Part II

Chapter 5

Introduction Part II

Overcoming AAV immunogenicity

I AAV vectors

Adeno-associated (AAV) vectors are used to achieve therapeutic gene delivery for the treatment of genetic and chronic disorders. Recombinant AAV vectors contain an expression cassette with a transgene of choice flanked by two non-coding viral inverted terminal repeats (ITR's) enclosed in a capsid which is composed of three proteins: VP1, VP2 and VP3. When AAV enters the cell, its genome is converted into double-stranded, transcriptionally active DNA, which predominantly persists in a non-integrated episomal form [1, 2]. These vectors efficiently transduce a wide variety of tissues and can provide the long term expression of the delivered gene after a single administration. Importantly, AAV vectors have not been associated with any pathology in humans and are replication-defective. AAV-based gene delivery has been successfully employed in treatment of genetic disorders in preclinical studies as well as in clinical trials [3-6]. The clinical studies included hundreds of patients and indicate an excellent safety record of AAV vectors for gene therapy in humans. The different safety aspects of AAV for the use in humans have recently been summarized elsewhere [7]. Immune responses have been assessed in clinical trials by measuring systemic and local cytotoxic reactions as well as neutralizing antibodies (NAB) against AAV and/or the expressed therapeutic protein [3, 5, 8-17]. The immunogenicity data reported so far show that immune responses against AAV capsid proteins can vary and are influenced by the target organ, route of delivery and dosing schedule.

II Cellular immune responses against AAV vectors

The first observation of a cellular immune response induced by AAV gene therapy occurred in patients with Hemophilia B who were treated with an AAV2 vector to deliver human coagulation factor IX [14, 18]. In this study, a cell mediated immune response to AAV2 capsid was reported, which was measured in parallel with a loss of transgene expression. Similar observations were reported in a more recent clinical study with AAV serotype 8 for FIX

delivery to the liver of Hemophilia B patients, when two patients receiving the highest vector dose required a short course of glucocorticoids which normalized serum aminotransferase levels and prevented the loss of transgene expression [6]. Whereas in patients with Hemophilia B a correlation between the induction of a $CD8^+$ T cell response towards the AAV capsid proteins and a loss of transgene expression was observed, this does not seem to be an issue in case of the intramuscular AAV vector delivery. In a clinical study in patients with α-1 antitrypsin deficiency in which the gene for α-1 antitrypsin was delivered by an AAV1 capsid, cellular immune responses were found against the capsid proteins from day 14 on in all subjects. However, the functional activities of those T cells, as well as the biological effects thereof are not clear since the expression of the transgene was sustained at sub-therapeutic levels in all subjects. These data suggest that the cellular immune responses to the AAV capsid did not eliminate the transduced cells [8]. Similarly, systemic and local cellular immune responses induced by intramuscular injection of alipogene tiparvovec did not impact on clinical efficacy and safety [4].

III Managing humoral immune responses against AAV capsid

A major challenge for successful tissue targeting of AAV vectors in patients is the presence of circulating neutralizing antibodies (NAB) against AAV vector capsid. There is little knowledge about the antigenic structures of AAV capsids and how exactly they interact with the antibodies that are raised against them. Nevertheless, antigenic epitopes have been identified and described for AAV capsids of serotypes 2 and 8 [19-21].

NAB can be present in the patient's blood prior to therapy due to naturally acquired infections with the wild type AAV virus (pre-existing NAB) pre-existing NAB against AAV are currently an exclusion criteria for participation in clinical trials that use AAV vectors. This is a major problem, because, depending on the AAV serotype, the reported prevalence of serotype-specific

pre-existing anti-AAV NAB in humans is considerable and there is a significant difference between AAV serotypes (**Figure 1**) [22].

