Molecular Therapy
Review

Lethal immunotoxicity in high-dose systemic AAV therapy

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High-dose systemic gene therapy with adeno-associated virus (AAV) is in clinical trials to treat various inherited diseases. Despite remarkable success in spinal muscular atrophy and promising results in other diseases, fatality has been observed due to liver, kidney, heart, or lung failure. Innate and adaptive immune responses to the vector play a critical role in the toxicity. Host factors also contribute to patient death. This mini-review summarizes clinical findings and calls for concerted efforts from all stakeholders to better understand the mechanisms underlying lethality in AAV gene therapy and to develop effective strategies to prevent/treat high-dose systemic AAV-gene-therapy-induced immunotoxicity.

Systemic gene delivery with the adeno-associated virus (AAV) vector is a powerful approach to treating diseases involving tissues throughout the body.[1] This therapeutic modality has significantly improved the quality of life of infants afflicted by spinal muscular atrophy (SMA) and was recently approved to treat 4- to 5-year-old patients with Duchenne muscular dystrophy (DMD).[2,3] Nonetheless, multiple cases of death occurred following high-dose intravenous AAV administration (Table 1).[4–9] Vector-induced liver failure, kidney failure, or heart failure has been implicated in these deaths.

Reporting in the *New England Journal of Medicine*, Terry Flotte and colleagues revealed lung failure as another cause of death in high-dose systemic AAV gene therapy.[10] The patient was a 27-year-old male with a 30-kb deletion mutation at the 5′ end of the *DMD* gene that abolished the expression of the muscle form of dystrophin. The patient showed a classic presentation of advanced DMD including muscle wasting, loss of ambulation, pulmonary dysfunction, and cardiomyopathy.[11] The therapy is intended to promote the expression of the cortical form of dystrophin in muscle using CRISPR-mediated transactivation. The vector (CRD-TMH-001) contains two expression cassettes: one expresses a VP64 (transcription activation domain)-fused dead *Staphylococcus aureus* Cas9 (dCas9-VP64) from the muscle-specific CK8e promoter, and the other expresses a gRNA targeting the cortical promoter of the *DMD* gene. The vector was packaged in AAV serotype 9 (AAV9), and 1×10^{14} vector genome (vg) particles/kg of the vector were delivered to the patient under transient immune suppression with corticosteroids (methylprednisolone and prednisolone), rituximab, and sirolimus. Prominent clinical findings after dosing (besides those commonly seen in patients with advanced DMD) included respiratory acidosis, pericardial infusion on echocardiography, and characteristic features of acute respiratory distress syndrome (ARDS) on the chest X-ray on days 3, 5, and 6, respectively. Treatment with eculizumab (C5 inhibitor), tocilizumab (interleukin-6 [IL-6] antagonist), anakinra (IL-1 receptor [IL-1R] antagonist), and intravenous immunoglobulins did not stop the progression. The patient died on day 8 from multi-organ failure. Laboratory examination showed a progressive decline in platelets, reduction in total protein, and hypoalbuminemia. IL-6 cytokine and cardiac markers were elevated on day 6. Excessive cytokines were detected in the pericardial fluid. However, liver and kidney function were normal. The autopsy revealed diffuse alveolar damage with hyaline membranes (an indicative finding of ARDS), pulmonary edema, and classic pathological lesions of DMD such as extensive fatty fibrotic infiltration in the heart and muscle. No signs of complement deposition or thrombotic microangiopathy (TMA) were detected. Immunological studies showed the absence of pre-existing or vector-induced AAV9 antibodies and the absence of AAV9- or dCas9-VP64-specific effector T cells. The highest amounts of vector genomes were detected in the liver (~700 vg/diploid genome), followed by lung (~120 vg/diploid genome), skeletal muscle (~100 vg/diploid genome), and heart (~40 vg/diploid genome). Transgene products (dCas9-VP64 transcript and protein, gRNA transcript) were detected in the liver in low amounts (but not in the heart or skeletal muscle). The authors proposed that cytokine-induced capillary leak syndrome underlies the observed fatal immunotoxicity in this patient.

Capillary leak syndrome is a serious condition caused by a generalized increase in vascular permeability leading to the transfer of protein-rich fluid from the circulatory system to the interstitial space and body cavity.[12] Capillary leak syndrome can be idiopathic, elicited by drugs (e.g., chemotherapy drugs), or associated with various disorders such as hemophagocytic lymphohistiocytosis (HLH). HLH is caused by the exaggerated response of the immune system and is

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Table 1. Fatality cases following high-dose systemic AAV delivery

