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REVIEW

Progress and prospects: immune responses to viral vectors

S Nayak and RW Herzog
Department Pediatrics, University of Florida, Gainesville, FL, USA

Viral vectors are potent gene delivery platforms used for the treatment of genetic and acquired diseases. However, just as viruses have evolved to infect cells efficiently, the immune system has evolved to fight off what it perceives as invading pathogens. Therefore, innate immunity and antigen-specific adaptive immune responses against vector-derived antigens reduce the efficacy and stability of in vivo *gene transfer. In addition, a number of vectors are derived from parent viruses that humans encounter through natural infection, resulting in preexisting antibodies and possibly in memory responses against vector antigens. Similarly, antibody and T-cell responses may be directed against therapeutic gene products that often differ from the endogenous nonfunctional or absent protein that is being replaced. As details and mechanisms of such immune reactions are uncovered, novel strategies are being developed, and vectors are being specifically engineered to avoid, suppress or manipulate the response, ideally resulting in sustained expression and immune tolerance to the transgene product. This review provides a summary of our current knowledge of the interactions between the immune system adeno-associated virus, adenoviral and lentiviral vectors, and their transgene products.*
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Prospects

- Advances in vector engineering (capsid engineering, microRNA-regulated expression cassettes)
- Advances in delivery techniques (administration to immune-privileged sites, taking advantage of organ-specific immune responses)
- Immune suppression and modulation regimens (using rapamycin, rituximab, cyclosporineA, anti-CD3 antibody and others)

Correspondence: Professor RW Herzog, Department Pediatrics, University of Florida, Cancer and Genetics Research Center, 1376 Mowry Road, Room 203, Gainesville, FL 32610, USA.
E-mail: rherzog@ufl.edu
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Introduction

Viral vectors are optimal vehicles for gene transfer because of their ability to efficiently infect host cells. Removal of the replicative and pathogenic ability of

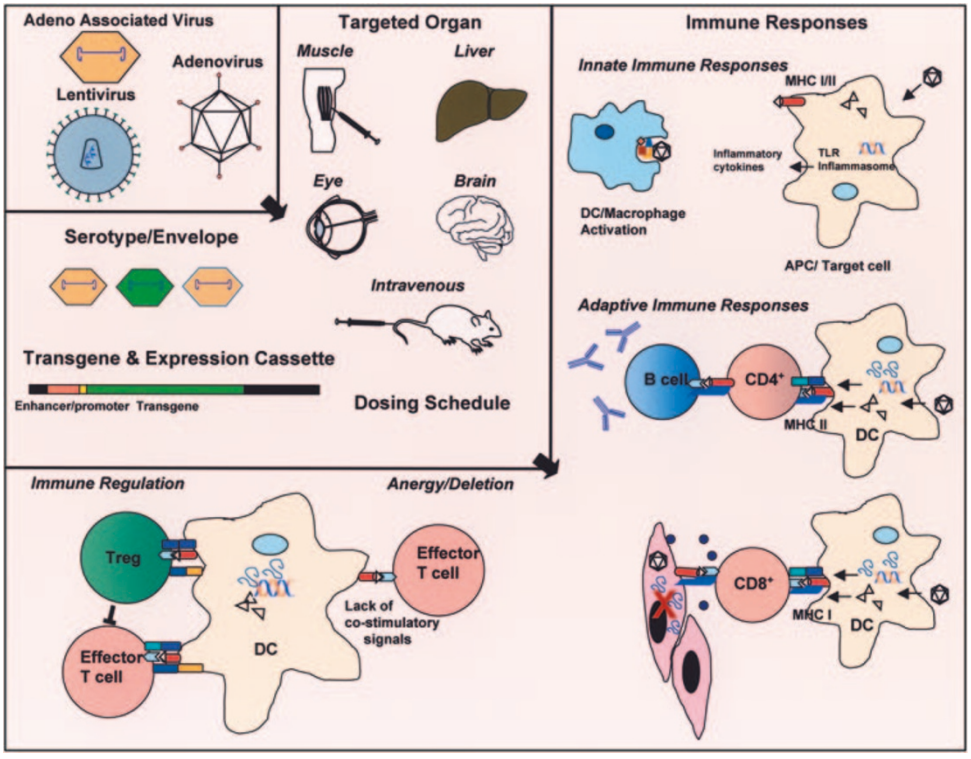

Figure 1 Overview of immune responses to viral vectors. Targeting specific organs, engineering viral envelopes, switching serotypes, modifying the transgene cassette, using tissue-specific promoters, or immune modulation regimens can result in immune avoidance to the viral vector and transgene product, and in some cases even induce tolerance to the therapeutic gene product.

