier *via* regulation of tight-junction proteins and recruitment of peripheral leukocytes (110). Ccl3, responsible for infiltration of leukocytes and CNS inflammation (111), was upregulated in perivascular macrophages in 25 DPI (**Figure 5C**).

We found that neurons had only a few differentially expressed genes at 25 DPI. Immediate early genes, such as Fos and Junb were upregulated in inhibitory neurons, while genes involved in modulating cell proliferation, such as Tafa1 and S1pr1, were downregulated at 25 DPI (79, 112).

By investigating the gene expression differences in subpopulations of cells post-injection, we found that endothelial cells upregulate genes linked to p53 signaling at 3 DPI (**Figure 5D**) which all return to control levels at 25 DPI. Immune cells such as microglia and perivascular macrophages upregulate genes involved in type I interferon response, MHC-I antigen processing, and chemokine signaling (**Figure 5D**). Inhibitory neurons display a subtle effect, consisting of differential expression of genes involved in stress response and cell proliferation at 25 DPI.

2.7 Larger Pools of Barcoded AAVs Recapitulate Complex Tropism Within a Single Animal

To showcase the capabilities of parallel characterization, we next designed a 7-variant barcoded pool that included the three previously characterized variants (AAV-PHP.eB, AAV-CAP-B10, and AAV-PHP.V1), AAV9 and AAV-PHP.B controls, and two additional variants, AAV-PHP.C1 and AAV-PHP.C2. For simplification of cloning and virus production, we designed a plasmid, UBC-mCherry-AAV-cap-in-cis, that contained both the barcoded cargo, UBC-mCherry, and the AAV9 capsid DNA (**Supplemental Figure 1B**). We assigned three distinct 24 bp barcodes to each variant (**Supplemental Table 5**). Each virus was produced separately to control the dosage, and 1.5 x 10^{11} vg of each variant was pooled and injected into a single animal.

After 3 weeks of expression, we performed single-cell sequencing on extracted cortical tissue. To increase the number of cells available for profiling, we processed two aliquots of cells, for a total of 36,413 recovered cells. To amplify the viral transcripts, we used primers that bind near the 3' end of mCherry such that the barcode was captured in sequencing (**Supplemental Table 1**).

Using our cell typing and viral transcript counting methods, we investigated the transcript counts and transduction bias of the variants in the pool. Compared with our previous profiling experiments, the log-transformed transcript abundance of UBC-mCherry detected per cell was lower than CAG-mNeonGreen-WPRE and CAG-tdTomato (adjusted $P < 0.0001$, $P=0.0767$, respectively, two-way ANOVA with multiple comparison correction using Tukey's method) and shifted towards vascular cells (adjusted $P < 0.0001$, $P=0.0004$, respectively, two-way ANOVA with multiple comparison correction using Tukey's method) (**Supplemental Figures 5B, C**). Next, we looked at the transduction rate difference for each variant compared with the rest of the variants in the pool for each cell type in our taxonomy (**Figures 6A, B**). Despite the lower expression rate and bias shift, the transduction rate difference metric captured the same tropism biases for AAV.CAP-B10 and AAV-PHP.V1 as determined from our previous experiments. AAV.CAP-B10 showed enhanced neuronal targeting relative to other variants in the pool, with this bias coming specifically from an increase in the transduction of glutamatergic neurons. All five variants with transcripts detected in neurons showed a decreased transduction rate in Vip+ neurons relative to other GABAergic neuronal subtypes (**Supplemental Figure 7C**). AAV-PHP.eB showed enhanced targeting of astrocytes (+5.9%, $P = 3.0 \times 10^{-10}$, 2-proportion z-test, multiple comparison corrected with Benjamini/Hochberg correction), and AAV-PHP.V1 showed strong bias for vascular cells (+49.7%, $p = 1.7 \times 10^{-45}$). In addition to confirming all our existing hypotheses, we were able to identify biases for the previously reported AAV-PHP.C2, which has not been characterized in depth. This variant, which was reported as having a non-neuronal bias similar to AAV-PHP.V1, showed significant transduction bias not only toward vascular cells (+15.7%, $P = 1.5 \times 10^{-7}$), but also toward astrocytes (+21.5%, $P = 3.0^{-28}$), and a bias away from neurons (−38%, $p = 4.5 \times 10^{-32}$).

3 DISCUSSION

The advent of NGS has enabled screening of large libraries of AAV capsids *in vivo* by extracting viral DNA from relevant tissue followed by sequencing of capsid gene inserts or DNA barcodes corresponding to defined capsids. To date, NGS-based screening has been successfully applied to libraries created by peptide insertions (28, 113), DNA shuffling of capsids (114–116), and site-directed mutagenesis (117). Although these NGS-based strategies allow the evolution of new AAV variants with diverse tissue tropisms, it has been difficult to obtain a comprehensive profiling for multiple variants across cell types, which is of utmost importance in organs with complex cell-type compositions, such as the brain (34, 45, 64–66). Towards this end, techniques such as IHC, fluorescent *in situ* RNA hybridization (98, 118–122) or *in situ* RNA sequencing (123–125) can be employed. Several limitations make it

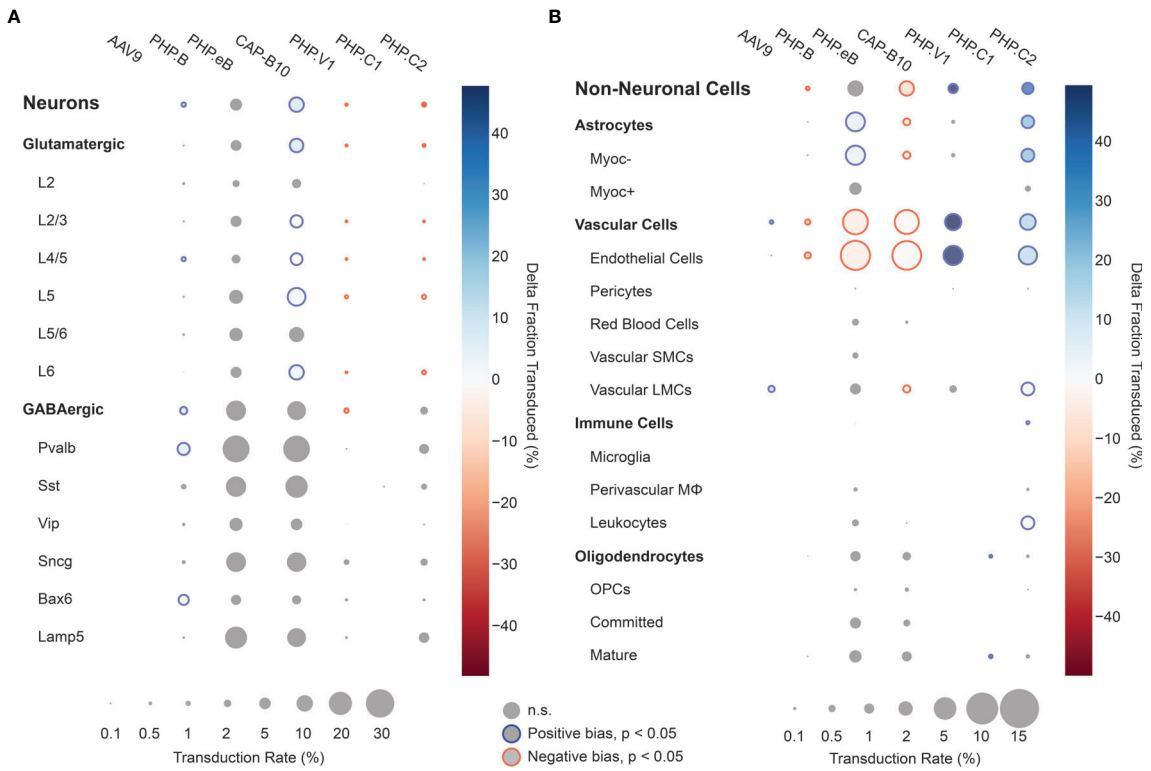

FIGURE 6 | Single animal injections of multiple barcoded rAAVs enables deep, parallel characterization. **(A, B)** Relative cell type tropism of 7 co-injected rAAVs for neuronal **(A)** and non-neuronal **(B)** cell types. The color scale indicates the difference in transduction bias of a variant relative to all other variants in the pool. The area of each circle scales linearly with the fraction of cells of that type with viral transcripts above background. For each variant and cell type, a 2-proportion z score was calculated to compare the number of cells of that type transduced by that variant relative to all other variants combined. Z scores were combined across two single-cell sequencing aliquots using Stouffer's method and corrected for multiple comparisons. Cell types with fewer than 10 transduced cells in either the variant or variants compared against were discarded. Only cell-type biases at an adjusted P-value < 0.05 are colored; otherwise they are grayed out.

challenging to apply these techniques as high-throughput, post-selection AAV tropism profiling methods. First, the limits of optical resolution and the density of transcripts in single cells pose challenges for full *in situ* transcriptome analysis and, until recently, have restricted the total number of simultaneously measured genes in single cells within tissue to several hundred (121, 123–126). By contrast, scRNA-seq with the 10x Genomics Chromium system enables detection of over 4000 genes per cell (95), fast transcriptomic analysis, and multiplexing across different tissue types (127, 128). Furthermore, the method is already widely used by the research community which can help with adoption of our proposed pipelines. Although droplet-based scRNA-seq methods lose spatial information during the dissociation procedure, analysis packages have been developed that can infer single-cell localization by combining scRNA-seq data with pre-existing information from ISH-based labeling for specific marker genes (129–134). Therefore, scRNA-seq techniques have great potential to rapidly profile the tropism of multiple AAV variants in parallel across several thousand cells defined by their entire transcriptome.

Here, we established an experimental and data-analysis pipeline that leverages the capabilities of scRNA-seq to achieve simultaneous characterization of several AAV variants across multiplexed tissue cell types within a single animal. To differentiate multiple AAV capsid variants in the sequencing data, we packaged variants with unique transgenes or the same transgene with unique barcodes incorporated at the 3' end. We added standard Illumina sequencing primer recognition sites (Read 2) to the viral transcripts using PCR amplification such that the barcoded region could be consistently read out from the Illumina sequencing data. Our computational pipeline demultiplexes viral reads found in the transcriptome according to which matching sequence is most abundant in a separate amplified viral transgene library. Comparing the distribution of viral transcripts by cell type to a null model of empty droplets, we could then determine the cell-type biases.

Our platform has corroborated the tropism of several previously characterized AAV variants and has provided more detailed tropism information beyond the major cell types. The fraction of transduced cells that are glutamatergic neurons was found to be markedly reduced for AAV-PHP.eB when compared with AAV.CAP-B10. Furthermore, within all the variants we tested, both Pvalb+ and Sst+ inhibitory neurons have greater transduction rates than Vip+ neurons. This bodes well for delivery to Pvalb+ neurons, which have been implicated in a wide range of neuro-psychiatric disorders (135), and suggests

Vip+ interneurons, which have recently been identified as being a sufficient delivery target for induction of Rett syndrome-like symptoms, as a target for optimization (136). Awareness of neuronal subtype biases in delivery vectors is critical both for neuroscience researchers and for clinical applications. Dissection of neural circuit function requires understanding the roles of neuronal subtypes in behavior and disease and relies on successful and sometimes specific delivery of transgenes to the neuronal types under study (1).

We further discovered that the vascular bias of AAV-PHP.V1 originates from its transduction bias towards endothelial cells. Interestingly, this is the only cell type we detected expressing Ly6a (**Supplemental Figure 8**), a known surface receptor for AAV variants in the PHP.B family (137–139). Given AAV-PHP.V1's sequence similarity to AAV-PHP.B and its tropism across mouse strains, this pattern suggests that AAV-PHP.V1 transduction may also be Ly6a-mediated. Finding such associations between viral tropism and cell-surface membrane proteins also suggests that full transcriptome sequencing data may hold a treasure trove of information on possible mechanisms of transduction of viral vectors.

We also revealed that AAV-PHP.C2 has a strong, broad non-neuronal bias toward both vascular cells and astrocytes. AAV-PHP.C2 also transduces BALB/cJ mice, which do not contain the Ly6a variant that mediates transduction by PHP.B family variants (137–139). This suggests that PHP.C2 may be the most promising candidate from this pool for researchers interested in delivery to non-neuronal cells with minimal neuronal transduction both in C57BL/6J mice and in strains and organisms that do not have the Ly6a variant.

All our tested variants with non-neuronal transduction have lower expression in Myoc+ astrocytes and pericytes. Astrocytes expressing Myoc and Gfap, which intersect in our data (**Supplemental Figure 8**), have been previously identified as having reactive behavior in disease contexts, making them a target of interest for research on neurological diseases (140, 141). Similarly, pericytes, whose dysfunction has been shown to contribute to multiple neurological diseases, may be an important therapeutic target (60, 142, 143). Both of these cell types may be good candidates for further AAV optimization but may have been missed with marker gene-based approaches. In both AAV characterization and neuroscience research efforts, different marker genes are often used for astrocyte classification – sometimes more restrictive genes such as Gfap, and other times more broadly expressing genes such as S100b or Aldh1l1 (144, 145). Similarly, defining marker genes for pericytes is still an active field (100, 102). Given the constraints of having to choose specific marker genes, it is difficult for staining-based characterizations to provide tropism profiles that are relevant for diverse and changing research needs. This highlights the importance of using unbiased, full transcriptome profiling for vector characterization.

We have shown that our combined experimental and computational platform is able to recover transduction biases and profile multiple variants in a single animal, even amidst the noise of ambient RNA. We have further shown that our method is robust to the variability inherent in delivery and extraction from different animals, with different transgenes, and with different regulatory elements. For example, we discovered lower overall expression from vectors carrying UBC-mCherry compared with CAG-mNeonGreen-WPRE. Such differences are not surprising since the WPRE is known to increase RNA stability and therefore transcript abundance (146). Furthermore, the shift in cell-type bias may come from the UBC promoter, as even ubiquitous promoters such as CAG and UBC have been shown to have variable levels of expression in different cell types (147). Despite these biases, looking at the differences in transduction between variants delivering the same construct within an individual animal reveals the strongest candidate vectors for on-target and off-target cell types of interest. While we show that our method can profile AAVs carrying standard fluorescent cargo, caution is needed when linking differences in absolute viral tropism to changes in capsid composition alone without considering the contribution of the transgene, regulatory elements, and distribution of cell types in recovered tissue. Therefore, for more robust and relative tropism between variants, we found it beneficial to use small barcodes and co-injections of pools of vectors. Our scRNA-seq-based approach is not restricted to profiling capsid variants but can be expanded in the future to screen promoters (148–150), enhancers (151, 152), or transgenes (86, 153), all of which are essential elements requiring optimization to improve gene therapy.

Finally, we have used scRNA-seq to understand how intra-orbital administration of AAV-PHP.eB affects the host cell transcriptome across distinct time points. Results from our study show genes pertaining to the p53 pathway in endothelial cells are differentially expressed 3 days after injection, an effect which vanishes at a later time point of 25 DPI. Though other cell types such as immune cells and neurons had a few differentially expressed genes pertaining to antigen presentation and cell differentiation, respectively, endothelial cells at 3 DPI are the only cells with a profound response signature. The highest number of differentially expressed genes being in endothelial cells suggests that vascular cells could be the initial responders to viral transduction and expression of the transgene. This is supported by Kodali et al., who have shown that endothelial cells are the first to elicit a response to peripheral inflammatory stimulation by transcribing genes for proinflammatory mediators and cytokines (154). With regards to p53 differentially expressed genes, Ghouzzi, et al. have also shown that the genes Phdla3, Aen, and Cdkn1a were upregulated in cells infected with ZIKA virus, signifying genotoxic stress and apoptosis induction (104). Upregulation of genes such as Bax and Cdkn1a could be a response to cellular stress induced by viral transduction (103, 155). However, the initial inflammatory responses did not escalate as shown by the low number of differential expressed genes across all cells (**Figure 5B**) at day twenty-five. Additionally, antigen presenting genes, such as Cd74 and H2-K1, returning back to control expression levels in microglia and a lack of proinflammatory cytokines being upregulated support that the event of infiltration of peripheral

leukocytes is unlikely, in agreement with prior studies (87, 88). Based on prior studies, the few genes that are differentially expressed at day 25 in excitatory and inhibitory neurons could also be due to transgene expression rather than the virion (89). Upregulation of immediate early genes such as Fos, Junb, and Ier2 in inhibitory neurons could indicate that the cells which are transduced and expressing viral transcripts could be under increased stress and metabolic demands, either directly in response to transgene expression, or in combination with the stresses of dissociation. For example, c-jun and c-fos were found to be upregulated by lung epithelial cells as part of the response to measles virus (156). Given that scRNA-seq is a sensitive technique, it can be prone to high false discovery rates if not properly controlled. To account for this, we used a highly conservative pseudo-bulk differential expression procedure, which has been shown to minimize false discovery rates compared to other batch correction methods (157). This conservative procedure, however, has lower relative power, and thus there may be additional effects in cell subtypes to AAV transduction. The confidence and statistical power of future scRNA-seq studies looking at AAV-related immune responses could be improved with increased sample size, such as *via* sample multiplexing strategies to pool multiple animals (127), or by increasing the sensitivity and specificity of viral transcript detection and performing differential expression within animals. It is also important to note that the findings discussed here are specific to the rAAV, transgene, and dosage. Nonetheless, our results highlight the power of single-cell profiling in being able to ascertain cell-type-specific responses at an early time point post-injection.