		AAV				Clinical profile						
	Drug name	Serotype	Dose (vg/kg)	Promoter	Transgene	Disease	Patient age	Time of death	Cause of death	Immunotoxicity	Clinical trial ID	Reference
Acute death	PF-06939926	AAV9	2×10^{14}	miniMCK	μDys gene	DMD	16 years	6 days post-dosing	heart failure	innate response	NCT03362502	Lek et al.,[8] Philippidis,[9] and Lek et al.[10]
	CRD-TMH-001	AAV9	1×10^{14}	CK8e	dCas9-VP64 and gRNA	DMD	27 years	8 days post-dosing	lung failure	innate response (cytokine-mediated)	NCT05514249	Lek et al.[10]
Subacute death	Zolgensma	AAV9	1.1×10^{14}	CBA	SMN gene	SMA	≤2 years (4 patients)	5–6 weeks post-dosing	liver failure	adaptive response	post-marketing	Philippidis, Whiteley, and Kishimoto and Samulski[6,19,20]
	Zolgensma	AAV9	1.1×10^{14}	CBA	SMN gene	SMA	6 months	8 weeks post-dosing	kidney failure	innate response (complement mediated)	post-marketing	Guillou et al.[7]
	AT132	AAV8	$1.3–3 \times 10^{14}$	DES	MTM1 gene	XLMTM	≤5 years (4 patients)	20–40 weeks post-dosing	liver failure	innate response?	NCT03199469	Shieh et al., Philippidis, Whiteley, and Kishimoto and Samulski[4,5,19,20]

miniMCK, CK8e, and DES are muscle-specific promoters. CBA is a ubiquitous promoter. μDys, microdystrophin; SMN, survival motor neuron; MTM1, myotubularin; DMD, Duchenne muscular dystrophy; SMA, spinal muscular atrophy; XLMTM, X-linked myotubular myopathy.

characterized by marked hypercytokinemia, also known as cytokine storm.[13] Interestingly, a case of HLH was reported in a 3-year-old patient with SMA within 36 h after infusion of Zolgensma (1.1×10^{14} vg/kg), an FDA-approved AAV9 vector expressing the survival motor neuron gene from the ubiquitous CBA promoter.[14]

Host factors may have also played an important role in the death of the patient with DMD reported by Lek et al.[10] Baseline serum cytokines are highly elevated in patients with DMD due to widespread muscle degeneration and inflammation.[15] Moreover, vascular structure and function are impaired in the absence of dystrophin.[16,17] In the advanced stage of the disease, ongoing respiratory insufficiency and cardiomyopathy reduced the patient's ability to cope with cardiopulmonary stress. Significant loss of lean muscle mass resulted in a relatively higher vector loading in muscle. Collectively, these host factors may have predisposed the patient to acute death following gene therapy.

Vector-induced immune responses have long been recognized as a major barrier in gene therapy.[18] An adenovirus-vector-induced lethal inflammatory response resulted in the tragic death of Jesse Gelsinger in 1999. The AAV vector, which was initially thought to be less immunogenic, is now widely recognized as posing a significant immunological risk to patients, especially when a high dose ($\geq 5 \times 10^{13}$ vg/kg) is injected intravenously. AAV-vector-related adverse (vomiting, nausea, and fever) and severe adverse (liver failure and kidney failure) events are frequently encountered in high-dose systemic AAV gene therapy trials. Most patients recover from these events following treatment with immune-modulating drugs, although death has occurred in some cases.

Hepatotoxicity is the most common adverse event following intravenous administration, likely because the liver is a sink for systemically delivered AAV vectors.[19] Liver toxicity usually starts between 1 and 4 weeks post-AAV injection. Elevation of liver enzymes (alanine aminotransferase and/or aspartate aminotransferase for non-muscle diseases and γ-glutamyl transferase and/or glutamate dehydrogenase for muscular dystrophy) indicate hepatocyte injury. Most patients respond to steroid therapy. Eight cases of fatality have been reported, including four patients with SMA treated with Zolgensma (1.1×10^{14} vg/kg) and four patients with X-linked myotubular myopathy (XLMTM) treated with an experimental AAV8 vector AT132 ($5 \times 10^{13}–3 \times 10^{14}$ vg/kg) (Table 1).[5,6,19,20] Different mechanisms have been proposed. The capsid-specific cellular response is likely the cause of liver damage in patients with SMA, as evidenced by infiltrating $CD8^+$ T cells in liver biopsy. In XLMTM, the lack of prominent inflammatory cell infiltration in the liver autopsy suggested that it is not due to the T cell response. Hepatic stress from high vector loading in the liver (and possibly an early innate response to the AAV vector) may have exacerbated the pre-existing hepatobiliary disease in patients with XLMTM and resulted in liver failure.