viruses, combined with their capacity to carry the therapeutic transgene and an ability to efficiently infect various mammalian cell types makes them amenable for use in gene therapy (Figure 1). However, the immune system has evolved to fight off invading pathogens, which makes viral vectors subject to immune responses that have to be either blocked or avoided to achieve therapeutic transgene expression. Administration of viral vectors can lead to the initiation of innate and adaptive immune responses against viral particles and gene products, leading to decreased efficiency of gene transfer or to the elimination of the transduced cells over a period of time (Table 1). Recent research has concentrated on various immune modulatory regimens using immune-suppressive drugs in combination with gene therapy, modification of viral capsids or choice of viral envelope. Immunogenicity of viral gene transfer can also provoke an immune response against the therapeutic transgene product, which may represent a neoantigen owing to the type of gene mutation present, rendering patients with, for example, null mutations, susceptible to recognizing the transgene product as a foreign antigen. Although there are similarities in immunity to different viruses, each vector contains its own set of activation signals, which are further modified by the environment of a specific tissue.[1]

Overview of immune responses in adeno-associated virus-mediated gene therapy

Adeno-associated virus (AAV) vectors are derived from a nonpathogenic replication-deficient parvovirus. The AAV vector genome is typically a $\leqslant$5-kb single-stranded DNA. Numerous serotypes, mostly isolated from humans or nonhuman primates, have now been characterized to improve the transduction of specific organs and to circumvent immune responses. The popularity of AAV as a vector stems from a broad host range, nonpathogenic nature, ability to transduce both dividing and nondividing cells, low innate immunity, as well as low efficiency of transduction of professional antigen-presenting cells (APCs), such as dendritic cells (DCs) or macrophages, possibly due to a post entry block which limits its immunogenicity.[2] Preclinical trials conducted in animal models of human disease have shown long-term correction of genetic disease using AAV vectors, and clinical trials have begun in a number of areas.

Innate immune responses to AAV; TLR-9 and complement

Adeno-associated virus is a weak innate immunogen; microarray studies have shown that AAV does not elicit the robust type I interferon (IFN) response as is seen for adenoviral vectors.[3] Similarly, cytokine and chemokine responses in the transduced tissue are limited and highly transient. AAV triggers Toll-like receptor (TLR) signaling (for example, TLR-9, which senses DNA).[4] AAV has also been shown to interact with complement.[5] The complement cascade, an important component of the innate immune system, leads to opsonization of foreign bodies and lysis of target cells. The three complement pathways include the classical, alternative and lectin binding pathways, all of which involve C3 convertases. Recent data have shown that the AAV2 capsid binds to the C3

Table 1 Summary of immune responses in viral gene transfer

Vector	*Innate immune response*	*Adaptive immune response*
AAV	Low and highly transient inflammation Complement activation TLR-9 dependent DNA sensing DC activation	Pre-existing NAB Memory CD8+ T-cell responses to capsid NAB formation after vector administration Antibody and T-cell responses against transgene product depending on route of vector administration and other factors Treg and immune tolerance induction to the transgene product for hepatic gene transfer
Adenovirus	Inflammation, immunotoxicity in target organ Thrombopenia, platelet activation Hemodynamic changes Inflammasome-dependent cell death Induction of inflammatory cytokines and IFN-α,β Activation of TLR-9-dependent and TLR-9-independent pathways of DNA sensing Activation of TLR-2 NK cell activation Endothelial cell activation Complement activation DC activation	Pre-existing NAB NAB formation after vector administration Transduction of APCs CTL responses against viral gene products (unless gutted vectors are used) and transgene product Antibody and T-cell responses against transgene products, especially if nonspecific promoters are used
Lentivirus	IFN-α,β production pDC and cDC activation TLR-7 signaling (?)	Highly efficient transduction of APCs CTL and antibody responses against transgene products unless micoRNA-regulated expression is used Tolerance induction to transgene products after hepatic gene transfer with micoRNA-regulated expression cassette

Abbreviations: AAV, adeno-associated virus; APC, antigen-presenting cell; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; IFN, interferon; NAB, neutralizing antibody; pDC, plasmacytoid DC; TLR, Toll-like receptor; Treg, T regulatory.

