Molecular Therapy
Commentary

AAV Immunogenicity: New Answers Create New Questions

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Adeno-associated viral (AAV) vectors have been successfully used to treat and even cure several genetic diseases. They are attractive tools for therapeutic *in vivo* gene transfer because of their wide tissue tropism and relatively low immunogenicity when compared to other viruses. Yet, immune responses to AAV continue to overshadow their otherwise impressive clinical record. For instance, cytolytic $CD8^+$ T cell responses against the viral capsid were identified in patients who received gene therapy. These may also be found in the general population as a result of natural infection with the virus. Whether the responses in gene therapy patients therefore represent memory T cell or primary immune responses remains controversial. Similarly, it has been puzzling that people with antibodies to AAV often show no evidence of $CD8^+$ T cell responses to the capsid. Now, a new study has uncovered a clear link between B and T cell responses to AAV capsid during natural infection that has not been fully appreciated in clinical studies where an interferon γ (IFNγ) assay was used to monitor immune responses. Interestingly, seronegative people tended to show a natural killer (NK) cell response to AAV, suggesting that some individuals mount an innate rather than adaptive response upon natural exposure to AAV.

The AAV capsid is a foreign antigen to the human immune system and is therefore a potential target for neutralizing antibodies (NABs) and $CD8^+$ T cells. Pre-existing NABs, which are prevalent in the human population, preclude some patients from enrollment in clinical trials. Following treatment, antibody formation prevents repeat dosing for months if not years and $CD8^+$ T cell responses to the capsid may cause immunotoxicities and eliminate transduced cells. These clinical observations raise several important questions. Why is there no apparent correlation between B and T cell responses in pre-existing immunity to AAV? What are the activation signals that trigger B and T cell responses against AAV? And why is the T cell response delayed until weeks to months after gene transfer? Recent studies address these critical issues.

In the new paper recently published in the *Journal of Clinical Investigation*, Kuranda and colleagues[1] provide some answers on AAV-elicited B cell activation in humans. The authors carefully analyzed lymphocytes and antigen-presenting cells from healthy volunteers following exposure to AAV or capsid-derived peptides. Importantly, they found that the presence of antibodies to AAV was more greatly correlated to the expression of tumor necrosis factor alpha (TNF-α), rather than IFNγ, by capsid-specific memory $CD8^+$ T cells.

Furthermore, the study provides some clues surrounding the origin of the B cell response. Monocyte-derived dendritic cells (moDCs, also referred to as "inflammatory DCs") from seropositive patients produced the cytokines interleukin (IL)-1β and IL-6 upon stimulation with AAV. *In vivo* experiments in mice showed that antibody formation was also dependent on these two cytokines. IL-6 production depended on IL-1β, indicating that IL-6 is induced secondarily to an initial IL-1β response. In several prior studies, $CD8^+$ T cell responses to the capsid and transgene products of AAV vectors were linked to sensing of the vector genome by the endosomal DNA receptor toll-like receptor 9 (TLR9). We showed that a collaboration of plasmacytoid DCs (which sense the genome via TLR9) and conventional DCs (which carry out antigen presentation) leads to $CD8^+$ T cell activation through a type I IFN-dependent mechanism.[2] Interestingly, TLR9 deficiency or removal of TLR9-stimulatory CpG motifs from the vector genome significantly reduces $CD8^+$ T cell activation without substantially impacting antibody formation. These new data show that a different DC subset, namely moDCs, may be the critical source of immune activation leading to B cell responses to the viral capsid. Taken together, the role of DCs in the initiation of adaptive immune responses to AAV infection and AAV-mediated gene transfer involves multiple DC subsets with discrete roles, leading to either B cell or $CD8^+$ T cell activation. However, the nature of the signal that leads to moDC activation by AAV is not yet clear. TLR9 can activate moDCs but is an unlikely candidate because TLR9 is not required for the antibody response. Klaudia Kuranda et al.[1] find evidence that capsid-derived peptides may stimulate the response. While there is literature supporting the notion that viral-derived peptides may be immune stimulatory, the innate immune system more typically senses molecular structures rather than specific sequences.

A similar unanswered question is why natural AAV infection leads to NK cell activation. This observation might be related to the circumstances of natural infection, such as the presence of other viruses, since AAV is naturally replication deficient and depends on a helper virus. Moreover, it is unknown whether $CD8^+$ T cell responses observed in healthy subjects correlate to those in AAV-treated patients. Such responses may have been missed in some gene therapy patients because TNF-α measurements were not performed. Assays measuring multiple cytokines are preferable but technically more challenging.

Another puzzle in the field has been that T cell responses to AAV capsid can occur

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months after gene transfer, long after the initial innate inflammatory responses. A possible explanation is that AAV particles might persist for some time and therefore could still be sensed by the immune system. Alternatively, it has also been shown that T cells can be activated initially, but only acquire IFNγ expression and full functionality later on.[3] Interestingly, Shao and colleagues,[4] in a recent paper in *JCI Insight*, show that transduced cells may provide innate immune signals related to expression from the vector rather than the viral particle. Human cell lines, primary hepatocytes, and chimeric mouse livers harboring human hepatocytes all expressed IFN that correlated with an upregulation of the cytosolic double-stranded RNA (dsRNA) sensor MDA5. Knockdown of MDA5 prevented IFNβ expression by transduced cells. The authors employed reverse transcription followed by PCR to provide evidence for the presence of dsRNA. The exact mechanism by which these molecules are generated requires further investigation but may be related to inverted terminal repeat (ITR) structure or intra-/inter-vector genome recombination. Whether and how dsRNA sensing relates to antigen-specific immune responses also remains to be defined. Nonetheless, these new studies find evidence for innate immune responses to AAV vectors that have previously not been appreciated. The hope is that, over time, these observations will begin to fit together into a bigger picture describing how immune responses to AAV are orchestrated and provide guidance for targeted intervention in the clinic.

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Gene Silencing in the Right Place at the Right Time

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The CNS in general and the brain, in particular, remain difficult to target for drug or gene delivery. This is mainly due to the presence of a highly restrictive barrier that lines the blood vessels within the brain, termed the blood-brain barrier (BBB). The BBB protects the brain from invading organisms and neurotoxins, regulates the uptake of essential nutrients, and also prevents the entrance of therapeutic agents.[1] In recent years, there has been a tremendous effort to develop new methods for delivering different types of therapeutic agents to the brain but, unfortunately, without much success.[1] In this issue of *Molecular Therapy*, Godinho et al.[2] report the delivery of small interfering RNA (siRNA)-lipid conjugates following BBB disruption as a means to deliver a therapeutic payload. Their work combines gene silencing, BBB disruption using mannitol, and specific brain targeting strategies. The strategy may pave the way for more efficient and selective delivery of therapeutics to the brain.

RNAi technologies, mainly siRNAs, have been widely studied as a therapeutic strategy to silence key genes in different diseases to reduce the expression of important proteins, including those considered "undruggable." On August 10th, 2018, the US Food and Drug Administration (FDA) approved the first ever RNAi drug, called Onpattro (Patisiran), to treat polyneuropathy in patients with hereditary ATTR amyloidosis, a potentially fatal condition that affects an estimated 50,000 people worldwide.[3] Nevertheless, siRNA faces multiple challenges for safe and effective delivery. These include protecting the siRNA from degradation in the bloodstream, avoiding rapid renal clearance, minimizing off-target effects, and limiting liver, kidney, and immune toxicity issues that could result in death.[3] To overcome these challenges, Godinho and colleagues[2] conjugated siRNA to phosphocholine (PC) docosahexaenoic acid (DHA), the most abundant

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