

# Humoral immune responses to AAV gene therapy in the ocular compartment

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## ABSTRACT

Viral vectors can be utilised to deliver therapeutic genes to diseased cells. Adeno-associated virus (AAV) is a commonly used viral vector that is favoured for its ability to infect a wide range of tissues whilst displaying limited toxicity and immunogenicity. Most humans harbour anti-AAV neutralising antibodies (NAbs) due to subclinical infections by wild-type virus during infancy and these pre-existing NAbs can limit the efficiency of gene transfer depending on the target cell type, route of administration and choice of serotype. Vector administration can also result in *de novo* NAb synthesis that could limit the opportunity for repeated gene transfer to diseased sites. A number of strategies have been described in preclinical models that could circumvent NAb responses in humans, however, the successful translation of these innovations into the clinical arena has been limited. Here, we provide a comprehensive review of the humoral immune response to AAV gene therapy in the ocular compartment. We cover basic AAV biology and clinical application, the role of pre-existing and induced NAbs, and possible approaches to overcoming antibody responses. We conclude with a framework for a comprehensive strategy for circumventing humoral immune responses to AAV in the future.

*Key words:* AAV, gene therapy, retina, ocular, neutralizing antibodies, immune response

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## I. INTRODUCTION

Adeno-associated virus (AAV) is a non-enveloped Parvovirus that was originally identified as a contaminant in adenoviral cultures (Hastie & Samulski, 2015). The virus exhibits a T = 1 icosahedral shape with a 25 nm diameter (Srivastava, Lusby & Berns, 1983). AAV capsid proteins contain a single-stranded DNA (ssDNA) genome (Rose *et al.*, 1969) flanked by inverted terminal repeats (ITRs) (McLaughlin *et al.*, 1988). Transcription mapping of the genome of AAV2 reveals three overlapping messenger RNA (mRNA) molecules that are produced from three promoters: p5, p19 and p40 (Laughlin, Westphal & Carter, 1979). The p5 and p19 promoters are known to mediate expression of *rep* genes required for AAV DNA replication and packaging of genetic cargo into AAV particles (Im & Muzyczka, 1990), whereas the p40 promoter drives transcription of *cap* gene mRNA, which is alternatively spliced to yield three distinct protein products, VP1, VP2 and VP3 in a 1:1:10 ratio (Becerra *et al.*, 1988). p40 also mediates expression of assembly-activating protein (AAP) in a different open reading frame (ORF) (Samulski & Muzyczka, 2014). AAP is not present in mature AAV capsids, but is known to play a key role in enabling production of high-titre AAV preparations for certain serotypes (Maurer *et al.*, 2018).

To produce recombinant AAV (rAAV) for gene therapy, the *rep/cap* genes are removed and replaced with a therapeutic gene and a promoter sequence to drive expression. The maximum size of a genetic sequence that can be cloned/synthesised between the ITRs is around 4.7 kbp, which limits the application of AAV in diseases requiring the delivery of large gene expression cassettes exceeding this capacity (Wu, Yang & Colosi, 2010). When rAAV genomes enter the cell

nuclei, second-strand synthesis occurs that converts a linear ssDNA molecule into a double-stranded DNA (dsDNA) episome, thus enabling the synthesis of therapeutic constructs (Choi, McCarty & Samulski, 2006). Achieving nuclear transfer of AAV genetic cargo is a complex multistep process, however. First, the virus must bind to a cell and undergo endocytosis by utilising a number of primary receptors and co-receptors, many of which have not yet been identified (Pillay *et al.*, 2016). AAV then ‘escapes’ from the endosome as it acidifies and it is thought to be trafficked to the nucleus *via* a nuclear localisation sequence (NLS) on the VP1 and VP2 capsid monomers (Xiao & Samulski, 2012). Recent studies have shown that nuclear import of rAAV is mediated by nuclear pore complexes (NPCs) and this process is dependent upon interactions with importin- $\alpha$  (Kelich *et al.*, 2015). After nuclear import, rAAV capsids uncoat, thereby releasing their genetic cargo into the target cell (Berry & Asokan, 2016).

## II. SUCCESSES IN AAV CLINICAL TRIALS

A number of successful clinical trials have now demonstrated the potential for using AAV as a gene transfer device for therapeutic purposes. AAV is an attractive vector for human gene therapy benefitting from several advantages. First, it is largely non-integrating, thereby reducing the risk of insertional mutagenesis *via* the disruption of tumour suppressor genes, which is a major concern for lentiviral gene therapies, for example (Moiani *et al.*, 2012). Second, AAV can be used to target a very broad range of cell types as a number of different serotypes exist, each with a unique tissue tropism (Srivastava, 2016). Whilst the packaging capacity of AAV is

relatively limited, it is thought to induce transgene expression in both dividing and non-dividing cells (Colella, Ronzitti & Mingozzi, 2018). Finally, although there is an emerging body of evidence implicating a negative impact of humoral and cellular immune responses on AAV-mediated gene transfer, AAV is largely considered a safe vector for human gene therapy, especially when delivered locally close to the site of pathology, such as the vitreous cavity of the eye (Khabou *et al.*, 2018).

At the time of writing, three gene therapy programmes have demonstrated efficacy in phase III studies. Zolgensma (onasemnogene abeparvovec-xioi) was approved by the USA FDA in 2019 for the treatment of spinal muscular atrophy (SMA) in patients harbouring biallelic mutations in the *smn1* gene. The therapy utilises the AAV9 serotype, which is unique in its ability to cross the blood–brain barrier (BBB), to deliver circular DNA (cDNA) encoding the *smn1* gene under control of the CAG promoter (Shahryari *et al.*, 2019). In one study, participants demonstrated remarkable improvements in motor function and quality of life, with 11/12 patients achieving full head control and two patients even walking independently (Rao, Kapp & Schroth, 2018; Al-Zaidy *et al.*, 2019). Luxturna (voretigene neparvovec) is an AAV2-based gene therapy for Leber's Congenital Amaurosis type 2 (LCA2). LCA2 is caused by biallelic (homozygous or compound heterozygous) mutations in the retinal pigment epithelium-65 (*rpe65*) gene, which encodes a critical enzyme essential for visual processing by converting all-trans-retinyl esters to 11-cis-retinol during phototransduction (Cremers, 2002). In an open-label, randomised phase III study, no therapy-related serious adverse events or unacceptable immune responses occurred, whilst 65% of treated patients demonstrated improvements in visual function (Russell *et al.*, 2017). Further assessment has corroborated the safety of the approach, demonstrating that Luxturna can deliver benefits to patients up to four years after administration (Maguire *et al.*, 2019). In summary, the successful gene therapy programme for LCA2 illustrates the merits of AAV-mediated gene delivery to the outer retina by subretinal injection, providing the impetus for other programs targeting disorders affecting photoreceptors and retinal pigment epithelium (RPE). Programs targeting the inner retina, and specifically the retinal ganglion cell (RGC) layer, have also demonstrated promising results for the amelioration of genetic disease. Lenadogene (GS010) is an AAV2-based treatment for Leber's Hereditary Optic Neuropathy (LHON), which is the most common cause of mitochondrial blindness, affecting at least 1 in 35000 of the population. The majority of patients carry one of three mitochondrial DNA (mtDNA) point mutations (m.3460A>G in MTND1, m.11778G>A in MTND4, and m.14484T>C in MTND6), which all affect genes encoding subunits of the mitochondrial respiratory chain complex I (Yu-Wai-Man *et al.*, 2014). In a phase III study, Lenadogene treatment resulted in improvements in best-corrected visual acuity (BCVA) with an increase of 26 Early Treatment Diabetic Retinopathy Study (ETDRS) letters in the treated eyes at

96 weeks following intravitreal injection, which is greater than expected from the natural history of LHON (Yu-Wai-Man, Moster & Sadun, 2019). Further, a secondary analysis of a phase I/II trial of patients receiving Lenadogene showed the treatment was safe and well tolerated, with mostly mild and transient intraocular inflammation in the anterior chamber and vitreous (Bouquet *et al.*, 2019). Overall, whilst Lenadogene is yet to obtain regulatory approval, the trials demonstrate the potential of AAV-mediated gene transfer to the inner retina. However, the unexpected observation that unilateral intravitreal injections given to patients resulted in improvements in visual acuity in the contralateral (untreated) eye is something that warrants further investigation (Yu-Wai-Man *et al.*, 2019).

### III. IMMUNE RESPONSES TO AAV – KEY CONSIDERATIONS

#### (1) How are immune responses initiated?

An organism mounts an immune response to protect themselves against foreign protein structures, termed antigens, that may be detrimental to their survival. Immune responses can be classified as innate or adaptive. In an innate immune response, pathogen-associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs). This drives a rapid and non-specific response that does not induce any immunological memory (Mogensen, 2009). During a wild-type AAV infection, PRRs can recognise viral nucleic acids and membrane glycoproteins, which leads to nuclear factor  $\kappa$ B (NF $\kappa$ B) and interferon regulatory factor (IRF) activation and synthesis of proinflammatory cytokines and type I interferons respectively (Vandamme, Adjali & Mingozzi, 2017). Adaptive immunity arises after the innate immune response and results in the development of 'immunological memory', which allows the organism to mount a faster and more efficient immune response when encountering the antigen for the second time. Adaptive immunity begins with the presentation of a particular antigen by an antigen presenting cell (APC) to T and B lymphocytes. These cells are then activated, and undergo clonal expansion (proliferation of T and B cells that are specific to the particular antigen that has been recognised). This is followed by T and B cell differentiation into effector cells [CD4+ or CD8+ T-cells, or plasma cells (PCs)] which act to eliminate the antigen *via* the destruction of virally infected cells or *de novo* antibody synthesis (Cui & Kaech, 2010; Pennock *et al.*, 2013; Tellier & Nutt, 2019). Finally, a population of memory T and B cells remain which are able to recognise the antigen rapidly if the organism encounters it a second time (Dorak, 2002).

#### (2) Is AAV non-pathogenic and non-immunogenic?

Until recently, wild-type AAV was not generally considered to be associated with any known human pathology. This is considered a key factor given that up to 90% of people are thought to be asymptotically infected with AAV

throughout their lifetimes (Kruzik *et al.*, 2019). However, recent evidence has suggested a role for wild-type AAV2 in the development of hepatocellular carcinoma (HCC). The authors observed AAV2 genome integration in 11/194 HCC samples tested, and concluded that wild-type AAV2 may have been a causative factor *via* oncogenic insertional mutagenesis (Nault *et al.*, 2015). It should be emphasised however, that their findings related to wild-type AAV2 and in later publications, the authors clarified that their findings should not be confounded with the clinical use of rAAV gene therapies (Nault *et al.*, 2016). Given the inherent similarities between wild-type AAV2 and rAAV2 however, more research into the possibility of oncogenic genome insertion is needed.

A key factor underpinning the progress of AAV-based gene therapy into the clinic is the safety profile of the vector. Depending on the route of administration, choice of serotype and dosage used, rAAV exhibits a relatively tolerable safety profile when compared to other gene transfer devices like adenovirus and lentivirus (Nayak & Herzog, 2010). A number of theories have been suggested to explain why wild-type and rAAV appear to be relatively non-immunogenic. First, most AAV serotypes may be poor transducers of professional APCs, such as dendritic cells, and may only result in minimal upregulation of major histocompatibility complex (MHC; a key molecular player in the presentation of antigens to the immune system) proteins in target cell types. This has been demonstrated in a number of publications investigating immune-competent sites like the liver and muscle (Mays *et al.*, 2014; Rossi *et al.*, 2019), however, there is little literature to support this claim in sites generally considered more immune-privileged, like the eye and central nervous system (CNS). A second theory suggests that a lack of viral DNA in rAAV vectors reduces recognition by PRRs. Whilst the removal of *rep* and *cap* genes from rAAV particles obviates the *in vivo* proliferation of the virus, thereby avoiding amplification of capsid antigens, this theory does not explain why rAAV is less immunogenic than adenoviral vectors, for example, which also have the genes required for capsid replication removed. One possible explanation for the differences in immunogenicity observed between AAV and adenoviral vectors is that AAV transduction induces lower and more transient innate immune activation (Wold & Toth, 2014).

### (3) Innate immune responses to AAV

Innate immune responses against AAV have recently been implicated as a possible cause for the toxicities that have been observed in gene therapy clinical trials. Innate immunity can be driven by anti-capsid and anti-nucleic acid pathways. It is now recognised that anti-capsid responses are likely derived *via* toll-like receptor 2 (TLR2) signalling, which is expressed on the cell surface (Hösel *et al.*, 2012). TLR9 signalling is responsible for the recognition of viral DNA sequences, and endosomal TLR9 has been implicated in the sensing of unmethylated CpG motifs in dendritic cell types (Rogers

*et al.*, 2017). Further, upregulation of TLR9 has been correlated with improved antigen presentation to the naïve CD8+ T-cells with MHC class I molecules, and signalling of this receptor with myeloid differentiating factor 88 (MyD88) has been implicated as a key pathway instigating immune responses against transgenes in the liver and muscle (Ashley *et al.*, 2019; Herzog *et al.*, 2019). The interaction between plasmacytoid dendritic cells (pDCs) and AAV may be a key factor mediating the efficiency of *in vivo* gene transfer in certain organs. Recognition of viral DNA sequences by TLR9 activates MyD88 and the type I interferon cascade, leading to NFκB-dependent cytokine and chemokine production in the liver (Jayandharan *et al.*, 2011), an effect that has been linked to progressive transgenic silencing over time (Suzuki *et al.*, 2013).

It has been established that the inhibition of key molecular players in the pathways mediating activation of the innate immune response can prevent anti-AAV CD8+ cytotoxic T-cell responses. Here, inhibition of TLR9 and type I interferon signalling has been shown to attenuate anti-AAV2 CD8+ T-cell induction (Rogers *et al.*, 2017), and blockage of type I interferons with monoclonal antibody therapy has demonstrated reduced cross-priming of anti-AAV8 CD8+ T-cells by pDCs (Shirley *et al.*, 2020).

Innate immunity against dsRNA molecules was also recently identified by Shao *et al.* (2018). Their findings implicated AAV ITR-driven dsRNA synthesis as a cause of type I interferon expression in transduced hepatocytes and also demonstrated that inhibition of MDA5 (a cytoplasmic RNA sensor) improved transgene expression in these cells (Shao *et al.*, 2018). This study in particular highlights our evolving understanding of innate immunity against AAV vectors but also provides a tangible example of how clarifying the molecular mechanisms underpinning AAV immunity can improve efficacy in preclinical and clinical studies.

The role of natural killer (NK) cells as determinants of *in vivo* gene therapy outcome is perhaps less well characterised than other arms of the immune system, however, one study has highlighted a possible role for these innate cytotoxic cells in seronegative individuals. Here, treatment of peripheral blood mononuclear cells (PBMCs) from seropositive patients with AAV capsids was associated with induction of an effector memory CD8+ phenotype (detected *via* granzyme B and CD107a degranulation marker expression), whilst PBMCs from seronegative patients treated with AAV was correlated with the activation of NK cells (Kuranda *et al.*, 2018).