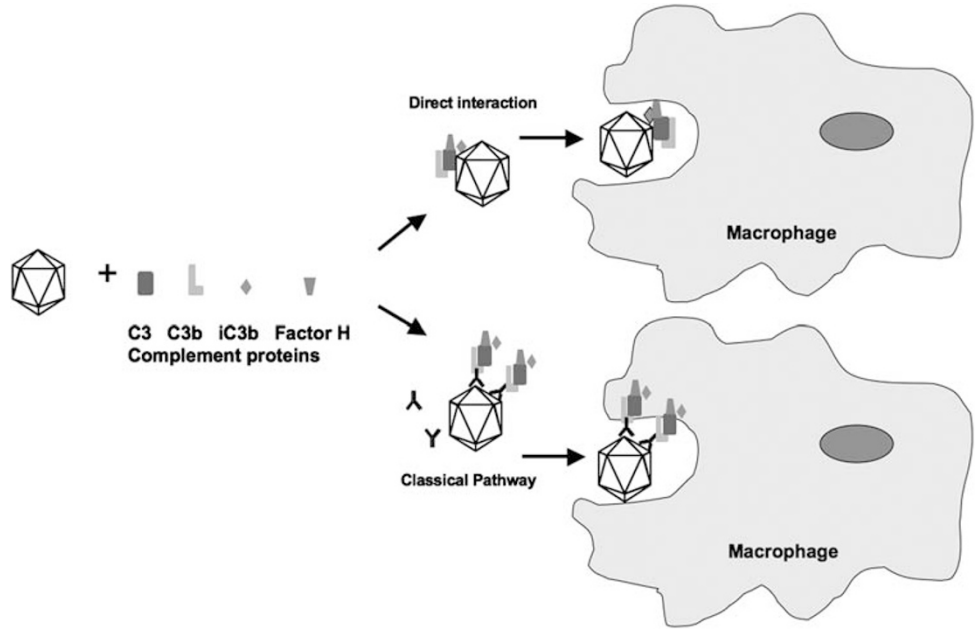

Figure 2 Activation of complement by viral vector particles. Adeno-associated virus (AAV) capsids bind to the C3 complement proteins C3, C3b, iC3b and complement regulatory factor H, thus increasing the uptake of virus into macrophages and enhancing their activation. C3–AAV capsid interactions are direct and can therefore occur independently of antibodies against capsid. However, the classical, antibody-dependent pathway also occurs. Similar interactions between adenovirus (Ad) vector particles and complement have also been identified.

complement proteins C3, C3b, iC3b and complement regulatory factor H, thus increasing the uptake of AAV into macrophages and enhancing their activation (Figure 2).[5] C3–AAV capsid interactions are direct and can occur independently of anti-AAV antibodies. However, complement activation by AAV is primarily antibody dependent (classical pathway). Complement-dependent activation of macrophages is not restricted

to the AAV2 serotype. For example, both AAV1 and AAV8 have been found to induce inflammatory gene expression in macrophages. Deficiency of C3 or complement receptor 1/2 results in the impairment of the humoral response to AAV.[5] C3 and CR 1/2 are essential for humoral but not for innate immune responses to AAV *in vivo*.

Adaptive immune responses to AAV vectors and their transgene products

Humoral immune responses against the AAV capsid or the transgene product can occur after exposure to AAV vectors. Such responses differ depending on the target organ, location within the target organ (in case of the eye and brain), route of administration, serotype, transgene and expression cassette, as well as the dosing schedule of injection[6] (Figure 1).[2] Humans are a natural host to AAV. A recent study regarding the prevalence of neutralizing antibody (NAB) titers to various AAV serotypes spanning humans in four continents has shown that the most prevalent NABs are to AAV2 followed by AAV1, whereas AAV8 and AAV7 have the least prevalent responses.[7] Interestingly, the structurally modified AAVrh32.33 serotype was rarely neutralized by the human sera.[7] However, different studies in mice and rhesus monkeys showed robust T-cell responses to the AAVrh32.33 capsid and transgene.[6,8]

IgG1 is the predominant antibody subclass response against the AAV capsid antigen in humans.[9] Preexisting NAB may not necessarily block *in vivo* gene transfer to some organs such as the skeletal muscle after intramuscular injection. However, injections into blood vessels, such as portal vein injections and direct injection into the liver parenchyma resulted in reduced transduction due to the presence of preexisting NAB. Local delivery of the vector outside the blood vessels may reduce exposure to NAB. In addition to the isolation of novel serotypes, shuffling of capsid sequences between serotypes and molecular evolution techniques are being used to create AAV particles that are more resistant to neutralization by the human sera. Although it is unlikely that such vectors can be readministered, these may improve initial gene transfer in humans.