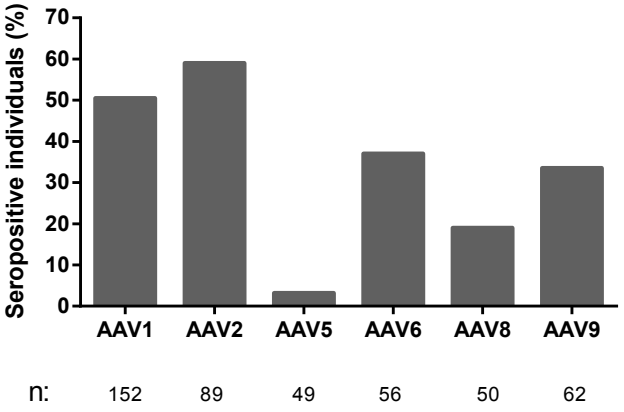

Figure 1. Adapted from Boutin et al. (Human Gene Therapy 2010). Prevalence of neutralizing factors in serum against AAV types 1, 2, 5, 6, 8 and 9, n indicates the number of serum samples tested.

Anti-AAV NAB are also formed after first administration of AAV vector gene therapy. Those NAB may reach high titers and have been reported to persist after treatment.

Although AAV-mediated expression generally is long-lived, re-administration may be necessary, during the lifetime of the patient, the naturally occurring turnover of cells might lead to loss of AAV transduced cells that episomally express DNA encoding the therapeutic protein, resulting in a decrease of the production of therapeutic protein. Repeated AAV treatment might also be needed if the initial treatment does not result in expression of sufficient therapeutic protein. In both cases, re-administration of the same AAV is thought not to be possible due to the presence of circulating NAB formed after the first administration. Those antibodies totally block the transduction and no efficacy of the treatment can be observed [1, 23].

In order to address these issues, numerous approaches have been explored to overcome or circumvent humoral immune responses directed at AAV vectors.

A. Cross administration of different natural AAV serotypes

Twelve AAV serotypes have been identified to date in humans and over 100 serotypes in nonhuman primates [24]. All serotypes studied present antigens that induce production of neutralizing antibodies (NAB). A potential strategy to avoid interference of NAB with primary or secondary AAV-based gene delivery is the use of AAV vector serotypes with different antibody reactivity profiles but similar affinity for the target tissue. When choosing AAV vector serotypes for such "cross-administration" approaches two pivotal aspects should be carefully considered.

Firstly, as would be predicted, homology between AAV serotypes might influence the possibility of NAB cross-reactivity [22, 25, 26]. Indeed, for closely related AAV serotypes 1 and 6 in *in vitro* studies, cross –neutralization of NAB from those serotypes was observed. AAV4, on the other hand, which is a divergent serotype, was only neutralized by the anti-AAV4 NAB from the spectrum of anti-AAV1-6 NAB, while AAV5 serotype showed to be inhibited by anti-AAV1, 2, 3, 5, and 6 NAB but not by anti-AAV4 NAB [26]. Additionally AAV7 and AAV8 have minimal cross-reactivity to other serotypes.

Secondly, different AAV serotypes have divergent tissue tropisms and different transduction efficiencies [27]. As a consequence, depending on the target tissue, the choice of alternative AAV serotypes might be limited.

Cross-administration approaches have proven to be successful in non-human primates, when AAV2/5 vector was administered intravenously in animal that had pre-existing anti-AAV8 NAB. In contrast, AAV8 vector administration in an animal with pre-existing anti-AAV8 NAB did not result in therapeutic protein expression [28]. Effective cross-administration strategy *in vivo* without a significant cross-inhibitory effect was also observed after cross-administration of AAV1, 2 and 5 serotypes in mouse skeletal muscle [29].

Although this approach has proven to be successful in the research setting, it should be noted that changing serotypes, also changes the drug from regulatory point of view and this would necessitate separate costly drug approval procedures for every serotype used.

B. Genetic AAV capsid

Extensive efforts have been directed towards the development of new artificial, less immunogenic AAV capsid variants. Conceptually, genetic modification of the regions that contain immunogenic AAV capsid epitopes could be an approach to escape pre-existing NAB binding and neutralization of modified AAV capsid. One of such strategies involves targeted modifications of identified antigenic regions of AAV capsid [21]. Such rational design was for instance investigated by Huttner at al. who demonstrated that insertion of different ligands at position 587 in AAV2 VP1 protein allow the transduction of cells in the presence of anti-AAV2 NAB [30]. Another group successfully employed a directed evolution strategy to generate large AAV2 mutant capsid library followed by high-throughput selection process to isolate mutants which evade NAB formation [31].