### (4) Cellular immune responses to AAV

The innate immune response is clearly linked to the development of cellular immunity against AAV vectors. Cellular immunity can be pre-existing (arises before administration of therapy; likely *via* asymptomatic infections by wild-type AAV) or arise *de novo* from the administration of an AAV gene therapy.

### (a) Pre-existing cellular immunity to AAV

Pre-existing cellular immunity to AAV is thought to arise during infancy following a wild-type AAV infection, which was recently evidenced by flow cytometry analysis demonstrating a memory T-cell phenotype (i.e. expression of differentiation markers) in subjects' lymphocyte populations. These memory T-cells expressed interferon- $\gamma$  (IFN $\gamma$ ), interleukin-2 (IL2) and tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and exhibit a cytotoxic phenotype (induce apoptosis of AAV-infected cells) as demonstrated by expression of granzyme B and CD107a (Verdera, Kuranda & Mingozi, 2020).

Whilst anti-AAV T-cells exhibit high cross-reactivity and demonstrate responses to a variety of AAV serotypes (Hui *et al.*, 2015; Kuranda *et al.*, 2018), the exact role of pre-existing cellular immunity is not well understood, and it is noted that the patterns of T-cell reactivity to AAV in gene therapy trials differ from those seen during a typical viral infection. As Verdera *et al.* (2020) suggest, this observation may reflect the fact that the mode of administration of AAV gene therapies is the injection of a large number of non-replicating recombinant vectors into the body, which does not match that of an ongoing viral infection with replicating virions. More research is required to further our understanding of this aspect of cellular immunity against AAV which may be useful to inform inclusion and exclusion criteria in clinical trials, for example.

### (b) Induction of anti-AAV cellular immune responses

As discussed above, a cellular immune response is mounted against AAV antigens following administration when APCs present immunogenic capsid proteins to cytotoxic CD8+ T-cells *via* MHC class I. These capsid-specific T-cells are thus directed against AAV-infected cells and can induce their apoptosis *via* cell-mediated cytotoxicity. This effectively clears AAV-transduced cells and thereby decreases expression of a particular transgene product (Mingozi *et al.*, 2007; Pien *et al.*, 2009). Presentation of viral capsid proteins on MHC class II can also occur, resulting in the activation of CD4+ T-cells, which are known to mediate both cellular and humoral immunity in response to AAV gene therapy (Chen *et al.*, 2006). CD4+ T-cells mediate a number of immune processes, but are thought to be critical mediators of the antiviral response given their role in activating B-cells to become antigen-synthesising plasma cells, and in facilitating class switching to immunoglobulin G1 (IgG1) neutralising antibody (NAb) subtypes (Janeway, Travers & Walport, 2001).

One of the first examples of cellular immunity against AAV2 in the clinic was seen during haemophilia B gene therapy trials. After an initial intravenous infusion of 2E12 gc (genome copies)/kg AAV2 carrying a functional copy of the factor IX (FIX) gene, transgene expression reached levels around 10% of that seen in healthy controls. After 4 weeks however, FIX levels decreased to baseline levels in tandem

with an increase in liver transaminase levels in which an anti-AAV2 capsid T-cell response was implicated (Manno *et al.*, 2006). Later studies confirmed this CD8+ T-cell response using enzyme-linked immunosorbent spot (ELISPOT) assays and flow cytometry in response to transduction of the liver, and showed that the T-cell response was directed against the AAV2 capsid protein (Mingozi *et al.*, 2007). In clinical trials investigating intramuscular delivery however, anti-AAV cellular immune responses have been observed, yet these have not apparently attenuated transgene expression in contrast to liver gene transfer studies (Brantly *et al.*, 2006, 2009). A number of factors may underpin these differences, including the serotype used (AAV1 was used in the muscle gene transfer studies cited above), the route of administration used, the number of viral particles administered, and the immune status of the enrolled patients at baseline (i.e. when initially recruited to the study).

Anti-capsid cellular immune responses can clearly have a detrimental impact on the efficacy of AAV gene therapy. Anti-transgene cellular responses have also been noted involving both CD4+ and CD8+ T-cells. As with anti-capsid CD8+ responses, anti-transgene cellular immunity can decrease transgene expression of AAV transduced cells *via* cell-mediated cytotoxicity mechanisms (Nidetz *et al.*, 2020). A number of factors have been implicated as key mediators of anti-transgene cellular immunity, including the route of administration, choice of promoter, frequency of CpG-rich sequences, and secretion of the transgene product (Verdera *et al.*, 2020). In the clinic however, anti-transgene cellular immune responses have only been observed in rare cases, usually in trials utilising the intramuscular delivery route, such as anti-Dystrophin T-cells in Duchenne's Muscular Dystrophy (DMD) trials, for example (Mendell *et al.*, 2010). More recently, however, this effect has also been observed in organs considered to be more immune-privileged. In a clinical study investigating mucopolysaccharidosis type IIIB, anti-transgene T-cells were documented following intracranial delivery of AAV5 (Tardieu *et al.*, 2017).

Interestingly, anti-capsid T-cell responses may not necessarily be deleterious for AAV-mediated gene therapy strategies. Initiation of tolerance to AAV capsids in two clinical studies has been observed which appeared to be mediated by the induction of regulatory T-cell (Treg) responses in which signalling *via* the programmed cell death protein-1 (PD1) and its cognate ligand PDL-1 was implicated (Mueller *et al.*, 2013; Ferreira *et al.*, 2014). In these trials, patients received AAV1 intramuscular injections which apparently led to the infiltration of Treg cells *in situ*. The capacity of Treg cells to attenuate immune responses, for example by inhibiting CD8+ cytotoxic T-cells (*via* induction of apoptosis *via* granzyme- and perforin-dependent pathways) and secretion of regulatory cytokines (e.g. interleukin-10 or interleukin-35), suggests that therapeutic augmentation of Treg responses may be beneficial for *in vivo* gene therapy (Gernoux, Wilson & Mueller, 2017).

#### IV. HUMORAL IMMUNE RESPONSES TO AAV

There is a clear role for the innate and cellular arms of the immune system in limiting the efficacy of AAV-based gene transfer strategies. It is now well established that the generation of antibodies against various viral components also represents a significant barrier for AAV gene therapy. This section will review the role of the humoral immune response in terms of pre-existing humoral immunity and the induction of anti-vector antibody responses. Understanding the fundamental biological concepts underpinning the production of antibodies and the mechanisms of neutralising antibodies is key to deriving counterstrategies.

##### (1) How are antibodies produced?

Antibodies are glycosylated proteins that can be presented on the surface of B-cells [and act as antigen receptors (B-cell receptors)] or secreted by B-cells to bind to and neutralise target proteins. Antibodies are composed of two 'light' and two 'heavy' chains which are linked by disulphide bonds. At the N-terminus on an antibody are the hypervariable regions which determine antigen specificity. Five classes of antibodies have been described (IgM, IgD, IgG, IgA, IgE), each varying according to their respective C-terminal domains, termed Fc regions. Fc regions mediate the effector functions of antibodies, for instance when facilitating antibody-dependent cell-mediated cytotoxicity by recruiting CD8+ T-cells (Hoffman, Lakkis & Chalasani, 2016).

Immature B-cells originate from the bone marrow in adult humans. They differentiate from haematopoietic stem cells into pro-B-cells, then pre-B-cells and then immature B-cells. This development follows rearrangement of immunoglobulin heavy and light chains on the cell surface, resulting in expression of an antigen-specific IgM B-cell receptor (Thomas, Srivastava & Allman, 2006). Immature B-cells migrate to the spleen and lymphoid organs where they undergo activation *via* antigen recognition by the B-cell receptor and helper signals from CD4+ T-cells. Antigen binding to B-cell receptors activates gene expression changes and internalisation of the antigen into an endosome. Antigen proteins are subsequently degraded and presented on the B-cell surface by MHC class II molecules, which facilitates interactions with helper CD4+ T-cells (Victora & Nussenzweig, 2012). Some activated B-cells develop into plasmablasts without entering the B-cell follicles within the spleen. Others return to the follicles where they undergo affinity maturation of antigen-binding sites [*via* somatic hypermutation (SHM)] and immunoglobulin class switching [*via* class-switch recombination (CSR)] to generate high-affinity antibody-producing plasma cells and memory B-cells with a diverse set of effector functions. Here, SHM induces point mutations in antigen-binding regions to enable selection of high-affinity clones whilst CSR replaces DNA sequences that dictate isotype classes. The latter permits the generation of antibodies with various effector functions without altering their antigenic specificity. These plasma cells

home to the bone marrow and produce antibodies independently of further antigen exposure (Muramatsu *et al.*, 2000; Chaudhuri & Alt, 2004; Shlomchik & Weisel, 2012).

##### (2) How do antibodies neutralise AAV?

Despite the relevance of pre-existing and induced humoral immunity against AAV, the precise mechanisms by which antibodies neutralise AAV have yet to be elucidated. Here we will summarise the main mechanisms that antibodies utilise to neutralise viral infections.

Antibodies can have a neutralising or binding (non-neutralising) function. A neutralising antibody (NAb) is defined as one that is capable of inhibiting the infectivity or pathogenesis of a virus. Binding, or non-neutralising antibodies, are thought to lack neutralising activity but may be involved in the recruitment of immune cells and the induction of antibody-dependent cellular cytotoxicity (ADCC) (Schmaljohn, 2013). ADCC involves the binding of antibodies to a target cell (for instance, one that is virally infected) which in turn initiates the lysis of that cell by an effector cell (Hashimoto, Wright & Karzon, 1983). This process can be mediated by NK cells, for example, which recognise the Fc portion of an antibody *via* cell-surface expression of Fc $\gamma$  receptors such as CD16 (Chen *et al.*, 2018). A comparable process is antibody-dependent cellular phagocytosis (ADCP), in which viral particles, or virally infected cells, are bound by antibodies whose Fc portions are recognised by Fc $\gamma$  on phagocytic cells (Tay, Wiehe & Pollara, 2019). The precise contribution of ADCC and ADCP to the clearance of transduced cells following administration of AAV requires further investigation, however.

Some NAb can impede a virus by inhibiting its function prior to its binding to a cell. IgA and IgM antibodies can induce aggregation of certain types of bacteria, and some IgG antibodies have exhibited this function in the context of polio virus infections, for example (Brioen, Dekegel & Boeyé, 1983). Other antibodies appear to induce loss-of-function conformational changes in their targets that destabilises their capsid structures. This has been demonstrated with NAb against Sinbis virus (Hernandez, Paredes & Brown, 2008) and human immunodeficiency virus-1 (HIV-1) (Klasse & Sattentau, 2002).

NAb can also interfere with the attachment of viruses to cells. For example, NAb can bind to HIV-1 gp120 and thereby prevent the binding of the virus to its cognate receptor, CD4 (Klasse *et al.*, 2012). This mechanism, thought to occur due to steric interference between virus cell-binding ligands and cell surface receptors, has also been shown to apply to flavivirus (He *et al.*, 1995), parvovirus (Booy *et al.*, 1998) and rotavirus (Ruggeri & Greenberg, 1991).

Other NAb may interfere with enveloped viruses like HIV-1 post-attachment by preventing fusion of viral and endosomal membranes once the virus enters its target cell (de Rosny *et al.*, 2004). Non-enveloped viruses enter cells *via* endocytosis and must 'escape' from endosomes in order to prevent their degradation in lysosomes. Antibodies against

polio virus have been shown to destabilise capsid proteins so that the virus cannot escape from the endosome (Wien *et al.*, 1995).

NAbs may also interfere with other essential viral intracellular processes. For instance, some NAb against human papilloma virus have been shown to prevent trafficking of viral DNA to the nucleus (Ishii *et al.*, 2010). Recently, a novel mechanism of intracellular virus neutralisation was described in which a cytosolic antibody receptor, called Trim21, binds to antibody-coated adenoviruses and facilitates their proteasomal degradation (Mallery *et al.*, 2010).

Clearly, NAb can utilise a number of mechanisms to neutralise virus infections. However, our understanding of how NAb interact with AAV is very limited, and only a few studies have investigated this aspect of gene therapy. A correlation between the levels of NAb and IgG titres has been established (Kruzik *et al.*, 2019), and it appears that IgG1, IgG2, and IgM are the main subtypes that are correlated with anti-AAV NAb (a similar correlation was not observed with IgG3 and IgG4 in this report) (Murphy *et al.*, 2009). However, it has not yet been characterised which subtype is primarily responsible for neutralising activity against AAV as opposed to binding activity against AAV. One study showed that NAb are associated with the accumulation of AAV in the lymphoid organs, whilst binding antibodies have been shown to increase transduction of the liver, highlighting the possibility that certain subclasses of antibody may be capable of partially redirecting vector tropism (Fitzpatrick *et al.*, 2018). Further, interactions between humoral immune responses and complement pathways have also been identified. In an *in vivo* study, mouse models deficient in complement receptor 1/2 and complement component 3 demonstrated a delayed antibody response to AAV2 vectors and significantly lower terminal NAb titres compared to wild-type controls (Zaiss *et al.*, 2008). Interactions between Ig subtypes and the cellular immune system have also been observed, with one study highlighting a possible correlation between IgG3 antibody levels and the development of T-cell reactivity against AAV capsid proteins (Mingozzi *et al.*, 2009).

### (3) Pre-existing humoral immunity

The prevalence of patient seropositivity (the harbouring of antibodies against an antigen) varies substantially depending upon the particular serotype in question and the geography in which the study was conducted (Calcedo *et al.*, 2009; Boutin *et al.*, 2010). In studies examining people from four continents, the prevalence of NAb against AAV1/2 ranged from 30 to 60%, which was greater than the 15–30% range observed for AAV7/8/9, and 2% seropositivity for AAV4 (Calcedo *et al.*, 2009). These disparities may reflect the likelihood of wild-type infections by a particular serotype, given that pre-existing humoral immunity, much like pre-existing cellular immunity described above, is thought to arise from asymptomatic wild-type AAV infections throughout a patient's lifetime. Anti-AAV NAb are also known to be highly cross-reactive, and patients are often observed to

harbour NAb against most, if not all, AAV serotypes; a finding that may reflect the conservation of epitopes across different AAV serotypes (Calcedo & Wilson, 2013).

In most liver gene transfer studies, AAV serotypes 2 and 8 have been utilised, however, in a recent clinical trial investigating haemophilia B, AAV5 was used. Of the 10 participants included in the trial, none were deemed to have pre-existing NAb against AAV5, and although some did demonstrate anti-AAV5 IgG and IgM, this had no detectable impact on the efficiency of gene transfer (Miesbach *et al.*, 2018). These findings have been corroborated by reports from Drygalski *et al.* (2019) who used a highly sensitive luciferase-based NAb assay to identify low anti-AAV5 NAb titres in haemophilia B patients undergoing gene therapy-based FIX replacement. They also showed that these low levels of NAb had no observable effect on the outcome of gene transfer in these patients. In this way, they were able to identify a new opportunity to include anti-AAV5 NAb seropositive patients in the clinical trial who were previously ineligible (Drygalski *et al.*, 2019).

