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Ocular Inflammation and Treatment Emergent Adverse Events in Retinal Gene Therapy

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1. Introduction

Viruses are the ideal vehicles to deliver therapeutic genes to target tissues. Viral vectors used for gene therapy differ from their natural parent viruses in that they are non-replicative, non-pathogenic, and are delivered as a high-titer bolus at an unnatural site.[1] However, similarities between the natural virus and viral delivery vector, such as capsid proteins, may result in host immune responses that may limit gene transfer or transgene expression. Additionally, the transgene product may also be targeted by the immune system resulting in ocular inflammation and removal of transduced cells. Finally, treatment emergent adverse events (TEAEs) may be associated with technical challenges of delivering viral vectors to the eye. Here, we review host immune responses to viral-mediated gene transfer, the role of ocular immune privilege, and factors that determine immunogenicity of retinal gene therapies. We discuss ocular inflammation and TEAEs from preclinical and clinical studies and explore strategies to mitigate these responses.

1.1 Viral Vectors for Retinal Gene Therapy

The effectiveness of a viral vector system for gene delivery depends on the target tissue, cellular tropism, packaging capacity, genome integration, and immunogenicity.[1] Three viral vectors that have been widely used in ocular gene therapy include adenovirus (Ad), lentivirus (LV), and adeno-associated virus (AAV). Although gene transfer can be accomplished *ex vivo* using an integrating virus such as lentivirus to introduce transgenes into stem cells, for example, for transplantation, this review will focus on *in vivo* gene transfer into postmitotic cells using viral vectors that enable either integrating or episomal DNA for long term expression.[2]

The Ad vector contains ~36 kilobases (kb) double-stranded DNA (dsDNA) in a viral capsid. First evaluated in respiratory conditions such as cystic fibrosis[3], its high packaging capacity and high transduction efficiency was thought to make it an ideal viral vector. However, its durability is limited by severe innate and adaptive host immune responses. A sobering reminder of this limitation is when an 18-year-old with partial ornithine deficiency who 18 hours after hepatic gene transfer went into multisystem organ failure due to disseminated intravascular coagulation.[3] When the viral capsid becomes tagged by coagulation Factor X,

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toll-like receptor (TLR) 4 on splenic macrophages are activated and set off a cascade of immune responses to create an anti-viral environment.[4] Ad also binds to complement component C3 and IgM antibodies resulting in neutrophil activation, while Ad-antibody complexes attract macrophages. An adaptive immune response also occurs with activation of CD8+ T cells and development of neutralizing antibodies (NAbs) which can interfere with viral vector re-administration.[4] This overwhelming immune response triggered by Ad rendered its application in *in vivo* gene transfer ineffective and unsafe. Alternatively, its short-term gene expression and ability to induce CD8+ T cells makes it ideal for use in vaccines and anticancer therapy, for which Ad could deliver cytotoxic genes to tumor cells and also induce a strong immune response against them.[4] Use of Ad allowed the scientific community to understand the intricacies of the immune response to viral vectors and laid down the foundation for exploring other viral gene therapies.

Lentivirus (LV), such as the human immunodeficiency virus (HIV), is an enveloped virus with single-stranded RNA which upon infection is reverse transcribed in the host cell's cytoplasm. It has a high packaging capacity of ~8 kb and can transduce multiple cell types, making it suitable for inherited retinal diseases (IRDs) such as Stargardt disease.[5] However, it primarily integrates into the host's DNA and can lead to insertional mutagenesis. [4] Although preexisting immunity to LV is low, high levels of T1 interferon (IFN) mediated by TLRs limits its efficacy for gene transduction *in vivo*. Furthermore, efficient transduction of macrophages and dendritic cells can trigger a strong immune response to the transgene product. Nevertheless, its stable integration allows for effective long-term gene expression for *ex vivo* gene transfer.

AAV is a small non-enveloped parvovirus with a single-stranded DNA genome of ~4.8 kb. AAV is not naturally pathogenic and does not replicate autonomously, relying on a helper virus such as adenovirus for replication. The removal of viral-coding sequences except for the inverted terminal repeats maximizes the packaging capacity of recombinant AAVs (rAAV) and contributes to their low immunogenicity and cytotoxicity *in vivo*.[6] Its low immunogenicity may be due in part to low antigen presenting cell transduction.[7] AAV also exists largely as extra-genomic episomal concatemers and seldom integrates into the host genome, thus reducing the risk of insertional mutagenesis.[8] Neuronal cells are also ideal targets for AAV vectors because episomal persistence is enhanced in nondividing cells. For these reasons, rAAV has emerged as the leading viral vector for *in vivo* ocular gene therapy.

1.2 Applications of Retinal Gene Therapy

rAAV has been evaluated for retinal gene therapy for various IRDs.[8] In 2017, the Food and Drug Administration (FDA) approved the first retinal gene therapy application with subretinal delivery of a rAAV vector carrying the RPE65 transgene (voretigene neparvovec-rzyl, Luxturna®, Spark Therapeutics, Philadelphia, PA) for the treatment of biallelic *RPE65*mutation–associated retinal dystrophy.[9] Among 37 subjects who received 1.5×10^{11} vector genomes (vg) in at least 1 eye in the phase 1 and 3 studies, performance on multi-luminance mobility testing and mean white light full-field light sensitivity threshold, which measures the lowest illumination perceived over the entire visual field, was stable out to 4 years.[9] All patients who received the intervention received perioperative systemic steroids

and the safety profile was consistent with the vitrectomy and subretinal injection procedure. Although one patient developed endophthalmitis, no other adverse immune responses were noted. These studies suggest that the effect of subretinal AAV-mediated gene therapy can be durable for at least 4 years without a major immune response.

Although the defective RPE65 gene in type 2 Leber Congenital Amaurosis (LCA) is naturally expressed primarily by retinal pigment epithelial (RPE) cells, other IRDs may result from genetic defects in other cell types such as photoreceptors, bipolar cells, Muller glia, or choroidal cells. Other retinal gene therapies currently under evaluation include transgene delivery of the rab-escort protein REP-1 for choroideremia,[10-13] rod photoreceptor ATP-binding cassette transporter ABCA4 for Stargardt disease,[14] cone photoreceptor cyclic nucleotide-gated channel subunits CNGA3[15] and CNGB3[16] for achromatopsia, retinoschisin RS1 for X-linked retinoschisis,[17] myosin MYO7A for Usher syndrome,[18] and the GTPase regulator RPGR for X-linked retinitis pigmentosa (XLRP).[8] Gene therapies can also be applied as a biofactory approach for treating neovascular and degenerative conditions such as age-related macular degeneration (AMD) by employing rAAV to enable sustained production of antibodies or inhibitory proteins targeting vascular endothelial growth factor (VEGF).[19-22]

2. Host Immune Responses

While host immune responses are typically directed against microbial pathogens such as AAV, they can also recognize transgene products as foreign in patients with inborn genetic mutations, as evident in AAV-based gene therapy trials for Duchenne's muscular dystrophy, where patients developed an adaptive immune response against the dystrophin transgene product.[23] Additionally, immune responses can be triggered not only by viral capsids and/or transgene proteins, but also by the viral genome or transgene itself. Although the eye is considered to be a site of immune privilege, the immunogenicity of ocular gene therapies using AAV varies with the viral capsid, vector dose, route of administration, biodistribution, as well as the specific promoter and transgene. Thus, while the impact of host immune responses on the efficacy, safety, and durability of viral gene transfer remain unclear, and likely varies with different approaches, they are critical to understanding ocular inflammation and TEAEs in retinal gene therapy.

