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REVIEW

Immune responses to gene therapy vectors: influence on vector function and effector mechanisms

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Circumventing the immune response to the vector is a major challenge with all vector types. Viral vectors are the most likely to induce an immune response, especially those, like adenovirus and AAV, which express immunogenic epitopes within the organism. The first immune response occurring after vector transfer emerges from the innate immune system, mainly consisting in a rapid (few hours) inflammatory cytokines and chemokines secretion around the administration site. This reaction is high with adenoviral vectors and almost null with AAV. It is noteworthy that plasmid DNA vectors, because of CpG stimulatory islets, also stimulate the innate immunity via the stimulation of TLR receptors on leukocytes. Specific immune response leading to antibodies production and T lymphocytes activation also occurs within a few days after vector introduction. Capsid antigens are mostly responsible for specific immunity toward adenoviruses, and are also involved in the response against AAV. In the former case only, however, viral gene-encoded proteins can also be immunogenic. The pre-existing humoral immunity coming from early infections with wild-type AAV or adenovirus can prevent efficient gene transfer with the corresponding vectors. In all cases, some parameters like route of administration, dose, or promoter type have been extensively described as critical factors influencing vector immunity. Strategies to fight against vector-induced immunity can come from the immunology field, since tolerance induction or immunosuppression are a possibility. Alterations to vector structure have also been extensively performed to circumvent the immune system and thus enhance gene transfer efficiency and safety.
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Introduction

Gene therapy vectors usually contain components of bacteria, viruses, or other microorganisms. Bacteria supply the plasmids used as small vehicles for transgenes. Viruses hold considerable appeal as gene therapy vectors because they are naturally able to incorporate foreign genetic material within the host cell genome. However, fighting infection by bacteria and viruses is among the key functions of the immune system. When a pathogen, or component thereof, penetrates within the body, an innate immune response develops promptly, causing cytokine production and an influx of nonspecific inflammatory cells (macrophages, dendritic cells, NK cells, and others). Toll receptors on the cell surface play a pivotal role in this innate response by allowing nonspecific inflammatory cells to recognize pathogenic epitopes in the viral capsids or bacterial membranes. This leads to the production of proinflammatory cytokines and to nonspecific global stimulation of the immune system. Adaptive immunity is stimulated later, when professional antigen-presenting cells (APCs) carrying antigens from the microorganisms migrate to the draining lymph nodes. These APCs present the antigens to lymphocytes and boost their response via costimulation molecules such as CD80, CD86–CD28, and CD40–CD40L. This activates the CD4+ and CD8+ T cells, which proliferate, produce cytokines, migrate out of the lymph nodes, and use their specific T-cell receptor (TCR) to recognize the pathogenic antigen epitopes presented in association with major histocompatibility complex (MHC) molecules at peripheral sites of infection. Production of proinflammatory cytokines (IL-1, IL-6, and TNF) is among the consequences of this peripheral epitope recognition. Simultaneously, CD8+ T cells eliminate the infected cells via cytotoxic effects involving secretion of interferon-gamma (IFNγ) and induction of apoptosis or lysis. Interaction of pathogens with B cells, with or without help from CD4+ T cells, leads to the production of antiviral or antibacterial antibodies capable of detecting intact microorganisms in the circulation. Antibodies bind to these microorganisms, often synergistically with complement, thereby triggering a chain of reactions that ultimately neutralizes the microorganism.