An alternative route of administration for gene therapy-based treatment of haemophilia B involves the injection of AAV vectors into the muscle. In 2003, rAAV vectors were used to deliver a functional copy of the FIX gene into male participants with severe haemophilia B. No evidence of local or systemic toxicity to the vector was observed up to 40 months post-injection, and the presence of antibodies directed against FIX protein were also not detected. In contrast to liver gene transfer studies that would be conducted in subsequent years, the presence of anti-AAV NAb in these patients did not have an apparent impact on the efficiency of gene transfer to the muscle (Manno *et al.*, 2003).

Another study investigated the possibility of utilising intrathecal injections as a treatment for mucopolysaccharidosis type III. The prevalence of NAb against AAV2 and AAV9 was assessed in the sera and cerebrospinal fluid (CSF) of healthy volunteers and enrolled patients. In the sera, anti-AAV2 NAb were detected at a higher level than anti-AAV9 NAb, and overall levels of both anti-AAV2 and -AAV9 NAb were higher in the sera than in the CSF. Upon vector administration *via* intrathecal delivery (which involves injection of a vector-containing solution into the spinal canal such that it reaches the CSF), the presence of these pre-existing NAb did impact the efficiency of gene transfer, but did not completely block transduction (Haurigot *et al.*, 2013). In a non-human primate study, the detection of pre-existing NAb at a 1:128 titre did not have any apparent effect on the efficacy of gene transfer when an AAV9.GFP gene therapy was used (Gray *et al.*, 2013). Taken together, these findings highlight the immune-privileged status of the CNS and demonstrate a role for the BBB in limiting the transfer of NAb from the systemic circulation into the CSF.

Studies utilising non-human primate models have demonstrated that pre-existing NAb against AAV may limit the

efficiency of transgene expression following an intravitreal injection (IVT). Pre-existing sera NAb titres of 1:10 or greater were found to reduce transgene expression, although some animals with 1:25–1:100 NAb levels still exhibited some degree of retinal transduction (Kotterman *et al.*, 2015). Data emerging from clinical trials, however, have suggested that pre-existing sera NAb titres may not represent a significant barrier to effective transduction of the retina *via* IVT. In one study, 2/5 patients demonstrated improvements in visual acuity following administration of an AAV-based treatment for LHON, in spite of the fact they harboured sera NAb titres of 1:5120 and 1:20480 at baseline (Feuer *et al.*, 2016). Further, in a follow-up study to this trial, the same investigators showed that, of the 14 patients enrolled in the clinical study, the four that demonstrated the greatest increases in visual acuity also had the highest (1:20480) NAb titres at baseline (Guy *et al.*, 2017). By contrast, a clinical trial investigating AAV2.sFLT1 [soluble FMS-like tyrosine kinase-1, an anti-vascular endothelial growth factor (VEGF) agent] IVT gene therapy for neovascular age-related macular degeneration (nAMD) showed that pre-existing NAb titres of 1:400 appeared sufficient to preclude effective vector administration and transduction of the retina. sFLT1 protein expression was also blocked in patients with 1:3200 NAb titres but no evidence of vector neutralisation was seen in the patient with a baseline titre of 1:100 (Heier *et al.*, 2017).

In summary, the role of pre-existing NABs in limiting the efficiency of gene transfer appears to vary according to the route of administration and choice of AAV serotype. Broadly, systemic intravenous injections appear to be more susceptible to NABs, whilst administration to immune-privileged sites such as the CNS may result in efficient gene transfer even in the presence of low titres of NABs. Whether the administration of AAV to a patient with pre-existing NABs may be more risky than a seronegative patient is an open question. However, it is possible that the former route may involve injection to a 'primed' immune system which could induce a significant immune response. By contrast, injection into seropositive individuals may simply result in rapid neutralisation of viral particles, circumventing any cellular transduction and concomitant immune responses. Pre-screening trial participants for NABs using serum neutralisation assays prior to administration to immune-privileged sites is a key factor in ensuring efficient gene transfer, however, greater standardisation of these assays may be required to establish threshold NAB levels at which administration of AAV can be successful in seropositive individuals (Meliani *et al.*, 2015).

#### (4) Induction of anti-AAV humoral immune responses

Pre-existing antibodies to AAV represent a key barrier to successful gene transfer in seropositive individuals. A related issue is whether the administration of AAV induces a robust NAB response that precludes re-administration of a gene therapy. The importance of repeated gene transfer varies according

to the route of administration and target organ, however, in our view, the ability to re-administer a gene therapy is a clinically relevant consideration for most, if not all, *in vivo* gene therapy programmes. Administration to young adults suffering from haemophilia, for example, is faced with the problem that the liver is a dividing tissue, leading to dilution of transgene expression over time. Further, as discussed above, CD8+ T-cell responses may clear transduced cells from target organs, especially after systemic or intramuscular delivery, again leading to reduced transgene expression over time. In addition, whilst many gene therapy programs are targeting monogenic recessive disorders, there is an increasing interest in developing AAV constructs for diseases exhibiting complex aetiologies. In these diseases, multiple pathogenic pathways are involved which may require sequential administration of different gene therapies. There is also the possibility that an initial gene therapy injection is sufficient to induce a robust NAB response but insufficient to rescue a clinical phenotype. Lastly, it is possible that methylation of viral promoter sequences may occur years after AAV administration, which may lead to transgenic silencing. In these scenarios, a repeat injection of AAV could be used to circumvent reduced therapeutic transgene expression and maximise clinical benefit. Therefore, a discussion of the possibility of NAB induction and possible strategies to circumvent these responses is warranted. In this section, we will briefly summarise NAB responses following administration of AAV to the liver, skeletal muscle and CNS. We then provide a detailed analysis of NAB induction observed in ocular gene therapy trials.

##### (a) Gene therapy trials targeting the liver and skeletal muscle

A number of clinical studies have reported increased anti-AAV NAB levels after vector administration to target the liver and skeletal muscle. In summary, these reports suggest that these two routes of administration lead to elevated NAB titres, which, in some cases, were observed up to 52 weeks after vector infusion. Further, the utilisation of immunosuppression in two of the trials discussed below did not appear to be sufficient to abrogate anti-capsid NAB responses. Given the role that pre-existing NABs may play in limiting the effectiveness of gene transfer, we suggest the induction of NABs in these trials may represent a possible barrier to vector re-administration in these individuals, which may limit the clinical utility of gene therapy. A summary of humoral immune responses in clinical trials targeting the liver and skeletal muscle for human gene therapy is provided in Table 1.

##### (b) Gene therapy trials targeting the central nervous system

Unlike studies investigating liver and skeletal muscle gene therapies, clinical trials administering AAV to the CNS have not reported the induction of NABs. However, the results of non-human primate and dog studies investigating the induction of NABs following delivery to the CNS indicate possible differences to other modes of administration. Principally,



Table 1. Summary of humoral immune responses in clinical trials targeting the liver and skeletal muscle for human gene therapy

Reference	Disease	Therapy	Delivery	IS	Number of patients with increased NABs
Manno <i>et al.</i> (2006)	Haemophilia B	AAV2.FIX	Intravenous	None	7/7
Nathwani <i>et al.</i> (2011)	Haemophilia B	scAAV2/8.FIX	Intravenous	None	6/6
Manno <i>et al.</i> (2003)	Haemophilia B	AAV2.FIX	Intramuscular	None	8/8
Brantly <i>et al.</i> (2006)	AAT deficiency	AAV2.AAT	Intramuscular	n/a	12/12
Brantly <i>et al.</i> (2009)	AAT deficiency	AAV1.AAT	Intramuscular	n/a	12/12
Flotte <i>et al.</i> (2011)	AAT deficiency	AAV1.AAT	Intramuscular	n/a	9/9
Stroes <i>et al.</i> (2008)	LPL deficiency	AAV2.LPL(S447X)	Intramuscular	None	8/8
Gaudet <i>et al.</i> (2013)	LPL deficiency	AAV2.LPL(S447X)	Intramuscular	CyA, MMF	14/14
Ferreira <i>et al.</i> (2014)	LPL deficiency	AAV2.LPL(S447X)	Intramuscular	CyA, MMF, Pred	5/5

AAV, adeno-associated virus; scAAV, self-complementary AAV; FIX, factor 9; AAT,  $\alpha$ 1-antitrypsin; LPL (S447X), low density lipoprotein variant harbouring single point mutation; IS, immunosuppression; CyA, cyclosporin A (T-cell inhibitor); MMF, mycophenolate mofetil (T- and B-cell inhibitor); Pred, prednisolone (steroid immunosuppressant); NAb, neutralising antibody.

pre-existing sera NAb titres appear to have less impact in neutralising vector administration, likely due to the impermeability of the BBB, however AAV injection to the CNS may still result in increased NAb titres.

Gene delivery to the CNS can be achieved *via* systemic vector delivery provided an appropriate AAV serotype (Vandenbergh, 2019) [or mutated AAV capsid (Hordeaux *et al.*, 2018; Hudry *et al.*, 2018)] is used. As outlined above, the potential of gene transfer for treating diseases affecting the CNS has been demonstrated in the context of SMA, and an FDA-approved therapy (Zolgensma) now exists for the condition which uses AAV9 expressing survival motor neuron-1 (SMN-1). The earliest clinical trials investigating SMA gene therapy *via* systemic delivery did not report the induction of antibodies against either the AAV9 capsid protein or the SMN-1 transgene (Mendell *et al.*, 2017; Al-Zaidy *et al.*, 2019; Lowes *et al.*, 2019). Similarly, a study conducted in non-human primates and piglets did not report the development of humoral immune responses against capsid proteins or transgene products, in spite of the observation of severe toxicity following high-dose intravenous administration of an AAV9 variant, AAVhu68, carrying the SMN-1 transgene (Hinderer *et al.*, 2018).

An alternative route of delivery to the CNS is intrathecal delivery. A study investigating intrathecal delivery of AAV9 as a possible treatment for mucopolysaccharidosis type III showed that vector administration induced a robust systemic humoral immune response against AAV9 capsids, despite all dogs demonstrating undetectable levels of anti-AAV9 NABs at baseline. Eight days after vector administration, NAB titres were greater than 1:1000. Interesting correlations between systemic and corresponding CSF NAB titres were also observed and appeared to be dependent upon the presence of inflammation in the CSF. In the absence of inflammation, CSF samples were negative or had NAB titres of <1:10, however, in dogs exhibiting signs of inflammation in the CNS, NAB titres of 1:100–1:1000 were seen, suggesting that the presence of

inflammation may compromise the integrity of the BBB (Haurigot *et al.*, 2013).

Injections can also be performed directly into the brain in order to transfer genes to the CNS. One non-human primate study examined injections of AAV9 expressing acid sphingomyelinase (ASM) into the cerebromedullary (CM) space of a Niemann-Pick disease type A model (a lysosomal storage disease). Slight increases in anti-AAV9 NAB levels were seen in both the sera (1:400–1:800) and CSF (1:100–1:200) at 1 and 3 month timepoints respectively, in response to vector administration (Samaranch *et al.*, 2019).

### (c) Gene therapy trials targeting the eye

The eye has been at the forefront of gene therapy research for a number of reasons. First, its compartmentalised nature and tightly regulated transport of molecules across the blood-retinal-barrier (BRB) reduces the risk of vector leaking into the systemic circulation, thereby mitigating a significant regulatory/safety concern (Cunha-Vaz, Bernardes & Lobo, 2011). Second, the eye is relatively accessible for vector administration, and several well-characterised routes of delivery, such as IVTs and subretinal injections (SRTs) are available for ophthalmologists. Third, a number of non-invasive tools can be used to assess clinical endpoints for a particular therapy, such as electroretinography (ERG) and optical coherence tomography (OCT). Anatomically, the eye is also smaller than other organs, such as the brain, which obviates the need for high vector doses to achieve robust transduction of target cell types. Lastly, the eye is generally considered to have an ‘immune-privileged’ status and generally exhibits lower immune responses than other organs. As mentioned, this is in part due to the impermeability of the BRB to humoral and cellular arms of the immune system which has been shown to limit access of circulating anti-capsid antibodies into the eye (Amado *et al.*, 2010). The phenomenon may also be accounted for by a process called anterior chamber-associated immune deviation (ACAID). During ACAID, immunogenic antigens

Table 2. Summary of humoral immune responses in clinical trials utilising SRT and IVT for human gene therapy

Reference	Disease	Therapy	Delivery	Number of patients	IS	Increased NAbS detected
Bainbridge <i>et al.</i> (2008)	LCA2	AAV2.hRPE65	SRT	3	Oral prednisolone	0
Bainbridge <i>et al.</i> (2015)	LCA2	AAV2.hRPE65	SRT	12	Oral prednisolone	6
Hauswirth <i>et al.</i> (2008)	LCA2	AAV2.hRPE65	SRT	3	n/a	1
Jacobson <i>et al.</i> (2012)	LCA2	AAV2.hRPE65	SRT	14	Topical steroids	6
Maguire <i>et al.</i> (2008)	LCA2	AAV2.hRPE65v2	SRT	3	Oral prednisolone	1
Maguire <i>et al.</i> (2009)	LCA2	AAV2.hRPE65v2	SRT	12	Oral prednisolone	2
Bennett <i>et al.</i> (2012)	LCA2	AAV2.hRPE65v2	SRT*	3	Oral prednisolone	0
Le Meur <i>et al.</i> (2018)	LCA2	AAV4.RPE65	SRT	9	Oral prednisolone	2
Ghazi <i>et al.</i> (2016)	RP	AAV2.hMERTK	SRT	6	None	2
Rakoczy <i>et al.</i> (2015)	nAMD	AAV2.sFLT1	SRT	9	Topical steroids	2
Constable <i>et al.</i> (2016)	nAMD	AAV2.sFLT1	SRT	21	Topical steroids	3**
Wan <i>et al.</i> (2016)	LHON	AAV2.ND4	IVT	9	Oral prednisolone	0
Feuer <i>et al.</i> (2016)	LHON	AAV2.P1ND4v2	IVT	5	None	1
Guy <i>et al.</i> (2017)	LHON	AAV2.P1ND4v2	IVT	14	None	2
Bouquet <i>et al.</i> (2019)	LHON	AAV2.ND4	IVT	15	None	14
Heier <i>et al.</i> (2017)	nAMD	AAV2.sFLT1	IVT	19	None	7

AAV, adeno-associated virus; LCA2, Leber's congenital amaurosis type II; RP, retinitis pigmentosa; nAMD, neovascular age-related macular degeneration; LHON, Leber's hereditary optic neuropathy; RPE65, retinal pigment epithelium-65; RPE65v2, retinal pigment epithelium-65 with enhanced Kozak sequence; MERTK, c-Mer protooncogene tyrosine kinase; sFLT1, soluble FMS-like tyrosine kinase; ND4, NADH dehydrogenase subunit 4; P1ND4v2, NADH dehydrogenase subunit 4 containing mitochondrial targeting sequence; SRT, subretinal injection; IVT, intravitreal injection; IS, immunosuppression; NAb, neutralising antibody.