Even though the AAV capsid engineering methods are promising, none of the currently identified AAV capsid mutants are completely resistant to NAB and accumulated mutations in AAV capsid may negatively affect efficiency of gene delivery. For example, AAV vector production, purification, stability, infectivity and tissue tropism might be affected. Finally, the immunogenicity of the new AAV capsids should be carefully investigated to assess whether they would be suitable for re-administration purposes.

C. Chemical modifications of the AAV capsid

Chemical polymers are used for steric stabilization of drug carrier systems such as lipoplexes, nanoparticles, and liposomes. Chemical polymers are coating materials that form a protective hydrophilic layer that limits the interaction with blood components. Coated nanoparticles are not efficiently absorbed by macrophages and therefore show reduced immune responses. The best characterized protein stabilizer material is polyethylene glycol (PEG) and its use has been approved in several therapeutic products. PEG is generally non-toxic and it extends the half-life of proteins with a reduction of immune responses. The attachment of chemical ligands to AAV capsid could potentially protect the AAV capsid from binding of NAB. Several groups have tested AAV capsid coating with PEG and other polymers [32-34] but this has resulted in a

moderate protection of AAV capsid against NAB at best, and in some cases the modification caused impaired AAV infectivity [34].

Hence, chemical conjugation of polymers to AAV capsids remains challenging and needs to be further developed. The current technology is not satisfactory as it does not fully prevent immunogenicity and may cause a loss of AAV infectivity.

D. Physical removal of circulating anti-AAV NAB

Plasmapheresis is an extracorporeal blood component separation technique which is currently used in the clinic to remove high-molecular-weight substances such as autoantibodies, immune complexes, cryoglobulins, and bacterial lipopolysaccharides [35]. Plasmapheresis may be used to remove antibodies from the bloodstream, thereby preventing them from binding to their targets. Due to the fact that plasmapheresis does not interfere with antibody production, the therapeutic effect generally is only transient. Plasmapheresis was used as a strategy to lower anti-AAV NAB, and different groups have reported promising results. Nonetheless, plasmapheresis by itself seems to be an option only for patients that have relatively low anti-AAV NAB titers [36, 37].

Physical contact between anti-AAV NAB in blood and injected AAV vector can be also prevented by specific delivery technologies. For delivery of AAV vectors to the liver in individuals with NAB, specific portal vein injection strategies were developed. The first approach involves portal vein branch flushing with saline that is directly followed with AAV8 vector injection. The second method is based on injection of the vector into the portal vein with the use of balloon catheter. Both of the methods proved to be similarly effective, but second is considered to be safer [38, 39]. This approach needs to be further investigated in order to determine the possibility of its application when high titers of NAB are present.

E. Immunosuppression

Immune suppression (IS) is used in the clinical practice to reduce or prevent immune response in organ transplantation and to treat autoimmune diseases. IS regimens are based on combinations of drugs such as glucocorticoids, anti-

proliferative and antimetabolite agents, calcineurin inhibitors, rapamycin inhibitors, and immune cell depleting or nondepleting monoclonal antibodies. Immunosuppressive drugs have many side effects and treatment with them increases susceptibility to opportunistic infections or chance of cancer occurrence.

For reduction of anti-AAV NAB titers B cell targeting drugs, as bortezomib or anti-CD20 monoclonal antibody have been investigated. Bortezomib, which is a proteasome inhibitor that is approved for the treatment of multiple myeloma, can eliminate both short- and long-lived plasma cells by activation of the terminal unfolded protein response [40]. Unfortunately the bortezomib-induced reduction in the levels of anti-AAV NAB was not sufficient to allow for re-administration of AAV vector. This limitation was related to residual anti-AAV NAB levels and bortezomib's inability to completely deplete memory B cells that are re-activated upon AAV vector re-administration (Karman 2010). The use of anti-CD20 antibody (rituximab) alone [41] or in combination with cyclosporine A [42] also showed only partial efficacy in lowering the anti-AAV NAB titer.