Although long-term expression in the skeletal muscle and lack of inflammatory responses were observed in a clinical trial in patients with hemophilia B using an AAV2 vector, in a subsequent trial, the initial therapeutic expression of the factor IX (F.IX) transgene declined starting 6 weeks after the hepatic gene transfer. This decline of the F.IX expression in a patient enrolled in the highest dose cohort was accompanied by transient elevations of liver enzyme levels, suggesting destruction of hepatocytes.[10] Another subject, who had a similar low titer of preexisting NAB to AAV2, was subsequently treated with a somewhat lower vector dose and showed a lower, but measurable, increase in liver enzyme levels, which correlated with the emergence of AAV2 capsid-specific $CD8^+$ T cells in peripheral blood, indicating T cell-mediated immunity.[10] Capsid-specific $CD8^+$ T cells may have been reactivated by the infused vector and eliminated vector-transduced hepatocytes.[11] Approximately 2.5 years after the initial vector infusion, capsid-specific functional $CD8^+$ T cells were still present and cross-reacted with a common epitope of AAV serotypes 1, 6, 7 and 8, suggesting that secondary infusions with different naturally occurring serotypes may not circumvent the T-cell response.[10] AAV capsid-specific $CD8^+$ memory T cells are present in humans at a very low frequency but may become reactivated upon AAV gene transfer. Hepatic AAV2 infusion over a range of doses in mice transgenic for human HLA-B*0702 MHC locus failed to elicit capsid-specific $CD8^+$ T-cell responses.[10] It is likely that natural infection with AAV in the presence of a helper virus causes T-cell responses in humans, which would not be the case in animals that are not natural hosts for AAV. However, although mice immunized with the AAV capsid or adenoviral vectors expressing the AAV capsid developed $CD8^+$ T cells against capsid epitopes, these failed to eliminate AAV-transduced hepatocytes in several studies.[12–15] This lack of an animal model that reproduces the observations in humans has hampered preclinical studies on immune responses to the AAV-transduced liver. Considering the fact that AAV vectors do not express capsid, input capsid derived from vector particles would have to be efficiently cross-presented by the transduced cell to $CD8^+$ T cells through MHC I molecules. The AAV capsid is ubiquitinated and degraded by proteosomes, which may occur over a period of time.[16] These observations support a model of cytotoxic T-lymphocyte (CTL)-mediated destruction of transduced cells, but do not explain the reason why capsid-specific $CD8^+$ T cells failed to attack the AAV-transduced liver in experimental animals.

Recently, several strategies to avoid CTL responses to the AAV capsid antigen have been suggested. For example, alternate serotypes that do not contain a heparin binding site are processed differently by DCs and activate $CD8^+$ T cells less efficiently.[17] Elimination of surface-exposed tyrosine residues on the capsid, enhances gene transfer to the nucleus and substantially reduces accumulation of ubiquitinated capsids in the cytoplasm.[18] AAV capsids with more rapid uncoating/degradation kinetics may also prove to be advantageous. Mycophenolate mofetil and cyclosporineA blocked T-cell responses at least at lower vector doses.[19,20] AAV1-mediated muscle gene transfer in patients with lipoprotein lipase deficiency resulted in capsid-specific and dose-dependent activation of $CD4^+$ and $CD8^+$ T cells.[21]

Disorders of the central nervous system (such as Parkinson's disease) are promising targets for AAV-based gene therapies, and despite the fact that the brain is an immune-privileged site, immune responses to gene transfer vectors have been observed. AAV vectors may cause transient innate immune responses at high vector doses in naive mouse brain parenchyma, and additional injections to the opposite hemisphere induce a significantly greater response and reduced transgene expression.[22] It has been speculated that the antigen that sparks a brain immune response to rAAV (recombinant AAV) is the capsid protein when injected into the brain striata. Consistent with this hypothesis, delayed readministration of vector, or switching serotype for a second gene transfer to the striata, resulted in no transgene loss or striatal inflammation, thus overcoming the danger of preexisting immunity. The authors of these studies also concluded that intracellular processing of the AAV capsid generates the immunogenic antigen, and that

capsid serotypes that are processed more quickly than rAAV2/2 are less immunogenic.[23]