2.1 Innate Immune Response

The innate immune response is the first line of host defense against viral infections. Pattern recognition receptors (PRRs) on immune cells such as macrophages, monocytes, granulocytes, natural killer (NK) cells and dendritic cells (DC) recognize conserved pathogen associated molecular patterns (PAMPs) on viruses. Critical PRRs include toll-like receptors, a family of transmembrane (TLR 1, 2,4, 5, 6, 10) and intracellular (TLR 3, 7, 8, 9) PRRs,[24] which upon recognizing PAMPs can trigger a signaling cascade to activate innate immune responses.[25] Surface membrane-bound TLR2 can sense viral capsid proteins,[26,27] while endosomal TLR9 binds to specific unmethylated DNA sequences such as cytosine-phosphate-guanine (CpG) motifs in viral genomes.[28] Transgenes with a significant number of CpG dinucleotides can also activate innate responses through TLRs as well.[29]

TLRs mediate a downstream signaling pathway via adaptor myeloid differentiation primary response gene 88 (MyD88) which leads to the release of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), an interferon regulatory factor, which activates transcription of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (see Figure 1).[24] Hepatic injections of AAV2 in mice triggers the TLR-MyD88 pathway in DCs and NF-kB-dependent release of cytokines, resulting in activation of $CD8^+$ T-cell responses to both AAV capsid and transgene product[30] and eventual loss of transgene expression.[31] MyD88 signaling also takes place in B cells and regulates the production of Th1-dependent antibodies to AAV.[32]

While TLRs are activated by extracellular and endolysosomal PAMPs, cytosolic PRRs respond to PAMPs in the cytoplasm. Examples of cytosolic PRRs include nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), retinoic acid-inducible gene-I-like (RIG-I-like) receptors (RLRs), and intracellular sensors of DNA like cyclic GMP-AMP synthase (cGAS).[28] NLRs and ALRs can induce inflammasome formation which triggers the release of IL-1β and IL-18.[33] RLRs contribute to a late innate immune response to double-stranded RNA, an AAV-derived replication intermediate, after long-term AAV transduction.[34] cGAS signaling can reduce AAV transduction in mouse embryonic fibroblasts, and may impact retinal gene therapy as it is upregulated after subretinal AAV delivery.[28]

Viral proteins can also interact with the complement system. Immunoprecipitation studies demonstrated binding of iC3b complement protein to AAV2 capsids in human serum, and both complement receptor 1/2- and C3-deficient mice display impaired humoral immunity against AAV2 vectors.[35] Although AAV2 capsids do not activate the alternate complement pathway, they bind to complement regulator protein H, which may protect the virus from the complement system.[35]

Together, the innate immune responses to AAV vectors lead to activation and release of multiple pro-inflammatory cytokines and chemokines, which then promotes vascular permeability and compromises the blood-retinal barrier (BRB). This results in recruitment and infiltration of innate immune cells including antigen presenting cells (APCs) to shape the adaptive immune response.[36]

2.2 Adaptive Immune Response

In contrast to the nonspecific nature of innate immunity, the adaptive host immune response is generated in response to specific pathogens and contributes to immunological memory. APCs first interact with naïve T cells in secondary lymphoid tissues, which then differentiate into antigen-specific T cells, including CD4+ helper T cells, which then interact with B cells to generate humoral responses and CD8+ cytotoxic T cells that mediate cellular immune responses. Because NAbs produced by B cells can inhibit vector transduction, while cytokine-secreting T cells can directly destroy infected cells, both humoral and cellular immune responses can contribute to limiting the effectiveness of AAV-mediated retinal gene therapy by eliminating vectors and transduced cells.

2.2.1 Humoral Immune Response—Humoral immunity to viral vectors is mediated by antibodies that interact with Fc receptors on immune cells, complement-activating antibodies, and NAbs.[37] While the first two types of antibodies can trigger death of the infected cells, NAbs limit viral infectivity by interfering with receptor interactions between the virus and host cells, and may also promote clearance of viral particles by sequestering them to the spleen.[38]

Because humans are natural hosts for wild-type AAV, pre-existing NAbs to certain AAV serotypes can be highly prevalent among individuals.[39] Serum NAb titers as low as 1:5 can inhibit transgene expression in mice[40,41] and nonhuman primates (NHPs).[42] Furthermore, many patients demonstrate NAbs to multiple AAV serotypes, indicating high co-prevalence and cross-reactivity which likely results from the highly-conserved amino acid sequence of the viral capsid. Although NAb titers are correlated with IgG rather than IgM levels, no correlation was found between serum NAbs and AAV-specific T cells, perhaps because T cells are tissue-resident and circulate at low levels. Beside NAbs, non-neutralizing binding antibodies (BAbs) may also be present, although their role is unclear. Some studies suggest that BAbs may enhance clearance of AAV vectors through opsonization.[38] However, another study showed that hepatic transduction with AAV8 was increased in mice that were passively immunized with BAbs compared to those immunized with NAbs.[43]

The clinical impact of NAbs on gene therapy remains an area of controversy. In clinical trials involving hepatic AAV gene transfer for hemophilia B patients, one subject with a pretreatment anti-AAV2 NAb titer of 1:17 experienced a reduction of factor IX levels to baseline after 4 weeks, while another subject with a NAb titer of 1:2 retained higher factor IX levels beyond 10 weeks following vector administration.[44] The immune response was found to be related to the AAV capsid rather than the factor IX transgene. Similarly, viral transduction by direct intraparenchymal injection of AAV2 and AAV5 vectors into mouse brains, an immune-privileged site, was also limited by circulating NAbs.[45] By contrast, circulating anti-AAV9 NAbs up to 1:20-1:40 did not compromise intracranial AAV9 gene delivery.[46] For retinal applications, Heier et al. showed that exudative AMD patients with higher baseline NAbs receiving intravitreal AAV2 to express soluble Flt1 to neutralize the VEGF receptor demonstrated reduced transgene expression.[20] However, NAbs may not have significant clinical impact for subretinal vector delivery, as patients who received subretinal rAAV2 carrying the RPE65 gene did not demonstrate significant elevation in anti-AAV2 NAbs. These patients therapeutically benefited from vector readministration to the second eye without clinically significant adverse effects.[47]