Novel IS approaches can specifically target $CD4^+$ T cells. Targeting of $CD4^+$ T cells in mice with non-depleting anti-CD4 antibody and cyclosporine A at the time of AAV delivery resulted in inhibition of the primary induction of anti-AAV NAB and it allowed for efficient re-administration of the same AAV serotype [43]. This IS strategy has to be further investigated for efficacy and safety in non-human primates before possible clinical application in AAV-based gene therapy. It also remains unknown whether this approach will prove to be effective in case of already established anti-AAV NAB titers (pre-existing anti-AAV NAB) which at the moment exclude many patients from AAV-based gene therapy.

F. Adsorption of NAB against AAV capsid

The principle of antibody adsorption was first demonstrated in gene therapy studies with adenovirus studies in which antibodies directed against adenoviral capsid were depleted from rabbit serum with the use of chromatography columns containing bound capsid proteins [44]. Such specific antibody depletion

proved to facilitate the transduction with adenovirus [45]. A similar principle of antibody adsorption was also studied in AAV-based gene delivery in mice by Scallan et al. who demonstrated that the presence of empty AAV capsids, which acted as decoys, significantly reduced the neutralization of AAV by anti -AAV NAB [46]. Mingozzi et al. have recently shown in mice and non-human primates that injection of therapeutic AAV vector together with empty AAV capsids allows liver transduction in the presence of even high titers of anti-AAV NAB. An additional factor in their experimental setup was the use of a mutant empty AAV capsid that cannot enter target cells but can adsorb anti-AAV NAB [47].

IV Handling cellular and humoral immune responses against transgene product

Next to the immune responses against the AAV capsid protein, cellular and humoral immune responses can be induced against the protein encoded by the transgene and it can cause a limited efficacy of the treatment [48]. The development of specific immune responses directed against the transgene product has been shown to be highly dependent on route of administration, AAV vector serotype, AAV vector dose, tissue specificity of the promoter and clinical profile of the patient.

Even though, promising pre-clinical experimental data have been obtained when targeting the liver [49-54], immune responses against the transgene product were reported in case of intramuscular AAV-based gene delivery which resulted in a limited efficacy of the treatment [55-57]. The cellular and humoral immune responses that can occur against the delivered transgene product might result in a loss of transgene expression, as reported in pre-clinical animal studies [58, 57]. Similarly, in the clinical trial for treatment of Duchenne's muscular dystrophy study in which children were injected intramuscularly with AAV-minidystrophin, immune responses against minidystrophin were observed and expression of the transgene product was lost over time [59, 60].

To circumvent the appearance of immune responses against transgene product several approaches can be considered.

A. Immune suppression

Clinical strategies to avoid immune responses directed at expressed proteins include the use of IS regimens. This approach proved to be efficient in pre-clinical studies using intramuscular AAV gene delivery. For instance, treatment with cyclophosphamide at the time of AAV administration and subsequently twice a week for next 6 weeks resulted in a sustained partial correction of haemophilia B in a null mutation dog model [61]. Another promising outcome was obtained when using immunosuppression with a combination of anti-thymocyte globulin (ATG), cyclosporine (CSP) and mycophenolate mofetil (MMF) which resulted in long-term expression of canine micro-dystrophin in skeletal muscle after intramuscular AAV-based delivery into canine X-linked muscular dystrophy model.

Although immune suppression may have some efficacy, in order to ensure long-term durability of protein expression, immune-modulating therapeutic approaches should facilitate tolerance induction towards the transgene product. Importantly, some IS regimens interfere with the induction or maintenance of tolerance towards the transgene. For example, it has been reported that administration of MMF, sirolimus and daclizumab resulted in a decrease of the Treg population which correlated with formation of inhibitory antibodies against human FIX while none of those was observed when the 2-drug IS consisting of MMF and sirolimus was used [62].

Many different IS regimen combinations for attenuation of transgene-directed immunity in AAV-based gene therapy have been tested in pre-clinical animal models with quite promising results [42, 43, 63, 64]. However, some IS drugs have significant side-effects and lack antigen-specificity [65], and it is desirable to be able to exclude those drugs from future IS approaches in gene therapy.