Preventing immune responses in AAV-mediated gene transfer

Immune responses against AAV-encoded transgene products vary substantially and are influenced by the target organ, route of delivery and dosing schedule. For example, in hemophilia B mice with a F.IX gene deletion, intramuscular vector administration caused a local immune response characterized by the activation of $CD4^+$ T and B cells to F.IX, which eliminated systemic expression. B-cell activation has also been a complication upon overexpression of erythropoietin in the skeletal muscle, which induced an autoantibody against erythropoietin, resulting in autoimmune anemia. Although $CD8^+$ T-cell responses occur and are of particular concern in inflamed muscle (which is typical for some forms of muscular dystrophy), these T cells are often not fully functional in the healthy muscle.[20,24,25] Recent studies have found that an intramuscular injection of AAV vectors often induces transgene product-specific $CD8^+$ T cells that express markers of T-cell functional exhaustion and T-cell suppression, and ultimately undergo programmed cell death in the skeletal muscle.[26,27] Several immune-suppression protocols have been successfully used in different animal models to block humoral and cellular immune responses to transgene products expressed in the skeletal muscle.[20] For example, the prophylactic use of immunosuppressant drug rapamycin in combination with interleukin (IL)-10 in the presence of factor IX (F.IX) dominant epitopes induces regulatory T cell (Treg) and long-term tolerance to the F.IX transgene product expressed in the muscles of hemophilia B mice.[28]

As opposed to muscle gene transfer, hepatic gene transfer with AAV vectors, has been shown to induce immune tolerance to a number of transgene products through the induction of Treg and other mechanisms.[29–32] In addition to direct tolerization of transgene product-specific $CD4^+$ T cells by induction of nonresponsiveness (anergy) or deletion, B- and T-cell responses (including CTL responses) are actively suppressed by the induction of Treg cells with an immune-suppressive phenotype.[30] These Tregs are phenotypically similar to naturally occurring Tregs and express, among others, the surface markers CD4 and CD25, and transcription factor FoxP3, the master switch for Treg development.[30] Antibody-mediated depletion studies have suggested that Tregs are critically required for tolerance to the transgene product after hepatic gene transfer.[30,33] IL-10 expression by Treg and Kupffer cells may directly suppress immune responses in the liver. Once tolerance is established, the transgene product can be safely expressed in sites that would otherwise predispose to immune responses.[34,35]

In some cases, one may simply avoid immunity by taking advantage of immune-privileged sites. The ocular disease Leber's congenital amaurosis, caused by an autosomal recessive mutation of RPE65, is being successfully treated in several ongoing clinical trials. Single eye injections of rAAV2-CBSB-hRPE65 resulted in an increase in visual sensitivity in several patients.[36–39] Owing to the immune-privileged nature of the eye, one should be able to improve treatment by reinjection in the previously injected eye or the partner eye, as has been suggested by animal studies in subretinal vector administration. This route causes a deviant immune response, resulting in a lack of NAB formation. However, intravitreal administration of the AAV2 vector in mice resulted in a humoral response against the capsid that blocked transgene expression on readministration of vector in a partner eye.[40]

Overview of immune responses in adenoviral vector-mediated gene therapy

Adenoviruses (Ads) are used in gene delivery and vaccine applications, for transducing various cell types, for incorporating large transgenes ($\sim$35 kb) with a high level of expression, and are easy to manufacture. Immune responses against Ad may be directed against the capsid, double-stranded DNA genome, viral proteins expressed from the vector backbone, or incorporated transgenes, and severely limit *in vivo* gene therapy. Systemic delivery of Ad vectors results in rapid physiological responses that include activation of innate immunity, induction of cytokines, inflammation, transient liver toxicity and thrombocytopenia.[41] Dose-dependent activation of innate and adaptive immune responses has been observed. Ad vectors transduce peripheral blood mononuclear cells and DCs.

Innate immune responses to adenoviral vectors; TLRs, inflammatory cytokines, inflammasome and complement

Adenoviral vector particles tend to elicit strong innate immune responses, and 90% of vector DNA is cleared from the tissue within 24 h of intravenous vector administration. Ads activate innate immunity through TLR-dependent and TLR-independent pathways, causing an upregulation of type I IFNs and inflammatory cytokines.[42] TLR-9 has been identified as a pattern recognition receptor for DNA containing unmethylated CpG motifs.[43,44] Both TLR-2 and TLR-9 have been implicated in the innate response to Ad.[45] TLR-2 is found on the cell surface, and TLR-9 is an endosomal receptor. The adeoviral ligand to TLR-2 is yet to be identified, in other viruses, glycoproteins can trigger this pathway. Signaling through these receptors typically leads to Th1 immunity, which may drive cellular and humoral responses to the vector and transgene. Sensing of Ad particles and genomes by these receptors results in the induction of inflammatory mediators and IFN-α,β. These type I IFNs activate NK cells and regulate the innate immune response against the vector.[46] Induction of cytokine IL-1, tumor necrosis factor and chemoattractant MIP-2 cytokines also occurs, promoting leukocyte migration and infiltration. Uptake by NK cells results in further release of cytokines and priming for an adaptive immune response. Ad vectors induce innate immune responses through MyD88/TLR-dependent and/or MyD88/TLR-independent pathways, depending on the cell type.[47] GM-CSF-stimulated DCs and conventional DCs use both MyD88 and TLR-9 for Ad vector-induced IL-6 and IL-12 production. However, neither MyD88 nor TLR-9 was crucial for Ad vector-induced IL-6 production in peritoneal macrophages. Ad vector-infected DCs can also mature through a MyD88-