Vectors derived from microorganisms have been modified to ensure safe gene transfer without inducing pathogenic effects. All viruses, for instance, can serve as vectors once their replicative and pathogenic potential is abolished and the gene of interest is inserted within their genetic material. The likelihood that a gene therapy strategy will be successful depends in large part on the ability of the vector to deliver the therapeutic gene within the target organ or cells, with adequate kinetics and minimal side effects. Circumventing the immune response to the vector is a major challenge with all vector

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types. Similar to wild-type viruses, viral vectors are detected by the immune system and generate an immune response that becomes effective before the virus infects its target cells. Thus, in theory and usually also in practice, vectors can induce an immune response directed against them. This response may be beneficial, for instance, when the goal is vaccination or tumor lysis. In most cases, however, the immune response is unwelcome. An immune response against a gene therapy vector may eliminate the vector and the transfected cells, decreasing both the intensity and the duration of transgenic protein expression. Furthermore, as with infection by microorganisms, the immune response to gene therapy vectors involves the production of proinflammatory cytokines and chemokines that have harmful effects. The adaptive immune response develops later than the innate response but also plays a crucial role. It includes a humoral response characterized by the production of neutralizing antibodies specific of the vector or transgene antigen and a cell-mediated response involving T cells and NK cells. Adaptive immunity not only contributes to eliminating the vectors and infected cells from the body but also results in a memory response that thwarts further efforts to use the same vector or transgene.

The objective of this review article is to define the various steps of the immune response (innate immunity then adaptive immunity with its humoral and cell-mediated components) to adenoviral vectors, adeno-associated viral (AAV) vectors, and naked DNA. Immunity against retroviral vectors will not be discussed because it chiefly targets the transgenes located within the vector rather than the antigens intrinsic to the vector itself. But briefly, lentiviral and retroviral vectors elicit very little immune responses when compared with other vectors even in an immune privileged area such as the eye. Nonetheless, when used *in vivo*, they are inactivated in the serum by complement activation and can also develop a citotoxic response. The former is more frequent when using vectors pseudotyped with the envelope glycoprotein from vesicular stomatitis virus (VSV-G).[1]

Immunity to the herpes simplex virus was the focus of an extensive review published in 2003.[2] Finally, a growing number of studies are investigating immunity to exogenous DNA, outside the gene therapy setting. Although designed to better characterize innate immune responses against bacterial DNA during bacterial infections, these studies have supplied valuable information for understanding the immune responses to plasmid vectors, which are derived from bacteria. Most of the vectors described in our review article trigger an innate immune response characterized primarily by the production of proinflammatory cytokines, followed by a specific humoral and/or cell-mediated immune response. These responses are diverse, as they may successively target various components of the vector (capsid proteins in viral vectors, viral gene products if present, plasmid DNA, transgene, and/or promoter). The intensity of the immune responses varies with a number of factors, which are discussed below. The nature of the vector influences the immune responses; among vectors used to date, adenoviruses are the most immunogenic. The vector dose, the route of administration, the nature of the transgene, and host-related factors responsible for interindividual variability (eg, the MHC genotype) influence the immune response. Strategies have been developed to sidestep antivector immunity and to enhance the safety and efficacy of gene therapy.

Immune mechanisms involved in responses to vectors

Activation of innate immunity

Induction of cytokine synthesis by adenoviral vectors.

Immunity to adenoviruses is a dose-dependent response induced by capsid proteins or by adenoviral gene products. First-generation adenoviral genes induce strong innate and adaptive responses. The adaptive response is far weaker with last-generation vectors, which are characterized by deletion of all or part of the viral genes. However, the capsid proteins in these vectors induce a noticeable innate response within 24 h of vector administration. An inflammatory infiltrate composed of neutrophils, NK cells, and macrophages develops within the sites of vector administration. Proinflammatory cytokines (most notably TNF-α) and chemokines secreted by these cells contribute to perpetuate the inflammatory response and to recruit additional inflammatory cells. Within 24 h, this response eliminates about 80% of the adenoviral particles.[3]

Activation of innate immunity by adenoviruses varies with the vector dose, as established *in vitro* with cultured peripheral blood mononuclear cells.[4] PBMC infection by adenoviruses induces cytokine production in a dose-dependent manner up to a threshold adenovirus dose, above which no further cytokine increase occurs. This plateau effect is probably ascribable to saturation of adenovirus receptors such as CAR. Thus, increasing the adenoviral vector dose would probably fail to increase transgene expression, as the adenovirus receptors would become saturated; in addition, the higher dose would induce a stronger inflammatory response responsible for increased elimination of the infected cells expressing the transgene. Several studies have shown that internalization of adenoviral vectors plays a crucial role in inducing the synthesis of proinflammatory cytokines.[5] These results further support the use of lower doses.