As shown throughout this work, there are several challenges we had to overcome to gain valuable insights from our droplet-based single-cell RNA sequencing approach. While we were able to overcome these in the context of our study, they hint at some important limitations of this method. First, droplet-based single-cell sequencing of tissues that are difficult to dissociate, such as brain, can lead to substantial background noise from debris. Alternative methods, such as single-nucleus RNA sequencing (158, 159), could potentially overcome this debris problem. Exploratory work would need to be performed to determine whether single-nucleus RNA sequencing captures a sufficient amount of immature viral transcripts, but, if effective, may obviate the need for computational detection of transduction above a background level. Another potential challenge of our method is scaling up to much larger numbers of variants. In order to establish high statistical confidence in tropism, many cells need to be transduced. However, given restrictions on the total dosage an animal can receive, adding more variants would require a lower dosage per variant. In simulation, we found that subsampling our 2-variant pool by 10-fold did not change the major tropism findings (**Supplemental Figure 9**). Given our current injections are 8-fold lower than the maximum allowed dosage, this suggests this method could scale up to 80 variants; however, further work would need to be done to validate whether this holds for a diversity of variants that may be competing for binding. Scaling higher would be challenging with current droplet-based single-cell RNA sequencing pipelines that process on the order of 10^4 cells per reaction. Alternative approaches, such as split-pool strategies, which can profile many more cells (160), may thus be appealing for larger variant pools.

In summary, our platform enables thorough tropism characterization of existing and emerging recombinant AAVs and helps uncover cellular responses to rAAV-mediated gene therapy, thus further guiding the engineering and use of gene delivery vehicles.

4 MATERIALS AND METHODS

4.1 Animals

Male C57BL/6J mice (Stock No: 000664) used in this study were purchased from the Jackson Laboratory (JAX). AAV variants were injected i.v. into the retro-orbital sinus of 6–7 week old mice.

4.2 Plasmids

In vivo vector characterization of AAV variant capsids was conducted using single-stranded (ss) rAAV genomes. pAAV : CAG-NLS-mNeonGreen, pAAV : CAG-NLS-mRuby2, pAAV : CAG-tdTomato, and pAAV : CAG-NLS-tdTomato constructs were adapted from previous publications (32, 45). To introduce barcodes into the polyA region of CAG-NLS-mNeonGreen, we digested the plasmid with BglII and EcoRI, and performed Gibson assembly (E2611, NEB) to insert synthesized fragments with 7bp degenerate nucleotide sequences 89 bp upstream of the polyadenylation site. We then seeded bacterial colonies and selected and performed Sanger sequencing on the resulting plasmids to determine the corresponding barcode.

The UBC-mCherry-AAV-cap-in-cis plasmid was adapted from the rAAV-Cap-in-cis-lox plasmid from a previous publication (34). We performed a restriction digest on the plasmid with BsmbI and SpeI to remove UBC-mCherry and retain the AAV9 cap gene and remaining backbone. We then circularized the digested plasmid using a gblock joint fragment to get a plasmid containing AAV2-Rep, AAV9-Cap, and the remaining backbone *via* T4 ligation. In order to insert UBC-mCherry with the desired orientation and location, we amplified its linear segment from the original rAAV-Cap-in-cis-lox plasmid. The linear UBC-mCherry-polyA segment and circularized AAV2-Rep,AAV9-cap plasmid were then both digested with HindIII and ligated using T4 ligation. In order to get the SV40 PolyA element in the proper orientation with respect to the inserted UBC-mCherry, we removed the original segment from the plasmid using AvrII and AccI enzymes and inserted AvrII, AccI treated SV40 gblock using T4 ligation to get the final plasmid.

To insert barcodes into UBC-mCherry-AAV-cap-in-cis, we obtained 300 bp DNA fragments containing the two desired capsid mutation regions for each variant and the variant barcode, flanked by BsrGI and XbaI cut sites. The three segments of the fragment were separated by BsaI Type I restriction sites.

We digested the UBC-mCherry-AAV-cap-in-cis plasmid with BsrGI and XbaI, and ligated each variant insert to this backbone. Then, to reinsert the missing regions, we performed Golden Gate assembly with two inserts and BsaI-HF.

4.3 Viral Production

To produce viruses carrying in trans constructs, we followed established protocols for the production of rAAVs (161). In short, HEK293T cells were triple transfected using polyethylenimine (PEI) with three plasmids: pAAV (see *Plasmids*), pUCmini-iCAP-PHP.eB (32), pUCmini-iCAP-CAP-B10 (90), or pUCmini-iCAP-PHP.V1 (45), and pHelper. After 120 h, virus was harvested and purified using an iodixanol gradient (Optiprep, Sigma). For our 7-variant pool, we modified the protocol to be a double transfection using PEI with two plasmids: UBC-mCherry-AAV-cap-in-cis and pHelper.

4.4 Tissue Processing for Single-Cell Suspension

Three to four weeks after the injection, mice (9-10 weeks old) were briefly anesthetized with isoflurane (5%) in an isolated plexiglass chamber followed by i.p. injection of euthasol (100 mg/kg). The following dissociation procedure of cortical tissue into a single-cell suspension was adapted with modifications from a previous report (162). Animals were transcardially perfused with ice-cold carbogenated (95% O_2 and 5% CO_2) NMDG-HEPES-ACSF (93 mM NMDG, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 5 mM Na L-ascorbate, 2 mM thiourea, 3 mM Na-pyruvate, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM kynurenic acid Na salt, pH adjusted to 7.35 with 10N HCl, osmolarity range 300–310 mOsm). Brains were rapidly extracted and cut in half along the anterior-posterior axis with a razor blade. Half of the brain was used for IHC histology while the second half of the brain was used for scRNA-seq. Tissue used for scRNA-seq was immersed in ice-cold NMDG-HEPES-ACSF saturated with carbogen. The brain was sectioned into 300-µm slices using a vibratome (VT-1200, Leica Biosystems, IL, USA). Coronal sections from Bregma −0.94 mm to −2.80 mm were collected in a dissection dish on ice containing NMDG-HEPES-ACSF. Cortical tissue from the dorsal surface of the brain to ~3.5 mm ventral was cut out and further sliced into small tissue pieces. NMDG-HEPES-ACSF was replaced by trehalose-HEPES-ACSF (92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM kynurenic acid Na salt, 0.025 mM D-(+)-trehalose dihydrate*$2H_2O$, pH adjusted to 7.35, osmolarity ranging 320–330 mOsm) containing papain (60 U/ml; P3125, Sigma Aldrich, pre-activated with 2.5 mM cysteine and a 0.5–1 h incubation at 34°C, supplemented with 0.5 mM EDTA) for the enzymatic digestion. Under gentle carbogenation, cortical tissue was incubated at 34°C for 50 min with soft agitation by pipetting every 10 min. 5 µl 2500 U/ml DNase I (04716728001 Roche, Sigma Aldrich) was added to the single-cell suspension 10 min before the end of the digestion. The solution was replaced with 200 µl trehalose-HEPES-ACSF containing 3 mg/ml ovomucoid inhibitor (OI-BSA, Worthington) and 1 µl DNase I. At room temperature, the digested cortical tissue was gently triturated with fire-polished glass Pasteur pipettes for three consecutive rounds with decreasing pipette diameters of 600, 300, and 150 µm. 800 µl of trehalose-HEPES-ACSF with 3 mg/ml ovomucoid inhibitor was added. The uniform single-cell suspension was pipetted through a 40 µm cell strainer (352340, Falcon) into a new microcentrifuge tube followed by centrifugation at 300 g for 5 min at 4°C. The supernatant was discarded and cell pellet was resuspended in 1 ml of trehalose-HEPES-ACSF. After mixing using a Pasteur pipette with a 150 µm tip diameter, the single-cell suspension was centrifuged again. Supernatant was replaced with fresh trehalose-HEPES-ACSF and the resuspended cell pellet was strained with a 20 µm nylon net filter (NY2004700, Millipore). After resuspension in trehalose-HEPES-ACSF, cells were pelleted again and resuspended in 100 µl of ice-cold resuspension-ACSF (117 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM kynurenic acid Na salt and 0.05% BSA, pH adjusted to 7.35 with Tris base, osmolarity range 320–330 mOsm). Cells were counted with a hemocytometer and the final cell densities were verified to be in the range of 400–2,500 cells/µl. The density of single-cell suspension was adjusted with resuspension-ACSF if necessary.

4.5 Transcriptomic Library Construction

Cell suspension volumes containing 16,000 cells–expected to retrieve an estimated 10,000 single-cell transcriptomes–were added to the 10x Genomics RT reaction mix and loaded to the 10x Single Cell Chip A (230027, 10x Genomics) for 10x v2 chemistry or B (2000168, 10x Genomics) for 10x v3 chemistry per the manufacturer's protocol (Document CG00052, Revision F, Document CG000183, Revision C, respectively). We used the Chromium Single Cell 3' GEM and Library Kit v2 (120237, 10x genomics) or v3 (1000075, 10x Genomics) to recover and amplify cDNA, applying 11 rounds of amplification. We took 70 ng to prepare Illumina sequencing libraries downstream of reverse transcription following the manufacturer's protocol, applying 13 rounds of sequencing library amplification.

4.6 Viral Library Construction

We selectively amplified viral transcripts from 15 ng of cDNA using a cargo-specific primer binding to the target of interest and a primer binding the partial Illumina Read 1 sequence present on the 10x capture oligos (**Supplemental Table 1**). For animals injected with a single cargo, amplification was performed only once using the primer for the delivered cargo; for animals with distinct cargo sequences per variant, amplification was performed in parallel reactions from the same cDNA library using different cargo-specific primers for each reaction. We performed the amplification using 2x KAPA HiFi HotStart ReadyMix (KK2600) for 28 cycles at an annealing temperature of 53°C. Afterwards, we performed a left-sided SPRI cleanup with a concentration dependent on the target amplicon length, in accordance with the manufacturer's protocol (SPRISelect, Beckman Coulter B23318). We then performed an overhang PCR on 100 ng of product with 15 cycles using primers that bind

the cargo and the partial Illumina Read 1 sequence and appending the P5/P7 sequences and Illumina sample indices. We performed another SPRI cleanup, and analyzed the results *via* an Agilent High Sensitivity DNA Chip (Agilent 5067-4626).

4.7 Sequencing

Transcriptome libraries were pooled together in equal molar ratios according to their DNA mass concentration and their mean transcript size as determined *via* bioanalyzer. Sequencing libraries were processed on Novaseq 6000 S4 300-cycle lanes. The run was configured to read 150 bp from each end. Sequencing was outsourced to Fulgent Genetics and the UCSF Center for Advanced Technology.

All viral transcript libraries except barcoded UBC-mCherry were pooled together in equal molar ratios into a 4 nM sequencing library, then diluted and denatured into a 12 pM library as per the manufacturer's protocol (Illumina Document #15039740v10). The resulting library was sequenced using a MiSeq v3 150-cycle reagent kit (MS-102-3001), configured to read 91 base pairs for Read 2 and 28 base pairs for Read 1. To characterize the effect of sequencing depth, one viral transcript library was additionally processed independently on a separate MiSeq run.

The UBC-mCherry viral transcript library, which was recovered with primers near the polyadenylation site, consisted of fragments ~307 bp long. Since this length is within the common range for an Illumina NovaSeq run, this viral transcript library was pooled and included with the corresponding transcriptome library.

4.8 Transcriptome Read Alignment

For transcriptome read alignment and gene expression quantification, we used 10x Cell Ranger v5.0.1 with default options to process the FASTQ files from the transcriptome sequencing library. The reads were aligned against the mus musculus reference provided by Cell Ranger (mm10 v2020-A, based on Ensembl release 98).

To detect viral transcripts in the transcriptome, we ran an additional alignment using 10x Cell Ranger v5.0. 1 with a custom reference genome based on mm10 v2020-A. We followed the protocol for constructing a custom Cell Ranger reference as provided by 10x Genomics. This custom reference adds a single gene containing all the unique sequences from our delivered plasmids in the study, delineated as separate exons. Sequences that are common between different cargo are provided only once, and annotated as alternative splicings.

4.9 Viral Transcript Read Alignment

For viral read alignment, we aligned each Read 2 to a template derived from the plasmid, excluding barcodes. The template sequence was determined by starting at the ATG start site of the XFP cargo and ending at the AATAAA polyadenylation stop site. We used a Python implementation of the Striped Smith-Waterman algorithm from scikit-bio to calculate an alignment score for each read, and normalized the score by dividing by the maximum possible alignment score for a sequence of that length, minus the length of the barcode region. For each Read 2 that had a normalized alignment score of greater than 0.7, we extracted the corresponding cell barcode and UMI from Read 1, and any insertions into the template from Read 2.

4.10 Constructing the Variant Lookup Table

For co-injections with multiple templates and injections of barcoded templates, we constructed a lookup table to identify which variant belongs to each cell barcode/UMI. For each template, we counted the number of reads for each cell barcode/UMI. For reads of barcoded cargo, we only counted reads where the detected insertion in the barcode region unambiguously aligned to one of the pre-defined variant barcodes. Due to sequencing and PCR amplification errors, most cell barcode/UMI combinations had reads associated with multiple variants. Thus, we identified the variant with the largest count for each cell barcode/UMI. We discarded any cell barcode/UMIs that had more than one variant tied for the largest count. Finally, each cell barcode/UMI that was classified as a viral transcript in the transcriptome (see *Transcriptome Read Alignment*) was converted into the virus detected in the variant lookup table, or was discarded if it did not exist in the variant lookup table.

4.11 Estimating Transduction Rate

To determine an estimate of the percent of cells within a group expressing viral cargo above background, we compared the viral transcript counts in that group of cells to a background distribution of viral transcript counts in debris (see *Droplet Type Classification*). First, we obtained the empirical distribution of viral transcript counts by extracting the viral counts for that variant in cell barcodes classified as the target cell type as well as cell barcodes classified as debris. Next, we assumed a percentage of cells containing debris. For each viral transcript count, starting at 0, we calculated the number of cells that would contain this transcript count, if the assumed debris percentage was correct. We then calculated an error between this estimate and the number of cells with this transcript count in the cell type of interest. We tallied this error over all the integer bins in the histogram, allowing the error in a previous bin to roll over to the next bin. We repeated this for all possible values of percentage of debris from 0 to 100 in increments of 0.25, and the value that minimized the error was the estimated percentage of cells whose viral transcript count could be accounted for by debris. The inverse of this was our estimate of the number of cells expressing viral transcripts above background.

To validate that this method reliably recovers an estimate of transduction rate, we performed a series of simulations using models of debris viral transcript counts added to proposed cell type transcript count distributions across a range of parameterizations. To get estimates of the background distribution of debris, we used diffxpy (https://github.com/theislab/diffxpy) to fit the parameters of a negative binomial distribution to the viral transcript counts in debris droplets within a sample. We then postulated 1,000 different parameterizations of the negative binomial representing

transcript counts in groups of cells, with 40 values of r ranging from 0.1 to 10, spaced evenly apart, and 25 values of p ranging from 0.001 to 0.99, spaced evenly apart. For each proposed negative binomial model, we drew 1,000 random samples of viral counts from the learned background distribution, and 1,000 random samples from the proposed cell distribution, and summed the two vectors. This summed vector was then used in our transduction rate estimation function, along with a separate 1,000 random samples of background viral transcripts for the function to use as an estimate of the background signal. We calculated the true probability of non-zero expression in our proposed cell negative binomial model (1 – P(X = 0)), and compared this value with the estimated value from the transduction rate estimation method.