independent pathway. The spleen is also a major contributor to Ad vector-triggered production of various cytokines and chemokines. Conventional DCs (in contrast to plasmacytoid DCs, pDCs) in the spleen have an important role in the induction of IL-6 and IL-12 after systemic administration of Ad vectors.[42]

Internalized adenoviral DNA induces maturation of pro-IL-1β in macrophages, which is dependent on the innate cytosolic molecular complex known as the inflammasome.[48] The inflammasome consists of NALP3/ASC adaptor proteins, which recruit the inflammatory caspase-1 into a molecular complex. This proinflammatory pathway functions independently of TLRs and IFN regulatory factors, and leads to cell death of macrophages.[48] The complement system also has a role in vector opsonization and clearance as a part of the innate immune system. Ad has been shown to bind C3-derived fragments directly or to activate the complement through antibodies in individuals having preexisting immunity (Figure 2).[49] Ad interactions with the mammalian complement system are significant and likely initiate inflammatory responses. Thrombocytopenia is caused by interactions between adenoviral particles and the coagulation system, resulting in platelet activation, binding to endothelial cell surfaces, and formation of platelet-leukocyte aggregates.[50] Finally, Ad vectors directly and indirectly activate endothelial cells.

Adaptive immune responses against adenoviral vectors

Rapid increases in IL-6, IFN-α, IFN-β, RANTES, IL-12 (p40), IL-5, G-CSF and GM-CSF are observed. Furthermore, a complex set of interactions between the innate and the adaptive immune system results in the activation of $CD4^+$ and $CD8^+$ T cells, and B cells.[41] Type I IFN signaling is important for T help-dependent antibody formation by B cells. IFNs also induce DC maturation by upregulating costimulatory molecules such as CD80, CD86 and CD40. Neutralizing antibodies against IFN-α and IFN-β have been found to be effective in blocking both innate and adaptive immune responses to the viral vector.[51]

Humoral immune responses preclude readministration of the vector by reducing efficiency of transduction *in vivo* due to NAB formation. Antiviral antibodies enhance the interaction and internalization of Ad with leukocytes through the Fc receptor and complement receptor. More than 97% of humans have preexisting antibodies against group C Ad, whereas ~50% have antibodies against the commonly used Ad serotype 2. Higher seroprevalence of neutralizing antibodies against Ad 5 is observed in Asian and African populations. Subgroup C infection is endemic in the human population; the majority of individuals seroconvert within the first 5 years of life as a result of natural infections. Preexisting immunity to Adhu5 virus strongly impairs B- and T-cell responses to the transgene product of vaccines that utilize the same vector serotype of the same serotype.[52] $CD4^+$ and $CD8^+$ cross-reactive T cells against different Ad serotypes were found in human peripheral blood mononuclear cells. Ad-specific $CD4^+$ T cells recognize conserved epitopes among different serotypes, with the majority of people developing long-lived $CD4^+$ T-cell responses to Ad, perhaps suggesting that alternate serotype vectors should be devoid of these epitopes. Ad-specific secretion of IFN-γ from peripheral blood mononuclear cells was found within 12 h of incubation, suggesting previous activation of Ad-specific CTLs. Most adults retain Ad-specific cellular memory after childhood exposure. Transduction of APCs by Ad vectors contributes to CTL responses, which are directed against viral gene products and transgene products and are dependent on T help.