In vivo, administration of adenoviral vectors into the respiratory tract rapidly causes the alveolar macrophages to produce MIP-1, MIP-2, TNF-α, and IL-6.[5] Interestingly, synthesis of adhesion molecules can also be induced by adenoviral infection. For instance, adenoviral infection of endothelial cells is followed by expression of adhesion molecules such as ICAM-1 and VCAM-1,[6] leading to increased leukocyte infiltration within transduced tissues. In monkeys, intraportal administration of adenoviral vectors is followed within a few hours by systemic synthesis of IL-6 and activation of splenic macrophages.[7] These swift and potent reactions induced by adenoviral vectors can have devastating consequences. In a clinical trial of gene therapy for ornithine transcarbamylase (OCT) deficiency, intrahepatic administration of 6×10^{13} adenoviral particles expressing the OCT gene was followed in several patients by an acute inflammatory response with high serum IL-6 levels.[8] One of the patients died, 48 h after the injection. This demonstrates not only the interindividual variability in responses to

vectors but also the need for a careful evaluation of vector doses.

Adeno-associated viruses induce little or no innate immunity. In contrast to adenoviruses, AAV vectors induce little or no innate immunity. A study comparing immediate effects induced by adenoviruses and AAVs *in vitro* and *in vivo* showed that infection of HeLa cells or epithelial renal cells by an adeno-lacZ vector caused massive expression of chemokines such as RANTES, IP-10, IL-8, MIP-1α, and MIP-2 in a dose-dependent manner. This effect was not seen with AAV, even in a 40-fold greater dose. *In vivo*, intravenous injection of AAV-lacZ rapidly induces the expression of messenger RNAs (mRNAs) for the cytokines TNF-α, RANTES, IP-10, MIP-1beta, MCP-1, and MIP-2, but this effect lasts only 6 h, as compared to more than 24 h with adenoviral infection.[9]

Innate response induced by naked bacterial DNA. The plasmids used for nonviral gene therapy, alone or in combination with liposomes or electrotransfer, can theoretically stimulate innate immune responses. Plasmids are composed chiefly of bacterial DNA that contains far greater amounts of unmethylated CpG motifs than does the DNA in eukaryotic cells. DNA devoid of CpG motifs does not induce proinflammatory cytokine synthesis by macrophages *in vitro*.[10] In contrast, CpG motifs have immunostimulatory effects including activation of the NFκB pathway, enhanced synthesis by monocytes of cytokines such as TNF-α or IL-6, and activation of NK cells.[11] Unmethylated CpG motifs also open up the adaptive immunity pathway by inducing the synthesis of IFNγ, IL-12, and IL-18 and by activating the Th1 pathway.[12] The molecular mechanisms underlying leukocyte activation by CpG involve the Toll-like receptors (TLRs), which activate the cells responsible for innate immune responses (eg, macrophages) after penetration of bacteria into the body. TLR 9 recognizes the unmethylated CpG motifs in immunostimulatory sequences (ISS) of bacterial DNA. In addition,[13] stimulation of human dendritic cells by bacterial components orients the T-cell response toward the Th1 pattern via secretion of IFN-γ and IL-12. A study published this year showed that a plasmid containing CpG sequences was capable of binding physically to TLR9, in a manner dependent on the CpG methylation status, and that the result was NFkB activation. One method for suppressing the inflammatory response induced by unmethylated CpG sequences in plasmids consists in eliminating or methylating these sequences. In an animal study published this year, this method markedly decreased the inflammatory response to *in vivo* injection of plasmids, so that the duration of transgene expression was substantially increased.[14]

