Commentary: J Fraser Wright

Human gene therapy – immunogenicity perspective

Recombinant viral vectors designed for human gene therapy are emerging as a central component of an enormously promising therapeutic paradigm, likely to rival the impact on human health and disease treatment realised by recombinant protein-based therapeutics. Challenges relating to viral vector manufacturing to support realisation of the potential of gene and gene modified cell therapies are well recognised.

In remarks made at the Alliance for Regenerative Medicine 2018 annual board meeting, then US Food and Drug Administration Commissioner Scott Gottlieb emphasised the challenges related to product issues in the context of the accelerating pace of development of these potentially transformative new medicines.[1] From my perspective, major 'product,' or chemistry, manufacturing and control (CMC), challenges include:

- Meeting vector manufacturing capacity requirements to support late-stage clinical development and commercial launch. This is especially true for larger indications, using existing production systems, which are complex and require similarly complex ancillary components, such as plasmid DNA or recombinant viruses for batch-wise generation of the vector product in production cell lines;
- Addressing the need for rapid manufacturing process development and validation. This is especially important when early clinical results demonstrate strong evidence of clinical benefit for severe diseases for which no other treatments are available; and
- Defining the product-critical quality attributes and a parallel need for rapid development and validation of analytical methods to accurately and precisely measure them for lot release and comparability studies.

In this commentary, my objective is to highlight a potential critical quality attribute of adeno-associated virus (AAV) vectors that is not well characterised, but that may seriously affect the potential for long-term durable transgene expression, a key requirement for the gene therapy 'one and done' therapeutic paradigm. AAV vectors inherently contain viral capsid proteins to achieve efficient delivery of the therapeutic transgene. Given that AAV vectors should avoid activation of the recipient immune response to the degree possible, it is important to ensure that other immunogenic signals are minimised. In this article, a perspective is given on the potential of unmethylated CpG motifs in AAV vector genomes, which are known to potentiate adaptive immune responses such as cytotoxic T lymphocytes (CTL's) that can limit long-term transgene expression, to be influenced by manufacturing methods.

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The potential role of immunostimulatory CpG motifs in AAV vector genomes contributing to unwanted, efficacy-limiting immune responses in clinical trials for haemophilia was the subject of an extended discussion at the 2018 National Hemophilia Foundation's 14th Workshop for Novel Technologies and Gene Transfer for Hemophilia convened in Washington DC. The discussion was catalysed by a presentation by Chapin and colleagues, who presented an analysis of the unexpected loss of F.IX expression in a clinical trial of BAX335 in 7/8 subjects and identified CpG-enrichment in the codon-optimised expression cassette as a potential cause.[2] They reported that their next-generation rAAV investigational products for Haemophilia A and B will be CpG-reduced. In the discussion that followed, other examples emerged AAV vectors in which CpG-enrichment in the vector genome led to poor outcomes i.e. short-term expression, and CpG-reduction led to good outcomes i.e. long-term expression, in human clinical trials.

Of note, in the design of the AAV vector genome used for the first haemophilia gene therapy trial recognised to achieve durable multi-year F.IX expression, the CpG content was deliberately reduced (Nathwani et al, 2006).[3] Given these recent reports and the need to understand the key product quality attributes and host immune response factors that influence long-term therapeutic transgene expression, it is important to fully explore the 'CpG as a key immune protagonist' hypothesis.

Unmethylated CpGs bind and activate toll-like receptor 9 (TLR9), the recognition component of an innate immunity signaling pathway for the recognition of 'non-self' pathogen associated molecular patterns (PAMPs) in humans (Hartmann et al, 1999; Hemmi et al, 2000).[4,5] The frequency of CpG dinucleotides in the human genome is about 1.5%, a value lower than the statistically expected value of 6.25% based on random nucleotide utilisation. In human cells about 75% of CpGs are methylation, and therefore the human genome contains unmethylated CpG motifs at a low frequency of about 0.4%. Microbial genomes exhibit markedly higher unmethylated CpG content, for example about 6% in bacteria, representing a distinct pathogen-associated molecular pattern that, unlike human DNA, binds the TLR9 in human antigen presenting cells, triggering innate pathways that potentiate defensive immune responses.

During the development and optimisation of recombinant protein expression in bioreactors, 'codon optimisation' algorithms were developed to increase the expression efficiency of protein production in heterologous production systems e.g. human proteins expressed in E. coli, yeast,

insect cells, and non-human mammalian cell lines (Gustafsson et al, 2004).[6] These algorithms increase CpG content in DNA sequences. While such codon optimisation has been used by some groups for AAV vectors, the rationale for this type of codon-optimisation of AAV expression cassettes designed for *in vivo* human gene therapy, involving expression of human proteins in homologous (i.e. human) cells in the presence of the human immune systems is questionable.

Any benefit achieved in transgene expression efficiency is outweighed by the increased risk of innate immune activation through TLR9 activation pathways, especially if the increased CpG is unmethylated. A related learning from the vaccine industry has been the use of unmethylated CpG-rich nucleotides as adjuvants, co-administered with vaccine antigens to activate the TLR9 pathway and potentiate cellular immune responses, for example to tumor antigens (Bode et al, 2011)[7], a feature not desired for a gene therapy vector. An AAV vector containing high levels of unmethylated CpGs in its genome represents a viral capsid antigen incorporated with an innate immune adjuvant, which should be avoided.

While CpG motifs in an AAV vector genome can be increased or decreased using codon modification strategies, they cannot be eliminated. Given that the actual ligand for TLR9 is *unmethylated* CpG motifs, an important consideration is whether the cell culture systems used for vector generation can affect the degree of methylation of extant CpG motifs. Using BrDU labeling, my lab previously reported data supporting that a significant subset of AAV2 vector particles generated by transient transfection of HEK293 cells contain expression cassettes excised directly from the vector plasmid DNA template (Hauck et al, 2006)[8], a finding that predicts a high level of unmethylated CpGs because plasmid DNA is normally not methylated.

Toth et al (2019)[9] described direct biochemical analysis of AAV vectors prepared by transient transfection, reporting that CpG in the AAV vector genomes were largely unmethylated. Undermethylation of CpGs is also a concern using insect cell-based rAAV production because low DNA methylation has been reported in Holometabola (Provataris et al, 2018).[10] Further improvements in AAV vector production systems should aim to minimise unmethylated DNA in AAV vectors, for example by modifications that incorporate methyl-transferases at appropriate steps. CpG methylation status in the genomes of AAV vectors prepared for human gene therapy is proposed to be a critical quality attribute that should be better characterised.

Human immune responses continue to represent a significant barrier for human gene therapy using AAV vectors. Host immunity constrains viral vector administration to 'one shot on goal' for systemic routes of administration, and human subjects who receive AAV vectors and subsequently lose therapeutic transgene expression due to immune responses are excluded from the benefit of future, improved AAV gene therapies because of their seroconversion to high titer vector antibodies.

Concerted optimisations aimed at ensuring avoidance of efficacy-limiting immune responses is important and should target AAV vector genome design – limiting CpG content, AAV manufacturing systems - maximizing CpG methylation, and immune suppression protocols – to dampen post administration immune responses and ensure long term, durable gene expression.

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