4.12 Calculating Viral Tropism

For each variant v_n and cell type of interest c_i, we estimated the percentage of cells expressing viral cargo. To calculate tropism bias, we used this estimated expression rate, t_{c_i,v_n}, to estimate the number of cells expressing viral transcripts in that cell type, T_{c_i,v_n} out of the total number of cells of that type, N_{c_i}. $T_{c_i,v_n} = t_{c_i,v_n} N_{c_i}$. Cell type bias, b_{c_i,v_n}, within a sample was then calculated as the ratio of the number of cells of interest divided by the total number of transduced cells, $b_{c_i,v_n} = \frac{T_{c_i,v_n}}{\Sigma_j T_{c_j,v_n}}$. Finally, to calculate the difference in transduction bias for a particular variant relative to other variants in the sample, δ_{c_i,v_n}, we subtracted the bias of the variant from the mean bias across all other variants, $\delta_{c_i,v_n} = \frac{T_{c_i,v_n}}{\Sigma_j T_{c_j,v_n}} - \frac{\Sigma_{m \neq n} T_{c_i,v_m}}{\Sigma_{m \neq n} \Sigma_j T_{c_j,v_m}}$.

4.13 Histology

4.13.1 Immunohistochemistry

The immunohistochemistry procedure was adapted from a previous publication (163). Brain tissue was fixed in 4% paraformaldehyde (PFA) at 4°C overnight on a shaker. Samples were immersed in 30% sucrose in 1x phosphate buffered saline (PBS) solution for >2 days and then embedded in Tissue-Tek O.C.T. Compound (102094-104, VWR) before freezing in dry ice for 1 h. Samples were sectioned into 50 µm coronal slices on a cryostat (Leica Biosystems). Brain slices were washed once with 1x phosphate buffered saline (PBS) to remove O.C.T. Compound. Samples were then incubated overnight at 4° C on a shaker in a 1x PBS solution containing 0.1% Triton X-100, 10% normal goat serum (NGS; Jackson ImmunoResearch, PA, USA), and primary antibodies. Sections were washed three times for 15 min each in 1x PBS. Next, brain slices were incubated at 4° C overnight on a shaker in a 1x PBS solution containing 0.1% Triton X-100, 10% NGS, and secondary antibodies. Sections were washed again three times for 15 min each in 1x PBS. Finally, slices were mounted on glass microscope slides (Adhesion Superfrost Plus Glass Slides, #5075-Plus, Brain Research Laboratories, MA, USA). After the brain slices dried, DAPI-containing mounting media (Fluoromount G with DAPI, 00-4959-52, eBioscience, CA, USA) was added before protecting the slices with a cover glass (Cover glass, #4860-1, Brain Research Laboratories, MA, USA). Confocal images were acquired on a Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany). The following primary antibodies were used: rabbit monoclonal to NeuN (Rbfox3) (1:500; ab177487; Abcam, MA, USA), rabbit monoclonal to S100 beta (1:500; ab52642; Abcam, MA, USA), and rabbit monoclonal to Olig2 (1:500; ab109186; Abcam, MA, USA). The following secondary antibody was used: goat anti-rabbit IgG H&L Alexa Fluor 647 (1:500; ab150079; Abcam, MA, USA).

4.13.2 Fluorescent *In Situ* Hybridization Chain Reaction

FISH-HCR was conducted as previously reported (99). Probes targeting neuronal markers were designed using custom-written software (https://github.com/GradinaruLab/HCRprobe). Probes contained a target sequence of 20 nucleotides, a spacer of 2 nucleotides, and an initiator sequence of 18 nucleotides. Criteria for the target sequences were: (1) a GC content between 45%–60%, (2) no nucleotide repeats more than three times, (3) no more than 20 hits when blasted, and (4) the ΔG had to be above –9 kcal/mol to avoid self-dimers. Last, the full probe sequence was blasted and the Smith-Waterman alignment score was calculated between all possible pairs to prevent the formation of cross-dimers. In total, we designed 26 probes for Gad1, 20 probes for Vip, 22 probes for Pvalb, 18 probes for Sst, and 28 probes for Slc17a7. Probes were synthesized by Integrated DNA Technologies.

4.14 Droplet Type Identification

scRNA-seq datasets were analyzed with custom-written scripts in Python 3.7.4 using a custom fork off of scVI v0.8.1, and scanpy v1.6.0. To generate a training dataset for classifying a droplet as debris, multiplets, neuronal, or non-neuronal cells, we randomly sampled cells from all 27 cortical tissue samples. We sampled a total of 200,000 cells, taking cells from each tissue sample proportional to the expected number of cells loaded into the single-cell sequencing reaction. Within each sample, cells were drawn randomly, without replacement, weighted proportionally by their total number of detected UMIs. For each sample, we determined a lower bound on the cutoff between cells and empty droplets by constructing a histogram of UMI counts per cell from the raw, unfiltered gene count matrix. We then found the most prominent trough preceding the first prominent peak, as implemented by the scipy peak_prominences function. We only sampled from cells above this lower bound. Using these sampled cells, we trained a generative neural network model *via* scVI with the following parameters: 20 latent features, 2 layers, and 256 hidden units. These parameters were chosen from a coarse hyperparameter optimization centered around the scVI default values (**Supplemental Table 3**). We included the sample identifier as the batch key so that the model learned a latent representation with batch correction.

After training, Leiden clustering was performed on the learned latent space as implemented by scanpy. We used default parameters except for the resolution, which we increased to 2 to ensure isolation of small clusters of cell multiplets. Using the learned generative model, we draw 5000 cells from the posterior distribution based on random seed cells in each cluster. We draw an equal number conditioned on each

batch. From these samples, we then calculated a batch-corrected probability of each cluster expressing a given marker gene (see *Cluster Marker Gene Determination*). For this coarse cell typing, we chose a single marker gene for major cell types expected in the cortex (**Supplemental Table 2**). If a cluster was expressing the neuron marker gene Rbfox3, it was labeled as "Neurons". If a cluster was expressing any of the other non-neuronal marker genes, it was labelled as "Non-neurons". Next, we ran Scrublet on the training cells to identify potential multiplets. Scrublet was run on each sample independently, since it is not designed to operate on combined datasets with potential batch-specific confounds. We then calculated the percentage of droplets in each cluster of the combined data that were identified as multiplets by Scrublet. We found a percentage threshold for identifying a cluster as containing predominantly multiplets by using Otsu's threshold, as implemented by scikit-image. All droplets in any cluster above the multiplet percentage threshold were labelled as "Multiplets". All other clusters were labelled as "Debris".

Next, we trained a cell-type classifier using scANVI on the droplets labeled as training data. We used the weights from the previously trained scVI model as the starting weights for scANVI. Rather than using all cells for every epoch of the trainer, we implemented an alternative sampling scheme that presented each cell type to the classifier in equal proportions. Once the model was trained, all cells above the UMI lower noise bound were run through the classifier to obtain their cell-type classification. Droplets classified as "Neurons" or "Non-neurons" were additionally filtered by their scANVI-assigned probability. We retained only cells above an FDR threshold of 0.05, corrected for multiple comparisons using the Benjamini-Hochberg procedure. Finally, since the original run of Scrublet for multiplet detection was performed on only the training data, and thus did not take advantage of all the cells available, we ran Scrublet on all droplets classified as cells, and removed any identified multiplets.

4.15 Cluster Marker Gene Determination

To identify which clusters are expressing marker genes, we determined an estimated probability of a marker gene being expressed by a random cell in that cluster. For each cluster, we randomly sampled 5,000 cells, with replacement. We used scVI to project each cell into its learned latent space, and then used scVI's posterior predictive sampling function to generate an example cell from this latent representation, and tallied how many times the gene is expressed. We repeated this for each batch, conditioning the posterior sample on that batch, to account for technical artifacts such as sequencing depth. Once we obtained a probability of expression of a marker gene for each cluster, we find a threshold for expression using Otsu's method, as implemented by scikit-image. Clusters that have a probability of expression above the threshold are considered positive for that marker gene.

4.16 Neuronal Subtype Classification

Cells classified as neurons were further subtyped using annotations from a well-curated reference dataset. We used the Mouse Whole Cortex and Hippocampus 10x dataset from the Allen Institute for Brain Science as our reference dataset (95). First, we filtered the reference dataset to contain only cell types that are found within the brain regions collected for our experiments. To ensure that, overall, enough cells per cell type were present in our datasets, we merged cell types with common characteristics, such as expression of key marker genes. We re-aligned our cell transcriptome reads to the same pre-mRNA reference used to construct the reference dataset, so that the gene count matrices had a 1:1 mapping. We then trained a joint scANVI model with all cells identified as neurons from our samples and the reference database to learn a common latent space between them. The model was trained to classify cells based on the labels provided in the reference dataset. Cells were sampled from each class in equal proportions during training. After the model was trained, all neurons from our sample were run through the model to obtain their cell type classification.

4.17 Non-Neuronal Subtype Classification

Cells classified as non-neuronal were further subtyped using automatic clustering and marker gene identification. We trained an scVI model using only the non-neuronal cells and performed Leiden clustering as implemented by scanpy on the latent space. We determined which clusters were expressing each of 31 marker genes across 13 cell subtypes. Marker genes were identified from a review of existing scRNA-seq, bulk RNA-seq, or IHC studies of mouse brain non-neuronal subtypes (**Supplemental Table 2**). Each cluster was assigned to a cell subtype if it was determined positive for all the marker genes for that cell subtype (see *Cluster Marker Gene Determination*). If a cluster contained all the marker genes for multiple cell subtypes, the cluster was assigned to the cell subtype with the greatest number of marker genes. Clusters that did not express all the marker genes for any cell subtype were labeled as "Unknown". Clusters that expressed all the marker genes for multiple cell subtypes with the same total number of marker genes were labeled as "Multiplets". For cell types that contained multiple clusters, we then calculated the probability of every gene being zero in each cluster (see *Cluster Marker Gene Determination*). We then compared gene presence between clusters of the same cell type to see if there were any subclusters that had a dominant marker gene (present in > 50% of samples), that was not present in any of the other clusters (< 10% of samples). For the three cell types that had unique marker genes, we named the cluster after the gene with the highest 2-proportion z-score between the sampled gene counts in that cluster *vs* the rest.

4.18 Quantification of Images

Quantitative data analysis of confocal images was performed blind with regard to AAV capsid variant. Manual quantification was performed using the Cell Counter plugin, present in the Fiji distribution of ImageJ (National Institutes of Health, Bethesda, MD) (164). Transduction rate was calculated as the total number of double positive cells (i.e. viral transgene and cell type marker) divided by the total number of cell type marker labeled cells. For each brain slice, at least 100 cells positive for the gene markers of interest were counted in the cortex.

4.19 Differential Expression

To calculate differential expression within cell types between groups of animals, we used the DESeq2 R package (165). For each cell type, the gene counts are summed across all cells of that type and treated as a pseudo-bulk sample. The summed gene counts from each animal are then included as individual columns for a DESeq2 differential expression analysis. We performed DE for 3 DPI and 25 DPI separately, testing each sample against saline-injected controls. For each cell type, only genes that were present in all samples of at least one condition are included.

4.20 Marker Gene Dot Plots

To generate dot plots for marker genes, we used scanpy's dotplot function (166). Gene counts were normalized to the sum of the total transcript counts per cell using scanpy's normalize_total function. Normalized gene expression values are log-transformed as part of the plotting function.

4.21 Statistics

Statistical analyses comparing the fraction of transduced cells and transduction rate in different cell types for **Figures 2**, **3**, **4D, E** and **5A** were conducted using GraphPad Prism 9. Statistical analyses comparing proportions of transduced cells within an animal in **Figures 4F** and **Figure 6** were performed using the Python statsmodels library v0.12.1. No statistical methods were used to predetermine sample sizes. The statistical test applied, sample sizes, and statistical significant effects are reported in each figure legend. The significance threshold was defined as a = 0.05.

DATA AVAILABILITY STATEMENT

All raw FASTQ files are available under the SRA BioProject PRJNA758711 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA758711/). Processed gene count matrices for droplets identified as cells, as well as the demultiplexed virus cargo counts, are available at CaltechData, doi: 22002/D1.2090 (http://dx.doi.org/10.22002/D1.2090).

ETHICS STATEMENT

Animal husbandry and all experimental procedures involving animals were performed in accordance with the California Institute of Technology Institutional Animal Care and Use Committee (IACUC) guidelines and reviewed and approved by the Office of Laboratory Animal Resources at the California Institute of Technology (animal protocol no. 1650).

AUTHOR CONTRIBUTIONS

DB, MA, TD, and VG conceived the project and designed the experiments. SC and MT provided critical single-cell RNA sequencing expertise. TD, MA, and DB prepared the DNA constructs and produced virus. MA performed the injections, tissue dissociation, histology, imaging and image quantification. DB and TD performed the single-cell library preparation and prepared samples for sequencing. DB and MA built the data processing pipeline. DB, MA, TD, and AW performed the analysis. All authors contributed to the MS as drafted by DB, MA, and VG. MT supervised single-cell RNA sequencing computational pipelines while VG supervised the overall project. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the NIH Pioneer DP1OD025535, NIH BRAIN R01MH117069, Beckman Institute for CLARITY, Optogenetics and Vector Engineering Research (CLOVER) at Caltech, the Single-Cell Profiling and Engineering Center (SPEC) in the Beckman Institute at Caltech, the Curci Foundation, the CZI Neurodegeneration Challenge Network (VG), and the Vallee Foundation (VG). VG and MT are Heritage Principal Investigators supported by the Heritage Medical Research Institute. DB was supported by PHS Grant Number 5T32NS105595-02.

ACKNOWLEDGMENTS

We thank the Gradinaru and Thomson labs for helpful discussions, Allan-Hermann Pool for advice on the mouse brain tissue dissociation procedure, Jeff Park for advice on 10X Genomics Chromium single-cell library preparation, Min Jee Jang for help in designing probes and troubleshooting FISH-HCR, and Ben Deverman and Ken Chan for early discussions on strategy. We also thank the software packages employed for visualization. **Figures 1**, **2**, and **Supplemental Figures 1, 2**, and **4** were partially created with Biorender.com. Bar graphs, scatter plots, and box plots were generated with the help of the Plotly Python graphing library.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.730825/full#supplementary-material

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Conflict of Interest: VG is a Co-founder and BoD member for Capsida Biotherapeutics, a Fully Integrated AAV Engineering and Gene Therapy Company in Southern California.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Immunology

REVIEW
published: 29 October 2021
doi: 10.3389/fimmu.2021.753467

Adeno-Associated Viruses (AAV) and Host Immunity – A Race Between the Hare and the Hedgehog

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Adeno-associated viruses (AAV) have emerged as the lead vector in clinical trials and form the basis for several approved gene therapies for human diseases, mainly owing to their ability to sustain robust and long-term *in vivo* transgene expression, their amenability to genetic engineering of cargo and capsid, as well as their moderate toxicity and immunogenicity. Still, recent reports of fatalities in a clinical trial for a neuromuscular disease, although linked to an exceptionally high vector dose, have raised new caution about the safety of recombinant AAVs. Moreover, concerns linger about the presence of pre-existing anti-AAV antibodies in the human population, which precludes a significant percentage of patients from receiving, and benefitting from, AAV gene therapies. These concerns are exacerbated by observations of cellular immune responses and other adverse events, including detrimental off-target transgene expression in dorsal root ganglia. Here, we provide an update on our knowledge of the immunological and molecular race between AAV (the "hedgehog") and its human host (the "hare"), together with a compendium of state-of-the-art technologies which provide an advantage to AAV and which, thus, promise safer and more broadly applicable AAV gene therapies in the future.

Keywords: AAV, antibody response, cellular response, capsid, engineering, immune evasion, pre-existing immunity, neutralizing antibodies

OPEN ACCESS

Edited by:
Nicole K. Paulk, University of California, San Francisco, United States

Reviewed by:
Eduard Ayuso, DiNAQOR (Switzerland), Switzerland
Domenico Tortorella, Icahn School of Medicine at Mount Sinai, United States

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *04 August 2021*
Accepted: *28 September 2021*
Published: *29 October 2021*

Citation:
Rapti K and Grimm D (2021) Adeno-Associated Viruses (AAV) and Host Immunity – A Race Between the Hare and the Hedgehog. Front. Immunol. 12:753467. doi: 10.3389/fimmu.2021.753467

1 INTRODUCTION

The hallmark of gene therapy is the delivery of exogenous nucleic acids to cells with the aim to replace missing or defective genes, or to suppress (RNA interference technology) or correct (genome editing) deleterious ones, in order to ultimately ameliorate genetic causes of disease. The ideal delivery vehicle or vector should safely, specifically and efficiently transport the therapeutic cargo and allow expression for the desired duration. Although the delivery of "naked DNA" has progressed all the way to clinical trials, the use of non-viral and viral vectors continues to dominate the field [reviewed in (1–3)]. Viral vectors rely on natural, evolutionary evolved properties of viruses to efficiently evade an organism's immune surveillance while delivering their cargo to specific cells. Several types of viral vectors are used in gene therapy today, mostly

adenoviruses, retroviruses and adeno-associated viruses (AAVs), of which the latter have emerged over the past 20 years as the leading platform for a myriad of applications (1, 2).