\*Participants received an SRT of AAV2 into the contralateral eye.

\*\*In this study, 3/9 patients seroconverted after receiving IVT of AAV2 (for the remaining 12 patients, NAb titres were not reported).

induce a tolerogenic immune response that involves regulatory T-cell and anti-inflammatory M2 macrophage induction, and increases synthesis of anti-inflammatory cytokines (Taylor, 2009; Zhou & Caspi, 2010; Keino, Horie & Sugita, 2018; Martínez-Alcantar *et al.*, 2019). An overview of the clinical studies reporting NAb titres for SRT and IVT gene therapies is provided in Table 2.

(i) Gene delivery to the outer retina by subretinal injection. A gene therapy for LCA2 was approved by the FDA in 2018. Efforts to develop an AAV-based gene therapy for LCA2 have helped to improve our understanding of AAV vector immunobiology when delivered into the subretinal space. These studies showed that the immune-privileged status of the eye, and the relatively non-immunogenic properties of AAV as a vector did not necessarily circumvent immune responses to vector administration.

Initial testing of humoral immune responses in C57BL/6 mouse models showed that a significant systemic antibody response against AAV capsid proteins could be induced by SRT. It was also found however, that the induction of systemic humoral immunity did not preclude vector re-administration into the ipsilateral eye (Anand *et al.*, 2000). The authors later characterised a deviant immune response upon SRT of AAV two years later, and highlighted similarities to ACAID. Here, the generation of a population of immunosuppressive Th2-type T-cells was reported post-SRT and they were identified as potential mediators of the immune deviation mechanism observed (Anand *et al.*, 2002). These data contrast with later reports which demonstrated SRT of AAV2 did not result in a detectable increase in NAb levels. This may reflect the

discrepancy in AAV serotypes used by the two investigators, however (Li *et al.*, 2008).

In 2008, the first report of AAV2.hRPE65.hRPE65 (using an RPE65 promoter) gene delivery to the subretinal space as therapy for LCA2 was published, however, only three patients were included in this study. None of the enrolled patients developed humoral immunity against AAV2 following vector administration, and sera NAb titres were either undetectable or <1:7 at baseline throughout the 12-month follow-up period (Bainbridge *et al.*, 2008).

A later report (Bainbridge *et al.*, 2015) examined the long-term safety of AAV2.hRPE65.hRPE65 gene therapy for LCA2, and provided evidence that intraocular inflammation and immune responses occurred when higher doses of vector were used. The study also highlighted a potential humoral immune response against AAV2. In 4/4 of patients receiving low-dose AAV2, circulating NAb titres increased throughout the 3-year follow-up period. In the high-dose group, four patients showed no changes in NAb levels throughout the study period. In the other two patients, however, vector administration apparently induced a humoral immune response. In both of these study participants, an asymptomatic episode of posterior intraocular inflammation was correlated with NAb titre increases. Throughout the study however, no anti-RPE65 transgene NAbS were detected. Crucially, this clinical trial demonstrated that humoral immune responses could be induced against AAV2 capsids even when a prophylactic oral glucocorticoid immunosuppressive drug regimen was given to patients before and after vector administration (Bainbridge *et al.*, 2015).

Other studies investigating SRT delivery of AAV2.CB.hRPE65 (chicken  $\beta$ -actin promoter) for LCA2 gene therapy have reported similar results. In a phase I trial, humoral immune responses were measured at baseline, and at days 14 and 90. Whilst 2/3 patients did not demonstrate an anti-vector NAb response, one patient exhibited a 7.5-fold increase in their 90-day antibody titre compared to baseline levels, however, this did not correlate with AAV2 capsid-specific reactivity of peripheral lymphocytes in which no changes were observed in any patients between baseline and days 14 or 90. In this study, anti-transgene NAb induction was not reported (Hauswirth *et al.*, 2008).

In a long-term assessment of the safety and efficacy of AAV2.CB.hRPE65, humoral immune responses were measured throughout a three-year follow-up period. 8/14 patients enrolled in the study actually demonstrated a decline in anti-AAV2 NAb titres throughout the study, whilst 6/14 demonstrated increases in NAb titres compared with baseline. Across all cohorts tested, no apparent correlation between AAV2 dosages and anti-AAV2 antibody titres was evident (Jacobson *et al.*, 2012). Interestingly, in the studies of Hauswirth *et al.* (2008) and Jacobson *et al.* (2012), no patients were given prophylactic glucocorticoid immunosuppressants, and this did not have an apparent effect on the induction of humoral immunity when compared to the patients in Bainbridge *et al.* (2008, 2015).

In a phase I trial published in 2008, AAV2.CB.hRPE65v2 [voretigene neparovec (Luxturna)] SRT administration was found to induce an anti-AAV2 NAb response in 1/3 patients enrolled in the study. The levels of this patient's NAb were elevated after 14 days, and did decrease by 30 days post-SRT but remained high compared to baseline. No evidence of anti-RPE65 NAb was observed (Maguire *et al.*, 2008).

The safety and efficacy of AAV2.CB.hRPE65v2 was then further investigated in a phase I dose escalation trial which introduced middle- and high-dose cohorts. In the middle-dose cohorts, 4/6 patients did not exhibit signs of an anti-AAV2 humoral immune response. In 2/6 patients however, significant increases in NAb levels were evident *versus* baseline values. Perhaps unexpectedly, in the high-dose cohort, no evidence of a humoral immune response was detected in any of the three patients. It should be noted however, that the two patients from the middle-dose cohort who exhibited the greatest increases in NAb levels also had the highest NAb levels at baseline. Therefore, it is possible that these patients had some pre-existing immunity to AAV2 that was sufficient to induce a humoral immune response upon vector administration but not sufficient to be excluded from the trial (Maguire *et al.*, 2009).

The investigators then tested whether re-administration of AAV2.CB.hRPE65v2 resulted in increased sera NAb levels. Of the three patients included in this follow-on trial, baseline NAb levels were 1:1–1:3.16 and remained at these low levels in spite of vector administration into the contralateral eye (Bennett *et al.*, 2012). Notably, in all studies examining AAV2.CB.hRPE65v2, systemic corticosteroid immunosuppressive treatment was given to patients to mitigate the

occurrence and severity of vector-mediated immune responses.

The majority of studies investigating gene therapy for LCA2 have utilised AAV2 for the delivery of therapeutic transgenes. One study has utilised the AAV4 serotype, however. Of the nine patients enrolled in this study, six did not exhibit detectable anti-AAV4 IgG at baseline or after vector administration. In two patients, a significant increase in anti-AAV4 IgG and NAb levels were seen. In one patient, a NAb titre of 1:50 was seen at baseline, but remained unchanged at 1:50 until 180 days follow-up, indicating that a humoral immune response was not induced by AAV4 SRT. In this study, prednisolone glucocorticoids were administered daily 1 week before and after the SRT of AAV4 prophylactically to inhibit immune reactions to the vector (Le Meur *et al.*, 2018).

Outside of LCA2, SRT of AAV vectors has been investigated for possible therapeutic effects in other genetic eye disorders like retinitis pigmentosa. More recently, the platform has also been applied to diseases with complex etiologies like nAMD.

Mutations in MER proto-oncogene tyrosine kinase (MERTK) cause retinitis pigmentosa. MERTK aberrations disrupt phagocytic activity of RPE cells, which in turn causes degeneration of rod and cone photoreceptors. AAV2 has been used to deliver functional MERTK genes into RPE cells *via* SRT. In a phase I study, 3/6 patients demonstrated improvements in visual acuity and all patients exhibited acceptable safety profiles. In 2/6 patients, anti-AAV2 humoral immune responses were also recorded and 10–26-fold increases in NAb titres were observed. However, no data were reported for these patients' NAb titres after one year of follow-up (Ghazi *et al.*, 2016).

The first reports of SRT gene therapy being used to treat nAMD in patients were published in 2015. In this phase I study, nine patients were enrolled and administered rAAV2.sFLT1 *via* SRT. The therapy was shown to reduce the requirement for 'rescue' injections of intravitreal anti-VEGF agents (Ranibizumab), whilst no drug-related adverse events were reported. Total anti-AAV2 and neutralising AAV2 antibodies were reported between baseline and one-year follow-up in six of the patients enrolled. In 4/6 patients, NAb titres remained unchanged at <1:20 throughout the study. In two patients, however, a robust humoral immune response was evident, these subject's NAb titres were <1:20 at baseline and increased to 1:20–1:100 at the 3-week timepoint (Rakoczy *et al.*, 2015).

Further testing of this gene therapy in a phase IIa study corroborated previous findings. Of the 21 patients studied, nine displayed no anti-AAV2 NAb at baseline, whilst 12 had titres between 1:20 and 1:100. In 3/9 patients with no detectable NAb at baseline, anti-AAV2 NAb was observed at completion of the study. In this publication however, data on NAb levels were not included, rendering further analysis challenging (Constable *et al.*, 2016).

(ii) Gene delivery to the inner retina by intravitreal injection. Compared to SRT, IVT is a much simpler route

of delivery for AAV-based gene therapies. IVTs can be performed in an ophthalmologist's office in a matter of minutes, and only requires topical analgesics for the injection. IVTs are also necessary to target inner retinal cells, principally RGCs. Recently, evidence has emerged suggesting that specific AAV serotypes can target the outer retina/photoreceptor layer *via* IVT. This has increased interest in this delivery route for treating common blinding disorders like nAMD and diabetic retinopathy (Dalkara *et al.*, 2013; Grishanin *et al.*, 2019). A number of preclinical and clinical studies utilising IVTs of AAV gene therapies have now been completed. These have highlighted similarities and differences between IVT and SRT in terms of the induction of humoral immunity against AAV capsid proteins.

One of the earliest reports contrasting the induction of NABs following SRT and IVT of AAV2 was published in 2008 (Li *et al.*, 2008). In a rodent model, it was shown that unilateral SRT did not trigger a humoral immune response against AAV2 capsid proteins. As a result, subsequent administration of AAV2 into the contralateral eye was permissible when delivered by SRT and IVT, a finding that supported clinical studies investigating SRTs and contralateral eye administration (Bennett *et al.*, 2012). When an initial injection of AAV2 was delivered *via* IVT however, the induction of humoral immunity was evident. The authors reported up to tenfold increases in total anti-AAV2 antibodies following IVT, a finding that correlated with the levels of NABs against AAV2 capsids. Upon re-administration of AAV2 to the contralateral eye, the authors found that SRT delivery resulted in robust transduction of the outer retina, however, they observed diminished expression if the IVT delivery route was used for vector re-administration (Li *et al.*, 2008). Their findings therefore highlighted possible differences between the immune mechanisms employed by the vitreous cavity *versus* the subretinal space, however, it should be noted that their observation that SRT did not result in a humoral immune response is in partial disagreement with some clinical (Hauswirth *et al.*, 2008; Jacobson *et al.*, 2012; Bainbridge *et al.*, 2015) and preclinical studies (Anand *et al.*, 2000, 2002).

In non-human primate studies, further evidence has arisen that IVT may induce stronger NAB responses than SRT. Kotterman *et al.* (2015) published data relating to the induction of anti-AAV NABs following IVT of multiple serotypes and mutagenised capsid protein AAV variants, and their analysis showed that IVT of all AAV variants caused a NAB response. They tested AAV2, AAV5, AAV9, AAV7m8 (peptide-insert mutant AAV) and AAV2 (Y-F tyrosine mutant AAV2), all of which were found to elevate NAB levels from 1:1–1:100 at baseline to 1:100–10000 after the injection. In some of the non-human primates, NAB levels were assessed over a period of 7 months. All injected animals demonstrated consistent NAB levels in the first 3 months following IVT, but some showed a 2–5-fold decline after 3 months. In general however, the persistence of elevated NAB titres for long periods after gene therapy administration was observed. The authors also reported cross-reactivity of NABs generated against multiple AAV serotypes. Here,

injection of AAV5 and AAV9 was found to increase anti-AAV2 NAB levels, and IVTs of AAV2, AAV7m8 and AAV2(Y-F) induced anti-AAV5, -AAV8 or -AAV9 humoral immune responses. The authors also noted an interesting but non-significant trend in which higher pre-injection NAB titres correlated with a smaller change between pre- and post-injection titres. It was postulated that this may be due to higher rates of vector neutralisation which attenuated retinal transduction and antigen presentation to the immune system (Kotterman *et al.*, 2015).

In a comparable non-human primate study, IVT and SRT of AAV capsid mutant variants AAV7m8 and AAV8BP2 (9mer peptide insert into AAV8 capsid) were found to induce humoral immune responses. Following IVT, AAV8BP2 and AAV7m8 increased sera NAB titres in non-human primate models post-IVT. These increases appeared to be slightly lower than that observed by Kotterman *et al.* (2015), however, this discrepancy may be explained by a multitude of confounding variables, such the different timepoints used in the study, difficulties in accurately titrating AAV preparations (rendering comparison between studies challenging) and of course the use of different AAV serotypes/mutant capsids. This study also compared sera NAB titres to vitreous NAB titres. After IVT of AAV8BP2, post-injection vitreous NAB titres remained unchanged at <1:3.16 in all animals. Following AAV7m8 IVT however, vitreous NAB levels rose in 2/6 non-human primates. Interestingly, this study also reported increases in NAB levels following SRT in some animals (1/3 after AAV8BP2 SRT and 4/4 after AAV7m8 SRT), a finding that reflects certain preclinical and clinical results (Anand *et al.*, 2000, 2002; Hauswirth *et al.*, 2008; Jacobson *et al.*, 2012; Bainbridge *et al.*, 2015) but disagrees with the results of investigations utilising mouse models (Li *et al.*, 2008).

Reports detailing the induction of humoral immunity in clinical trials following IVT of AAV-based gene therapies arose sometime after those utilising the SRT route of administration. In 2016, one study described the use of AAV2 expressing mitochondrial reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (ND4) as a possible treatment for LHON. Patients enrolled in this clinical trial were given oral prednisolone for 1 week prior to and 8 weeks after the administration of the therapy. At baseline and 6 months post-IVT, NAB titres in all nine patients remained unchanged and at levels below 1:20, however, it should be noted that no improvements in visual acuity were observed in this study (Wan *et al.*, 2016).