Interestingly, cells involved in adaptive immunity are also activated by DNA. Thus, mobilization of CD8+ T cells occurs after intramuscular electrotransfer of plasmids in mice.[15] Furthermore, a role for B cells in anti-DNA immunity was suggested by a study in which an intramuscular injection of plasmid DNA induced integration of the transferred genetic material into B cells from bone marrow and lymph nodes.[16]

Role for adaptive immunity

Adenoviruses induce a specific cell-mediated response. Expression of adenoviral genes by first-generation adenoviral vectors results in an immune response specifically directed against the products of these genes. Gutless adenoviral vectors, which are devoid of viral genes, induce a weaker T-cell response.[17] However, even in the absence of viral transcription, adenoviruses can induce a cytotoxic T-cell response[18] as well as infiltration by CD4+ and CD8+ T cells.[19] This response may be ascribable to the immunogenicity of adenoviral capsid proteins, which may result in elimination of the infected cells that express the antigen. The mechanism probably involves the following sequence: internalization and priming by dendritic cells of capsid antigens associated with Class II MHC antigens, presentation of these antigens to CD4+ T cells, which become activated, and in turn CD8+ T cell activation by these CD4+ T cells.

One of the main components of adenovirus-induced immune responses may be the ability of these vectors to infect professional APCs such as dendritic cells and macrophages. Adenoviruses can infect mature and immature dendritic cells without altering their maturation or their antigen-presenting capabilities.[20] The earliest effect is cell activation manifesting as the production of cytokines such as IL-6, IL-12, or TNF. Then, these APCs directly present the viral antigens to CD4+ and CD8+ T cells, generating a cytotoxic response that precludes readministration of the vector. This mechanism is the key to the generation of cell-mediated responses against adenoviruses.

AAVs induce a specific cell-mediated response. The adaptive cell-mediated response is far weaker with AAV vectors than with adenoviral vectors. A major reason is probably the inability of AAVs to efficiently infect APCs such as dendritic cells and macrophages. More specifically, dendritic cell infection by AAVs may depend on the maturation stage of the cells, with the vectors being unable to efficiently infect mature dendritic cells. In contrast, immature dendritic cells can incorporate the vector *in vitro* and, after adoptive transfer *in vivo*, they can generate a specific T-cell response responsible for decreased transgene expression.[21] However, this response develops only when immature dendritic cells *in vitro* are infected by a large amount of AAV particles or when adoptive transfer *in vivo* is carried out using a large number of immature dendritic cells. Thus, during simple *in vivo* administration of AAV vectors, a cell-mediated response may develop only when immature dendritic cells are present in abundance at the site of AAV infection; in all likelihood, this is rarely the case, as the modest inflammatory response induced by AAV vectors does not increase the number of immature dendritic cells, in contradiction to the strong response induced by adenoviruses. Taken in concert, these data suggest that AAV vectors may be capable of infecting immature dendritic cells, but only when large doses of vector are used. In addition, the modest number of dendritic cells present at sites of AAV infection *in vivo* usually fails to induce a T-cell response of sufficient magnitude to eliminate the infected cells and, therefore, to decrease the duration of transgene expression. Interestingly, however, the ability of AAVs to infect immature dendritic cells

has been used to generate cytotoxic responses to tumor or virus antigens in antitumor vaccination strategies,[4,22] or anti-HIV vaccination.[23]

A T-cell response specific of the transgene and responsible for elimination of the infected cells can occur, however, in some situations. In mice transgenic for the hemagglutinin (HA) TCR, infection of muscle cells by AAV-HA is followed after several weeks by partial elimination of the transduced muscle fibers via activation of an HA-specific T-cell response. In addition, the CD4+ T cells recognize an HA antigen presented by dendritic cells via an indirect mechanism, without transduction by AAV-Ha but with priming by HA antigens.[24] These results contrast with those obtained under similar conditions with an adenovirus-HA: in this situation, prompt and massive muscle fiber elimination occurs as a result of presentation of the HA antigen, both directly by dendritic cells transduced by adeno-HA and indirectly by priming.