Overcoming immune responses in adenoviral gene transfer

Ad vectors with modified capsid sequences such as Ad5/f45 appear to have significantly reduced seroprevalence. Other serotypes, for example, Ad-35, may have less NAB and T-cell cross-reactivity with Adhu5. Preexisting immunity to Adhu5 completely inhibited induction of transgene product-specific antibodies by an Adhu5 vector, but did not affect antibody responses to chimpanzee-derived Ad vectors.[53] Mastrangeli *et al*.[54] showed that alternating serotypes Ad5 (subgroup C), Ad4 (subgroup E) and Ad30 (subgroup D) for repeated respiratory administration showed some success in circumventing humoral responses in a cystic fibrosis model. The high innate immunogenicity of Ad vectors has a negative effect on their use in gene therapy, but made these vectors more attractive as vaccine carriers. For example, Ad vectors that are being developed for use as cancer vaccines and as vaccine prototypes for human immunodeficiency virus (HIV) type 1 based on E1-deleted Ad recombinants are in phase II clinical trials.[53] A rAd5-based HIV-1 vaccine in a clinical trial resulted in increased HIV infection by 2.3-fold in Ad-seropositive individuals. One initial hypothesis was that the vaccination of seropositive individuals caused the expansion of $CD4^+$ Ad-specific T cells, which served as targets for HIV. However, activated Ad5-specific lymphocytes were determined not to be the cause of increased susceptibility; hence, the area remains under investigation.[55] This surprising development in HIV vaccine biology shows the importance of studies of the immune systems' interactions with vectors in disease models.[55,56]

The removal of all viral coding sequences from Ad vectors and their replacement with human noncoding intron (stuffer) sequences has resulted in helper-dependent, high-capacity (gutted) vectors, which show reduced CTL responses and therefore increase the potential for stable transgene expression; gutted Ad vectors still leave much to be desired for readministration. The use of organ-specific promoters (for example, the hepatocyte-specific human α1-antitrypsin promoter when targeting the liver) reduces expression of the transgene in professional APCs, thereby further reducing the risk of CTL responses. Utilization of strong promoters for improved transgene expression, in combination with limited vector doses, may also prove to be helpful in curtailing Ad vector-directed innate immune responses and inflammation. Innovative delivery techniques, such as hydrodynamic injections and other approaches toward local delivery of low vector doses directly into the liver, diminish systemic vector dissemination and reduce inflammatory responses and immunotoxicity.[57] Other strategies to subdue immune responses associated with Ads include the use of immune suppression, pretreat-

ment with glucocorticoid, use of lipid bilayer envelopes and preventing binding to the CAR receptor by masking the adenoviral fiber knob.[52,58,59]

Overview of immune responses to lentiviral vector-mediated gene transfer

Lentiviral vectors can transduce nondividing cells and integrate into the host cell genome, hence being advantageous for long-term expression of the therapeutic transgene (but posing a risk for insertional mutagenesis).[60] The duration of transgene expression and potency of immune responses to LV-encoded transgene products have varied substantially for different studies on *in vivo* transfer. LV delivery in immunocompetent mice results in efficient transduction and transgene expression in several cell types such as retina, muscle and hematopoietic cells, whereas the transduction of hepatocytes is relatively inefficient. However, potent immune responses directed against the transgene product have been shown to clear the transduced liver cells within 4 weeks after the injection. Efficient LV-mediated transduction of APCs is responsible for the induction of potent CTL and antibody responses against the transgene product. Pseudotyped LV containing glycoproteins from Ebola Zaire Virus, Lymphocytic choriomenengitis virus, mokola virus and from the vesicular somatitis virus G (VSV) delivered to the mouse lung resulted in the activation of transgene-specific T cells against the GFP transgene and the vector itself. VSV-pseudotyped LV showed large reduction in transgene expression within 90 days.[61] In contrast, a Baculovirus GP64 pseudotyped feline immunodeficiency vector (AcGP64-FIV) showed persistent (50 weeks) expression of luciferase in nasal epithelia when delivered to the nostril; similarly, LacZ was expressed for 90 days, as observed by Sinn *et al*.[62] However, studies by Kremer *et al*.[63] showed that in a different strain of mice, and using a HIV-derived LacZ vector, pseudotyped with GP64, the transgene expression declined to undetectable levels by 6 months. The difference in results may lie in the different vector designs, routes of administration and dosage of injection, etc. Different routes of administration (nasal vs intratracheal) may affect the longevity of gene expression due to differential activation of the immune system. Nasal application of peptides is known to produce Treg to the applied antigen and may indicate a mode of tolerization in nasally applied LV vectors. One would also expect the nature of the transgene and the underlying mutation to exert an effect. Although CTL responses to envelope proteins are also a potential concern, data in mice thus far suggest that CTL-mediated elimination of transduced cells is (1) caused by T cells specific to the transgene product, (2) a result of transgene expression in APCs even if a tissue-specific promoter is used and (3) the envelope has a role in the infection of APCs.