In the same year, data from another clinical trial investigating LHON gene therapy were published. In this study however, a self-complementary AAV2 (scAAV2; contains dsDNA genome) harbouring tyrosine to phenylalanine substitutions and an ND4 gene expression cassette (AAV2.CBA.PIND4v2) was utilised, and no immunosuppressants were given to the five patients. Only one patient experienced an increase in NAB levels after 7 days, which then decreased back to baseline levels after 90 days follow-up. It should be noted however, that the other four patients enrolled

exhibited very high (1:5120–1:20480) NAb levels at baseline, but this did not appear to inhibit transduction efficiency/therapeutic efficacy (Feuer *et al.*, 2016). In a follow-up to these initial results, similar data were reported that corroborated the previous findings (Guy *et al.*, 2017). In accordance with other studies investigating SRT of AAV gene therapies (Hauswirth *et al.*, 2008; Jacobson *et al.*, 2012; Bainbridge *et al.*, 2015), there was no obvious correlation between baseline NAb titres and the induction of a humoral antibody response. Another comparison between this study and data from SRT investigations is that the observation of a significant humoral immune response correlated with an episode of anterior uveitis, a finding similar to that reported by Bainbridge *et al.* (2015).

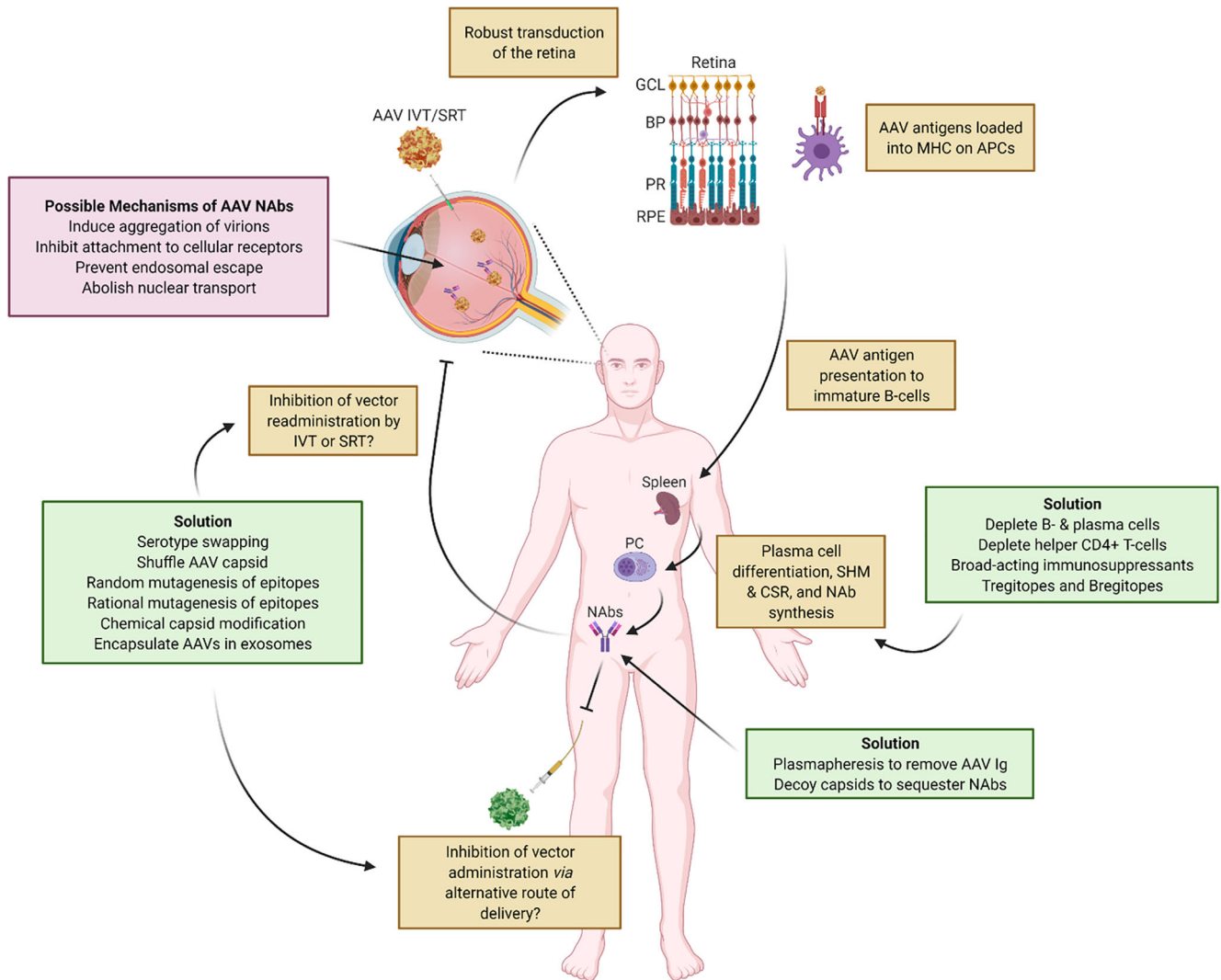
Induction of humoral immune responses in a subset of patients administered with AAV2 gene therapies in the absence of prophylactic immunosuppressants has also been shown in a phase I/II study investigating the use of AAV2.ND4 in LHON patients. In this trial, 13/15 patients exhibited episodes of anterior uveitis and vitritis, two of which were administered immunosuppressants to counteract this ocular inflammation. The authors also concluded that there was no observable correlation between the ocular inflammation score (OIS) and the vector dose administered. They also reported no apparent association between the humoral immune response and vector dose, nor the humoral immune response and OISs. Most patients did demonstrate an increase in their NAb titres after AAV2 IVT, however, 3/3 patients receiving the lowest dose (9E9 vector genomes/eye) and 3/3 patients receiving 3E10 vector genomes/eye showed increases in NAb titre levels from baseline which largely persisted throughout the 96-week follow-up period. In patients who received 9E10 viral genomes/eye, however, the results were less clear, and whilst a transient increase in NAb titres was observed in 5/6 patients, these resolved back to baseline levels in two cases, but persisted until week 96 in the remaining three cases. Finally, in patients who received 1.8E11 viral genomes/eye, 3/3 showed elevated NAb titres shortly after receiving an IVT of AAV2.ND4. Overall, this study provided evidence that AAV2 IVTs may induce NAb responses, however, it should be emphasised that these patients did not receive prophylactic immunosuppressant therapy (Bouquet *et al.*, 2019). Here, more research is required to understand whether glucocorticoid therapy is able to circumvent the induction of humoral immunity in patients receiving IVTs of AAV2 and what the importance of this finding may be, for instance, in enabling re-administration of the vector to the ipsilateral or contralateral eye.

Whilst clinical studies utilising AAV2 IVT have mostly targeted genetic diseases, the approach has also been used to target nAMD. AAV2.sFLT1 (delivered by IVT) was studied in 19 patients, and 6/19 demonstrated substantial reductions in retinal oedema and improvements in vision. There was some evidence of the induction of humoral immunity against AAV2, however, this was not associated with the vector dose

administered. 6/6 patients in the low-dose groups (2E8-2E9 vector genomes/eye) showed no detectable increase in NAb levels. In patients who received 6E9 vector genomes/eye, 2/3 demonstrated anti-AAV2 NAb responses. For patients in the first 2E10 cohort, 3/3 exhibited increased NAb titres throughout the one-year investigation period. In the other cohort of patients receiving the highest dose of AAV2.sFLT1 (2E10 vector genomes/eye) 2/7 demonstrated increases in NAb titres. The utilisation of prophylactic immunosuppressants was not reported in this study, however, topical steroids were used to treat episodes of pyrexia and intraocular inflammation in two patients in the high-dose cohort (Heier *et al.*, 2017).

(iii) Is the eye immune-privileged with respect to AAV gene therapies?. Despite the assumption that the eye is a relatively immune-privileged environment and therefore favourable for testing gene therapies, the above discussion highlights a number of key findings suggesting that humoral immunity to AAV, either pre-existing *via* wild-type infection or induced by vector administration, may represent a significant hurdle for successful gene transfer to the inner and outer retina. The number of patients enrolled in the clinical trials described above is limited, reflecting the small patient populations for their respective indications. However, in our view, a number of trends may be evident in the data. These patterns highlight unanswered questions in the field and identify possible research questions for the future. A summary of a possible role of humoral immunity in response to ocular gene therapies is summarised in Fig. 1.

- (1) Only a subset of patients demonstrate increases in anti-AAV NAb levels after vector administration *via* SRT (Bainbridge *et al.*, 2015; Constable *et al.*, 2016) or IVT (Heier *et al.*, 2017; Bouquet *et al.*, 2019). Some of these patients exhibit very low/undetectable NAb titres at baseline, others had high NAb titres, yet evidence of the induction of humoral immunity to AAV was occasionally observed in both cases post-injection. One possible explanation is that patients that are seronegative at baseline may harbour AAV reactive T-cells (Mingozzi *et al.*, 2007; Veron *et al.*, 2012) which facilitate a strong humoral immune response upon vector administration.
- (2) One trend is that the significant increases in NAb levels observed in some patients appear to correlate with episodes of intraocular inflammation. A number of studies investigating SRT (Bainbridge *et al.*, 2015) and IVT (Guy *et al.*, 2017; Heier *et al.*, 2017; Bouquet *et al.*, 2019) have shown that the onset of events such as vitritis and anterior uveitis was seen in patients who exhibited the greatest changes in their NAb titres between baseline and the weeks/months following vector administration. It should also be noted that some of the patients who developed intraocular inflammation had very low/undetectable NABs at baseline. More research is required to understand why this subset of patients



**Fig 1.** The humoral immune response in relation to ocular gene therapy. Our review suggests that a subset of patients develop anti-AAV NABs after SRT or IVT of AAV, however, it is not clear whether this can be circumvented *via* administration of perioperative systemic steroids. Further studies are needed to determine whether the induction of anti-AAV NABs affects re-administration into the eye by IVT/SRT or into other organs. In addition, the precise mechanisms utilised by AAV NABs remain unknown, and more research is needed to elucidate the basis of their neutralising activity. AAV, adeno-associated virus; APC, antigen presenting cell; BP, bipolar cell; CSR, class switch recombination; GCL, ganglion cell layer; IVT, intravitreal injection; MHC, major histocompatibility complex; NAb, neutralising antibody; PC, plasma cell; PR, photoreceptor; RPE, retinal pigment epithelium; SHM, somatic hypermutation; SRT, subretinal injection.

developed much stronger NAb responses compared to other participants. Further, patients who developed high NAb titres and intraocular inflammation may be at increased risk of CD8+ T-cell infiltration into the retina and the clearance of transduced cells over time, which may eventually lead to the loss of therapeutic efficacy. We suggest that patients who develop intraocular inflammation are closely monitored for signs of diminished transgene expression. In this regard, considering the apparent failure of AAV reactive T-cells to recirculate in peripheral blood (Vandamme *et al.*, 2017) (rendering their

*ex vivo* analysis *via* blood sampling/ELISPOT assay challenging), episodes of intraocular inflammation could serve as a surrogate biomarker for patients most at risk of deleterious CD8+ T-cell infiltration into the retina.

- (3) There is no apparent correlation between baseline NAb titres and the magnitude of increase in NAb titre observed after vector administration. Of the patients that did exhibit significant increases in NAb levels after SRT (Bainbridge *et al.*, 2015; Rakoczy *et al.*, 2015; Constable *et al.*, 2016) and IVT (Guy *et al.*, 2017; Heier *et al.*, 2017; Bouquet *et al.*, 2019), high and

low/undetectable baseline NAb titres were observed. This evidence may discount a possible theory that high baseline NAb levels lead to rapid neutralisation of vectors upon injection, which in turn precludes the presentation of their capsid proteins to the immune system (Kotterman *et al.*, 2015). It also suggests that a 'primed' immune system (i.e. one harbouring NAb/B-cell epitopes against AAV) may not necessarily lead to stronger NAb responses compared to a patient who has no detectable NAb at baseline.

- (4) It is unclear whether AAV administration *via* SRT or IVT induces NAb responses in a dose-dependent manner, which may simply reflect the small sample sizes used in these studies. However, it is notable that two studies (Maguire *et al.*, 2009; Bennett *et al.*, 2012) investigating SRT of AAV2 found no evidence of a dose–response effect. Further, in studies using IVT for vector delivery, one reported higher frequencies of elevated NAb titres in the low-dose group than the medium-dose group (Guy *et al.*, 2017), and another detected NAb responses in both low- and high-dose groups (Bouquet *et al.*, 2019). In the largest study reported in the literature, no NAb response was reported in the low-dose group, but equivalent increases in NAb levels were seen in the medium- and high-dose groups (Heier *et al.*, 2017). One possible explanation is that delivery of AAV above a certain threshold is sufficient to induce NAb production, but this might not be further enhanced by administration of additional capsid antigens.
- (5) The administration of perioperative steroids may not be sufficient to attenuate production of anti-AAV NAb. Of the 16 clinical studies highlighted herein, seven reported the use of oral prednisolone glucocorticoid steroids prophylactically to treat the immunological complications of AAV gene transfer. Of these seven however, four reported increased NAb levels in some of the patients enrolled, suggesting that steroid therapy may not have been sufficient in circumventing humoral immunity in these individuals (Maguire *et al.*, 2008, 2009; Bainbridge *et al.*, 2015; Le Meur *et al.*, 2018).
- (6) Further research is required to understand how the induction of anti-AAV NAb may affect vector re-administration in ocular gene therapy trials, especially for the IVT route of delivery. Some of the studies discussed showed that high NAb titres can persist for years after delivery of AAV *via* SRT and IVT in a subset of patients (Jacobson *et al.*, 2012; Bainbridge *et al.*, 2015; Heier *et al.*, 2017; Bouquet *et al.*, 2019), and whilst most studies suggest that high pre-existing sera NAb levels limit the efficiency of gene transfer to the retina (Kotterman *et al.*, 2015; Heier *et al.*, 2017), others showed that patients with high NAb titres at baseline actually demonstrated the greatest therapeutic response to the gene therapy (Guy *et al.*, 2017). In this regard, one possible line of investigation could be to determine the effect that IVT or SRT AAV administration has on vector re-administration *via* other

routes, e.g. intravenous infusion for liver indications. In the future, this may be pertinent to patients who exhibit multiple morbidities affecting different organs and require several gene therapy treatments.

## V. POSSIBLE STRATEGIES TO CIRCUMVENT HUMORAL IMMUNITY TO AAV

As discussed above, an increasing body of evidence implicates a deleterious role for pre-existing and induced NAb in the context of retinal gene therapy. Further, it is unclear whether oral prednisolone is effective at inhibiting NAb responses. This suggests that the development of strategies to circumvent humoral immunity to AAV will likely be helpful in improving the outcomes of ocular gene therapy trials. In this section we review a number of possible strategies that may be applicable to this problem.