AAVs are small, non-enveloped, non-pathogenic viruses endemic in humans and multiple vertebrate species. They belong to the genus *Dependoparvovirus* within the family *Parvoviridae* and are amongst the smallest animal DNA viruses [(4), reviewed in (5–7)]. They carry a ~4.7 kb single-stranded genome that is flanked by two 145 bp ITRs (inverted terminal repeats) forming a characteristic T-shaped hairpin and is packaged in a capsid of T=1 icosahedral symmetry and ~26 nm diameter. Their genome consists of two main open reading frames (ORFs), *rep* and *cap* [reviewed in (7)], and two additional ones encoding the assembly-activating protein (AAP) (8) and the recently discovered membrane-associated accessory protein (MAAP) (9). While the three viral capsid proteins VP1-3 share their C-terminal region, it is VP3, the shortest and most abundant of the three, that determines tissue tropism through receptor binding and interaction with factors in the circulation and interstitial tissue, including but not limited to antibodies [(10–12), reviewed in (2)]. These properties are mainly attributed to nine variable regions (VRI-IX) within VP3 (13).

So far, at least 13 naturally occurring primate serotypes and hundreds of variants have been identified, and countless engineered AAVs with specialized properties are constantly generated [(14–18), reviewed in (1, 2, 19)]. AAVs infect cells by binding to cell surface molecules, identified either as receptors, attachment or viral entry factors, and typically glycans or proteinaceous in nature. Some of these are serotype-specific while others are not, such as AAVR [(20–23), reviewed in (19)]. Binding to these factors is followed by receptor-mediated endocytosis, intracellular trafficking, endosomal escape, nuclear transport, capsid uncoating and finally second-strand genome conversion in the nucleus [reviewed in (19, 24)].

Reasons for the attraction of AAVs as vectors include their broad tropism, low immunogenicity as compared to other vectors, and apathogenicity. Moreover, they are easily engineered as gene delivery vector, by replacing the viral genome with a therapeutic expression cassette, yielding a recombinant AAV (rAAV) with the ITRs as the only essential *cis* elements. Recombinant AAVs transduce cells akin to an infection with their parental wild-type (wt)AAVs, but they cannot integrate into the host cell chromosome in a site-specific manner or integrate at very low frequency, due to the lack of the *rep* gene (25). Still, they can establish long-term transgene expression in both, animals and humans (26–29). Encouraging data in preclinical animal models and in clinical trials [reviewed in (30)] have led to the approval of several gene therapies in recent years, starting with Glybera, a rAAV1 carrying the lipoprotein lipase gene, whose intramuscular delivery aimed at the treatment of lipoprotein lipase deficiency. However, due to the high cost, the scarcity of the disease and the lack of approval in the US, it was withdrawn five years later (2017), despite its therapeutic efficacy (1, 2, 19, 31, 32). The first AAV gene therapy approved by the US Food and Drug Administration (FDA) in 2017 was Luxturna™ or voretigene neparvovec, followed by its approval in Europe end of 2018. Luxturna is an AAV2 vector carrying the *RPE65* gene, which is delivered to the retina to treat an inherited form of blindness caused by a mutation in this gene (1, 2, 19, 31). The second gene therapy approved in the US in 2019 was ZOLGENSMA® (onasemnogene abeparvovec-xioi), i.e., an AAV9 vector carrying the human survival motor neuron (*SMN*) gene and used for the one-time treatment of children under the age of 2 who suffer from spinal muscular atrophy (SMA) (1, 2, 19, 31). Overall, the future of the field is bright, with a 2019 FDA report estimating the yearly approval of 10-20 new cell and gene therapy products by 2025 (2, 33).

Despite the three approved AAV gene therapies and the success of numerous clinical trials with AAVs (1, 2, 30, 31), challenges or impediments remain. This is exemplified by the first clinical trials for the treatment of severe hemophilia B, using an AAV2 vector carrying the gene for coagulation Factor IX (FIX) that was delivered either intramuscularly (34) or infused through the hepatic artery (35). While the first trial was hampered by low-level, short-term expression of <1% of FIX (34), immune responses against the AAV vector were noted in a second trial (35), which were not predicted by any of the preclinical studies in small or large animals (36). No long-lasting systemic toxicity was observed and therapeutic levels of FIX were obtained, but they rapidly declined to background levels, accompanied by a transient increase in liver transaminases. It was later determined that this was due to cytotoxic T-cell (CTL) responses from memory CD8+ T-cells against hepatocytes presenting AAV epitopes *via* major histocompatibility complex (MHC) class I (36–39). In another clinical trial using AAV8 to deliver a codon-optimized *fIX* gene, a short-term supply of immunosuppressants sufficed to block cellular immune responses and enabled long-term expression (27). In additional clinical trials, the presence of pre-existing anti-AAV antibodies governed the efficiency of the gene transfer (40, 41), as will be discussed in more detail below.

The experience and knowledge from these initial clinical trials shaped subsequent efforts to create new generations of AAV vectors that would perform better in humans. In particular, it quickly became evident that immune responses are a major roadblock and require thorough investigation, in order to develop novel, urgently needed strategies to evade or alleviate them. This review will first explore the mechanisms of anti-AAV immune responses and methods to measure them, before focusing on the multifaceted approaches to escape them in a (pre)clinical setting.

2 IMMUNE RESPONSES AGAINST AAV

Immunity is the ability of higher organisms to protect themselves from pathogenic invaders, such as viruses or bacteria. Although AAVs are non-pathogenic and vectors derived thereof no longer express any viral proteins, their viral nature renders them a target for the immune system. On top, the fact that AAVs were

discovered in human tissues explains why humans carry immunologic memory against them (3, 30, 42, 43).

Generally, the immune system consists of two major arms, the innate and adaptive immunity, which are intertwined and closely regulate one another (44). Adaptive immunity, also called acquired immunity, includes humoral immunity (B-cells, neutralizing antibodies) and cell-mediated immunity (T-lymphocytes, macrophages, natural killer cells).

The following chapters briefly summarize the current knowledge of the role of these arms in anti-AAV immune response in humans before we focus on clinically relevant countermeasures.

2.1 Innate Immunity Against AAVs

The innate system is the first line of defense against invaders and, therefore, is relatively non-specific. Professional antigen-presenting cells (APCs), which are present in most tissues, express pattern recognition receptors (PRRs). These recognize structural features on molecules, such as glycans and viral nucleic acids, which are shared between microorganisms and named pathogen-associated molecular patterns (PAMPs) (45). Toll-like receptors (TLRs) are PRRs that are critically involved in immune responses against AAVs together with myeloid differentiation primary response 88 (MyD88), i.e., their universal adaptor [reviewed in detail in (42)]. They are type I transmembrane proteins, which contain leucine-rich repeats and which are located on the cell surface (TLR1, 2, 4-6 and 10) or the endosome (TLR3, 7-9) (46), where they recognize the AAV capsid or the viral nucleic acid (CpG-containing viral genomes and double-stranded (ds)RNA), respectively [reviewed in (42, 45, 47)]. Triggering of PRRs results in nuclear translocation of nuclear factor κB (NF-κB) and interferon regulatory transcription factors (IRFs), which subsequently induce expression of pro-inflammatory cytokines and type I interferons [IFN, reviewed in (3, 42)]. Type I IFNs are the essential link between innate and adaptive immunity [(48–51), reviewed in (3, 47)].

The importance of innate immune responses in AAV gene transfer is subject to intense ongoing investigation. Early studies in mice showed low levels of chemokine induction, at least compared to adenoviral vectors, and the duration was also transient and did not lead to liver necrosis (52). One of the first factors identified to play a role in inhibiting AAV transduction is apolipoprotein B mRNA editing complex 3A (APOBEC3A) (53), a component of the intrinsic immunity. However, the link to innate immunity remains unclear (54). Further studies, mostly performed in the liver, revealed important roles for TLR2 and, most prominently, TLR9 receptors in the early-phase activation of the innate immune system, involving different cell types. TLR2 can sense the capsid of rAAVs (serotypes 2 and 8) on the surface of human non-parenchymal liver cells, such as Kupffer and liver sinusoidal endothelial cells (LSECs). This results in NFκB-mediated immune responses and activation of several interleukins and tumor necrosis factor α (TNFα), but not type I IFN (55). Extensive studies support the role of the viral genome and in particular of the presence of unmethylated CpGs in triggering the innate immune system through the TLR9-MyD88 pathway in different cell types (56, 57), not only in Kupffer cells (58), but also in dendritic cells (DCs) including plasmacytoid (59–61), conventional (59) and monocyte-derived DCs (59, 62). Different TLRs appear to be activated and have distinct effects on different DCs, all of which participate in linking innate and adaptive immunity (45). Still, TLR9 seems to be the most efficient in perpetrating downstream events, such as humoral responses (59) (see below for the link to the adaptive immunity).

Unmethylated CpGs, which are present in the ITRs but also in vector expression cassettes, were shown early on to play a central and enhancing role in the aforementioned TLR9-MyD88 activation (48, 52, 57, 61, 63) [reviewed in (56)]. The detrimental role of CpGs in expression cassettes became evident in clinical trials by the stronger immune responses triggered by codon-optimized transgenes that contained higher CpG levels compared to the wild-type sequences (57), which highlights the importance of the encapsidated nucleic acid. Similarly, the presence of self-complementary rather than single-stranded AAV vector DNA (scAAV vs ssAAV) also results in stronger induction of the innate immune system through TLR9 (58).

In addition to the role of the DNA, the role of double-stranded (ds)RNA produced from AAV vectors has most recently been identified as a factor governing the success of AAV gene therapy (64). Late rather than early innate immune responses are likely to explain the decline in transgene expression that is often observed in patients weeks after AAV delivery. In a recent study, it was hypothesized that the dsRNA produced by the inherent promoter activity of the AAV ITRs was sensed by the PRR melanoma differentiation-associated protein 5 (MDA5). Together with the signaling adaptor mitochondrial antiviral signaling protein (MAVS), MDA5 induces expression of IFN-β, a type I IFN. This induction was seen in different cell lines but, more importantly, also *in vivo* in the humanized liver of mice (64). However, another dsRNA PRR, TLR7, did not show a similar effect (59). Hence, the role and the mechanisms of dsRNA sensing in the innate immune responses require further study (42).

The aforementioned studies of innate immune responses against AAVs yield insights into the connection to the adaptive immunity arm. Indeed, the innate system is considered the key player in the induction of the adaptive responses, which is further corroborated by the fact that transient immune suppression of inflammatory cytokines in clinical trials also suppressed the adaptive immune responses (30). The TLR9-MyD88 pathway is central in this association with both, humoral (51, 59, 62, 65) and cellular immunity (48, 51, 56, 60, 61, 63, 65). In particular, the role of MyD88 in neutralizing antibody induction against the capsid was underlined (51), as well as its role in B-cell induction (59, 65), T helper 1 induction (3, 51, 65), or the shift from Th1 to Th2 (51). In contrast, TLRs are involved in the induction of CD8+ T-cells against the transgene product (51). B-cell induction can also be mediated by cytokines from monocyte-derived DCs (moDCs) (66). The role of TLRs in cell-mediated responses is strongly corroborated by multiple studies. CpG DNA in AAV vectors can induce CD8+ T-cell responses (63), and TLR9 was implicated in capsid antigen presentation

through MHCI (51, 60, 63). This process was also shown to require type I IFN (60, 67), next to TLR9, which is secreted by plasmacytoid DCs (pDCs) and binds to its receptor on conventional DCs (cDCs). Subsequently, licensing of the latter activates CD8+ T-cells. Inhibition of this pathway reduced antibody production against the capsid (47, 48). Intramuscular AAV injection, with or without a TLR9 agonist depending on the mouse strain, elicited T-cell responses against the transgene product (59, 62). Similar effects were also observed following systemic administration (68). Finally, at lower doses, AAVs can interact directly with members of the complement system, especially iC3b, which can enhance humoral responses through the classical pathway. Yet, they also bind the complement regulatory protein factor H, which hinders the onset and intensity of antibody formation. At higher doses, AAVs can activate the complement and macrophages, in an antibody-dependent manner (69) [reviewed in (70)].

Together, a wealth of data supports the role of the innate immune system in animals or humans and especially in the induction of adaptive immune responses, as discussed in more detail below.

2.2 Adaptive Immunity Against AAVs

Adaptive immune responses follow, and are activated by, the innate system. The adaptive immune system is sophisticated and highly specific to the pathogens. The main actors of the two major branches of adaptive immunity, humoral and cellular, are the B- and T-cells. During development, they produce a vast amount of receptors by rearranging their DNA that recognize the pathogens in an initial encounter. Subsequently, this system generates the so-called immunological memory, which is more robust and is maintained for years after the first invasion (45). The key steps towards immunity include antigen capture and presentation by APCs to lymphocytes, which are in turn activated, clonally expanded and differentiated to effector cells. The effector functions include (1) activation of B-cells and production of antibodies against the pathogen, (2) activation of inflammation, of macrophages as well as of B- and T-cells by helper T-cells, (3) CTL responses to eliminate the pathogen, and (4) induction of regulatory T-lymphocytes to suppress immune responses. The effector phase is followed by contraction of lymphocytes by apoptosis that restores homeostasis and by survival of antigen-specific cells to yield immunologic memory (44, 45).

AAV vectors that are presently in clinical evaluation or used as basis for gene therapeutics are typically derived from wild-type AAVs with no or minimal modifications to the capsid, such as peptide insertions or point mutations. As humans are exposed to these viruses early in life, it comes as no surprise that adaptive immunity is a major challenge for gene therapy, as discussed below. Adaptive immune responses against AAVs (overview in **Figure 1**, **Table 1**) have been well documented in clinical trials and investigated in animal models [reviewed in (3, 30, 43, 95, 113)]. The next chapter will explore both, humoral and cellular immune responses against the capsid or the transgene product, as well as methodologies for their detection.

2.2.1 Humoral Immune Responses

Humoral immunity against AAVs, either exhibited by the prevalence of anti-AAV antibodies in the human population (71, 114, 115) but also in animals (116–118), or triggered by AAV vector administration, has been investigated since the early days of AAV vector engineering (119), and it has since been viewed and intensively discussed as a major impediment and exclusion criterion in AAV gene therapy clinical trials. Exacerbating this challenge is that not only up to 90% of individuals in certain areas of the world are seropositive for AAV and that 30-70% are believed to carry neutralizing anti-AAV antibodies (nAbs), but there is also significant cross-reactivity among the known naturally occurring AAV serotypes as well as their synthetic derivatives (115, 120, 121). As the topic of human seroprevalence against AAVs, induced by natural infection or by gene therapy, has already been covered extensively in a flurry of previous reviews including an excellent recent article by Weber in this journal (122) (also references therein), we kindly refer the reader to this literature for more background information. Below, we will instead focus on methodologies for the detection of humoral immune responses against AAVs and then later (chapter 3) discuss experimental strategies to circumvent these.