Humoral response induced by adenoviruses and AAVs. Administration of adenoviral or AAV vectors *in vivo* leads to presentation of the viral capsid antigens to the B cells present within lymph nodes. This results in CD4+ T-cell activation, which in turn induces differentiation of B cells to plasma cells via cell cooperation mechanisms involving costimulatory molecules CD40–CD40L and cytokines such as IL-6 or IL-4. Antibodies produced by plasma cells are specific of viral capsid proteins; when they have neutralizing effects, these antibodies can prevent infection by the vectors during subsequent gene therapy attempts. In humans, the problem is compounded by pre-existing immunity developed against wild-type adenovirus or AAV. Viral infections due to group C adenoviruses are endemic in virtually every part of the world. Specific antibodies to adenoviruses are detectable in 97% of individuals; in addition, more than 50% of individuals have pre-existing humoral immunity to type 2 adenovirus, a serotype widely used for gene therapy.[25] These antibodies can neutralize transduction by adenoviral vectors *in vivo*,[26] activate the complement system, and induce an inflammatory response in the presence of adenoviruses.[27] Molecular analysis of inhibition by neutralizing antibodies present prior to adenovirus administration shows differences among humoral responses directed against the three main components of the adenoviral capsid (fiber, penton, and hexon). Kinetic studies conducted after vector administration show that antifiber antibodies develop first, followed by antipenton antibodies and, finally, by antihexon antibodies. In addition, non-neutralizing sera contain only antifiber antibodies, suggesting that these do not have neutralizing effects. In contrast, antipenton antibodies are neutralizing and can act synergistically with antifiber antibodies.[28] This type of study has led to the development of strategies for gene delivery via adenoviral vectors in which the neutralizing effects of the humoral response are attenuated, for instance by conjugating the vector to polyethylene glycol in order to mask the capsid proteins.[29]

Humoral responses also occur against AAV vectors. Infection by the nonpathogenic AAV2 is common, and the prevalence of anti-AAV2 antibodies ranges from 35 to 80% according to the age group and geographic location.[25,30,31] Several studies have shown that anti-AAV antibodies have neutralizing effects that decrease the efficiency of *in vivo* vector infection in the liver[32] or lungs,[31] and therefore limit the chances of success with repeated administration of these vectors. Other studies, in contrast, have established that this humoral response has no influence on the efficiency of infection with the vector administered within the muscle[33] or lungs.[34] Similarly, the development of anti-AAV antibodies is minimal or nonexistent after administration of AAV into the brain[35,36] or retina.[37] However, these studies were conducted in animals, which do not have pre-existing anti-AAV immunity, in contrast to humans. This difference highlights the need for caution when interpreting animal studies, which may be of little relevance for predicting effects in humans.

In humans, anti-AAV antibodies are found in serum and other body fluids such as joint fluid[38] and amniotic fluid.[39] Anti-AAV2 IgGs were found in joint fluids from 21 (100%) patients with a variety of joint diseases; these antibodies consistently neutralized chondrocyte infection by AAV-2 *in vitro*. The neutralizing effect of joint fluid was correlated with the anti-AAV IgG level and was lifted when the number of infecting AAV particles was increased.[38] Similarly, neutralizing anti-AAV antibodies have been found in human amniotic fluid[39] and may limit the effectiveness of *in utero* gene therapy. Furthermore, serum anti-AAV IgMs have been found in humans after recent AAV infection or reactivation of a latent form of the virus.[30]

Antibodies to AAV serotypes 1, 3, and 5 are common in humans, and their prevalences increase with age. However, their neutralizing effects seem less potent than those of anti-AAV2 antibodies. In a study of 77 healthy individuals, neutralizing anti-AAV1 antibodies were found in 20% of individuals, as compared to 27% for anti-AAV2.[40] Neutralizing anti-AAV5 antibodies are uncommon: they were consistently negative in a population of 85 healthy individuals.[41] In aggregate, these data highlight one of the problems raised by phase I/II clinical trials of AAV-vector gene therapy. The patients included in these studies, having developed an antibody response to the AAV vectors, would be at a disadvantage should treatment with improved AAV vectors become available subsequently.