### (1) Overcoming pre-existing AAV NAb

As outlined above, pre-existing NAb against AAV represent a major hurdle to the successful application of gene transfer technologies, and may even have a detrimental effect in immune-privileged sites like the CNS and retina. To overcome this problem, a number of approaches have been reported, including modifications to the vectors and the clinical procedures. These are summarised in Table 3.

#### (a) Altering the route of administration

This strategy may be applied to some gene therapies targeting certain organs/tissues. For instance, we have discussed how intramuscular (Manno *et al.*, 2003) and intravenous (Miesbach *et al.*, 2018) injections of AAV can be used to treat haemophilia B. Data from one study would suggest that injections into the muscle are less susceptible to the presence of pre-existing NAb than intravenous infusions, highlighting a possible strategy to administer AAV to haemophilia B patients who would normally be excluded from clinical trials (Manno *et al.*, 2003). Similarly, intrathecal delivery of AAV to the CSF has been shown partially to circumvent pre-existing anti-AAV9 NAb (Haurigot *et al.*, 2013), suggesting this mode of administration may be preferable to systemic administration of AAV9 for delivering genes to the CNS (Jackson, Dayton & Klein, 2015). However, it should be noted that altering the route of administration may attenuate effective transduction and therapeutic efficacy in a target organ.

#### (b) Use of alternative AAV vectors

In spite of the high rates of cross-reactivity exhibited by anti-AAV NAb (Boutin *et al.*, 2010), alternative AAV vectors with differing epitopes might be able to evade pre-existing or induced humoral immunity. This may involve the utilisation

Table 3. Summary of possible strategies to overcome pre-existing NABs against AAV

Strategy	Advantages	Disadvantages	Translational barriers
Alter the route of administration	Clinically translatable approach	May alter the pattern of transduction, reducing gene delivery to target cell type or tissue. Limited number of routes of administration depending on the target tissue	Low: possible to draw on examples from other clinical trials demonstrating safe vector delivery for a given route for some serotypes/organs
Use alternative AAV vectors	Increasing number of novel AAV vectors identified which may be effective at NAB evasion. Increasing understanding of AAV epitopes is enabling rational mutation of antigenic regions could be applied to any vector. <i>In vitro</i> and <i>in vivo</i> screens available to identify resistant vectors	Engineering novel AAVs can be expensive and technically challenging, especially when applying multiple rounds of <i>in vivo</i> selection. Novel AAVs may be able to circumvent NABs but may have unwanted and unintended properties such as high toxicity	High: extensive safety and efficacy testing likely required by regulators to demonstrate benefits of novel capsid in preclinical models and patients. High economic cost associated with regulated clinical trials
Chemical modification of AAV	Non-genetic modifications require relatively simple chemistries amenable to scalable manufacturing	Data suggest limited resistance of PEGylated AAV to NABs. Limited number of examples of other biological polymers applied to AAV to date	High: safety of biological polymers may be established, but extensive safety testing would be required for novel formulated vectors
Use decoy capsids	Clinically translatable if 'known' serotype (e.g. AAV2) decoy capsids are used. Possible to use higher/lower ratio of decoy:full capsids depending on pre-existing NAB titre	Inclusion of additional decoy capsids may increase immune responses, possibly resulting in (i) CD8+ T-cell activation and destruction of transduced cells, and (ii) stronger induction of NAB responses. Would require production of more AAV capsids which could create a manufacturing bottleneck	Medium: AAV vectors (e.g. AAV2 or 8) used for decoys have established safety profiles in humans, but some additional safety/toxicity studies may be required as higher overall capsid titres will be used
Plasmapheresis	Effective at reducing pre-existing NAB levels if multiple rounds are used. Relatively non-invasive and safe procedure; routinely used in other applications to deplete antibody levels	May not be effective if pre-existing titres are very high. 'Rebound' phenomenon may limit effectiveness and possibility of repeated use	Low: routinely used for treatment of autoimmune conditions. Relatively safe and non-invasive procedure
Use broad-acting immunosuppressants	Possible to utilise FDA-approved drugs to suppress immune responses; highly translatable. Well-characterised safety and efficacy profiles and mechanisms of action	May be ineffective at completely depleting memory B-cells in the bone marrow, which may be required to overcome humoral immunity. Little evidence the approach may enable vector administration in spite of reducing NAB levels in pre-immunised models	Low: many immunosuppressants are FDA-approved, are routinely used in patients, and have favourable safety/toxicity profiles
Use antibody-degrading enzymes	Effective in preclinical studies at enabling efficient gene transfer in low and moderate NAB titre animals. Clinical evidence suggests the approach is safe in patients undergoing graft rejection	Patients may harbour pre-existing humoral immunity to, or develop humoral immunity against this microbial enzyme. Strategy may only be partially effective against high titres of NABs	Medium: IdeS is not FDA/EMA approved, however, regulatory barriers to successful translation may be lessened given positive safety data pertaining to IdeS in the clinic

AAV, adeno-associated virus; NAB, neutralising antibody; PEG, polyethylene glycol; IdeS, an immunoglobulin-cleaving enzyme.



of naturally occurring AAV isolates or the engineering of novel AAV vectors.

Approximately 100 naturally occurring AAVs have now been identified in humans and non-human primates, each of which harbours a distinct tissue tropism, immunogenicity and susceptibility to NAb. AAVrh32.33, for example, was isolated from Rhesus macaques, and can transduce a variety of human cell types (Vandenbergh, 2010; Mays *et al.*, 2013), yet only 2% of humans are thought to harbour pre-existing NAb against the virus (Calcedo *et al.*, 2009). Whilst this may prove helpful in circumventing humoral immunity, it should be noted that AAVrh32.33 may be highly immunogenic when injected intramuscularly, possibly limiting its application as a gene therapy vector (Mays *et al.*, 2013). Another natural AAV isolate is AAVrh10, which has been shown to be largely safe and effective at delivering genes to the CNS, lung, liver and heart in animal models. One study reported that 21% of humans are seropositive for AAVrh10, and harbour pre-existing NAb against this virus, which is lower than that reported for AAV2 (20–90% humans are seropositive), for example, suggesting that AAVrh10 may be an effective means of transferring genes to patients refractory to AAV2-based gene therapies (Thwaite *et al.*, 2015).

Ancestral sequence reconstruction (ASR) *via* phylogenetic analysis of AAV capsid sequences has also been proposed as a tool for identifying ancestral AAVs with favourable vector immunobiology properties. Anc80L65 is one such vector that is an ancestor of AAVs 1, 2, 8 and 9, and can deliver genes to the retina, liver, and muscle. Analysis of the structure and sequence alignment of Anc80L65 with extant AAVs demonstrated that Anc80L65 was structurally distinct from AAV2, 8 and rh10, exhibiting 12.2, 9.1 and 8.6% *cap* sequence divergence respectively. As a result, it was found that the vector was significantly more resistant to neutralisation by cross-reactive anti-AAV2 and anti-AAV8 sera than AAV2 and AAV8 vectors. However, in an *in vivo* model utilising an initial intravenous injection and sequential intramuscular injection, escape from neutralisation was only observed in 3/5 animals tested. Overall, Anc80L65 is a promising candidate vector for circumventing humoral immunity (Zinn *et al.*, 2015).

Other means of engineering the AAV capsid to overcome neutralisation by NAb involves the modification of antigenic epitope regions on the capsid surface, using molecular biology techniques like error-prone polymerase chain reaction (PCR) and DNA shuffling, to generate diverse capsid libraries which can subsequently be screened for capsids resistant to neutralisation by anti-AAV NAb (Kotterman & Schaffer, 2014).

An early demonstration of the potential of this approach was reported in 2008, when an adapted DNA family shuffling technology was used to create hybrid AAV capsid proteins. Two rounds of selection were used. The first used *in vitro* hepatocyte cultures to identify AAVs effective at transducing target cells. Pooled human immunoglobulin G (IVIg) was then used to isolate an AAV2/8/9 chimera, termed AAV-DJ, that was found to be more resistant to NAb than AAV2 in IVIg-immunised mice (Grimm *et al.*, 2008).

This approach was explored further to identify AAV capsids that were resistant to neutralisation. A number of AAV capsid libraries were utilised, including a randomly mutagenised AAV2 library, an AAV2 library with specific residues subject to saturation mutagenesis, a ‘shuffled’ AAV cap library (generated from AAVs 1, 2, 4, 5, 6, 8, and 9), and an AAV library in which surface hypervariable loops had been swapped between serotypes. Two rounds of selection were then used to identify AAV capsids resistant to neutralisation by NAb by IVIg and sera samples from patients excluded from haemophilia B clinical trials harbouring high-titre AAV NAb. The authors conclude that the novel AAV capsids generated may be used to treat patients with high titres of pre-existing NAb and repeated gene transfer (i.e. re-administration of a gene therapy) in patients who have developed NAb against AAV (Bartel, Weinstein & Schaffer, 2012).

Aside from directed evolution, AAVs can also be rationally designed *via* the mutagenesis of epitope regions. This first requires the identification of regions important for binding/neutralisation by antibodies. One method utilises peptide scanning, in which short linear epitopes are used in enzyme-linked immunosorbent assay (ELISA) screens to identify capsid proteins that bind to anti-AAV antibodies. Peptide insertion into AAV capsids can also be used to disrupt NAb epitopes and thereby identify antibody binding regions on the vector (Tse, Moller-Tank & Asokan, 2015). *In silico* modelling and systematic mutagenesis of IgG2a antibodies to AAV2 has also been used to identify residues important for antibody binding (Wu *et al.*, 2014). These discoveries have aided the development of NAb-resistant AAVs using a structure-guided approach that does not require the use of IVIg or high NAb titre patient sera to exert selective pressures on large capsid libraries for identifying resistant clones. In one report, synthetic AAVs were evolved *via* rational mutation of AAV1 antigenic epitopes, which were found to evade polyclonal anti-AAV1 neutralising sera without impacting the tissue tropism or transduction efficiency of the vectors in mouse models and non-human primates (Tse *et al.*, 2017).

Whilst most modifications to AAV to improve NAb resistance have focussed on modification of *cap* gene/capsid protein sequences, recent reports have shown that the encapsulation of AAVs in exosomes can also reduce their susceptibility to humoral immune responses. Termed exosome-associated AAV (exoAAV), these novel vectors have demonstrated improved transduction across a range of cell types and tissues, in addition to enhanced resistance to NAb in IVIg-immunised mice. This reduced sensitivity to NAb has been shown for exoAAV8 in the liver (Meliani *et al.*, 2017) and exoAAV9 in the CNS (Hudry *et al.*, 2016).

### (c) Chemical modification of AAV

The engineering of AAV to avoid neutralisation by NAb has resulted in the development of exciting novel vectors capable of circumventing humoral immune responses *via* the

disruption of antigenic epitopes. An alternative approach to preventing NAb-AAV binding/neutralisation is to shield epitopes *via* chemical modification of AAVs.

Polyethylene glycol (PEG) can be chemically conjugated to AAVs to mask them from NABs, for example. Whilst PEG-coated AAVs have been shown to exhibit greater resistance to NAB-mediated neutralisation, it should be noted that only partial protection was inferred *via* this chemical modification. It was also noted by the authors that the addition of PEG above a certain threshold attenuated the AAVs transduction potential, likely *via* steric hindrance between cell surface receptors and cognate AAV capsid proteins (Lee *et al.*, 2005).

Alternative approaches have sought to encapsulate AAVs in polymer gels like polylactic glycolic acid and alginate that gradually degrade *in vivo*. This approach has been applied to adenoviruses (Beer *et al.*, 1998; Sailaja *et al.*, 2002) and has shown to increase the resistance of these vectors to NABs, suggesting that this approach may also be effective for AAV vectors. However, the development of anti-polymer antibody responses may pose a significant challenge to this technology (Hoang Thi *et al.*, 2020).

#### (d) Use of decoy capsids

The use of decoy capsids to circumvent anti-AAV NABs involves the administration of empty capsid AAVs or infection-deficient AAVs to sequester NABs prior to or at the same time as therapeutic vector administration. In non-human primates, this approach has been shown to block circulating NABs competitively in a dose-dependent manner and concomitantly restore transduction efficiency (Mingozzi *et al.*, 2013b), however, other studies have shown that this approach is not always effective at circumventing humoral immune responses (Monahan *et al.*, 2015). The authors also showed that the presence of empty capsids in their AAV8 vector preparations did not prevent neutralisation of FIX.R338L delivery in their models *via* a so-called ‘decoy capsid’ action (Mingozzi *et al.*, 2013b; Monahan *et al.*, 2015). In contrast to previous reports, which utilised IVIg (Mingozzi *et al.*, 2013b), Monahan *et al.* (2015) opted for a passive transfer strategy involving the generation of anti-AAV8 NABs in one group of mice which were then collected and transferred to a different group of mice receiving the FIX.R338L gene therapy. The authors reported that 1:2 or 1:16 NAB titres were sufficient to attenuate transfer of FIX.R338L, but this effect was not rescued by the inclusion of empty capsids in the AAV8 preparation. This was in opposition to previous findings suggesting that ‘decoy capsids’ may be an effective means of circumventing anti-AAV NABs (Mingozzi *et al.*, 2013b). Several key differences between these studies are evident, however. First, the source of NABs [use of hIVIg (Mingozzi *et al.*, 2013b) *versus* passive transfer of mouse sera (Monahan *et al.*, 2015)] is a key disparity. Further, in Mingozzi *et al.* (2013b), a higher ratio of decoy vector to full vector was used, both of which may explain the different outcomes.

The use of capsid decoys may represent a possible strategy for overcoming NAB responses, however, this approach may

have certain drawbacks. The inclusion of empty capsids in vector formulations has been shown to reduce transduction efficiency and increase vector-related immunotoxicity in one study investigating liver gene transfer with AAV8 (Gao *et al.*, 2014), however, in a non-human primate study investigating AAV2-mediated gene delivery to the retina, the removal of empty capsids had no detectable impact on the generation of anti-AAV lymphocyte or NAB responses (Timmers *et al.*, 2020).