2.2.2 Cell-mediated Immune Response—While humoral responses originate from B cells, cellular adaptive immune responses are mediated by T cells, which are triggered by AAV or transgene antigens that have been either passively transduced or actively phagocytosed by APCs. The presentation of proteasomally-degraded cytosolic antigens on class I major histocompatibility complex (MHC) molecules by most nucleated cells induces CD8+ cytotoxic T cells, while presentation of endosomally-processed antigens on class II MHC molecules on immune cells, such as dendritic cells, macrophages, monocytes, as well as RPE cells, trigger CD4+ T cells. The ability of APCs to cross present viral or transgene antigens to either MHC class I or II drives the T cell response, as interaction of APCs with

CD4+ T cells can induce antigen-specific B cell responses, while also stimulating the CD8+ T cell response that directly destroy transduced cells.[48]

Unlike humoral immunity, the prevalence of pre-existing T cells directed against AAV is less common. This due to the fact that capsid-reactive T cells are more often localized to the spleen than the peripheral blood,[49] and because methods to detect T cell responses are not as sensitive as NAbs, which accounts for the discrepancy between prevalence of AAV-specific NAbs and circulating CD8+ T cells.[39] Similar to the cross-reactivity of NAbs to different AAV serotypes, CD8+ T cells can respond to multiple AAVs due the shared processing and presentation of capsid epitopes.

The impact of AAV-specific T cell responses was noted in AAV-mediated gene therapy trials for Hemophilia B, where similar to the effect of NAbs, increases in AAV2-specific CD8+ T cell responses were associated with elevated transaminases and reduced gene expression after 4-6 weeks.[44] In another study using AAV1-mediated gene transfer to muscle, capsid-specific CD8+ T-cells resulted in a decrease of transgene expression and a rise in creatine phosphokinase, an indicator of muscle cell damage.[50] While stronger adaptive immune responses have been noted in hepatic and muscle gene transfer, gene transfer to immune-privileged sites such as the brain and eye have exhibited little to no detectable immune response to capsid or transgene in peripheral blood.[2]

3. Ocular Immune Privilege

Like other parts of the central nervous system, the eye benefits from ocular immune privilege due to 1) the presence of physical blood-ocular barriers, 2) an immunosuppressive molecular microenvironment, and 3) deviant immune responses mediated by immunosuppressive regulatory T cells (Tregs) known as anterior chamber associated immune deviation (ACAID).

3.1 Blood-Ocular Barriers

Blood-ocular barriers consists of tight junctions between RPE cells, and between endothelial cells of retinal and corneal capillaries. Along with the lack of direct lymphatic drainage, these barriers sequester ocular antigens from the developing immune system and limits immune cell entry into the eye. Importantly, the blood-retinal barrier separates different compartments of the eye with unique immunological properties, including the vitreous, subretinal, and suprachoroidal compartments to which viral vectors for gene therapy can be delivered.

3.2 Immunosuppressive Ocular Environment

In addition to physical barriers, the eye expresses high levels of soluble immunomodulatory factors including α-melanocyte stimulating hormone (α-MSH),[51] transforming growth factor-β (TGF-β),[52] soluble Fas ligand,[53] and macrophage migration inhibitory factor (MIF),[54] which are potent inhibitors of neutrophils, macrophages, and NK cells. The eye also expresses high levels of immunomodulatory cell-surface molecules such as membrane-bound Fas ligand[53] and complement regulatory proteins[55] among others, which together

creates an immunosuppressive microenvironment to limit the inflammatory responses in the eye.

3.3 Anterior Chamber Associated Immune Deviation (ACAID)

Much our initial understanding of ocular immune privilege came from introducing antigens into the anterior chamber (AC), as in corneal transplantation, which results in an acquired immune response known as anterior chamber-associated immune deviation (ACAID). This is a process by which tolerance-inducing F4/80+ APCs bearing the immunogens migrate through the trabecular meshwork to the spleen, where instead of triggering a typical adaptive immune response, they induce systemic immune tolerance to the antigen by stimulating splenic CD1-reactive natural killer T (NKT) cells.[56] These cells then produce immunosuppressive factors such as IL-10 and TGF-β and generating antigen-specific Tregs cells.[57-59]

A similar mechanism of immune deviation occurs in the vitreous and subretinal compartments.[60-62] Unlike antigens in the anterior and vitreous chambers which drain into systemic circulation, those in the subretinal space are thought to exit through the choroid and travel through lymphatics to cervical lymph nodes.[62] While F4/80+ APCs are key mediators in ACAID, DCs in the choroid and microglia in the neurosensory retina may play a similar role for immune deviation in the subretinal space. In particular, the RPE may serve an important role in subretinal space immune deviation. The RPE secretes immunosuppressive factors such as TGF-β, somatostatin, thrombospondin and pigment epithelial derived factor (PEDF)[63] and constitutively expresses membrane-bound Fas ligand[64] which induces apoptosis in inflammatory cells.[65] RPE disruption with intravenous sodium iodate results in loss of immune deviation in the subretinal and vitreous compartments, but not the AC, [62] highlighting the importance of the RPE barrier in subretinal gene therapy. However, blood-retinal barriers such as the RPE may be compromised by the underlying retinal pathology in patients with IRDs or surgical maneuvers associated with delivering the viral vector. Thus, the risk for ocular inflammation and host immune responses to retinal gene therapy depends not only on the viral vector, but also on the route of delivery to different ocular compartments, especially with respect to blood-ocular barriers, as well as the therapeutic transgene and underlying retinal condition.

4. Factors Affecting Host Immune Responses

Factors that affect host immune responses to viral-mediated gene therapy include 1) the viral vector and dose, 2) the mode of delivery and vector biodistribution, 3) promoter and transgene, and 4) the disease to be treated.