There are two major *in vitro* methodologies, *i.e.*, cell-based assays and ELISAs (enzyme-linked immunosorbent assay), which are used for screening of anti-AAV nAbs and each exhibiting distinctive advantages (123–125). Cell-based assays are more widely used as they are robust and fast. Furthermore, they can distinguish between neutralizing and non-neutralizing, binding antibodies (123, 126). Recently, a variation of these assays has been reported, *i.e.*, a cell-binding assay. This assay, albeit being fast, cannot make this distinction and can only detect neutralization at the level of receptor binding (127). ELISAs, on the other hand, are easy, relatively sensitive and highly useful for determining the immunoglobulin subclasses (114). However, they are typically used to measure binding, not necessarily neutralizing antibodies. There is a high but not absolute degree of correlation between the two assays (121, 128). In cell-based assays, serial dilutions of blood serum or plasma are mixed with equal amounts of an AAV vector, preincubated and then transferred to cells. Transduction efficiency is determined at a distinct time point and expressed as percent to a no-serum/plasma control. The titer is determined as the first dilution at which inhibition exceeds 50% (123, 124, 129, 130), which makes this assay similar to a half-maximal inhibitory concentration (IC_{50}) assay. These assays have been performed in numerous variations, using different transgenes (green fluorescent protein (GFP), LacZ and luciferase), cell types (HEK293T or Huh7), serum or plasma, heat-inactivation or not, with adenovirus superinfection or not etc. The sensitivity of the assay was found to decrease with lower cell densities or with GFP [reviewed in (123, 131)]. Of these options, luciferase and HEK293T cells are the most widely used (123, 124, 131), although a need for optimization remains (132). It should also be noted that different AAV purification methods produce different full/empty capsid ratios, which could also impact data

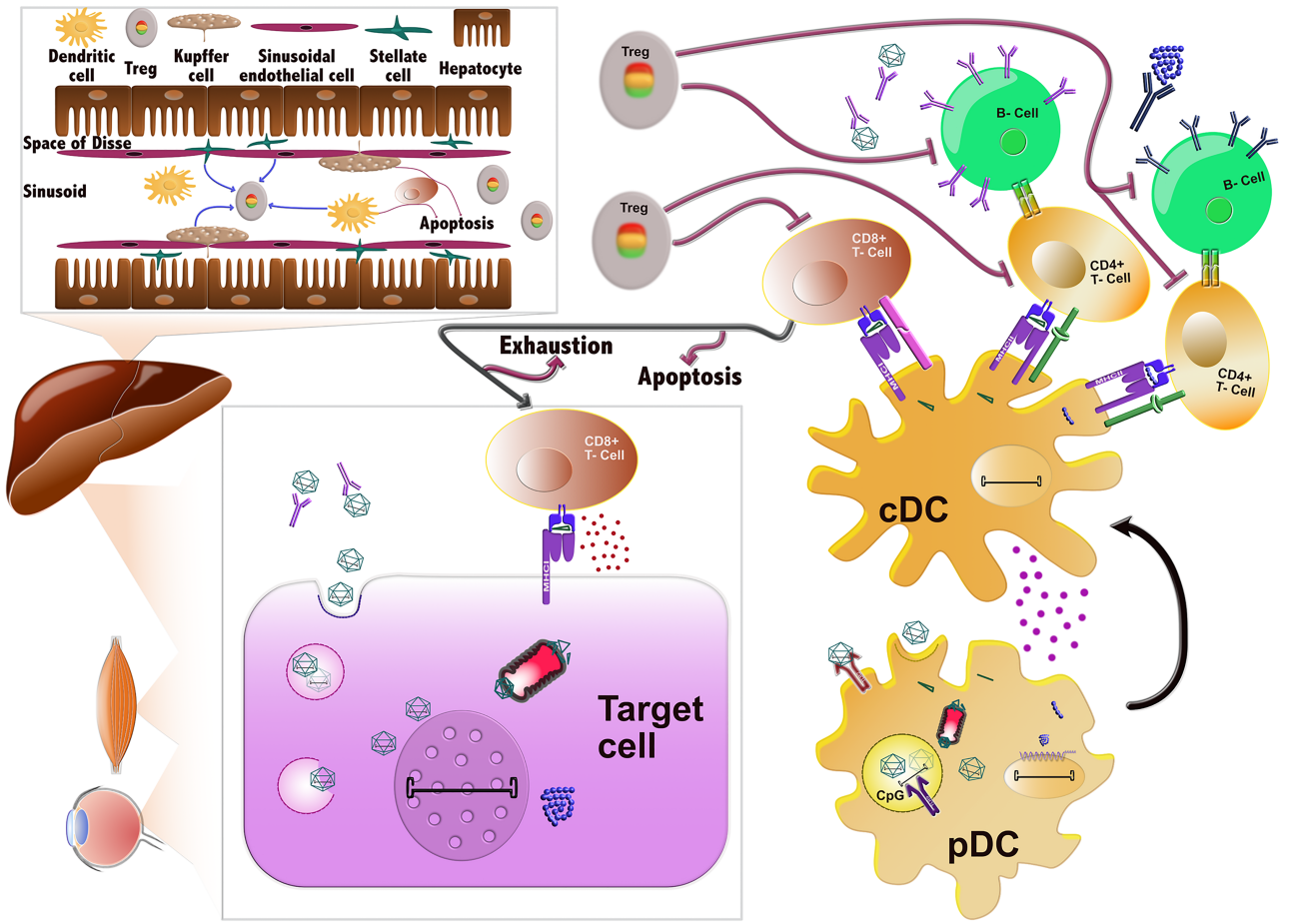

FIGURE 1 | Immune responses against AAVs. AAVs delivered systemically can be neutralized by pre-existing antibodies prior to entering the cells. If they evade nAb binding, they enter the cells through endocytosis and can then be degraded in the endosomes. In certain cell types, such as DCs, their genome or capsid can be sensed by TLR9 and TLR2, respectively, which induces the innate immune response. Alternatively, AAVs can successfully escape the endosome and traffic in the cytoplasm, where they can be ubiquitinated, resulting in capsid degradation. The ensuing peptides can next be loaded to MHC class I molecules and presented on the surface of the cells, which are then targeted and possibly eliminated by CD8+ T-cells (CTL response). After endosomal escape, AAVs can also successfully transduce the cell and deliver their viral genome to the nucleus, where the transgene is expressed. Any misfolded protein encoded by the transgene can be degraded by the proteasome and the ensuing peptides can be loaded onto MHCI and also provoke a CTL response. Sensing of AAV vector components in plasmacytoid DCs (pDCs) by the innate immune system leads to the activation of conventional DCs (cDCs). cDCs employ antigen presentation to cross-prime CD8+ T-cells towards an effector type (Teff) and to activate CD4+ T-cells. The latter can activate B cells, which in turn produce the nAbs against the capsid and transgene product. After prolonged or inadequate stimulation, the CD8+ T-cells can be eliminated by different mechanisms including T-cell exhaustion, anergy or apoptosis. The tolerogenic environment in the liver can also stimulate the production of regulatory T-cells (Tregs), which can suppress the aforementioned immune responses at different stages.

transferability efforts (133). Several studies have also attempted to determine the correlation between *in vitro* and *in vivo* assays, but this proved to be challenging and tedious (129, 131, 134). Despite the fact that humoral immunogenicity remains a major impediment for gene therapy in humans and in animal models (3, 30, 47, 116, 135, 136), an international standard assay that takes into consideration key parameters, such as sensitivity and specificity, has yet to be established (135). This underscores the importance of reporting these assays in sufficient detail to allow comparisons between studies and provides the opportunity for a call to the community for additional standardization efforts (78, 122, 137).

2.2.2 Cellular Immune Responses

2.2.2.1 Cellular Immune Responses Against the Capsid

Intriguingly, even though the challenges posed by humoral immunity, predominantly against the AAV capsid, were well established in larger animal models and screened for in patients in clinical trials, the detected cellular immune responses were not as anticipated [reviewed in (3, 30, 43, 137, 138)]. In the first liver-directed clinical trial to treat hemophilia B, hepatic intra-arterial delivery of a recombinant AAV2 vector expressing the human blood-coagulation factor IX resulted in a limited duration of transgene expression and a transient, asymptomatic elevation of liver transaminases in the high-dose group. This was attributed to

TABLE 1 | Summary of immune responses to AAV gene therapy and evasion/prevention strategies.

Response	Strategy to evade them
Pre-existing nAbs against the capsid (35, 71, 72) • previous exposure to wtAAVs	Host • exclusion from clinical trials (71) • route of administration (73), saline flushing (74) • immune-privileged organ (75, 76) • nAb depletion [plasmapheresis (77), immunoadsorption (78), (IgG)-degrading enzymes (79)] Vector • novel serotype selection (80) • AAV capsid engineering (81, 82) • chemical modification of the capsid (83)
Pre-existing nAbs against the transgene • previous exposure to recombinant or truncated protein (84)	Host • targeting of tolerogenic organs (85)
Activation of the innate system • vector capsid (55)/genome (51, 58)/dsRNA (64) sensing • CpG containing vector genome (57, 59, 63)	Vector • CpG depletion (67) • TLR9-inhibitory sequences addition (86) • suppression of ITR promoter activity (70)
nAbs against the capsid after gene therapy (79, 87)	Host • targeting of tolerogenic organs (88) • induction of tolerance (89) • B-cell depletion (90) Vector • vector engineering to avoid antigen presentation (91)
nAbs against the transgene after gene therapy (59, 62, 87) • null or missense mutations (92–94) • route of administration (92)	Host • targeting of tolerogenic organs (37, 95–97) • immune suppression (98) • B-cell depletion (90) Vector • tissue-specific expression (promoter, miRs) (99, 100)
Cellular immune responses against the capsid (39, 101) • route of administration (102, 103) • vector dose (101)	Host • immune suppression (27, 98, 101) • targeting of immune-privileged organs (102, 103) • targeting of tolerogenic organs (104), induction of tolerance (89, 105) • cell-type specific expression (promoter, miRs) Vector • vector selection/engineering to avoid antigen presentation (106–108)
Cellular immune responses against the transgene • route of administration (92, 109) • vector dose (110)	Host • immune suppression (98, 111) • targeting of immune-privileged organs (37) • targeting of tolerogenic organs (104), induction of tolerance (97) • restriction of transgene expression (promoter, miRs) (100) Vector • vector engineering to avoid antigen presentation (112)
Vector dose (toxicity)	Host • lower vector dose • capsid/transgene optimization for increased expression

cellular immune responses towards the capsid that were targeting transduced hepatocytes, as the rise of transaminases was accompanied by a rise in capsid-specific CD8+ T-cells (35). In a subsequent trial using rAAV8 to systemically deliver a self-complementary genome encoding a codon-optimized FIX variant (scAAV2/8-LP1-hFIXco), an asymptomatic increase in serum transaminases or liver-enzyme levels was also observed in the medium- and high-dose groups, together with an increase in capsid-specific CD8+ T-cells in peripheral blood. Still, in patients treated with glucocorticoids, FIX expression was maintained years after vector application (27, 101). It was further established that humans carry capsid-specific T-cells against AAVs (38, 139), and that the elicited immune response is dose-dependent (35, 101). However, immune suppression strategies seem capable of obviating this obstacle, at least to some extent (27, 38, 98, 140–142) [reviewed in (43, 72, 143, 144)]. Besides dose, the route of administration may also play a role in the induction of T-cell immune responses. Intramuscular delivery typically induces stronger cellular

responses, although these seem to only account for a reduction of transgene expression, but not for vector elimination (72, 102, 103). They are concomitant with the infiltration of T-cells that do not have a cytolytic (CTLs) but rather a regulatory phenotype, Tregs (145). The latter are formerly known as suppressor T-cells, which are responsible for tolerance to self-antigens. These results once again highlight the complexities of immune responses against AAVs.

The experience gained by the early clinical trials has motivated significant research on the characterization of these immune cells and the mechanisms underlying their induction, stimulation and regulation. APCs are professional, such as DCs, or non-professional, with the former expressing MHC class II molecules and the latter MHCI. Class I MHC molecules, expressed by almost all nucleated cells, mostly present antigens to cytotoxic T-cells, as opposed to MHCII that trigger helper (CD4+) and regulatory T-cells (45). After AAV administration, APCs intracellularly process transgene peptides or proteolytic products through proteasomal degradation of the AAV capsid (146) in the cytosol and cross-present them onto MHCI (60, 147, 148). This, in turn, flags the transduced cells for destruction (39, 149–151). Presentation on MHCII molecules facilitates humoral and cellular immune responses (48, 87, 105). The epitopes on the AAV capsid that are recognized by CD8+ cells are conserved across serotypes (38, 147). These cells are limited in peripheral blood mononuclear cells (PBMCs) (41, 147), which are typically screened during clinical trials, but are more abundant in lymphoid organs, such as the spleen, and recognize epitopes presented *via* major MHCI (147). Capsid-specific T-cells have been found in splenocytes from children (38, 147), which points to the induction of not only humoral, but also cellular immunity early in life after AAV infection and to the maintenance of memory T-cells in secondary lymphoid organs [reviewed in (3, 43)]. These cells express IFNγ, TNFα, IL-2, perforin and the degranulation marker CD107α (66, 147, 152), which equips them with a T-effector phenotype and the ability to exhibit cytolytic activity [reviewed in (3, 43)]. From the aforementioned research, it was long thought that the cellular immune responses against the capsid are mediated by memory CD8+ T-cells generated during childhood after natural infections. Recently, however, the presence of CpG motifs has been linked to the expansion of naïve T-cells directed against epitopes on the capsid. In contrast, memory T-cells react more vigorously to AAV vectors largely depleted of the CpG motifs as well as to empty capsids (63).

Another key aspect is the linkage of humoral to cellular immunity (48, 66). Typically, T-cells are identified by their ability to produce IFNγ upon stimulation with capsid peptides. One notable study that went a step further provided a link between seroprevalence and T-cell reactivity. Seropositive individuals had TNFα-secreting memory CD8+ cells, whereas seronegative individuals showed transient activation not of naïve T-cells, but of natural killer (NK) cells that secrete both, IFNγ and TNFα (66). Additionally, antibody formation requires the CD40-CD40L axis in CD4+ cells (48) and IL-1β and IL-6 in moDCs (66).

Finally, a contribution of the capsid itself in the induction of the cellular immune responses has been reported (87, 153, 154). In more detail, it was shown that AAVrh32.33 can induce stronger humoral and cellular immune responses than AAV8 (87) or other commonly used serotypes. This can be attributed to structural differences and, in particular, to the surface-exposed variable regions, mainly IV (153). This serotype is of particular interest for vaccine applications, due to its low seroprevalence. AAVs as a vaccine have multiple advantages: a single intramuscular application suffices for long-term expression, immunogenicity and protection, and their high thermal stability reduces the thermal-chain requirements, as shown recently for the AAVCOVID vaccine. However, even though large-scale production is feasible, it is challenging to meet the needs of a pandemic (155, 156). The capsid tropism, trafficking and transduction efficiency of APCs also seem to be factors contributing to vector immunogenicity (106–108, 154).

Several studies explored the cellular immune mechanisms in non-human primates (NHPs) and revealed that natural infections with AAVs also produce cellular and humoral responses. Capsid-specific CD4+ and CD8+ cells in rhesus macaques display distinctive differentiation status and function, as well as cell-subset frequencies, with higher proportions of T-effector (Teff) cells as compared to humans (139). Furthermore, unlike chimpanzees, human immune cells do not express CD33-related Siglecs (sialic acid-binding immunoglobulin-type lectins), which are inhibitory signaling molecules thought to downregulate immune cell activation (157).

Cellular immunity against AAV is predominantly evaluated by determining the frequency of capsid-specific T-cells. Two major assays are currently in use, namely, IFNγ enzyme-linked immune absorbent spot (ELISpot) assay and, more recently, flow cytometry combined with intracellular cytokine staining (ICS) (3, 158). ELISpot measures the frequency of T-cells that produce a cytokine, such as IFNγ or, as recently suggested, also TNFα (66), upon stimulation with the appropriate antigen. As a first step, peripheral blood mononuclear cells or splenocytes (usually from humans or from animals, respectively) are isolated, cultured and plated on ELISpot plates that contain membrane bottom wells pre-coated with antibodies against the target cytokine. Afterwards, the cells are stimulated with peptide pools from different AAV capsids. Upon stimulation, the immune cells, granted they have receptors recognizing the antigen, produce cytokines that are captured by the underlying antibodies. Cells are then removed and the cytokine is detected with another antibody, producing spots on the membranes corresponding to each cell that produced cytokines. Use of serial dilutions allows to determine the number of positive cells in a population (139, 147) [reviewed in (3, 113)]. For higher sensitivity, ICS of these immune cells after stimulation with AAV capsid peptide pools can be quantified using flow cytometry (66, 147) [reviewed in (3, 113)], which also allows for multifunctional analysis of T-cells (147). Additionally, because AAV-specific circulating T-cells are rare, an enrichment step based on MHCI tetramers or pentamers and magnetic beads can enhance the detection sensitivity for both assays (41, 66, 147).

As noted initially, the cellular immune responses observed in the first clinical trials, which led to rejection of AAV-transduced

cells, were not predicted in any of the small or preclinical animal studies (36). Over time, multiple explanations have been proposed, such as the primate/human source of AAVs, immunological memory in humans, or differences in the immune system [reviewed in (3, 36, 43, 47, 113)]. Extended efforts were then dedicated to develop suitable animal models [reviewed in (113)], including incorporation of a highly immunogenic peptide (SIINFEKL) of ovalbumin in the AAV capsid. To study primary or secondary responses, AAV capsid-specific CD8+ T-cells, derived either from OT-1-transgenic animals (they carry the T-cell receptor for this peptide), or from mice immunized using adenoviral gene transfer of this peptide, are adoptively transferred to recipient mice that are injected with AAVs (60, 63, 159). The adoptive transfer can also be performed without the presence of the peptide or by including an *in vitro* expansion step and further stimulation of the immune system. However, these methods still fail to fully recapitulate the CTL responses [reviewed in (42, 113)].