#### (e) Plasmapheresis

Plasmapheresis is a clinical procedure that involves *ex vivo* removal of NABs from an individual’s blood by using filtration- or centrifugation-based techniques, before the blood is transferred back (Derksen *et al.*, 1984). The approach can be used to reduce the build-up of antibodies in conditions like haemorrhagic lupus pneumonitis (Erickson, Franklin & Emlen, 1994) and Guillain-Barre syndrome (Buzzigoli *et al.*, 2010), both of which involve elevated levels of pathologic antibodies. It is also used in patients with visual loss from atypical optic neuropathies mediated by antibodies against aquaporin-4 (AQP4) and myelin oligodendrocyte glycoprotein (MOG) (Sato *et al.*, 2014). In terms of gene therapy, plasmapheresis provides a possible means of achieving a transient drop in NAB titres below a certain threshold to enable vector administration. It could, therefore be applied to an individual with high pre-existing NAB levels or one who develops high NAB titres after receiving a gene therapy. This approach was tested in a non-human primate model, and found to be effective with six rounds of plasmapheresis over a 2-day period effectively reducing NAB levels in seropositive animals. Following vector administration, animals that had undergone plasmapheresis exhibited the same transduction levels as seronegative animals following intravenous injection of a microDystrophin-expressing AAV construct (Chicoine *et al.*, 2014). Plasmapheresis is usually well tolerated from a safety perspective (Vucic & Davies, 1998). However, one possible limitation to using plasmapheresis is a phenomenon known as ‘rebound’, in which IgG levels quickly return to the same or even higher levels following treatment. In a clinical trial, it was shown that multiple rounds of plasmapheresis were required to reduce NAB levels for gene therapy, which could be partly explained by this ‘rebound’ effect. Whilst reductions to <1:5 NAB titres were achieved in some individuals across multiple serotypes (AAV1, 2, 6 and 8), it should be noted that such an outcome required five rounds of plasmapheresis in some instances. Furthermore, in those individuals exhibiting very high pre-existing NAB titres, for example, 1:12800 anti-AAV2 in one particular case, successive rounds of plasmapheresis did not reduce the titre below 1:200. This suggested that the process may be ineffective in enabling efficient gene transfer in all patients with pre-existing NABs (Monteilhet *et al.*, 2011).

Recently, a novel plasmapheresis protocol was described, in which the specific depletion of anti-AAV IgG from plasma could be achieved without depleting total IgG levels. Using

this approach, high-titre human IgG pools and plasma samples were tested, and near-complete removal of anti-AAV IgG was achieved using an N-hydroxysuccinimidyl sepharose column onto which AAV8 particles were grafted. The process was able to reduce anti-AAV8 IgG to levels that enabled efficient gene transfer in mouse models. This study highlighted the possible advantages to specific depletion of AAV IgG using plasmapheresis, which has distinct safety advantages, mitigating the risks of leaving an individual vulnerable to opportunistic pathogens following pan-IgG depletion (Bertin *et al.*, 2020).

#### (f) Use of pharmacological immunosuppressants

The use of immunosuppressant drugs, which may impact both the innate and adaptive arms of the immune system, appears to be largely effective in managing anti-AAV T-cell responses when administered prophylactically or once a rise in liver transaminases is detected. Completely circumventing pre-existing anti-AAV humoral immunity with broad-acting immunosuppressants is more challenging however, likely due to the inability of these compounds effectively to deplete memory B-cells in the bone marrow (Vandamme *et al.*, 2017). One report utilising a mouse model of AAV immunity has shown that pharmacological intervention can be used to reduce pre-existing NAb levels, however. Pre-existing immunity was generated by an intravenous injection of AAV9 vectors. A combination of rapamycin and prednisolone administered daily for 8 weeks reduced anti-AAV9 NAb levels by 85–93%, and in addition, decreased levels of B-cells and plasma cells were observed. This combination was found to selectively inhibit helper T-cell-mediated B-cell activation in the spleen, leading to effective anti-AAV9 NAb depletion. Although such an approach could prove an attractive strategy for circumventing humoral immune responses to AAV, this study did not confirm whether vector re-administration was possible in their model after treatment with rapamycin and prednisolone (Velazquez *et al.*, 2017).

#### (g) Use of NAb degrading enzymes

One recent report outlined the possible use of an immunoglobulin-degrading enzyme, IdeS, to reduce pre-existing NAb levels and enable efficient gene transfer in mouse and non-human primate models. The authors showed that reductions in NAb levels from 1:10 to <1:1 in C57BL6/J mice was possible following a 25 µg injection of IdeS, which was associated with increased FIX expression after delivery of AAV8.FIX, and a concomitant 50% reduction in bleeding time (measure of therapeutic efficacy in haemophilia B models). Similarly, in cynomolgus macaques (*Macaca fascicularis*), a 1:17.2 to <1:5 reduction in NAb titre was achieved after two injections of 500 µg/kg IdeS. This was associated with a 4–10-fold increase in FIX levels after delivery of AAV8.FIX. The authors suggest that the utilisation of IdeS in human trials may be safe, given that its use has not yet been associated with significant toxicities or increased risk of opportunistic infection in healthy subjects (Winstedt *et al.*, 2015) or patients undergoing graft transplantation

(Jordan *et al.*, 2017). However, the possibility of patients harbouring pre-existing humoral immunity to IdeS, or developing humoral immunity to IdeS following administration, may require further investigation. The authors tested whether IdeS could enable repeated gene transfer using sequential injections of AAV-LK003.FIX. Whilst 10-fold reductions in NAb levels were seen in some of the non-human primate models, only two subjects appeared to permit transduction after the second injection of AAV-LK003, and even in these animals, the level of FIX peaked at ~100 ng/ml, which is four times lower than that observed in the IdeS-treated animal in their earlier experiments. In summary, IdeS appeared to be effective at circumventing humoral immunity when faced with low to moderate NAb levels, but may not be completely effective at overcoming high NAb titres (Leborgne *et al.*, 2020).

## (2) Preventing the induction of AAV NAb

Pre-existing NAb represent a significant barrier to the success of gene therapies in the clinic. As outlined above, the delivery of AAV into most, if not all, sites in the body appears to result in the development of humoral immunity against the capsid, which may limit the possibility of vector re-administration. This is an important aspect of AAV gene therapy for several reasons. First, gene therapy constructs may display reduced efficacy over time as cells epigenetically downregulate ‘foreign’ gene expression cassettes *via* CpG island hypermethylation (Gray *et al.*, 2011). Second, in dividing tissues such as the liver, transduced cells are lost over time, thereby reducing therapeutic efficacy (Verdera *et al.*, 2020). In a number of non-dividing tissues, such as the inner retina/RGC layer, many pathologies exhibit a degenerative phenotype which may lead to the loss of transduced cells over time (Levin & Gordon, 2002). For many diseases, titrating the vector dose may prove a useful strategy towards ‘tailoring’ a gene therapy to a particular patient due to disparities in age and/or stage of disease progression, for example. In all of these scenarios, efficient vector re-administration is required, in which preventing the development of anti-AAV NAb is key. Here we will discuss a number of possible strategies that could be used to achieve this goal in clinical trials and preclinical studies. These are summarised in Table 4.

#### (a) Inhibition of T-cell activation

CD4+ ‘helper’ T-cells can indirectly affect the humoral immune response by regulating the function of B-cells and their antibody-producing progeny plasma cells. Downregulating CD4+ T-cell function can therefore be utilised as a means of reducing anti-AAV NAb levels. The promising results discussed below suggest that the inhibition of T-cell activity could prove an effective means of preventing the induction of anti-capsid humoral immune responses in the eye.

Anti-CD4 antibodies have been used to deplete (*via* induction of apoptosis in target cells) CD4+ T-cell levels and

Table 4. Summary of possible strategies to prevent induction of anti-AAV Nabs

Strategy	Advantages	Disadvantages	Translational barriers
Inhibit T-cell activation	Several pathways can be targeted to prevent anti-AAV NAb responses using FDA-approved drugs such as Cedelizumab. Tregitopes may be used to induce tolerance of AAV antigens	T-cell inhibition may leave patient vulnerable to infection and cancer development. Fewer FDA-approved modalities than small molecule-based approaches to immunosuppression	Medium: some FDA-approved anti-CD4 mAbs are available for creating immunosuppression in patients. Few clinical data supporting safety of approach in humans
Inhibit B-cell activation	Inhibiting B-cell function may prevent NAb formation whilst retaining cytotoxic T-cell function, against tumourigenic cells for example. FDA-approved drugs are available for this application. Preclinical evidence suggests generation of tolerogenic B-cells is possible	B-cell levels may take 6–12 months to recover, leaving patients vulnerable to opportunistic pathogens and tumourigenesis. Fewer FDA-approved modalities than small molecule-based approaches to immunosuppression	Medium: some FDA-approved anti-CD20 mAbs are available for creating immunosuppression in patients. Few clinical data supporting safety of approach in humans
Use broad-acting immunosuppressants	Possible to utilise FDA-approved drugs to suppress immune responses; highly translatable. Well-characterised safety and efficacy profiles and mechanisms of action	Preclinical evidence suggests this approach may not be sufficient to prevent NAb formation or enable vector re-administration. Outcomes of clinical trials investigating gene delivery to the retina also suggest this approach may not be effective in all patients	Low: many immunosuppressants are FDA-approved and are routinely used in patients. Many immunosuppressants have favourable safety/toxicity profiles

AAV, adeno-associated virus; NAb, neutralising antibody.

abolish anti-AAV NAb levels following a tail vein injection of AAV in a mouse model. However, this study also demonstrated that delivery of the vector directly into the portal circulation produced a humoral immune response that was only partially T-cell-dependent, thereby reducing the effectiveness of the anti-CD4 depletion strategy. Overall the study demonstrated that CD4 depletion may be an effective means of circumventing NAb responses for certain routes of delivery (Xiao *et al.*, 2000).

Alternative approaches have described the use of non-depleting anti-CD4 antibodies to prevent NAb responses. Here, intravenous administration of an anti-CD4 monoclonal antibody (mAb) prior to delivery of an AAV2/9 vector demonstrated reductions in anti-vector and anti-transgene [acid  $\alpha$ -glucosidase (GAA)] NABs in a mouse model of Pompe disease. The authors concluded that the mAb prevented co-receptor (CD4) stimulation of T-cells, in turn rendering them ineffective as B-cell activators (Han *et al.*, 2015). T-cell-dependent activation of B-cells can also be inhibited by blocking other signalling pathways. Antibodies and fusion proteins against CD40-CD40L and CD28-CD80/86 receptors have been shown to reduce NAb levels against AAV proteins and thereby enable re-administration to the lung in a rabbit model (Halbert *et al.*, 1998). One study sought to

combine a non-depleting anti-CD4 mAb therapy with the T-cell immunosuppressive drug, cyclosporin A (CyA), and showed that a 20-fold reduction in NAb levels was possible using this approach (McIntosh *et al.*, 2012). Other studies have utilised 'Tregitopes' (De Groot *et al.*, 2008) (IgG-derived MHC epitopes) to activate Treg responses in order to inhibit NAb production. In a mouse model, Tregitopes were fused to AAV capsid peptides, and subsequently used to induce proliferation of Treg cells that suppressed CD8+ T-cell cytotoxic function against AAV-expressing cells (Hui *et al.*, 2013). This demonstrated an interesting proof-of-concept and suggests that Tregitopes could be applied to reducing NAb levels after vector administration.

#### (b) Inhibition of B-cell activation

The inhibition of T-cell activation can prevent anti-AAV antibody production by B-cells/plasma cells. Efforts to inhibit activation and/or induce apoptosis of B-cell/plasma cells have also shown promise as tools for overcoming humoral immunity to AAV. The activation of B-cells and their maturation into antibody-producing plasma cells is a complex process. With AAV gene therapy, APCs are thought to display AAV capsid peptides to immature B-cells which

initiate downstream signalling pathways resulting in the endocytosis of AAV antigens (Carrasco & Batista, 2006; Harwood & Batista, 2008). The B-cell then presents the antigen on its surface with MHC class II molecules, which can be recognised by CD4+ helper T-cells, causing them to proliferate and migrate to the germinal centres, where somatic hypermutation and isotype switching occur. Most activated B-cells will become plasma cells and produce NAb against AAV, whilst others develop into memory B-cells (Basner-Tschakarjan, Bijjiga & Martino, 2014). Inhibition of B-cell activation for circumventing humoral immune responses to AAV capsids has shown promise in a number of preclinical models, highlighting possible application to ocular gene therapy programmes.

Understanding the process by which B-cells are activated therefore helps to inform strategies to prevent NAb responses. Bortezomib, an FDA-approved therapy for multiple myeloma (plasma cell tumour), is a proteasome inhibitor that attenuates antigen processing and presentation of epitopes on the surface of B-cells. Administration of Bortezomib has been shown to reduce NAb titres by 8–10-fold by depleting AAV2/8-specific IgG-producing plasma cells in the lymphoid organs and bone marrow. However, this reduction was not sufficient to allow vector re-administration, which the authors attribute to residual anti-AAV8 NAb due to the inability of Bortezomib completely to eradicate anti-AAV8 plasma and memory B-cells (Karman *et al.*, 2012).

Depletion of activated B-cells can also be utilised as a means of reducing NAb levels. Rituximab, an anti-CD20 antibody, can induce B-cell apoptosis by (i) recruiting cytotoxic natural killer and macrophage cells to antibody-bound B-cells, or (ii) activating the cytotoxic complement cascade *via* C1q binding to induce B-cell lysis (Smith, 2003). In a clinical trial, Rituximab treatment was shown to reduce circulating NAb levels up to 24 weeks after two intravenous infusions, however the authors noted that these reductions were only observed in a subset of patients with titres of <1:1000, and only a minority of subjects' titres were reduced to <1:5 (Mingozzi *et al.*, 2013a). This finding was corroborated by reports that a combination of rituximab and rapamycin in a 45-month-old patient with Pompe disease was an effective strategy to mitigate anti-AAV immune responses. The authors conclude that the strategy allows for the possibility of vector re-administration in the future, but no data were presented in relation to this, and it should also be noted that this was a single-subject clinical trial (Corti *et al.*, 2014). In a non-human primate study, CyA (calcineurin inhibitor, inhibits T- and B-cells) was trialled in combination with Rituximab. The study showed that anti-transgene (FIX for haemophilia B) NAb levels could be significantly reduced with this strategy. Further, in one animal, anti-AAV6 NAb titres dropped to undetectable levels, which was subsequently shown to be permissive of vector re-administration. In the other animal however, the dual immunosuppression strategy did not appear to be effective and re-administration appeared to be blocked by anti-AAV6 NAb (Mingozzi *et al.*, 2012).

Clearly, B-cell depletion strategies may be an effective means of overcoming humoral immunity to AAV, however, the approach is not without its drawbacks. For instance, following Rituximab treatment, patients are immunocompromised for a period of around 6–12 months as their B-cell levels return to normal, which can leave them vulnerable to opportunistic pathogens. In two severe cases, activation of dormant human polyomavirus was associated with the onset of progressive multifocal leukoencephalopathy following administration of Rituximab (Chakraborty *et al.*, 2011). Further, it should be noted that anti-CD20 antibodies will not deplete plasma cells, which are the antibody-producing cells of the immune system, possibly limiting the ability of the strategy to reduce NAb levels whilst still posing a significant safety concern (Leandro, 2013).

As an alternative to B-cell depletion, the induction of B-cell tolerance can be utilised to prevent the generation of anti-AAV NAb and thereby allow for vector re-administration. For instance, antigen-specific immunotherapy can be used to inhibit B-cell activation by inducing Treg cells for a particular antigen. In one study, an immunogenic protein was fused to an immunoglobulin heavy chain, and transferred into activated B-cells *in vitro* using a retroviral vector. The generation of tolerogenic B-cells was observed which were found to attenuate immune responses against multiple epitopes of the cloned protein. Mechanistically, this effect was linked to the stimulation of Treg cells by the transduced B-cells (Skupsky *et al.*, 2007). Whilst this approach has not yet been applied to AAV gene therapy, these results suggest it might be effective at limiting humoral immune responses.