4.1 Type of Viral Vector

The choice of viral vectors used for retinal gene therapy has largely been guided by host immune responses and safety considerations. Intravenous Ad triggered greater chemokine and cytokine expression with widespread liver inflammation compared with AAV, which only induced a transient response, likely due to differences in surface receptor usage, virus

internalization, or intracellular trafficking.[66] Furthermore, the dsDNA in Ad is a stronger agonist for TLR9 and thus may stimulate a greater innate immune response. [67]

Among AAV vectors, different serotypes not only exhibit distinct tropism for different types of retinal cells but are also impacted by different prevalence of pre-existing NAbs and degrees of immunogenicity. NAbs against AAV1 and AAV2 have the highest prevalence, with reported rates of 30-70%, while NAbs against AAV5, AAV7, AAV8 and AAV9 are lower at 15-30%.[39,68] The impact of pre-existing NAbs on retinal gene delivery is controversial, however, and likely depends on the degree of immune privilege of the ocular compartment to which the virus is delivered. Structural differences between AAV8 and AAVrh32.33 viral capsids also produce different adaptive immune responses to the capsid and transgene product,[69] while AAV1 transduces DCs more efficiently than AAV8 and triggers a stronger immune response.[70] Strategies to reduce immunogenicity include the use of proteosome inhibitors to reduce AAV2-capsid presentation MHC I molecules, [71] and amino acid alterations in the AAV2 capsid to evade ubiquitination and cell death.[72] Interestingly, the use of self-complimentary (sc) viral genomes with dsDNA rather than the native single-stranded DNA to improve transduction efficacy actually led to more severe adaptive immune responses, independent of capsid serotype.[73] Other novel variants such as AAV7m8 and AAV8BP2 also appear to exhibit different levels of immunogenicity, with studies in nonhuman primates (NHPs) suggesting that AAV8BP2 may have a better safety profile compared to AAV7m8.[74]

4.2 Route of Administration & Biodistribution

The route of viral vector delivery into the eye has significant impact on the immunogenicity of the ocular gene therapy. Different ocular compartments not only preferentially expose the viral particles to different retinal cell types, but also exhibits different local and systemic biodistribution, as well as exposure to host immune surveillance. Intravitreal injections can be easily performed in an outpatient setting, and the technique is familiar to most retinal specialists. Viral vectors delivered into the vitreous cavity could theoretically transduce broad areas of the retina, but are inhibited from reaching the photoreceptors and RPE by the internal limiting membrane (ILM) barrier. By contrast, subretinal injections must be performed in an operating room and requires the use of a subretinal cannula, which necessitates vitreoretinal surgical maneuvers. The therapeutic effect of the viral vector is also limited to the area of the subretinal bleb that is created, though the viral particles can readily access and transduce photoreceptors and RPE.

Another important distinction between intravitreal and subretinal AAV delivery is the difference in vector outflow and systemic biodistribution. Seitz et al. compared the biodistribution of subretinal and intravitreal AAV8 in NHPs and found that intravitreal delivery showed higher levels of virus in aqueous humor, while subretinal delivery resulted in higher levels in the retina and downstream tissues such as the optic nerve and chiasm.[75] Interestingly, compared to subretinal injections, the intravitreal AAV8 group showed a 464-fold higher virus load in the bloodstream, as well as higher levels in the spleen and draining lymph nodes.[75] These findings suggest that intravitreal delivery or leakage of vector into the vitreous cavity results in viral vector egress through Schlemms canal into systemic

circulation and lymphatic tissues, where it could activate effector immune cells to trigger the host immune response. Indeed, Reichel et al. showed that intravitreal AAV8 produced higher antibody titers as compared to subretinal delivery at same or lower doses.[76] This strong humoral response appears to be independent of viral serotype, transgene, vector dose, or number of eyes injected, and increases after a second AAV injection.[77] In mice, the antibody response to intravitreal AAV can inhibit vector expression upon subsequent administration via the same route to the fellow eye, but had no effect on subretinal vector readministration.[78] In contrast to intravitreal delivery, delivery of rAAV2 vector into subretinal space did not generate any humoral response against the viral capsid.[78] In fact, sequential bilateral subretinal injections of AAV2 carrying RPE65 in dogs and NHPs demonstrated an elevation of NAb after the first treatment, and additional increase after the second eye was treated, but showed minimal inflammatory changes or other adverse effects.[79] Subretinal injections of the same AAV2 vector into the second eye of human patients also appear to be safe and effective, with no evidence of functional deficit.[47] Thus, the ocular immune privilege and decreased outflow to systemic circulation from the subretinal compartment may be less impacted by either pre-existing immunity or adaptive responses to a therapeutic vector than the vitreous cavity, and better enable sequential bilateral AAV treatments.

A novel mode of vector delivery involves injection into the suprachoroidal space.[80,81] The suprachoroid is a potential space outside the blood-retinal barrier[82] that is located between the choroidal vasculature and scleral wall of the eye. This space could be accessed using different methods, including transscleral microneedles that has been successfully employed to deliver triamcinolone for the management of macular edema.[83,84] Ding et al evaluated suprachoroidal delivery of AAV8 using conventional 30-gauge needles, and demonstrated widespread green fluorescent protein (GFP) expression across various cell types in rats, pigs, and NHPs.[85] No major adverse inflammatory reactions were noted, although all three NHPs showed elevated NAbs at 21 days after injections despite being seronegative at baseline.[85] Yiu et al. employed transscleral microneedles similar to those in human studies, and compared suprachoroidal, subretinal and intravitreal delivery of AAV8 expressing GFP in NHPs with longitudinal imaging, and found that suprachoroidal delivery produced mostly peripheral transgene expression in RPE that was highest after 1 month but subsequently declined at month 3.[86] The loss in transgene expression was attributed to chorioretinal infiltration of inflammatory cells which was accompanied by greater humoral and cellular responses to the GFP transgene, whereas intravitreal injection of the same vector and dose produced minimal transgene expression but greater sequestration to the spleen and stronger antibody responses to the AAV8 capsid.[87] Interestingly, GFP expression after suprachoroidal vector delivery appears to persist in scleral fibroblasts at month 3. Together, these studies suggest that the greater trabecular outflow of viral vectors from the vitreous cavity to the bloodstream and spleen can trigger stronger host immune responses to the AAV capsid, compared with the slower uveoscleral outflow of virus from the suprachoroidal space. However, the greater transgene expression in scleral tissues outside the blood-retinal barrier after suprachoroidal delivery could also elicit a greater immune response to the transgene (see Figure 2).

4.3 Transgene and Promoter

Beside the type of viral vector and route of delivery, the specific transgene and promoter[88] may also contribute to ocular inflammatory and immune responses. For example, GFP is a foreign protein to the host, and is intrinsically linked to immunogenicity and cytotoxicity.[89] Although fluorescent proteins enable *in vivo* visualization of transgene expression, they are prone to generating oxidative damage when expressed at high levels. [89] For example, comparison studies have shown that retinal expression of GFP is more toxic that retinoschisin, a mammalian protein.[90]

Interestingly, different promoters can also impact retinal toxicity after viral transduction. Xiong et al. found that AAV vectors with broadly-active promoters such as cytomegalovirus immediate-early promoter (CMV), human ubiquitin C promoter (UbiC), chicken beta actin promoter (CAG), and RPE-specific Bestrophin promoter (Best1 or VMD2), resulted in dose-dependent retinal inflammation, toxicity, and function loss, whereas vectors with photoreceptor-specific promoters, including human red opsin (RedO), human rhodopsin (Rho), human rhodopsin kinase (RK), and mouse cone arrestin (CAR) were not toxic.[88] This pattern of toxicity was found across various capsid types including AAV8, AAV5 and engineered capsids, and did not depend on the transgene, as AAV-CMV-null displayed similar toxicity as AAV8-CMV-GFP.[88] The authors hypothesized that RPE and/or microglia are the primary activators of this immune-mediated retinal toxicity, although the precise mechanism remains elusive.[88]

Despite these concerns, voretigene neparvovec-rzyl (Luxturna) has been FDA-approved with use of the CAG promoter, with no clear evidence of toxicity reported in clinical trials.[91] Similarly, phase 1 studies using an AAV2 with VMD2 promoter for MERTK mutation-related retinitis pigmentosa (RP) have not shown major adverse effects.[92,93] Local immune dysregulation or impaired RPE function may account for this relative sparing, but additional studies are needed to investigate the relative safety of different promoter and transgenes for retinal gene therapy.