Recently, a patient with SMA was diagnosed with lethal TMA following Zolgensma treatment (Table 1).[7] TMA (also referred

to as atypical hemolytic uremic syndrome [aHUS]) is characterized by microangiopathic hemolytic anemia, thrombocytopenia, kidney injury, and microthrombi.[21,22] This condition is triggered by endothelial injury. The first case of AAV-induced TMA occurred in a teenage patient with DMD who received 5×10^{13} vg/kg of an AAV9 microdystrophin vector. Laboratory studies suggested complement activation, and the patient recovered with anti-complement therapy. Since then, TMA has been observed in systemic AAV gene therapy for many diseases (DMD, SMA, Fabry disease, Danon disease, and methylmalonic acidemia) involving wild-type or engineered AAV capsids. TMA usually occurs within 1–2 weeks after AAV dosing and can happen in patients of any age. The lowest reported dose that triggered TMA was 1×10^{13} vg/kg. It is believed that the antibody-mediated classic pathway plays a critical role in complement activation, but direct binding of AAV to components of the complement system may also contribute to complement activation through the alternative pathway.[23] Notably, genetic and disease-associated alterations in the complement system are important predisposing factors in AAV-induced TMA. Anti-complement therapy (such as C5 inhibitor eculizumab and ravulizumab) and hemodialysis are effective strategies for treating TMA. In the lethal case of TMA in the infant patient with SMA,[7] secondary *Staphylococcus epidermidis*-induced sepsis aggravated TMA and led to death.

Cardiac involvement has also been observed in patients with DMD treated with systemic AAV microdystrophin gene therapy. Five 7- to 9-year-old patients developed myositis at 3 to 6 weeks following AAV infusion. Of these patients, three also developed myocarditis. The vectors used in these studies were AAV8, AAV9, and AAVrh74, and the doses ranged from 1×10^{13} to 2×10^{14} vg/kg. Immunological studies suggested that myositis and myocarditis were due to the cytotoxic T lymphocyte response against an antigenic epitope present in microdystrophin but absent in patients due to deletion mutations.[24] One acute cardiogenic death occurred in a nonambulatory patient 6 days after receiving 2×10^{14} vg/kg of an AAV9 microdystrophin vector (PF-06939926) (Table 1).[8,10] The death of this patient was thought to involve an innate immune response to the AAV capsid. Cardiac toxicity is a prominent finding in Zolgensma mouse studies, but results in mice do not always translate to human patients.[25] Nevertheless, an acute cardiac death was observed in a 15-month-old patient with SMA 3 days after AAV infusion.[26] Since no autopsy was performed, it is unclear whether the death was related to the AAV vector.

The reported cases of fatality, though infrequent, suggest that our understanding of immunotoxicity in high-dose systemic AAV gene therapy is limited and that the current mitigating strategies are insufficient. There is an urgent need to investigate the underlying mechanisms of lethality observed in high-dose systemic AAV gene therapy. Unfortunately, the existing animal models have not been very useful. Autopsy can help determine the cause of death but is not always performed. The development of novel, creative means to study AAV-induced death is warranted.

Given the rarity of these deaths, host factors are likely critical contributors. In current AAV gene therapy trials, patients are screened for pre-existing AAV antibodies (total binding antibody and neutralizing antibody) and some for pre-existing capsid-specific T cells. Expanding screening criteria to include more immune-related components, such as the HLA type and complement-related genetic changes, may provide important insights into understanding and preventing lethal immunotoxicity.

Immune-modulating drugs are commonly used in AAV clinical trials to manage immune-related adverse and severe adverse events. However, specific guidelines are rarely established for each type of AAV-induced toxicity. Optimization and standardization of the management protocols (e.g., which drug/drug combinations, when, how much, and how long to use) would help reduce AAV-related mortality.

Another highly promising strategy to minimize AAV-induced fatality is to improve the vector itself. Capsid engineering has the potential to significantly enhance potency and tissue specificity (hence, substantially reduce the dose). An excellent example is the recently developed AAVMYO and MyoAAV capsids.[27] Vector genome engineering is another effective approach. Several strategies, such as eliminating or reducing CpG motifs and including immune-modulating elements like Toll-like receptor 9 (TLR9) inhibitory oligos and microRNA-binding sites, have already shown promise in preclinical studies and clinical trials.

Several gene therapy modalities are in development to treat DMD. These include exon-skipping, microdystrophin, CRISPR, and dystrophin-independent gene therapies.[28] Several exon-skipping drugs and one microdystrophin drug have received regulatory approval. Lek et al. reported the first case of CRISPR therapy in a patient with DMD.[10] The short course prevented the authors from drawing any conclusions on the therapeutic efficacy of CRISPR transactivation and the Cas9-induced immune response in the patient. However, we previously showed that AAV-mediated Cas9 expression induced a strong cytotoxic T cell response and loss of edited muscle cells in canine DMD models.[29] AAV-capsid-induced innate and adaptive responses and transgene-product-induced humoral and cellular responses remain critical barriers and present potentially life-threatening risks in systemic AAV gene therapy.

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DECLARATION OF INTERESTS

D.D. is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences, a member of the scientific advisory board for Sardocor Corp., and an inventor of several issued and filed patents on DMD gene therapy and AAV vectors.

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