Another means of preventing B-cell activation is to target inhibitory co-receptors present on the surface of B-cells. CD22 and SIGLEC-G are two sialic acid-binding co-receptors known to play a role in mediating the induction of tolerance of self-antigens. Recent data have shown that high-affinity ligands for these co-receptors can induce antigen-specific B-cell tolerance, highlighting a possible strategy for treating autoimmune diseases which may be applied to preventing anti-AAV humoral immune responses in the future (Müller & Nitschke, 2014). For instance, immunisation of mice with nanoparticles conjugated to human factor VIII and CD22 has been shown to induce tolerance to and antibody production against factor VIII (Macauley *et al.*, 2013), suggesting a similar approach could be applied to AAV capsid proteins.

#### (c) Use of pharmacological immunosuppressants

In addition to targeting specific arms of the immune system with anti-CD4 or anti-CD20 antibody-based depletion strategies, the use of broad-acting small molecule immunosuppressants has been trialled, either alone or in combination. Many of these are FDA-approved and routinely used in patients, making this approach highly clinically translatable for ocular gene therapy.

In a mini-pig model of advanced heart failure, AAV1 encoding sarcoplasmic reticulum calcium ATPase gene

(SERCA2a) was delivered by intravenous administration. A combination of immunosuppressive drugs was tested to see whether this could circumvent NAb responses. Here, oral mycophenolate mofetil (MMF) and rapamycin were administered daily from 2 weeks before to 3 months after vector infusion, and methylprednisolone sodium succinate was administered daily by intramuscular injection up to 3 months after AAV1 delivery. In spite of the utilisation of this aggressive immunosuppressive regimen, the induction of AAV1 NAb was still observed in both the immunosuppressed and non-immunosuppressed groups, and comparison of these groups revealed no apparent effect of immunosuppression on the development of NAb (Greenberg *et al.*, 2016).

Studies examining immunosuppression in non-human primates have suggested the approach may not be effective for liver gene therapy with AAV5. Here, animals were injected intravenously with AAV5 to generate an immune response. Over the next 12-week period, anti-thymocyte IgG, methylprednisolone, tacrolimus and rituximab were given in combination. These were found to attenuate anti-AAV5 NAb during the 12 weeks, but NAb levels rose significantly once this treatment stopped. The authors then demonstrated that this 'rebound' in NAb levels completely inhibited repeated vector re-administration of AAV5 (Unzu *et al.*, 2012).

These results were corroborated by another study in non-human primates investigating microDystrophin delivery to the muscle. Animals receiving (i) prednisolone alone, (ii) a prednisolone, tacrolimus and MMF combination, and (iii) no immunosuppressants were compared. No differences in transgene expression were observed in seropositive animals between these three groups, indicating that the immunosuppressants given may have been ineffective at reducing anti-AAV NAb levels. This was in spite of the fact that the immunosuppressants were shown to downregulate lymphocyte proliferation, a finding which may highlight the challenge in attenuating pre-existing humoral immunity to AAV (Chicoine *et al.*, 2014).

The complexity of overcoming the induction of NAb against AAV following delivery to the CNS has been demonstrated in clinical studies. One study tested an AAV2 vector expressing aspartoacylase and delivered by intracranial injection in patients with Canavan disease. In this study, only 3/10 patients developed low to moderate sera NAb titres *versus* baseline, in spite of the fact that no patients were given perioperative immunosuppressants (McPhee *et al.*, 2006).

In another trial, an AAVrh10 vector encoding arylsulphatase A was delivered by intracerebral injection in patients with metachromatic leukodystrophy. Steroids were administered 1 day prior and 10 days after the surgery, however, in all patients enrolled in the study, the development of anti-AAV antibodies was observed in the sera and CSF. This suggestion that steroids may not be effective in preventing anti-AAV NAb responses has been supported by recent data investigating AAV9 gene therapy. Here, AAV9 vectors were used to transfer gigaxonin into the CNS of cross-reactive immunologic material (CRIM)-negative giant axonal neuropathy patients, and a combination of methylprednisolone,

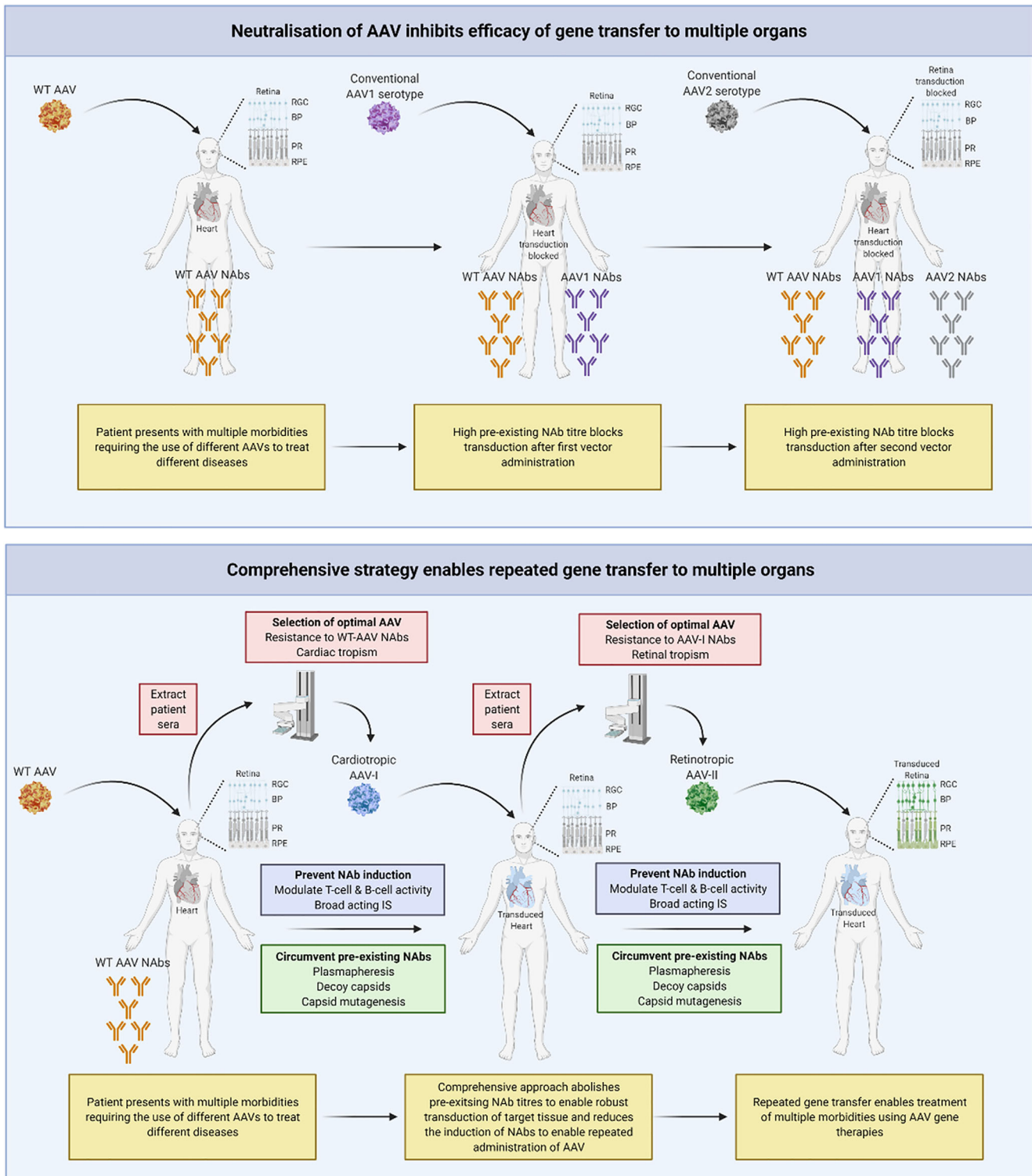
prednisone, tacrolimus and rapamycin was used. However, anti-AAV9 NAb were still observed in the sera and CSF after vector infusion, in spite of the fact that the combination of immunosuppressants was effective at preventing inflammation (pleocytosis; elevated CSF lymphocyte counts) (Verdera *et al.*, 2020). This is possibly in accordance with clinical studies investigating AAV gene transfer to the retina reviewed above in which some patients developed NAb responses in the absence of intraocular inflammation.

Recently, small molecule immunosuppressants have been modified to improve their properties *in vivo*. In a study utilising non-human primate and mouse models, rapamycin was encapsulated in poly-lactic acid nanoparticles, termed SVP rapamycin (rapa). SVPrapa was found to prevent anti-AAV cellular and humoral immune response induction, thereby permitting re-administration of the vector, when co-administered with the initial injection of AAV. The drug mitigated antigen-specific activation of T-cells and B-cells, prevented CD8+ T-cell infiltration into the liver, and inhibited memory T-cell responses. Interestingly, the authors showed that the adoptive transfer of splenocytes from treated to naive mice transferred the immunomodulatory properties of SVPrapa. They then demonstrated that anti-CD25 antibody depletion partially rescued SVPrapa's effect on anti-AAV8 IgG levels, suggesting that Treg cells may be involved in the mechanism of action (Meliani *et al.*, 2018).

### (3) Overcoming humoral immune responses to AAV – the need for a comprehensive approach

The studies discussed above highlight the possible challenges of circumventing humoral immune responses to AAV. It is clear that some approaches have shown promise in preclinical models and human patients. In our view, however, effective strategies to overcome pre-existing NAb and prevent the induction of humoral immunity against AAV gene therapies are still under development. A summary of possible advantages, disadvantages and barriers to clinical implementation for each approach is provided in Tables 3 and 4. Here we will suggest a comprehensive strategy that could be used to circumvent both pre-existing and induced humoral immunity in the future. We aim to provide a framework for future investigations so that robust and repeatable vector administration to previously untreatable patients is possible. Our proposed approach is outlined in Fig. 2.

Completely to eliminate pre-existing humoral immunity against AAV, especially if a patient presents with a high NAb titre, may require a combinatorial approach. AAV-specific plasmapheresis may initially be used to reduce NAb titres, followed by a vector infusion incorporating a novel vector with either (i) mutated capsid epitopes (*via* a random or computational approach), (ii) NAb-resistant chemical modifications, or (iii) a combination of both. The inclusion of decoy capsids may also be utilised to sequester any NAb that were not cleared by plasmapheresis. In conjunction, a combination of broad-acting small molecule immunosuppressants may be administered perioperatively, along with a



**Fig 2.** Possible approaches to enable repeated gene transfer. Top panel: an individual with a high pre-existing NAB titre that results in neutralisation of conventional AAV serotypes in the absence of immunomodulation. Bottom panel: Pre-existing NABs are depleted and immunomodulation is used to reduce induction of anti-AAV NABs post-administration. Patient sera is extracted and used to screen an AAV library for NAB-resistant and target tissue-tropic clones. This enables repeated gene delivery to multiple organs in a patient presenting with multiple morbidities. AAV, adeno-associated virus; BP, bipolar cell; IS, immunosuppression; PR, photoreceptor; RGC, retinal ganglion cell; RPE, retinal pigment epithelium; NAB, neutralising antibody.



T- or B-cell-targeted peptide immunotherapy approach to induce antigen-specific tolerance to an AAV antigen and prevent the induction of anti-AAV NABs that may arise in response to certain gene therapies.

This example, whilst hypothetical, demonstrates the range of possible solutions currently under investigation and highlights the need for a range of approaches to ensure efficient and repeatable vector administration to target organs. However, there is a need for more research into what combination of possible solutions provides maximal benefit depending on the target organ and route of administration. In particular, investigation into possible redundant or counterproductive combinations (e.g. effective T-cell/B-cell depletion negating the need for small molecule immunosuppressants, or successful capsid modifications eliminating the use of decoy capsids).

Further studies are also needed to establish safety and efficacy profiles of novel vectors in particular, which will require regulatory approval before routine clinical use is feasible. In the future, a library of FDA-approved, antigenically distinct engineered AAVs with highly specific tissue tropisms would likely be beneficial, which may enable repeated ‘swapping’ of vector capsids for a particular therapeutic construct to overcome pre-existing and/or induced humoral immunity whilst ensuring robust and tissue/cell-specific transduction. Here, high-throughput NAB assays could enable selection of an AAV variant from a library that is resistant to neutralisation by a particular patient’s sera. A peptide scanning based-approach (Moskalenko *et al.*, 2000) could even be used subsequently to identify epitopes on the selected capsid, allow rational mutagenesis of these regions and ensure maximal resistance of the engineered virion to neutralisation, and to facilitate ‘individualised’ gene delivery to a particular patient based on a thorough characterisation of their NABs.

## VI. CONCLUSIONS

- (1) AAV is a promising vector for delivering therapeutic genes to diseased cells. A number of clinical trials have shown that AAV is a safe and effective vehicle for gene therapy, however, the deleterious role of NABs remains a significant barrier in a proportion of treated individuals.
- (2) Pre-existing NABs limit the efficiency of gene transfer to the cells being targeted with a potential negative impact on treatment efficacy. This is an especially important consideration for strategies that involve intravenous AAV infusions into the circulation where very low or even undetectable NAB titres may completely abolish transduction of the target tissues.
- (3) After vector administration, an increase in AAV NABs has been documented in some individuals recruited into clinical trials investigating muscular, hepatic and neurological disorders. In the ocular compartment, the increased NAB titres observed after IVT or SRT does not appear to be correlated with the vector dose or baseline NAB titre. Interestingly, intraocular inflammation appears to be more common in those individuals who do exhibit significant increases in NAB titres. More research is, therefore, needed to understand why this particular subgroup exhibit greater NAB responses, with early evidence pointing towards AAV reactive T-cells playing a role in these seronegative individuals. The longevity of the therapeutic effect of AAV vectors in individuals with intraocular inflammation could be monitored with long-term follow-up, as this group may be most at risk of CD8+ T-cell clearance of transduced cells and eventual loss of efficacy.
- (4) In our view, circumventing AAV NABs to enable repeated dosing to multiple organs, including the retina, will require a comprehensive strategy capable of overcoming both pre-existing and induced humoral immune responses. More research is also required to understand whether prophylactic steroid administration prevents NAB induction, and the application of other T-cell and B-cell immunomodulators to block this critical step could also prove beneficial. To overcome pre-existing high titres of NABs, the potential application of plasmapheresis and decoy capsids prior to intraocular injection requires further evaluation. Utilising patient sera to select NAB-resistant clones from a capsid library, followed by rational mutation of capsid epitopes could prove a useful strategy in the future, enabling individualised intraocular gene delivery to a particular individual.

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