5. Ocular Inflammation in Retinal Gene Therapies

5.1 Preclinical Studies

Pre-clinical and clinical studies demonstrate a viral dose-dependent relationship with host immune responses and intraocular inflammation, depending on the route of administration (see Table 1). Intravitreal injection of AAV2 expressing sFlt1 in NHPs demonstrated mild to moderate, though self-resolving, vitreous inflammation in the high dose group (2.4×10^{10} vg) but not in the low dose group (2.4×10^{9} vg).[94] Intravitreal delivery of a tyrosine-mutant variant of AAV2 to express human retinoschisin 1 (rAAV2tYF-CB-hRS1) in macaques also showed anterior and posterior inflammatory responses at the 4×10^{10} and 4×10^{11} vg doses with development of serum anti-AAV antibodies and evidence of mononuclear infiltrates on histological examination, but no adverse effects on electroretinography or visual evoked potentials.[95]

Like intravitreal injections, subretinal delivery of AAV vectors also exhibit dose-dependent inflammation, but generally shows less immunogenicity at similar vector doses. In NHPs,

Vandenberghe's team compared subretinal injection of AAV2 and AAV8 to express GFP with dose-ranging studies from 1 x 10^8 to 1 x 10^{11} vg. They found that serum NAbs were correlated with degree of GFP expression, and were mostly detected at viral titers of 10^{10} vg or greater.[96] NAbs were also detected in the AC, but only in animals receiving the highest dose (1 x 10^{11} vg) of either serotype.[96] Similar doses of subretinal AAV2 (1 x 10^{10} - 5 x 10^{10} vg)[91] did not elicit ocular inflammation in preclinical NHP studies for rAAV2-VMD2-hMERTK for treatment of MERTK-associated RP, but subretinal delivery of AAV8 for CNGA3-based achromatopsia (1 x 10^{11} – 1 x 10^{12} vg)[97] and AAV2tYF expressing human CNGB3 (1.2×10^{11} – 1.2×10^{12} vg)[95] were associated with posterior segment inflammation in NHPs. AAV also triggered intraocular inflammation in dogs, with subretinal AAV5 expressing CNGB3 showing multifocal retinitis from 5×10^{11} – 1.65 x 10^{13} vg,[16,98] AAV2 expressing RPGR showing inflammation at 2.1 x 10^{11} vg,[99] and a comparison of AAV2/5 expressing GFP under 3 different promoters (mOP, hGRK1, and CBA) all showing ocular inflammation and retinal toxicity at the highest doses (1.51 x 10^{11} – 4.79 x 10^{12} vg).[100] Interestingly, in one study by MacLachlan et al., subretinal AAV8 carrying the human RLBP1 gene triggered ocular inflammation in NHPs at doses as low 3.3 x 10^8 vg, with photoreceptor thinning and reduced ERG signal,[101] highlighting the variability of immune responses between these preclinical studies.

Because the suprachoroidal space is outside the blood-retinal barrier, this ocular compartment may not afford the same degree of immune privilege as the subretinal or even vitreous space. In NHPs, suprachoroidal AAV8 expressing GFP triggered AC cells, vitritis, and peripheral chorioretinitis that resolved after a 2-week oral course of steroids, while intravitreal injections of the same vector and dose showed no notable intraocular inflammation.[87] An important drawback of animal studies is that expression of human transgenes or proteins such as GFP are foreign to the host and could contribute to ocular inflammation regardless of the viral vector. Moreover, the host immune responses in these species may differ from humans. For example, while prevalence of NAbs against AAV2 are higher than AAV7, AAV8, or AAV9 in humans,[102,103] the prevalence NAbs against AAV7, AAV8, and AAV9 are much higher in NHPs.[104] Thus, evaluating inflammatory and immune responses in human studies, using vectors that express therapeutic human proteins, may provide greater insight on the safety of these treatments.

5.2 Human clinical trials

In an early phase 1 dose-escalation study evaluating the safety of intravitreal AAV2-sFlt01 for neovascular AMD (2 x 10^8 - 2 x 10^{10} vg), two patients who received the highest dose developed intraocular inflammation that resolved with topical steroids, and anti-AAV2 antibodies developed in most of the higher-dose cohorts.[20] A more recent phase 1 trial using the novel AAV.7m8 vector to express the anti-VEGF agent aflibercept (2 x 10^{11} – 6 x 10^{11} vg) also showed ocular inflammation across all cohorts, despite the use of perioperative steroids.[105]

In human trials with X-linked retinoschisis (XLRS) patients, intravitreal AAV2-RS1 demonstrated dose-dependent intraocular inflammation which responded to topical and oral steroids. An increase in serum NAbs noted in all groups except for the lowest dose (1 x 10^9

vg), and substantial titers against AAV8 (1:320–1:2,560) noted in the highest dose (1 x 10^{11} vg), despite being screened for the absence pre-existing NAbs.[17] Patients with Leber's Hereditary Optic Neuropathy (LHON) who received 1.0 – 2.0 x 10^{10} vg intravitreal AAV2 expressing the NADH dehydrogenase subunit 4 (ND4) gene demonstrated no NAbs after 6 months.[106] A later phase 1/2 dose-escalation clinical study with the same vector up to 1.8 x 10^{11} vg demonstrated vitritis in all patients. No dose response relationship was noted between viral dose or NAb levels and the degree of inflammation.[107] Although the threshold for host immune responses to intravitreal AAV delivery varies between vector, disease, and study, less inflammation is generally noted at <1x10^{10} vg.