2.2.2.2 Cellular Immune Responses Against the Transgene Product

Immune responses to the transgene products are influenced by multiple factors, which can be divided into (1) host-specific, such as the underlying mutations (missense, stop codon), the genetic background, disease-related inflammation and pre-existing immunity and (2) vector-specific, such as the AAV capsid and genome, delivery route, tissue-restricted promoters, vector dose, or transgene [reviewed in (30, 42, 95)]. Gene therapy in patients that lack a given protein, due to *e.g.*, a stop codon, is likely to induce a transgene product-specific response. However, underlying mutations (92), even single-amino acid substitutions or transgene-derived cryptic epitopes (84, 93) can induce transgene product-specific cellular responses restricted to tissue-resident T-cells (109). Another determining factor is the genetic background of the patient (37, 94, 160). Disease-specific conditions, such as the dystrophic environment characterized by inflammation, contribute to transgene rejection (161). Pre-existing immunity against the transgene due to protein replacement therapy is also a limiting factor (84). Regarding vector-specific responses, the contribution of the capsid (107, 162, 163) and the vector genome (64, 67, 164) has already been elaborated on in the previous sections. Additionally, the route of administration and promoter-restricted expression are major determinants of immune responses to transgene products. Intravenous delivery results in concurrent expression in the liver and induction of immune tolerance (37, 95), as detailed in the next section. However, intramuscular injection or restriction to this tissue *via* muscle-specific promoters typically results in a stronger immune response (37, 88, 92, 162, 165). This example again highlights the critical role of the promoter in AAV vector constructs and concurrently illustrates the possibilities to reduce immune responses through a meticulous selection of promoters and other regulatory elements. Ideally, this results in the detargeting of vector gene expression from APCs and, thus, avoids presentation of transgene peptides on MHCI and subsequent CTL-mediated clearance of the transduced cells.

Such regulatory elements also comprise binding sites for tissue- or cell-specific mi(cro)RNAs, which can be easily included in the 3' untranslated region of an AAV vector expression cassette and which will shut down unwanted gene expression in cells expressing the selected miRNA(s). For instance, this strategy has been exploited in the past to purposely detarget AAV transgene expression from the liver, by incorporating binding sites for the liver-specific miR-122 into the recombinant AAV genome (166–168). Most relevant in the context of anti-AAV immune responses are miRNAs that are abundantly expressed in professional APCs, especially miR-142 (169) or, as reported most recently, miR-652-5p (99). As shown repeatedly, inclusion of binding sites for these miRNAs can diminish both, antibody formation and CTL responses, in turn boosting transgene expression and extending its persistence in mice. Impressively, combination of binding sites for miR142 and miR652-5p even enabled robust expression of the highly immunogenic ovalbumin *in vivo*, through detargeting from APCs, inhibition of CTL activation and suppression of Th17 responses (99). The fact that incorporation of miRNA binding sites into AAV vectors is technically simple and that saturation of the endogenous miRNA/RNAi pathway is unlikely (due to the artifical design of these sites that bind miRNAs with perfect complementarity) makes this strategy very appealing and versatile. Finally, vector dose also plays a significant role in defining the T-cell immune response (110) against the capsid and the transgene (84, 170, 171).

Despite the possibility of immune responses against the transgene product, there are few reports of clinical trials encountering this limitation. This could be attributed to residual natural protein expression, to the type of application (*e.g.*, gene replacement therapy), to the preconditions of the individuals, to vector delivery to immune-privileged organs, to the induction of immune tolerance and/or exhaustion, or to the application of immune suppression [reviewed in (3)]. Nonetheless, some transgene product-specific immune responses were observed in vector-treated individuals. In a phase I/II clinical trial, six Duchenne muscular dystrophy patients received the mini-dystrophin transgene intramuscularly. Dystrophin-specific cytolytic CD8+ T-cells were observed in all patients after treatment and in two before (84). T-cell responses were also observed in a separate clinical trial to treat another monogenic disorder, α-1-antitrypsin (AAT) deficiency. Intramuscular delivery resulted in AAT-specific T-cell responses in two participants as well as in a reduction in expression in one of them (172). Finally, in a phase I/II clinical trial to treat mucopolysaccharidosis type IIIB syndrome (Sanfilippo type B syndrome), an rAAV2/5 vector carrying the human α-N-acetylglucosaminidase (NAGLU) was delivered intracerebrally. In three of the four patients, circulating T-cells that produced TNFα upon stimulation with NAGLU peptides were detected but later subsided, indicative of the development of tolerance (173).

2.2.3 Immune Tolerance and Exhaustion

Immune tolerance is a vital part of the immune system, which encompasses a broad spectrum of processes that result in a state of

non-reactivity towards antigens or immune homeostasis. This ensures protection from harmful, excessive immune responses inside the host, such as against self-antigens or against chronic infections and the ensuing inflammation that can cause significant tissue damage. The major mediators of immune tolerance are the regulatory T-cells, which include the natural and the induced, also called adaptive, Tregs (nTreg and iTreg), located in the thymus and in the periphery, respectively [reviewed in (143, 174)]. Tregs are the major actors involved in inducing systemic tolerance through liver-directed gene transfer, with the liver being a long recognized tolerogenic organ (175). Different markers are used to identify Tregs, most frequently CD4 and CD25 extracellularly and FoxP3 (forkhead box P3) intracellularly (CD4+CD25+FoxP3+ T-cells) (143, 175). The transcription factor FoxP3 is central to establishing the regulatory lineage. iTregs have a transient expression of FoxP3, whereas it is stable in nTregs (176). Tregs mediate tolerance *via* interaction with CD8+ T-effector cells, whereby they can either inhibit proliferation and IFNγ secretion, or induce cell death through granzyme or perforin (177–179). Tolerance through Tregs is also mediated in the liver draining lymph nodes *via* secretion of immunosuppressive cytokines, such as IL2 or IL10 that cause Teff anergy, exhaustion or suppression (88, 180–182), or differentiation of naïve CD4+CD25- T-cells into Tregs (182) [reviewed in (138)].

Different APCs in the liver are critical for the induction of tolerance (96, 110). Kupffer cells (KCs), *i.e.*, liver-resident macrophages, have a less mature phenotype and can induce expansion of Tregs or the conversion of Teff to Tregs, *via* programmed death ligand-1 (PD-L1) expression and IL-10 production (180, 182, 183). Liver sinusoidal endothelial cells (LSECs) are one of the first liver cells to recruit lymphocytes. Yet, due to high levels of IL-10, priming of T-cells is inefficient, thereby promoting Tregs (175, 184) [reviewed in (3, 95, 143, 175)]. Hepatic DCs also have the capacity to induce and maintain tolerance [reviewed in (175)]. Defective antigen presentation in the liver lymph nodes also results in T-cell exhaustion (110). T-cell exhaustion, that is CD8+ T-cells without effector functions, can be caused by interactions with Tregs, cytokines or by activation of inhibitory receptors, such as PD-1, and can also mediate long-term transgene expression (88, 185, 186). Remarkably, these cells persisted in human muscle biopsies even five years post-vector delivery (185).

3 STRATEGIES TO EVADE IMMUNE RESPONSES AGAINST AAVS

AAV vectors have been used successfully in numerous clinical trials and in gene therapies. Still, as detailed above, the host immune system poses a substantial barrier to their broad and effective application. A major but unsatisfactory solution thus far has been the identification of patients with pre-existing, typically humoral immunity and their exclusion from participation. Importantly, several additional approaches were also widely explored to include more patients for whom gene therapy might be a preferred, if not the only therapy available. These can be classified into two major categories, *i.e.*, modulation/suppression of the immune system or engineering of the AAV vector on the level of capsid and/or transgene (**Figure 2**, **Table 1**).

Below, we will discuss a selection of these approaches that have been published in a large body of work and that range from basic research all the way to the clinic.

3.1 Modulation/Suppression of the Immune System

3.1.1 Route of Administration

The route of administration has been strongly implicated in the inhibition by, and the induction of, immune responses. The exact choice is usually determined by the type of disease. Examples for administration routes include intravenous for hemophilia or liver/heart diseases, intramuscular or intravenous for muscle diseases, or intracerebral, intraparenchymal, intrathecal, or intravenous for neurological diseases [reviewed in (1)]. Direct injection into the target organ, such as intramuscular injection (73), is the most straightforward method to avoid circulating antibodies. Additionally, saline flushing to avoid contact with nAbs in combination with direct or balloon catheter-guided vector injection has shown promise (74). Nonetheless, this does not obviate the generation of immune responses after gene therapy (187), which could eliminate transgene expression later on, as explained in detail before. Fortuitously, several delivery methods exist today that facilitate the evasion of these responses (immune-privileged organs) or their manipulation (immune tolerance or T-cell exhaustion).

3.1.2 Immune-Privileged Organs

The intravascular or intravenous route of administration has been predominantly associated with inactivation of AAVs by nAbs in the circulation and interstitial tissues (35, 40, 116, 117, 135, 188). Thus, it is encouraging, albeit not a panacea, that certain tissues provide shelter from the immune system. Three organs are considered immune-privileged, namely, the brain, the eye and the liver.

3.1.2.1 Brain

The CNS, and in particular the brain, have long been considered to be isolated from the immune system by the physical blood-brain barrier (BBB). Moreover, the brain lacks classical draining lymph nodes and APCs in the parenchyma. While these concepts have been challenged (189), CNS gene transfer, either to the cerebrospinal fluid (CSF) (75, 190) and, to a lesser extent, after intraparenchymal injection (191–193), remains comparatively successful notwithstanding the presence of circulating nAbs. Still, in patients with high titers of circulating antibodies, even CNS gene transfer is inhibited (134, 194). This was exemplified in a study in which preimmunization of mice after intramuscular injection hindered subsequent brain delivery. On the other hand, passive transfer of NHP serum containing nAb to mice did not impact gene transfer to the hippocampus or the thalamus (134).

3.1.2.2 Eye

The ocular immune privilege, akin to the one in the brain, was first described in the middle of last century. It is mediated by a

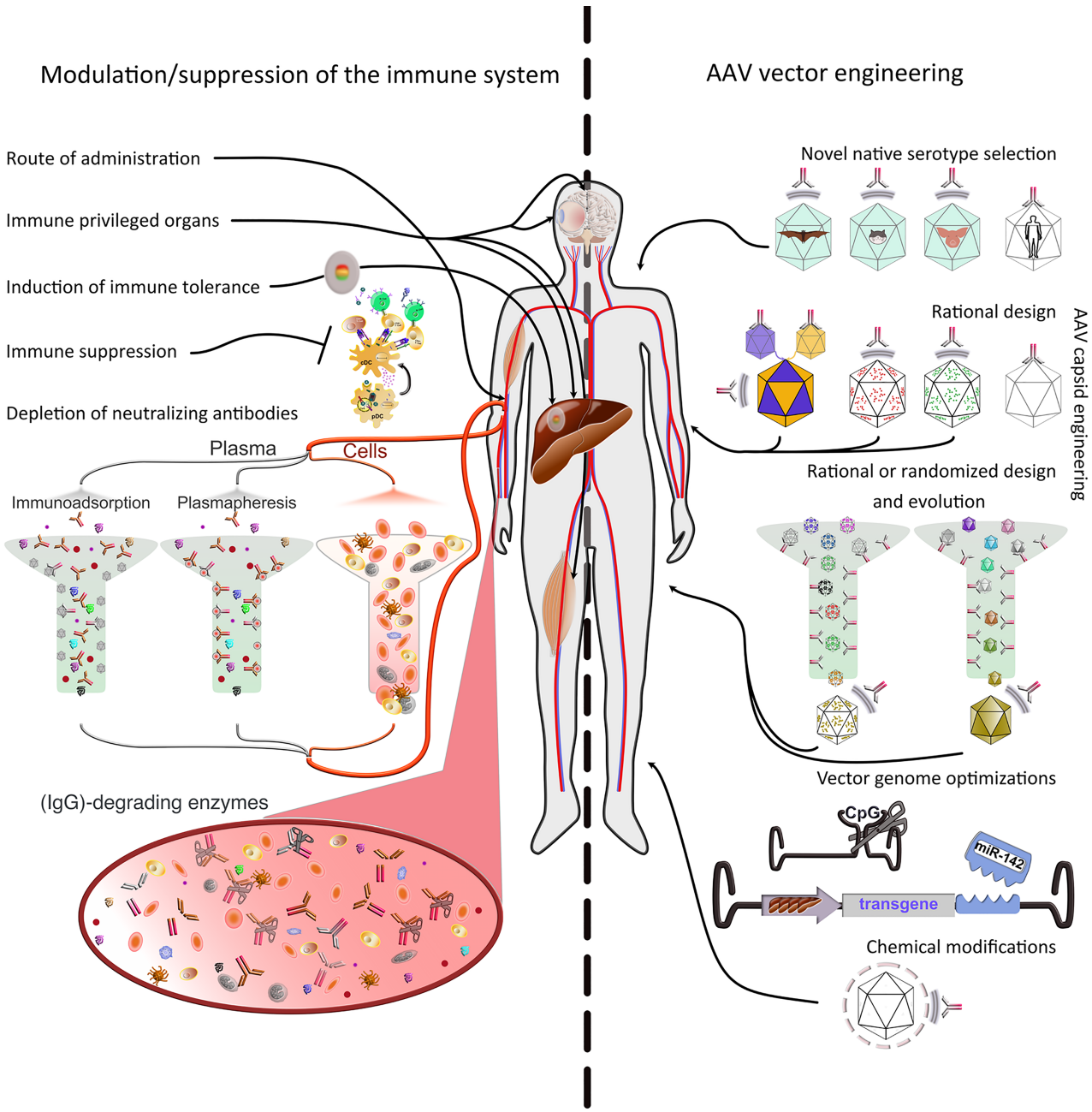

FIGURE 2 | Strategies to evade immune responses. Several approaches to evade immune responses have been applied in the clinic or explored on a basic research level. They can be roughly divided into two main classes, *i.e.*, 1) modulation or suppression of the subject's immune system and 2) AAV vector engineering. 1) Modulation/suppression of the immune system. The route of administration is decisive in evading pre-existing immunity. Targeting immune-privileged organs, when possible, provides protection not only by evading immune responses, but also by inducing regulatory responses. The latter can also be induced through multiple exogenous interventions. Additionally, pharmacological immune suppression has been used extensively in the clinic. Pre-existing immunity (nAbs) is particularly difficult to evade or suppress. Promising approaches include plasmapheresis or the use of immunoadsorption columns *ex vivo* after separation from the cellular parts of the blood. Furthermore, recent studies have used IgG-degrading enzymes *in vivo*. 2) AAV vector engineering. The use of native serotypes from species other than humans or NHPs, or engineering the capsid of existing serotypes (predominantly from primate species) are advantageous strategies to evade immunity. The current serotypes can be modified in defined positions (rational design) based on acquired knowledge about sequence-structure-function relationships. To increase variability, the rational design strategy can be used to generate libraries, which then can be evolved/selected *ex* or *in vivo*. Libraries can also be fully randomized and interrogated for multiple properties, not only immune evasion. Moreover, the viral genome can be optimized to minimize antigen presentation (e.g., *via* cell-type specific promoters, miRNAs to prevent expression in APCs or peptides to inhibit antigen presentation) or immune system induction (CpG depletion). Finally, the capsid can be protected from the immune system by coating with molecules, such as PEG, engulfment in exosomes, or display of immune evasion peptides on its surface.

blood-retina barrier (195), absence of efferent lymphatics, presence of elevated concentrations of immunomodulatory molecules and immunoinhibitory factors, and finally the ACAID system (anterior chamber-associated immune deviation), which is typically stimulated after perturbation of the ocular integrity. ACAID induction by intraocular antigens results in induction of Tregs, immunomodulators in the aqueous humor, anti-inflammatory cytokines and F4/80 macrophages that present the antigen to clusters of immune cells in the spleen (196, 197). The induction of a deviant immune response has already been reported for adenoviral and AAV gene transfer to the subretinal space (198). In combination with other mitigating factors, such as the low effective dosage and hence minimal toxicity and low manufacturing burden, this well-characterized immune privilege has catapulted ocular gene therapy to the forefront of the field [reviewed in (30)], with multiple clinical trials concluded or underway, culminating in the approval of Luxturna™ (199). There are two major delivery methods, subretinal and intravitreal, of which the latter is slightly more immunogenic (76). Although pre-existing immunity can pose a challenge to intravitreal delivery (200, 201), several clinical trials were successful due to the immune-privileged status of the eye (202) [reviewed in (30)].