Immune response against the therapeutic gene. In gene replacement therapy a gene that is not expressed in the patient is introduced *de novo*. CD4 T cell clones specific for the therapeutic gene have not been deleted in the thymus and thus an immune response may be forthcoming and help may be given to B cells to produce antibodies and CD8 T cells to develop a cytotoxic response against the therapeutic gene. In the periphery, the ability of T cells to mount an immune response or, on the other hand, tolerate an antigen depends on the expression of costimulatory molecules on the cells that present the antigen to the T cell. Expression of such costimulatory molecules on antigen-presenting cells (APC) in turn depends on the presence of the antigens of certain 'danger signals' normally harbored by pathogens. Thus, T cells distinguish 'infectious non-self' from 'non-infectious self'. In the gene substitution scenario, the transgene by itself is devoid of 'danger signals', and will not be likely to cause upregulation of costimulatory

molecules on APCs, but the viruses used as vectors or even sequences of nonhuman origin present in the plasmid vector, when using naked DNA, can induce a strong host immune reaction.[42] Under such a situation, tolerance can be broken in a similar manner as the breakage of tolerance to self-antigens that happens in association with viral infections. Maintained expression of the transgene or repeated administration of the vector carrying the therapeutic gene will boost the immune system, with the final outcome of gene therapy acting as a 'vaccination' against the therapeutic gene that will ensue in destruction of treated cells, limiting the persistence of the therapeutic gene and efficacy of the therapy.

Factors influencing immune responses against the vector

Route of administration

Many studies have established that humoral and cell-mediated immunity to AAV components or to transgenes vary with the route of administration of the vector. Antivector immunity has been found in C57Bl/6 mice that were given AAV-ovalbumin (OVA) injections intraperitoneally, intravenously, or subcutaneously. Antibodies were produced both against OVA and against AAV, and cytotoxic T cells specific of OVA were generated. Conversely, after intramuscular injection of the vector, the humoral response was of similar intensity but the cytotoxic cell-mediated response was far weaker.[43] Numerous experimental therapeutic studies and one clinical trial have evaluated the treatment of hemophilia by AAV-factor VIII (FVIII) or factor IX (FIX). There is abundant evidence that strong immune responses, in particular to transgene products, occur in some cases. Again, the response may depend on the route of administration of the vector. A humoral response to human factor IX has been found in mice after intramuscular administration, but not intrahepatic administration, of AAV-human FIX.[44] One possible explanation is that tolerance to FIX may occur with the high FIX levels produced after intrahepatic injection but not with the lower levels obtained after intramuscular administration. In addition, FIX is produced naturally in the liver but not in the muscle. Conceivably, in this setting, mistakes may occur in the processing of the transgenic protein produced by muscle, leading to the generation of neoepitopes that induce a specific immune response. A study reported this year shows that intravenous administration can obviate the need for more invasive routes, such as intrahepatic injection. The intravenous route seems to be associated with similar transgene expression efficiency, suggesting a low level of immunity against the vector.[45] Finally, the neutralizing effect may vary with the degree of accessibility of the antibodies within the target tissues. This may explain the strong neutralization seen in the liver and lungs and the far weaker neutralization in the retina and brain, as antibodies have limited access to these last two sites.

Dose of vector

The production of proinflammatory cytokines induced by adenoviral vectors depends on the vector dose, both *in vitro*[4] and *in vivo*, as does the intensity of the specific humoral anti-AAV response. In addition, high doses of vector can induce tolerance to the transgene product, as shown with an AAV encoding human FIX and injected to mice via the intrahepatic route. Induction of tolerance seems to depend on the amount of transgene expressed, as is often the case with induced tolerance. This effect probably involves the generation of regulatory CD4+ T cells that produce TGFβ and other compounds.[46] However, in another study, mice given a vector by the intramuscular route produced neutralizing anti-F IX antibodies across the entire dose range investigated.[47] Humoral immunity to the AAV capsid, in contrast, can be sidestepped by using low doses of vector.[48,49] When the transgene is capable of inducing an immune response, it seems that a balance must be achieved between the responses developed against the transgene product itself and the response to the vector antigens, most notably those located in the capsid.