B. Alternative AAV vector delivery methods

An approach that has been recently developed and might be an attractive alternative to IS regimens when targeting the muscle with AAV-based gene de-

livery are regional intravenous (RI) deliveries instead of intramuscular (IM) injections. Toromanoff et al. have demonstrated that IM injections of AAV in non-human primates are frequently associated with presence of inflammatory infiltrates and the destruction of transduced myofibers necessitating the use of IS. In contrast, RI AAV delivery resulted in a stable transgene expression without immunosuppressive treatment. It seems that the local vector distribution in the skeletal muscle is a key factor that triggers the immunogenicity after IM delivery, and this route of administration results in a high number of vector genome copies per cell. On the other hand, RI route of AAV delivery resulted in homogenous and wide spread vector distribution and lower vector genome copies per cell [66]. Arruda et al. have demonstrated similar results in canine skeletal muscle for FIX after RI delivery by AAV2 vector [67].

C. Tissue-specific promoter use

Avoiding the overexpression of the transgene product in non-target tissues and especially in antigen presenting cells (APCs) is a very important method to avoid transgene directed immune responses. Tissue-specific promoters have been demonstrated to have an important role in reducing the immune responses to the transgene products in pre-clinical animal models, especially in muscle directed AAV-based gene therapy for muscular dystrophy [68, 69].

D. B-cell mediated tolerance induction

B lymphocytes can act as antigen presenting cells (APCs) and via this pathway they may induce of antigen-specific tolerance [70, 71]. Genetically modified B cells have proven to be excellent tolerogenic APCs in several animal models. Expression of peptide-immunoglobulin fusion proteins by B cells can induce tolerance towards the fused peptide and this results in reduced cellular and humoral immune responses against that peptide upon immunization in CFA [72-74]. Therefore, transgene product specific tolerance induction using autologous B-cell gene therapy could be a potential strategy to avoid transgene-directed immune responses in AAV-based gene therapy.

E. Exploiting the endogenous microRNA machinery for regulation of transgene-directed immune responses

MicroRNAs are small non-coding RNAs that are endogenously expressed in a tissue-specific manner and play an important role in maintaining their functions and differentiation [75, 76]. They are also important in innate and adaptive immunity as they control differentiation of various immune cell subsets and their functions [77-79].

MicroRNAs are capable of post-transcriptional regulation of gene expression when they bind to their specific target sequence. Modification of viral vector cassettes with microRNA targets is the latest pursuit to regulate gene expression in gene therapy. A recently explored strategy to avoid immune responses against transgene products in gene therapy took advantage of the activity of mir-142-3p, which is a miRNA specifically expressed in antigen presenting cells (APCs). Incorporation of mir-142-3p target sequences within a transgene sequence has been shown to mediate inhibition of transgene expression in haematopoietic lineage cells, including APCs in both *in vitro* and *in vivo* setup [80]. The use of mir-142-3p target sequences prevented immune responses towards the transgene product in mice when a lentiviral vector was used for gene delivery targeting the liver [81, 82]. Additionally, our group provided evidence that humoral and cellular immune responses against the transgene product can be efficiently reduced by use of mir-142-3p target sequences in AAV-based intramuscular gene delivery [83]. Boisgerault et al. also applied that strategy in intramuscular AAV-based gene delivery settings and confirmed our findings [84].

V Concluding remarks

Significant progress has been made in the use of AAV vectors in human gene therapy and the first AAV vector product for treatment of lipoprotein lipase deficiency received market authorization in Europe [85, 86]. Immune responses observed during AAV gene therapy trials in humans do not appear to be a safety risk. However, for future indications, host immune responses against AAV vector capsid and the transgene product will need to be more effectively addressed. There is a need for the development of safe and effi-

cient strategies that would allow treatment of patients with pre-existing NAB against AAV capsids, re-administration of AAV vectors in case transgene expression is lost due to natural turnover of the transfected cells and that prevent or treat possible immune responses against the transgene product.

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