The majority of past and ongoing gene therapy clinical trials employ subretinal injections, which generally exhibit less inflammatory and immune responses. In the phase I study of 15 patients with the RPE65 form of LCA who received subretinal AAV2 therapy up to 17.78 x 10^{10} vg by Jacobson and colleagues, all patients demonstrated anti-AAV2 antibodies at baseline, but a majority showed no increase in antibody titers greater than two-fold up to 3 years after treatment.[108] One patient experienced three-fold increase at day 14 which returned to baseline at day 90, but spiked again at year 2, presumably due to re-exposure to wild type AAV.[108] Although some patients had pre-existing T cell memory, no increase in response was noted in any patient.[108] In another phase I/II trial of 12 patients with RPE65 mutations in the United Kingdom, Bainbridge et al. noted inflammatory or immune responses in 5 out of 8 patients who received the higher dose (1 x 10^{11}vg), while none were noted in the 4 who received the lower dose (1 x 10^{10}vg) after subretinal injections.[109] One patient developed mild anterior uveitis and focal pigmentary changes in the macula, with sustained visual acuity reduction of 15 letters, and was found to exhibit AAV2 NAbs and some AAV2-specific T cell response at 4 weeks, although the other participants with intraocular inflammation were largely asymptomatic and showed no sustained functional impact.[109] In a later study by Le Meur et al. which evaluated both subretinal AAV2 and AAV4 for RPE65-related LCA in 9 patients, no intraocular inflammation was noted up to 4.8 x 10^{9} vg.[110] Long-term safety data from phase 3 studies of the FDA-approved voretigene neparvovec-rzyl by Maguire et al. showed no adverse immune or inflammatory responses out to 4 years.[9]

A series of clinical trials have also been conducted employing subretinal AAV2 delivery of REP-1 for choroidermia. In Xue et al., 1 in 14 patients who received the higher 1 x 10^{11}vg dose developed significant retinal inflammation that was attributed to vector reflux into the vitreous cavity.[10] In the Alberta study by Dimopoulos and colleagues, 1 in 6 subjects receiving the same dose showed localized intraretinal inflammation, loss of photoreceptor layer, and significant visual decline, which upon review of the surgical video, was noted to have developed intraretinal and subretinal hemorrhage during the injection procedure, as well as introduction of subretinal air bubbles.[11] A later study using microscope-integrated optical coherence tomography (OCT) in 6 patients using the same vector and dose showed improved safety profile, with only one patient showing mild vitreous cell and one showing mild AC cell, both of which resolved within 1 week of treatment.[12] The most recent phase 2 study of 6 patients did not report any notable adverse effects.[13]

Other recent human trials using subretinal AAV injections include the use of rAAV2-VMD2-hMERTK for MERTK-associated RP which showed no inflammation or toxicity up to 2 years despite some patients developing a rise in anti-AAV2 antibodies,[92] as well as rAAV.sFLT-1 for neovascular AMD, in which patients did not exhibit significant intraocular inflammation, and only 3 of 9 seronegative patients developed NAbs that did not appear to impact protein production.[21,22] Together, these studies confirm that vector delivery to the subretinal space may be less likely to trigger ocular inflammation or a pronounced host immune response.

6. Other Treatment Emergent Adverse Effects

Beside intraocular inflammation, TEAEs of retinal gene therapy may also be related to the injection procedure itself. Intravitreal injections are commonly performed by retinal specialists and are generally well tolerated. The risk of endophthalmitis after intravitreal injections are generally low with proper antiseptic techniques.[111] Patients with XLRS who received intravitreal injections of rAAV8 noted minimal pain and subconjunctival hemorrhage associated with the procedure.[17]

Unlike intravitreal injections,[111] subretinal viral vector delivery typically requires pars plana vitrectomy, where risks include elevated intraocular pressure (IOP), glaucoma, hypotony, endophthalmitis, hemorrhage, retinal detachment, cataract progression, epiretinal membrane formation, and macular hole formation. Although most gene therapy clinical trials used 23-gauge vitrectomy systems, smaller 25- or 27-gauge vitrectomy instruments may provide lower rates of surgical complications.[5]

Delivery of the viral vectors requires the insertion of a 39- to 41-gauge cannula through the retina to access the subretinal space. This can be accomplished in 2 steps with a “pre-bleb” of balanced salt solution, or by direct subretinal injection. A saline pre-bleb theoretically minimizes inadvertent vector injection into the vitreous or choroid, but passage of a second cannula may stretch the retinotomy causing vector reflux into the vitreous also. Use of intraoperative OCT and automated injectors may enhance the accuracy and precision of surgical technique and prevent complications.[12] A novel subretinal injection device that is deployed from a flexible cannula passed through the suprachoroidal space may overcome the risk of vitreous spillage altogether.[112,113]

In the clinical trials leading to the approval of voretigene-neparvovec-rzyl, 68% of patients experienced ocular TEAEs, most of which were related to the surgery itself.[9] One patient had loss of foveal function due to the procedure, and one had elevated IOP with optic atrophy in setting of endophthalmitis. Seven (18%) patients developed cataracts, 7 (18%) had elevated IOP, 3 (8%) experienced ocular inflammation, of which one was endophthalmitis, 2 (5%) with epiretinal membrane, 1 (3%) with choroidal hemorrhage, and other patients reported eye pain, irritation and pruritus.[9] A meta-analysis of 164 eyes of 82 patients with RPE-associated LCA across 6 prospective clinical trials revealed central retinal thinning of 19.2 μm in treated eyes at 2-3 years post-treatment as compared to controls, which may be attributed to the temporary retinal edema or detachment caused by subretinal injection.[114]

In the choroideremia trials, TEAEs besides ocular inflammation include retinal stretch during surgery and subsequent retinal thinning in 1 patient,[9] and intravitreal, intraretinal and subretinal hemorrhage that accompanied the introduction of subretinal air bubbles.[10] The use of intraoperative OCT in a later trial helped investigators identify preexisting retinal thinning and avoid excess foveal stretching and macular hole formation.[12]

In other gene therapy trials for IRDs, 1 in 6 patients who received rAAV2-VMD2-hMERTK developed a shallow foveal detachment without macular hole formation that persisted until 6 weeks.[92] Filamentary keratitis, oscillopsia and cataract progression were also reported in the other patients. In a phase I/II study of 18 X-linked RP patients, no severe ocular TEAEs were reported, though mild corticosteroid-responsive inflammation related to viral vector, hemorrhage, ocular hypertension, pain, corneal abrasion and suture granuloma were noted. Similar minor procedure related adverse outcomes were noted in 9 patients with CNGA3 mutation-related achromatopsia.[115]

7. Mitigation Strategies

7. 1 Mitigation of Humoral Immune Responses

One approach employed in clinical trials to avoid complications of pre-existing immunity is to screen for and exclude patients with pre-existing NAbs. However, due to the endemic nature of AAV and high seroprevalence of NAbs, this strategy may exclude a large portion of patients.[68,116] AAV vectors isolated from other species such as the AAV hybrid rh32.33 isolated from rhesus macaques exhibit low prevalence of NAbs among human subjects.[116] Methods such as directed evolution may also be used to engineer AAV capsids to evade antibody neutralization,[117] although such strategies may result in altered tropism and still be affected by antibody cross-reactivity.