Patient-related factors

Among factors that influence the immune response to the vector, the genetic background should be borne in mind. In mice, for instance, after administration of the AAV vector encoding a human transgene, antitransgene responses varied widely according to the genetic background.[50] Mouse strains that are susceptible to autoimmunity (NOD, NZW, MRL-lpr) may develop stronger immune responses to AAV vectors than nonsusceptible strains.[51]

Promoters

Interactions exist between transgene promoters and the immune system, most notably the cytokines present at the site of transgene expression. These cytokines either inhibit or activate the promoters. A study of endothelial cells transduced by an adenovirus-lacZ under the control of the cytomegalovirus promoter showed that IFN-γ and IL-10 downregulated promoter activity whereas, in contrast, TNF-α, IL-1β, and IL-4 increased promoter activity.[52] IFN-γ had the same inhibitory effect on the SV40 promoter but stimulated the MHC Class I promoter.[53] This is not surprising, as IFN-γ is known to affect the expression of MHC molecules. Regardless of the mechanisms involved, the cytokine pattern should be taken into account when selecting the promoter, with special attention to the cytokines induced by the vector and to those that are overexpressed in the disease of interest.

Pathways that modulate antivector immunity

Development of viral vectors belonging to various serotypes

With both adenoviruses and AAVs, the development of vectors belonging to serotypes other than those most commonly used (AAV-2 or group C adenoviruses, mainly serotypes 2 and 5) improves gene transfer efficiency, both *in vitro* and *in vivo*. Serotypes may differ in their affinities for differentiated cell types and, as mentioned above, pre-existing humoral immunity is not the same for all serotypes. With AAV vectors for instance, AAV-1 is the most efficient vector for infecting muscle and liver, followed by AAV-5, -3, -2, and -4, in that

order.[54] AAV-8, which has been used recently for gene therapy, is more efficient than AAV-2, -5, and -7 for infecting liver cells, and AAV-8 encoding FVIII is also more efficient in the treatment of hemophilia in mice.[55] Studies have shown that neutralizing antibodies to AAV-7 and AAV-8 are rare in human sera,[56] so that these serotypes are good candidate vectors for humans. Similarly, most humans have no neutralizing antibodies to the group B adenovirus serotype 35.[57] In addition, serotype 35 has limited tropism for the liver and may therefore be safer than other serotypes.

Immunosuppression by costimulation pathways inhibition and tolerance induction

Specific tolerance has to be induced, before any contact with the therapeutic gene takes place as a conditioning regime for gene therapy in all cases where an immune response has been reported. The goal may be easier than in other clinical situations where tolerance is a therapeutic goal (such as autoimmune or transplanted patients), considering that in the gene therapy scenario, we know the gene we are introducing, controlling the moment and the context in which we are going to deliver it.

Considering that we can dissect the 'specific signal' given by peptides from the antigen assembled in MHC molecules on the surface of APCs; from the 'danger signal', consisting of costimulatory signals on the APC upregulated by the presence of certain patterns in the vector. We may have the possibility of inducing tolerance towards the therapeutic gene by administering it before hand, devoted of any non-human sequence that could boost the immune system. In this manner, the therapeutic gene would be presented in the absence of costimulatory signals and thus induce in the specific T cells a state of unresponsiveness to any subsequent stimulation, even if it includes costimulation.[58] There are several strategies to induce peripheral tolerance that have been assayed in transplanted or autoimmune patients and that can be applicable for gene therapy. Eventually, in the paper from Ginhoux and Davoust in this review, vector-induced tolerance by regulatory T cell is extensively overviewed.