In contrast to pre-existing immunity, induction of the immune system after gene therapy in the eye was observed more frequently (203), albeit not to the same extent reported for other delivery routes (detailed in previous sections). Induction of a dose-dependent inflammatory response was observed in animal models (204–207) and in humans (208–212). In several clinical trials using either of the two delivery methods (subretinal or intravitreal), inflammation occurred but could be treated with immune suppression regimes. Likewise, transient antibody response against the capsid and cellular immune responses were also noted [reviewed in (30, 76, 213)]. The low to mild and transient immune responses allowed repeated administration of AAV vectors, either contralateral or in one eye (214, 215) or the delivery of two boluses in the same eye (216). Finally, long-term expression could be achieved in most clinical trials, with a decline observed in some after a few years (202, 210) [reviewed in (30, 76, 213)].

3.1.2.3 Liver

Liver is the largest organ in the body, whose sinusoids filter an antigen-rich blood. In order to protect itself from the antigenic overload of nutritional components, chemicals and drugs, liver promotes immune tolerance rather than reaction (217). Even though systemically-delivered liver-targeted gene therapy cannot evade pre-existing immunity which challenges the immune-privileged status of the liver, the ability to harness the tolerogenic hepatic microenvironment has encouraged substantial research in the gene therapy field (145), especially regarding the expression of transgenes that are absent in subjects prior to the injection (187) [reviewed in (95, 175)]. Tolerance is dose-dependent, with higher dosage ensuring immune tolerance through Tregs, IL-10 expression, Fas-L and depletion of Teff cells as enabling factors (88, 110, 218). Another important consideration is whether the transgene product is secreted or not. Secreted proteins are presented in multiple organs and thus need a much lower threshold to produce Tregs. Interestingly, intracellularly restricted expression, e.g., using proteins located in the cytoplasm, results in antigen presentation by liver-draining lymph nodes (celiac and portal) and production of Tregs that are then disseminated to the periphery (96). This tolerance can be harnessed even with gene therapy targeted to other organs. Simultaneous expression of the transgene in the liver can induce expression of Tregs and tolerance to the transgene product expressed in muscle (104, 186). Most interestingly, the induction of immune tolerance through liver can be achieved despite the established presence of inhibitors (219) or even after gene delivery to another organ (88), hence reversing existing immunity [reviewed in (3, 30, 85, 138)].

3.1.3 Induction of Immune Tolerance

Induction of immune tolerance can be achieved by targeting the liver, even at a later timepoint, as elaborated on in previous sections. Alternatively, Tregs can be *ex vivo* reprogrammed and adoptively transferred (97), or they can be induced through molecules (Tregitopes) or chemically (rapamycin). Tregitopes (Treg epitopes) are peptides found within human IgGs and exhibit high-affinity binding to MHCII. They were initially identified in humans (220) and are conserved in mammalian species (221). *Via* a mechanism requiring cell-to-cell contact, Tregitopes can trigger the proliferation of Tregs. Fusion of Tregitopes to the capsid proteins could also reduce CD8+ T-cell reactivity by fostering proliferation of Tregs *in vivo* (105). Alternatively, immunomodulatory drugs, such as rapamycin, can be used to induce Tregs. The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K-Akt-mTOR) pathway regulates thymic and peripheral Treg generation (222). Rapamycin, an immunosuppressing compound used in graft rejection, can expand CD4+CD25+FoxP3+ Tregs (223). A hallmark study showed that simultaneous administration of synthetic vaccine particles encapsulating rapamycin [SVP(Rapa)] with AAV vectors alleviated anti-capsid humoral and cellular immune responses, thus enabling vector re-administration. Adoptive transfer of splenocytes to naïve mice transferred this immunomodulatory property while depletion of CD25+ cells neutralized this effect (89). Gene transfer to seropositive rhesus macaques after rapamycin treatment was only successful using subcutaneous, but not intravenous delivery (224), implying that route and dosing schedule may be key to success (225). Further studies in preclinical models are necessary to assess the efficacy of these treatments in the clinic as well as their safety, as they compromise the immune system and make the host vulnerable to infections [reviewed in (85, 138)].

3.1.4 Immune Suppression

Immune modulation involves a broad range of approaches, of which the earliest and most widely applied in human clinical trials is transient immune suppression predominantly of T-cells with corticosteroids, such as prednisolone (188) [reviewed in (30, 85, 98, 226)]. Immune suppression can also be mediated using alternatives to steroids, such as mycophenolate mofetil (MMF) and tacrolimus (227), or MMF and rapamycin (98). Immune

suppression has had varying effects in studies and clinical trials. Despite positive results (27, 101, 142, 228, 229), it often did not sustain long-term expression of the transgene (88, 230). Of note, sustained immune suppression with daclizumab is associated with a reduction in Tregs (111). Therefore, a recent study proposed new protocols using a meticulously timed T-cell-directed IS, with an early administration of MMF and rapamycin and a delayed delivery of anti-thymocyte globulin (ATG), to determine the balance between immunogenicity and tolerance (98). Immune suppression regimens were also used to deplete B-cells and thereby nAbs, such as rituximab with cyclosporine (231), rituximab with sirolimus (90), or rituximab alone (232), rapamycin without or with prednisolone (89, 233), and anti-CD20 with rapamycin (234). However, one should be aware that such regimens target the immune system of the host as a whole and are not specifically tailored to the gene therapy. Finally, another intriguing approach that has shown great promise but requires further studies is inhibition of proteasomal processing of internalized capsids, which is necessary for antigen presentation and part of the immune reaction process, using a variety of molecules (146, 148) some of which are also approved for use in humans (146) [reviewed in (138)].

3.1.5 Depletion of Neutralizing Antibodies

Immune suppression offers only moderate capabilities to evade pre-existing immunity, which would otherwise disqualify 15-50% of the population from clinical trials. Accordingly, additional approaches are urgently needed (235). Recently, a hallmark study illustrated the potential of Imlifidase (IdeS), a cysteine endopeptidase derived from the immunoglobulin G (IgG)-degrading enzyme of *Streptococcus pyogenes*. IdeS cleaves human IgG into $F(ab')_2$ and Fc fragments, thus eliminating its Fc-dependent effector functions. In this study, the authors successfully showed the cleavage of human intravenous immunoglobulin (IVIg, a cocktail of serum from thousands of human individuals) *in vitro*, as well as in pre-immunized mice and seropositive NHPs *in vivo*. IdeS administration prior to AAV gene transfer *in vivo* reduces the levels of IgGs, thereby allowing efficient transduction. Treatment with IdeS also enabled readministration of AAVs. Finally, the efficiency of IdeS was also successfully validated with human sera (236). In a similar study, IdeZ, an IdeS homolog isolated from *S. equi* ssp. *zooepidemicus*, showed efficient IgG cleavage of dog, monkey and human sera and facilitated *in vivo* gene transfer in passively immunized mice and macaques (237). Moreover, a large protein, protein M, was identified in *Mycoplasma* that binds Igs with broad reactivity by using a different mechanism. *In vitro* and *in vivo* studies showed the efficiency of this approach to protect AAVs from nAbs and to allow readministration (235, 238).

Removal of anti-AAV nAbs has also been attempted through plasmapheresis, an extracorporeal method in which a device separates plasma from the cellular component of the blood. Afterwards, the plasma is filtered through various techniques, remixed with the blood cells and returned to the subject. Alternatively, the plasma can be substituted by a replacement solution. Plasmapheresis was used to remove immunoglobulins against the capsid in humans (239) and in NHPs (77). In the latter study, AAV gene transfer after plasmapheresis resulted in efficient transduction (77). While promising, this approach has several limitations including that multiple rounds are needed. A further concern is the so-called "antibody rebound" effect, which means that the antibody pool is readily replaced. Also, certain patients with weak physical conditions might be more adversely affected by the procedure, and the complete removal of immunoglobulins leaves the patients vulnerable to infections (77, 239) [reviewed in (47, 138)]. Recently, elegant studies have thus combined plasmapheresis with immunoadsorption columns loaded with empty AAV capsids, in order to selectively deplete nAbs against the AAV capsid (78, 79). In a similar manner, a prior study exploited empty AAV capsids that were injected to mice together with the AAV vectors to act as decoys for AAV-specific nAb (240). However, a concern is that excess AAV capsids could elicit stronger cellular immune responses or immunotoxicities (151) [reviewed in (42)]. It should also be noted that these procedures non-discriminately remove all AAV-binding antibodies, some of which do not neutralize but actually enhance transduction or alter distribution (126, 241).

3.2 AAV Vector Engineering

3.2.1 Novel Serotype Isolation

The majority of the AAV serotypes that are currently in clinical use are human or NHP isolates from liver or spleen [reviewed in (1, 2)]. However, a concern with their use is that epidemiological studies show a high seroprevalence of those serotypes in the human population (114, 115, 121, 242, 243). A rational approach to overcome this concern is to isolate novel natural AAV serotypes from other species, which are expected to exhibit lower seroprevalence in humans but may also display lower transduction efficiencies. To this end, novel native AAVs have already been isolated from avian (244), rat and mouse (14), caprine (245), porcine (246, 247) and bat (80, 248) species. Some of these showed promise in *in vivo* biodistribution studies where they were found capable of targeting multiple tissues (246), such as heart (246), muscle (246, 247, 249), lung (245) or retina (247). Despite these promising results, further studies are necessary in larger, preclinical animal models to better characterize and ideally validate their potential for clinical application.

3.2.2 AAV Capsid and Transgene Engineering

3.2.2.1 Rational Capsid Design

There is a growing amount of information on immunogenic epitopes on the AAV capsid, based on cryo-EM structural mapping studies (250–255) [reviewed in (253)], single point mutants (256–258), or barcoded (9, 259) or not (260, 261) libraries of pooled vectors carrying point mutations. For the cryo-EM studies, monoclonal antibodies (mAbs) predominantly from hybridoma screens have been used, although recently, the isolation of human mAbs that are more clinically relevant has also been pursued (262). These studies facilitate the rational design of vector capsids or the engineering of libraries directed at the immunogenic epitopes, in order to render the complexity of the library technically attainable and to concurrently circumvent

unwarranted changes to essential properties, such as tropism. Such information was applied to enhance nAb evasion by mutagenizing single [V719M (263), S671A (264), 265T (256)] or multiple positions (265, 266). A similar approach was used to modify surface tyrosines to avoid ubiquitination (267), proteasomal degradation and antigen presentation (149, 268, 269). This can be extended to other amino acids (serine, threonine, and lysine) (270, 271) and serotypes (272). Polyploid vectors, also called mosaic, combine capsid subunits from different serotypes and are formed by simply mixing the production plasmids at different ratios. This methodology identified a triploid AAV2/8/9 vector that could evade immunity more than the parental vectors (273).

A conceptually different approach is to display peptides on the surface of the capsid to evade or quench the immune system. Display on the AAV capsid of a self-peptide (SP), a 21-amino-acid long truncated bioactive form of CD47, whose binding to SIRPα (Signal regulatory protein α) on macrophages acts as a "don't-eat-me" signal for macrophages, was shown to reduce macrophage uptake (91). Likewise, fusion of Tregitopes on the AAV capsid protein VP1 reduced CD8+ T-cell responses and increased Tregs (105).

3.2.2.2 Rationally Designed Capsid Libraries and Directed Evolution

The wealth of knowledge regarding the immunogenic epitopes on the AAV capsid has been mostly implemented in rational engineering and directed evolution of viral libraries, to create novel, engineered immune evading AAVs. In an early study, five immunogenic amino acid positions (449, 458, 459, 493, 551) were randomized on AAV and the library was evolved under negative selection pressure using IVIg, producing immune-escaping variants (260). By studying the AAV1 complexed with four different Fabs of mouse anti-AAV1 mAbs, the cryo-reconstructed structures revealed three capsid antigenic footprints: region IV (456-AQNK-459), region V (492-TKTDNNNS-499), and region VIII (588-STDPATGDVH-597). Each region was separately randomized, and the libraries subjected to iterative rounds of evolution on vascular endothelial cells that are highly permissible to AAV1, in order to evolve the library for properties other than efficient transduction. The top variants were then combined albeit some could not be juxtaposed, as observed previously (274). In this case, the variant was combined with a library based on another footprint and a new evolution was applied. One of the evolved CAMs (Capsid Antigenic Motifs), CAM130, was significantly enhanced compared to the parent vector and, whilst maintaining tropism, showed an advantageous, immune-evading serological profile (81). A similar study evolved an AAV8-based variant, AAVhum.8, that exhibits mouse and human hepatocyte tropism and sera-evading properties (275). Another group harnessed a mAb PAV9.1 selected from a panel of hybridoma clones to identify a conformational epitope on AAV9 for further mutagenesis that comprises 494-TQNNN-498 and 586-SAQAQ-590. These Fab complementary determining regions (CDRs) were mutated using serotype swapping, alanine replacement, and additional point mutations. The resulting CDRs were efficient at evading the mAb binding, but they could not evade binding or neutralization by polyclonal serum or plasma from mice, macaques, or human donors (276). Another recent rational approach focused on residues identified to be different among 150 AAV3B variants, selected the surface-exposed ones and mutagenized them only to naturally occurring residues in this position. The library was evolved in human hepatocarcinoma spheroid cultures and the top variant, AAV3B-DE5, was further evaluated. It exhibited reduced seroreactivity against IVIg and individual human samples as well as tropism towards human, but not mouse hepatocytes in chimeric livers, similar to the parental serotype (277). Rational design strategies have contributed significantly to the field. However, screening of completely randomized libraries without *a priori* knowledge of immunogenic epitopes also offers valuable solutions, as discussed next.

3.2.2.3 Randomized Capsid Libraries and Directed Evolution

Complementing the efforts outlined in the prior chapter that rely on limited antecedent knowledge to facilitate some degree of rational design followed by further AAV evolution, many groups have devised and applied experimental forward-oriented strategies in order to identify immunoevasive AAV variants. To this end, comprehensive libraries of synthetically engineered AAV capsid variants are first generated and then subjected to a negative selection pressure, which ideally eliminates all candidates that cross-react with, and are neutralized by, anti-AAV antibodies. The methodologies to create such diverse capsids libraries are manifold and have been extensively reviewed in the past by us and others (2, 278), hence it may suffice below to name some of the most widely used approaches including DNA family shuffling, peptide display, error-prone PCR and ancestral reconstruction.

One of the first studies to illustrate the power of directed AAV library screens and antibody-mediated selection was reported by the Kay lab in 2008 (279), which exploited the fact that IVIg contains a mixture of anti-AAV antibodies that is a good proxy for the human population. Accordingly, the group first created a library of ~7×10^5 shuffled AAV capsid variants from eight parental viral serotypes and then iteratively amplified this capsid pool on human liver cells in the presence of IVIg, with the aim to eliminate all variants that were recognized by the anti-AAV antibodies. Indeed, this strategy enabled the isolation of a single chimeric AAV variant, called AAV-DJ, that at least partially resisted antibody neutralization *in vitro* and *in vivo* to a much greater extent than AAV2, which is one of its dominant parental serotypes and which has been used extensively in humans to date. The ability of shuffled AAV capsids to partially evade IVIg neutralization was later also confirmed by several other groups including notable work from Koerber et al., albeit this group only used IVIg during the stratification of already isolated chimeric AAVs and not for selection (280).

Similarly, in another representative example from the Schaffer lab (261), negative selection *via* a neutralizing anti-AAV2 rabbit serum was harnessed to enrich antibody-resistant AAV capsid variants from libraries that had been created through error-

prone PCR amplification of the entire AAV2 *cap* gene. Interestingly, their lead candidate had not only become more antibody-resistant than wild-type AAV2 but had also acquired additional properties, such as altered DNA packaging efficiency, heparin affinity or cell specificity, likely explained by the well-known pleiotropic roles of many residues within the AAV capsid proteins.

These conclusions were confirmed in a flurry of more recent work from several labs, which cannot be covered comprehensively here; hence we will only highlight examples below that are representative for numerous other studies. One such example is another pivotal study by the Kay lab (82) in which Paulk and co-workers performed a multiplexed AAV library screen combining iterative *in vivo* selection of a shuffled library in "humanized" mice (*i.e.*, mice xenotransplanted with human hepatocytes), followed by two rounds of *ex vivo* AAV capsid depletion on IVIg-coated beads. Of the shuffled capsid variants enriched by this procedure, NP59 is remarkable as it combines robust and specific *in vivo* transduction of human hepatocytes with good performance in seroreactivity and transduction neutralization assays, including the use of individual serum samples from macaques or humans (healthy or hemophilia B patients), or again IVIg. A very similar approach has also been reported more recently by the Li lab (281), using a different starting library and resulting in unique shuffled AAV variants. As a last example in this category, a study by the Samulski lab should be pointed out, which is remarkable for the fact that Li et al. selected a shuffled AAV capsid library in the presence of neutralizing sera not only in cultured cells, but directly in the muscle of mice (282, 283). Furthermore, rather than using an IVIg pool for selection, the group harnessed individual sera from human patients who had participated in a clinical trial for Duchenne muscular dystrophy with an AAV2.5 vector. A particularly notable conclusion in this work was such a stringent selection strategy may be beneficial, since pools such as IVIg are a mixture comprising sera without neutralizing anti-AAV antibodies, hence AAV capsid variants emerging from IVIg selection may only escape neutralization in subjects with high individual antibody titers, whereas those selected with a stringent serum may be more broadly resistant.