Preventing immune responses in lentiviral gene transfer

Hepatic LV administration in mice has been observed to induce a rapid and transient IFN-α,β response, which is dependent on infectious vector particles.[64] *In vitro* challenge of APCs suggested that pDCs drive this response. Despite the delivery of replication-defective vectors, type I IFN responses effectively interfere with transduction in a cell type-specific manner and promote functional CTL responses. Consequently, a high level of stable LV GFP gene transfer to hepatocytes was observed in mice deficient in IFN-α,β signaling, whereas wild-type mice showed only transient (<25 days) transduction in a small proportion of hepatocytes, which were rapidly targeted by CTLs.[64] These responses were seen despite the fact that LV elicit weaker IFN-α responses from pDC compared with the parent HIV-I virus. Systemically injected LV is preferentially sequestered by liver macrophages. Chimeric GP64/Sendai envelope proteins pseudotyped LV vectors have been shown to reduce macrophage transduction, thereby possibly helping to evade the immune system.[65] Stimulation of murine pDC by lentivirus particles is weak compared with other single-stranded RNA viruses such as VSV, influenza or Sendai virus, which activate murine pDC by ligation of TLR-7, the pattern recognition receptor that senses single-stranded RNA molecules in endosomes.[66] LV induces low levels of cytokine secretion by pDC, possibly through a TLR7-dependent pathway.[64] VSV-G protein-pseudotyped LV may also contain tubulovesicular structures with DNA fragments that can stimulate TLR9.[66] However, the blocking of TLR-7 or TLR9 pathways has been observed to be insufficient to prevent LV from inducing IFN-α responses.[60]

Similar to adenoviral vectors, the use of a hepatocyte-restricted promoter reduces the risk for immune responses to the transgene product in liver-directed gene transfer. However, depending on the mouse strain tested, this effect is not robust enough, possibly because of leaky expression and the high transduction efficiency of APCs with LV. For example, LV efficiently infects sinusoidal cells in the liver, including resident macrophages (Kupffer cells) and also splenic macrophages and DCs. Brown and Naldini developed an innovative strategy to solve this dilemma. Incorporation of several repeats of a target sequence for a hematopoietic cells lineage-specific microRNA into the 3′-end of the transcript is highly effective in blocking transgene expression in professional (that is, bone marrow-derived) APCs. The therapeutic value of this approach has been documented in hemophilia B mice. After LV transfer of a F.IX gene to the liver, sustained correction of coagulation was achieved using the miRNA regulated expressing cassette, and formation of inhibitory antibodies against F.IX were avoided (ultimately results in tolerance induction).[67,68]

Prospects

Each vector system faces its own set of immunological hurdles for gene therapy, some more related to innate immunity, others to adaptive immunity (including memory and preexisting immunity). Nonetheless, similar to transplantation biologists, gene therapists are learning to circumvent, manipulate or suppress unwanted immune responses. Advances in vector engineering (such as capsid engineering, miRNA-regulated expression cassettes, etc) and delivery techniques, administration to immune-privileged sites, taking advantage of organ-specific immune responses, immune

suppression and modulation regimens represent promising strategies to overcome immunological hurdles. Although translation of knowledge gained from preclinical studies in animal models to human therapeutics in gene therapy is not always straightforward, immune modulation protocols are nonetheless being refined and tailored toward specific vector/target tissue/disease combination.

With regard to AAV, more clinical results are expected to emerge in the near future from ongoing or yet to be initiated clinical trials. These will help us understand the effect of the target organ (such as the brain, central nervous system, eye, muscle, liver) on T-cell responses to capsid and its consequences for long-term expression. Interestingly, a recent paper has described a patient who received intramuscular AAV1-mediated gene transfer and continued to express the transgene despite a detectable CD8 response to capsid.[69] However, a different study suggested transient expression as a result of capsid-specific response in muscle.[21] More human data are required to resolve these issues.

In the case of AAV and Ad, continued engineering of capsids should establish whether preexisting NAB/cross-reactive antibodies to the vector can be avoided without the loss of gene transfer in humans.[70] Ad vectors are an excellent model for obtaining a detailed understanding of innate immune responses to DNA viruses. A fairly complete picture of the interactions between Ad and the innate immune system is emerging. These vectors remain attractive vaccine carriers, and new vaccines based on human and primate serotypes are being developed. With regard to using Ad vectors for the treatment of genetic disease, limited doses and local delivery of gutted vectors will likely be required to avoid immunotoxicity.

Results obtained from LV-based treatment of genetic disease in large animal models are expected to be produced in the near future. It will be of interest to compare efficacy and immune responses using optimized envelope and miRNA-regulated expression cassettes to current data from rodent models. Effective detargeting of professional APCs will decrease immune responses to LV gene transfer. In general, additional clinical experience with viral vectors combined with advances in the laboratory should generate a more complete assessment of immune responses to these gene transfer vectors and their transgene products.

Conflict of interest

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