Costimulation pathways inhibition. Inhibition of costimulation pathways involving T cells and APCs holds potential as a means of suppressing the antivector immune response. CD40/CD40L and CD28-CD80/CD86 interactions are the main costimulating pathways in the specific immune response mediated by T cells and APCs. The CD40/CD40L pathway blockade by CTLA4-Ig, for instance, increases the duration of transgene expression in mice, without inhibiting the production of neutralizing antiadenovirus antibodies.[59] Similarly, persistent expression of a transgene encoded by an adenovirus has been found in *CD40L* knockout mice, and administration of anti-CD40L antibody diminishes the production of neutralizing antiadenovirus antibodies.[60] Interestingly, cell-mediated immunity against adenoviral vectors can also be inhibited by intravenous injection of adeno-CTLA4-Ig, the main mechanism being a decrease in Th1 responses. Finally, the immune response against AAV can be suppressed by blocking the costimulation pathways, as shown with CTLA4-Ig combined with nasal instillation of AAV.[48] Also, administration of anti-CD4 antibody diminishes the production of neutralizing anti-AAV antibodies in mice.[49]

Use of tolerogenic dendritic cells

DCs are the key APCs involved in antigen presentation to T cells,[61] being also the major means to garnish antigens in either a tolerizing or an activating manner. DCs have been extensively used to induce tolerance towards self- or transplantation-antigens and may also be used to induce tolerance towards the therapeutic gene used in gene therapy.[61] Dendritic Cells express costimulatory molecules as they mature, thus tolerogenic DC are immature DCs. Tolerogenic DCs can be generated by incubation with inhibitory cytokines such as IL10 or TGF-β[62] or immunosuppressive drugs.[63,64] It is interesting to note that whereas the immunosuppressant CsA inhibits anergy induction when acting directly on T cells[65] it might have a tolerogenic effect through their inhibition on DC maturation.[63]

All these strategies can be used to blunt or suppress the response to a vector to which the subject has not been exposed to previously, as is the case with animal models. They cannot overcome pre-existing immunity in humans. In this context, prenatal gene delivery of the therapeutic gene has also been proposed as a means to favor tolerance induction.[66]

Alterations to vector structure

The vector itself, in addition to the transgene, induces an immune response. Therefore, changing the antigen structure of the vector may be an effective method of weakening the antivector immune response. Antigen changes can be achieved by modifying the capsid proteins (when the vector is a virus), by modifying or eliminating the largest possible number of viral genes encoding immunogenic viral proteins, or by modifying the biochemical structure of the DNA in plasmid vectors. First-generation adenoviral vectors were the first candidates to these improvements, as they were both highly efficient in terms of cell infection and highly immunogenic. Many groups worked on improving these vectors by removing as many adenoviral genes as possible. These efforts ultimately produced 'gutless' or last-generation adenoviral vectors, which are minimally immunogenic and therefore ensure more prolonged transgene expression.

With AAV vectors the problem is different, as these vectors are probably devoid of coding viral genes. Immunity against AAV vectors is probably related solely to antigens in the capsid of the infecting particles. Researchers sought to identify the capsid protein epitopes that generate neutralizing antibodies in human sera[31] with the goal of subsequently modifying these epitopes in order to decrease the immunogenicity of the AAV particles.

Finally, it has been shown that the immune response generated by the introduction of plasmid DNA *in vivo* (see above) is chiefly due to the unmethylated CpG motifs in the DNA. One strategy used to blunt this induced innate response consists in reducing the number of unmethylated CpG motifs,[14] in particular by inducing their methylation[67] (see above).

Conclusion

When creating new vectors and developing gene therapy strategies for a specific disease, the consequences of antivector immunity should be borne in mind. Manipulation of the immune system to induce tolerance of the vector by the body may deserve consideration, although the most satisfying approach consists in developing vectors with little or no potential for inducing an immune response.

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