Similar conclusions were also drawn in parallel work in which capsids were diversified not *via* DNA shuffling, but rather *via* insertion of short peptides (typically 7 to 14 amino acids long) on the capsid surface. While the primary purpose of these peptides is binding to (usually unknown) receptors on target cells, several groups have reported that display of these short additional peptides on the AAV shell can also modulate its recognition by neutralizing antibodies. As a representative example, one of the first studies reporting this finding should be highlighted (284), in which Huttner and colleagues found that insertion of peptides selected in previous screens in AAV2 amino acid positions 534 and 573 substantially reduced capsid affinity for neutralizing anti-AAV2 antibodies in human sera.

In general, it should be an interesting goal for future work to compare the lead candidates from these and other studies side-by-side in genuine or humanized mouse livers in the presence of anti-AAV antibodies, ideally using a vector DNA/RNA barcoding strategy (285) to enable a fair comparative analysis in the same animal(s). Besides, the aforementioned research, along with the rational design studies, further corroborates the notion that antigenic, tropism and potency determinants overlap in the structural context of the AAV capsid and thus perfectly complements the alternative strategies noted above, not only on a technical but also on the biological level.

3.2.2.4 Chemical Capsid Modifications

Rather than engineering the capsid itself, either *via* directed evolution or rational design, numerous groups have pursued an alternative and complementary strategy to mask AAV from neutralizing antibodies, by either chemically modifying the shell or encapsulating the particles in extracellular vesicles (exosome). As with the capsid engineering approaches, the diversity of strategies is so substantial and the literature so complex that we can only highlight a few representative examples below, and we apologize to all colleagues whose work we had to omit for space reasons.

One particular strategy that has been reported frequently is AAV modification *via* chemical conjugation with polyethylene glycol (PEG), which is a simple, cheap and effective means to cover and protect the capsid from neutralization, but which may also come at the cost of (steric) interference with AAV transduction and tropism. This dilemma was exemplified, for instance, in one of the first reports of AAV PEGylation by Lee and co-workers (286) who found that there is only a small window of PEGylation, *i.e.*, PEG:lysine conjugation ratio and PEG molecular weight, that enables effective antibody protection while maintaining infectivity. Subsequently, other used various alternative strategies for AAV PEGylation, such as AAV2 modification with a series of activated PEGs, some of which yielded protection from neutralization without severely impeding transduction efficiency (287), or use of genetic code expansion for insertion of a lysine mimic into the AAV2 capsid that enabled site-specific PEGylation and escape from neutralization (288).

Instead of coating the AAV particle *via* chemical modification, other groups, most notably the one of Casey Maguire, rather encapsulate the vector particles in naturally occurring cellular vesicles, resulting in what was originally called "vexosomes" and later exo-AAVs. As, for instance, demonstrated by György et al. in 2014 (83), exosome-encapsulated AAV9 vectors were significantly more resistant to both, pooled human serum as well as IVIg, and they also performed better than naked AAV9 in passively IVIg-immunized mice. Importantly, this method is not restricted to a particular serotype, since escape from neutralization in cultured cells was also observed for AAV1 and AAV2. These encouraging results were corroborated and extended in a series of more recent studies, including a notable piece of work from Meliani et al. who showed that exo-AAV8 vectors (and also exo-AAV5) performed better than the wild-type counterpart at liver-directed human factor IX expression in mice, perhaps owing to a faster nuclear translocation rate and autophagy-independent trafficking. Remarkably, in turn, the higher expression was also correlated with an increased frequency of Tregs in lymph nodes

of exo-AAV8-treated mice, suggesting that improved induction of immunological tolerance may be an additional benefit of exosome-encapsulated AAVs. Moreover, exo-AAV8 was also more resistant to neutralizing anti-AAV8 antibodies in human sera and in a passive immunization mouse model of liver gene transfer, further illustrating the potential of this strategy to expand the proportion of human subjects who are eligible for AAV gene therapies.

Finally, another intriguing approach worth mentioning has recently been reported by Katrekar and colleagues (289), who combined genetic code expansion and click-labeling to precisely tether oligonucleotides to the surface of the chimeric AAV-DJ capsid (279). When incubated with lipofectamine, this resulted in so-called "cloaked" AAVs that were much more resistant to neutralizing anti-AAV antibodies and concurrently yielded higher CRISPR gene editing efficiencies than an unconjugated AAV-DJ control, reminiscent of the dual benefit observed with the exo-AAV strategy (see above).

While these and other, equally compelling studies that could unfortunately not be mentioned here are very promising, it is also clear that additional, meticulous work is needed and that a number of challenges have to be overcome before chemically modified AAVs can be used more broadly and even clinically. This includes the need to optimize and standardize large-scale production and purification protocols, as well as the requirement of a thorough characterization of possible impurities and contaminations especially in cell-derived exo-AAVs. In addition, immune responses against PEG or lipofectamine will have to be studied and, if detected, may limit the widespread application of certain formulations. Last but not least, cloaking or coating the viral shell may inadvertently and negatively impact *in vivo* features such as biodistribution, kinetics and blood clearance, which would be particularly detrimental for synthetic capsids that have been genetically engineered to have a more defined tropism or other advantageous properties.

3.2.2.5 Vector Genome Optimization

Next to the viral capsid, also the cargo, *i.e.*, the recombinant genome consisting of the transgene expression cassette flanked by the ITRs, offers multiple opportunities for engineering and alleviation of immune responses, including its structure and components.

Regarding structure, a favorable design with respect to efficiency are self-complementary (sc) or double-stranded AAV vector genomes, which——after replication and encapsidation——carry two inverted copies of a transgene that rapidly and effectively self-anneal in the transduced cell. Thereby, scAAV genomes alleviate the rate-limiting step of second-strand DNA conversion that normally restricts transduction with conventional single-stranded AAV vectors and that explains their slow kinetics of transgene expression that is typically observed *in vivo*. While beneficial in this aspect, a drawback of scAAV vector genomes that has become apparent over the last decade is their higher propensity to trigger an innate immune response *via* the endosomal DNA receptor TLR9, as observed in several tissues including the liver and the muscle. This includes early data in mice where scAAV vectors led to increased expression of various innate immune-related genes and induced innate responses in a dose-dependent manner *via* TLR9 signaling. This, in turn, enhanced adaptive immune responses to the capsid but not the transgene product, probably due to the short-lived and self-limiting nature of the innate response (58).

Still, in independent work, the Ertl lab showed that scAAV vectors, as a result of their faster transgene expression kinetics, are also more prone than ssAAV to induce transgene product-specific CD8+ T-cell and B-cell/antibody responses in mice (164). The extent of these responses depended on the capsid serotype, with AAV7 yielding much stronger effects than AAV2, probably owing to the higher efficiency of AAV7 in the muscle.

While both studies unanimously concluded that AAV genome configuration governs the immunogenicity of AAV particles, the authors also concurred that lowering doses may allow scAAV vectors to dodge the immune system. Alternatively, or in addition, as demonstrated consistently by the Wilson, Ertl and Church labs, innate immune responses can also be blunted by directly engineering the vector genome. First, Faust and colleagues reported that vector genomes depleted of CpG islands, which are typically sensed by TLR9, evade the innate and adaptive immune response and establish persistent transgene expression in skeletal muscle in mice in the absence of T-cell infiltrates, even when delivered by a highly immunogenic AAVrh32.33 capsid (67). Concurrent with this, and as already noted above in chapter 2.2.2.1, the Ertl lab found that CpG depletion in AAV vector genomes can diminish a primary, *de novo* T-cell response, by reducing expansion of naïve CD8+ T-cells against AAV capsid epitopes (63). In contrast, these engineered vectors triggered a secondary response, by driving proliferation of anti-AAV capsid-specific memory CD8+ T-cells, a phenomenon that was also observed with empty AAV capsids. The latter is particularly relevant in view of the fact that spiking in empty capsids into AAV vector preparations has previously been shown to dampen the humoral immune response to the viral particles, implying that the empty capsids acted as antibody sponges. Hence, the findings by Xiang et al. (63), that empty capsids do not stimulate a primary T-cell response to antigenic capsid epitopes and also do not boost T-cell activation triggered by full AAV particles, are pivotal for our understanding of the role of full *versus* empty capsids and for the future optimization of strategies to circumvent humoral and cellular anti-AAV immune responses.

Most recently, Chan and co-workers moreover showed that TLR9 activation can also be dampened through incorporation of short oligonucleotides that antagonize TLR9 activation, dubbed TLR9i (i for inhibitory) (86). Such TLR9i sequences were found to cloak the AAV vectors in multiple, but not all tested animal models and tissues, confirming the pivotal role of TLR9 sensing but concurrently implying the existence of other, TLR9-independent immune mechanisms. The latter may also have contributed to the results of clinical trials using AAV8 for expression of the human blood coagulation factor IX in hemophilia B patients, in which capsid-specific CD8+ T-cell responses were observed despite the use of CpG-reduced vector genomes (101). In this context, another original hypothesis that is noteworthy and that was presented by Li and Samulski (1) suggests that the intrinsic promoter activity of the AAV ITRs

might drive the generation of dsRNA at later stages of transduction, in turn activating cellular RNA sensors and thus stimulating an innate immune response. If true, this would imply a solution whereby this promoter activity is diminished or blocked, by deliberately engineering the ITRs and/or insulating the expression cassette from the ITRs (1).

Interestingly, the authors of the latest work on TLR9i postulated that these sequences may act by outcompeting CpG islands in the vector genome for TLR9 binding, potentially *via* their higher TLR9 affinity and their ability to prevent TLR9 dimerization and activation upon binding (86). While not tested in this work, this raises the intriguing question whether combining TLR9i with the aforementioned depletion of CpG islands may synergize and further dampen innate immune responses to the AAV vector genome. Additional topics for future work should include the persistence of the effect and the impact on vector transgene expression, as well as on clinically relevant immune responses, including humoral or cellular immunity to the capsid proteins or the transgene product. Furthermore, it will be prudent and informative to assess this strategy in more animal models and with other targets, using different clinically applicable administration routes. Until then, the fact that Chan et al. noted beneficial effects on the anti-AAV immune response in a variety of experimental settings and largely independent of the other vector components, such as capsid or promoter, is already highly encouraging as it suggests a large degree of versatility, modularity and translatability.

4 TOXICITY

Despite the success of AAV-based gene therapeutics and the prevailing view that AAV is less immunogenic than other recombinant virus platforms, rapidly mounting evidence from preclinical work in large animals and clinical studies in humans implies that AAV vectors can cause inflammatory and immune responses as well as other dose-dependent toxicities and pathologies. This includes observations of severe adverse events, possibly related to innate and cellular immune responses, in patients suffering from Duchenne muscular dystrophy (DMD) or spinal muscular atrophy type 1 who had been treated with high AAV vector doses (290, 291). In the DMD trial (IGNITE), treatment of a boy with a high dose ($2x10^{14}$ vg/kg) of the therapeutic vector (SGT-001, encoding micro-dystrophin) resulted in lower red blood cell and platelet counts (thrombocytopenia), caused kidney damage, and activated the complement system. Fortunately, these complications were all resolved and the sponsoring company (SOLID) improved their manufacturing protocol (to reduce the number of empty capsids), eventually allowing the FDA to lift their initial ban on this trial.

Similar findings were reported in a phase I trial (sponsored by Rocket Pharmaceuticals) for treatment of Danon disease, a devastating and rare X-linked autophagic vacuolar myopathy that results from mutations in the LAMP-2 gene and that can cause dysfunction of the musculature and other organs, frequently triggering early mortality. In this trial, one patient treated with the high dose ($1x10^{14}$ vg/kg) and who had a pre-existing anti-AAV9 immunity, experienced adverse events that were presumably immune-related and that also comprised thrombocytopenia and acute kidney injury. Luckily, also this patient ultimately recovered and regained normal organ function. Still, the serious and consistent adverse events in these two trials clearly raise a warning flag about possible toxicity from *in vivo* application of excessive AAV vector doses.

In addition, AAV dose-dependent pathology was observed in dorsal root ganglia (DRG) in vector-treated non-human primates that seemed to be largely independent of capsid or cargo (292, 293). The effects were only mild to moderate and not associated with fatalities; moreover, a possible mitigation has recently been proposed in which the inclusion of binding sites for miR-183 a miRNA largely restricted to DRG neurons, can alleviate DRG toxicity (294). Nonetheless, the possible link between AAV delivery and sensory neuropathies in DRGs requires further investigation and careful monitoring in ongoing or future clinical studies.

However, most dire and most alarming is the outcome of the recent ASPIRO trial, in which three children affected by X-linked myotubular myopathy (XLMTM) and given a very high dose of $3x10^{14}$ AAV8 vg/kg bodyweight developed progressive liver dysfunction, bacterial infection and sepsis (two of the three patients), eventually resulting in death of all three individuals (295–298). Also here, a critical role of the immune system has been suspected, including the presence of anti-AAV antibodies in these patients that could have triggered an innate response or activated the classical arm of the complement system. Notably, none of the patients in this trial who had received a lower vector dose developed liver-related adverse events. Instead, several children regained the ability to sit, stand or walk and no longer needed ventilator support, clearly illustrating the great potential of this gene therapy approach and of the vector used. Of note is that no such toxicities had previously been observed with the same vector in mice or non-human primates, even using higher doses of $8x10^{14}$ AAV8 vg/kg, and that very encouraging efficacy data had been obtained in murine and canine XLMTM models (299, 300). This highlights the species-specific differences in AAV-host interactions and the urgent need for better characterization including a possible contribution by immune mechanisms, with the clinically highly relevant aim to control and overcome, or ideally altogether prevent, such adverse events in patients.

Last but not least, we note another very recent, serious adverse event in an AAV gene therapy clinical trial (INFINITY, company sponsor Adverum), this time the loss of sight in a patient with diabetic macular edema who was treated with a high dose ($6x10^{11}$ vg/eye) of a vector based on a synthetic AAV capsid (AAV2.7m8). The fact that the eye is more immunoprivileged than other organs (see also chapter 3.12.2.) and that the effect occurred long (30 weeks) after dosing may argue at least against an acute anti-AAV immune response, but the mechanisms are still unclear.

5 CONCLUSIONS

The historic fable "The race between the hare and the hedgehog", originally published by the Brothers Grimm in 1843, describes

the race of two animals, one fast (hare) and one slow (hedgehog), of which the latter has no chance of winning the contest. Still, the hedgehog seemingly outcompetes the hare through cheating, as he places his lookalike wife at the finish line and thereby ultimately frustrates the hare to an extent that it dies. For many reasons, one can readily apply the same image and draw comparisons to the incessant arms race between AAV and its human host, starting with natural infections and nowadays significantly accelerated by the growing clinical use of recombinant AAV vectors. Akin to the fable, there is substantial hope that the hedgehog in this analogy, *i.e.*, the AAVs, will eventually win this race against the host (the hare), assisted by a little cheating in the form of our ever expanding understanding of AAV-host interactions and the concurrent advent of powerful new technologies to blunt or alleviate anti-AAV immune responses, a collection of which has been highlighted and discussed in this article. It is also evident that this looming happy ending requires a considerable body of additional, concerted work from the field, aimed at a better characterization of the new, genetically engineered, evolved or designed, and/or chemically modified AAV particles along with improvements in the technology for their clinical-grade manufacturing including production, purification, quality control and batch release. Still, in view of the remarkable pace which the race between AAV and the host has picked up in recent years, there is every reason to hope and believe that the fairy tale of AAV gene therapies in the absence of adverse immune responses will eventually become a reality.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

KR was supported by a Marie Curie International Incoming Fellowship PIIF-GA-2013-627329. DG greatly appreciates support by the German Research Foundation (DFG) through the DFG Collaborative Research Centers SFB1129 (Projektnummer 240245660) and TRR179 (Projektnummer 272983813), as well as by the German Center for Infection Research (DZIF, BMBF; TTU-HIV 04.819). DG is moreover grateful for support from the ANR/DFG-funded project MATRIXNASH and the DLR-funded project KARTLE.

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Conflict of Interest: DG is a co-founder and shareholder (CSO) of AaviGen GmbH. DG and KR are inventors on a pending patent application related to the generation of immune-evading AAV capsid variants.

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