Another approach to evade the humoral response is to co-administer empty AAV capsids to competitively inhibit NAbs. Mingozzi et al. showed that inclusion of empty capsids reduces neutralizing activity of NAbs and increases genetic transduction in a dose-dependent manner.[118] To address the higher antigen load and presentation on MHC class I molecules, they mutagenized the AAV capsid so that it could not enter the cell, but noted that other modes of engulfment of capsid such as pinocytosis may result in eventual antigen presentation and immunogenicity.[118]

Use of immunosuppressive agents to target B cells and plasma cells has also been used to limit humoral responses.[119] Anti-CD20 treatment was shown to be partially effective in reducing AAV NAbs in some rheumatoid arthritis patients,[120] but the use of immunosuppression must be weighed against potential risks and side effects. Plasmapheresis may also help reduce serum NAb levels,[121] although this type of therapy does not completely eliminate T cell responses unless combined by T cell-directed immunosuppressive agents.

7.2 Mitigation of Cell-Mediated Immune Responses

Few clinical trials exclude patients for pre-existing T cell immunity due to the limited sensitivity of methods for detection. Assays to identify antigen-specific T cells from

peripheral blood mononuclear cells (PBMCs) may not detect T cells that circulate at very low frequencies.[122] An approach used in hepatic gene transfer involves the engineering of a hyperactive variant of the transgene, which would allow the vector dose to be reduced and thus less likely to stimulate a T cell response.[123] This approach may not be feasible for some forms of retinal gene therapy, however, where the transgene may not encode a catalytic protein.

Immunosuppressive agents such as proteasome inhibitors can decrease T cell activation and proliferation, decrease antigen presentation and enhance AAV transduction.[72] Although immunosuppression can reduce cytotoxic responses, it can also downregulate anti-inflammatory regulatory T cell responses.[124] Novel AAV capsids could be engineered to minimize antigen presentation but may still be limited by immune cross reactivity.[49]

8. Conclusion

Gene therapy using viral vectors such as AAV has been shown to be effective for many IRDs and some acquired conditions such as AMD. However, host immune responses, ocular inflammation, and other TEAEs continue to pose barriers to widespread adoption. A variety of factors contribute to the safety of each therapeutic platform. Different viral capsids determine cellular tropism, but also immunogenicity. The route of administration to different ocular compartments determines local and systemic biodistribution and exposure of viral or transgene product to immune surveillance. Degenerative retinal conditions may compromise blood-ocular barriers depending on the severity of the condition, while certain promoters and transgenes may contribute to immunogenicity and retinal toxicity. Variations in study design, patient population, surgical techniques, and detection assays for immune responses contribute to the difficulty of cross-study comparisons.[125] Nevertheless, our mounting experience across past and ongoing clinical trials is providing greater insight into host immunity, ocular inflammation, and technical challenges of gene delivery. Innovations in engineering novel vectors, surgical instrumentation, and mitigation strategies will help provide a path to broaden the application of viral gene therapy to treating retinal diseases.

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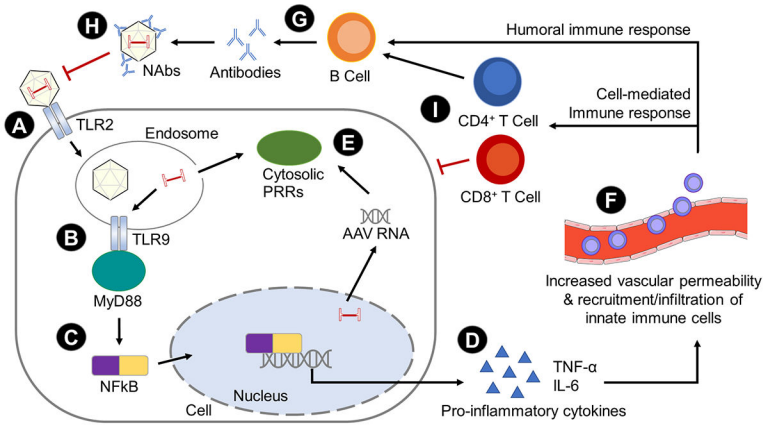


Figure 1. Cellular and molecular mechanisms of host immune responses to viral vectors.

(A) TLRs can recognize PAMPs on viruses and trigger a signaling cascade to activate innate immune responses. Surface-bound TLR2 can sense viral capsid proteins.

(B) Endosomal TLR9 binds to specific unmethylated DNA sequences like CpG motifs on viral genomes.

(C) TLRs connects with signaling adaptor myeloid differentiation primary response gene 88 (MyD88) and leads to release of nuclear factor NF-kB.

(D) This then activates transcription of pro-inflammatory cytokines like TNF-α and IL-6,

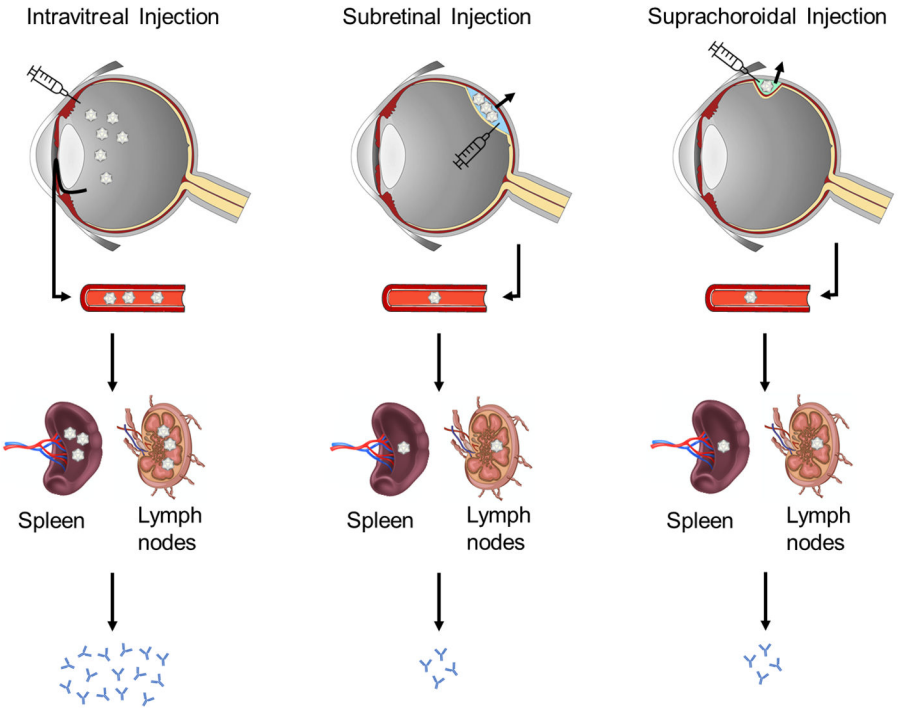
(E) Cytosolic PRRs respond to PAMPs in the cytoplasm, including NLRs, ALRs, and RLRs.

(F) The pro-inflammatory cytokines can also promote vascular permeability and disruption of the blood-retinal barrier.

(G) $CD4^+$ helper T cells interact with B cells to generate humoral responses. B cells produce antibodies that interact with Fc receptors on immune cells, complement-activating antibodies, and NAbs.

(H) Pre-existing NAbs to certain AAV serotypes can limit viral infectivity by interfering with receptor interactions between the virus and the host cells, thereby inhibiting transgene expression.

(I) Cellular adaptive immune responses are mediated by T cells, which are triggered by AAV or transgene antigens. The presentation of proteasomally-degraded antigens on class I MHC molecules induces CD8+ cytotoxic T cells, while presentation of endosomally-processed antigens on class II MHC molecules induces $CD4^+$ helper T cells. Interactions of APCs with CD4+ T cells induces antigen-specific B cell responses, while APCs stimulate CD8+ T cells to directly destroy transduced cells.



	Intravitreal	Subretinal	Suprachoroidal
Retinal transgene expression	Variable	High	Variable
Scleral transgene expression	Low	Low	High
AAV egress to circulation	High	Low	Low
Cellular immune response to transgene	Low	Low	High?*
Humoral Nab response to AAV	High	Low	Low

*for foreign transgenes, unknown for native transgenes

Figure 2. Routes of viral vector delivery.

Effects of different routes of viral vector delivery on transgene expression, egress to circulation and immune response.

Table 1.

AAV vector, promoter and doses used in preclinical and clinical studies.

	AAV vector	Promoter	Gene	Dose-range without inflammation (vg/eye)	Dose-range with inflammation (vg/eye)	Reference
Intravitreal studies						
NHP	AAV2	Chicken β-actin	sFLT01	2.4×10^{9}		MacLachlan et al 2011
	AAV8	Arrestin 3	CNGA3	1×10^{12}	--	Reichel et al 2017
	AAV8	CMV	GFP	7×10^{12}	--	Chung et al 2020
Human	AAV2	CMV	ND4	1×10^{10} - 2×10^{10}	--	Wan et al 2016
	AAV2	Chicken β-actin	sFLT01	2×10^{8} - 6×10^{9}	2×10^{10}	Heier et al 2017
	AAV8	RS1	RS1	1×10^{9}	1×10^{10} - 1×10^{11}	Cukras et al 2018
	AAV2	CMV	ND4	--	9×10^{9} - 1.8×10^{11}	Bouquet et al 2019
	AAV.7m8	CMV	anti-VEGF	--	2×10^{11} - 6×10^{11}	Khanani et al 2020
Subretinal studies						
Rat	AAV8	CB7	GFP	1.2×10^{7}	--	Ding et al 2019
Dog	AAV5	PR0.5	CNGB3	1.53×10^{14}	--	Komáromy et al 2010
	AAV5	3LCR-PR0.5	CNGB3	1.73×10^{12} - 7.48×10^{12}	--	Komáromy et al 2010
	AAV5	PR2.1	CNGB3	8.47×10^{10} - 9.39×10^{12}	9.43×10^{12} - 1.65×10^{13}	Komáromy et al 2010
	AAV2/5	mOP500	GFP	3.27×10^{9} - 3.27×10^{10}; 3.27×10^{12}	3.27×10^{11}	Beltran et al 2010
	AAV2/5	hGRK1	GFP	1.51×10^{9} - 1.51×10^{10}	1.51×10^{11} - 1.51×10^{12}	Beltran et al 2010
	AAV2/5	CBA	GFP	4.79×10^{9} - 4.79×10^{11}	4.79×10^{12}	Beltran et al 2010
	AAV5	PR2.1	CNGB3	7.5×10^{9} - 1×10^{11}	5×10^{11} - 1.1×10^{13}	Ye et al 2017
	AAV2tYF	GRK1	RPGR	8.4×10^{9} - 4.2×10^{10}	2.1×10^{11}	Dufour et al 2020
NHP	AAV2	CMV	GFP	--	1×10^{10} - 1×10^{11}	Vandenberghe et al 2011
	AAV8	CMV	GFP	1×10^{8} - 1×10^{10}	1×10^{11}	Vandenberghe et al 2011
	AAV2	VMD2	MERTK	1×10^{10} - 5×10^{10}	--	Ghazi et al 2016
	AAV2tYF	PR1.7	CNGB3	--	1.2×10^{11} - 1.2×10^{12}	Ye et al 2016
	AAV8	Arrestin 3	CNGA3	--	1×10^{11} - 1×10^{12}	Reichel et al 2017
	AAV8	CPK850	RLBP1	3.3×10^{6} - 3.3×10^{7}	3.3×10^{8} - 3.3×10^{9}	MacLachlan et al 2017
	AAV8	CMV	GFP	--	7×10^{12}	Chung et al 2020
Human	AAV2	CBA	RPE65	5.96×10^{10} - 17.88×10^{10}	--	Jacobson et al 2012
	AAV2/2	RPE65	RPE65	1×10^{10}	1×10^{11}	Bainbridge et al 2015
	AAV	CMV	sFLT01	1×10^{10} - 1×10^{11}	--	Rakoczy et al 2015
	AAV2	VMD2	MERTK	5.96×10^{10} - 17.88×10^{10}	--	Ghazi et al 2016
	AAV	CMV	sFLT01	1×10^{11}	--	Constable et al 2016
	AAV2/4	RPE65	RPE65	1.22×10^{9} - 4.8×10^{9}	--	Le Meur et al 2018
	AAV2	CAG	REP1	1×10^{10}	1×10^{11}	Xue et al 2018
	AAV2	Chicken β-actin	REP1	--	1×10^{11}	Dimopoulos et al 2018

	AAV vector	Promoter	Gene	Dose-range without inflammation (vg/eye)	Dose-range with inflammation (vg/eye)	Reference
	AAV2	Chicken β-actin	REP1	1×10^{11}	--	Lam et al 2019
	AAV2	Chicken β-actin	RPE65	1.5×10^{11}	--	Maguire et al 2019
	AAV2	Chicken β-actin	REP1	1×10^{11}	--	Fischer et al 2019
Suprachoroidal Studies						
Rat	AAV8	CB7	GFP	1.2×10^{7}; 2.85×10^{9}	--	Ding et al 2019
Pig	AAV8	CB7	GFP	4.75×10^{10}	--	Ding et al 2019
NHP	AAV8	CB7	GFP	4.75×10^{10}	--	Ding et al 2019
NHP	AAV8	CMV	GFP	--	7×10^{11} - 7×10^{12}	Chung et al 2020