Challenges and Strategies: The Immune Responses in Gene Therapy

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Abstract: The host immune responses, including T lymphocytes mediated immune response and humoral immune responses are the important parts of the challenges in gene therapy. There are some potential immunostimulants in gene delivery systems, such as viral and non-viral vectors. Viral gene products, transgene products, viral proteins derived from viral particles required by dead-end infection, and CpG DNA in plasmid may play important roles in inducing the host immune responses when foreign genes are transferred into the targeted tissues. The immune responses should lead to many problems in gene therapy: transient expression of therapeutic gene, non-efficient re-administration of the same vectors, and severe side-effects in clinical trials. Although RNAi may act as gene therapeutic agent for suppression of specific gene expression, little attention has been given to the potential non-specific effects that might be induced. It was reported that small interfering RNAs (siRNAs) can induce the host interferon response following transfected to mammalian cells. Facing these challenges, a number of studies have been focused on taking measures to solve them, such as immunosuppression, selection of different administration routes and dose of the vectors, using the tissue-specific promoters and modifying the vectors.
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1. INTRODUCTION

Gene therapy means introducing therapeutic genes into the targeted cells to cure or slow down the progression of diseases. It has the potential applications of a wide array of genetic, degenerative, and infectious diseases or cancer. Gene therapy requires a certain number of elements: gene delivery systems with less toxicity and immunity, high efficiency in gene transfer and the therapeutic gene expression in the targeted cells or tissues at functional level in a controllable manner. To date,

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however, the gene delivery systems, including non-viral vectors and viral vectors, have somewhat immunogen inducing the host immune responses in gene therapy, which is one of the challenges of gene therapy. Because both non-viral vectors and viral vectors contain potential immune-stimulators, including CpG DNA in the plasmid from bacterial DNA or in the viral genomes, and viral protein from viral particles needed by dead-end infection of recombinant viruses, transferring these vectors into targeted cells or tissues may induce not only T cell-mediated immune responses to transduced cells, but also humoral immune responses by generating antibodies to viral proteins and transgene products. These immune responses against vectors, transgene products, and transduced cells could lead to transient expression of the therapeutic genes and re-administration inhibition of the recombinant vectors, even lead to severe side-effects in clinical trial. Of course, the immune-gene therapy strategies for cancer are based on the application of gene transfer techniques to enhance the immune responses against tumor cells. Augmenting immunogenicity by genetically modifying tumor cells or immune cells to express co-stimulatory molecules, tumor antigen or cytokines has proven to be a priming therapeutic strategy in animal tumor models and currently under investigation in clinical trials for cancer. A full description of the immune-gene therapy of cancer described to date is beyond the scope of this review. In this review, we will focus on discussing the challenges caused by the immune responses in gene therapy and the strategies for avoiding them.

2. GENE DELIVERY SYSTEMS FOR GENE THERAPY

The gene delivery systems available for gene therapy now fall into two categories—viral vectors and non-viral vectors. Viral vectors are derived from viruses by replacing its genetic components with the therapeutic genes. Generally, the viral vectors can be divided into two types: integrating and non-integrating viral vectors. The former, such as, retroviral, lentiviral, and adeno-associated viral vectors, can integrate into the human genome; whereas the non-integrating vector (e.g., adenoviral vector) is maintained in the nucleus without integrating into the chromosomal DNA, so that the transgene is apt to lose during cell division and expression of the foreign gene is transient. In a packaging cell, the essential components for further propagating of viruses can be provided *in trans*, which enable the viral vectors to be packaged as the viral particles and to deliver genes to the targeted cells. Certainly, this is a dead-end infection, because the vectors lack the essential components for viruses' propagation. Recombinant viral vectors can lead to the generation of infectious parental viruses. This is a principle frequently used in viral vector design in gene therapy.

Non-viral vectors include naked-DNA and liposomes. They are based on plasmid which is a closed, circular DNA strand. Therapeutic genes can be inserted directly into the plasmid, and then this recombinant plasmid can be introduced into cells in a variety of ways. For example, it can be injected directly into targeted tissues as naked-DNA. However, because of low efficiency of gene transfer, several approaches have been developed to enhance gene transfer efficiency via naked-DNA including gene gun and electroporation. The plasmid DNA may also be formulated with cationic lipids/liposomes or polymers into virus like particles and injected directly into targeted tissues, these formulation condense the plasmids into small particles and facilitate its entry into cells. Even though non-viral vectors can be produced in relatively large amounts, and they are likely to present less toxic, they suffer from inefficient gene transfer at present, and from degeneration of the recombinant DNA.

3. POTENTIAL IMMUNOSTIMULANTS IN GENE DELIVERY SYSTEMS

A. Viral Proteins

Even though the gene delivery vehicles for gene therapy are designed to avoid its toxicity and immunogen, there are some potential immunostimulants in these systems, including viral proteins

derived from viral particles, viral genes, and CpG DNA in plasmid. These potential immunostimulants may play important roles in inducing the host immune responses when foreign genes are transferred into the targeted tissues.[1] Generally, viral vectors containing the genes of interest are packaged into the viral capsid, which then bud out of the cell and become an infectious viral particle. These particles are composed of certain viral proteins that can target the host cells; however, the viral proteins, which are recognized as non-self by host immune system, could induce the host immune responses. The integrating viral vectors are thought as less or non-immunogen gene delivery systems. In fact, some studies found that these vectors also induce different degrees of the immune responses.

Replication-incompetent retroviral vectors have been employed in experimental settings and clinical trials. But a potential problem with the use of such vectors may be the host immune responses directed against the vector particles themselves. McCormack et.al.[3] examined immunoglobulin (Ig) responses specific for retroviral vectors derived from murine leukemia virus (MLV), anti-MLV Ig was seen following intramuscular administration of retroviral vectors in mice and in nonhuman primates.[2,3] The envelope proteins derived from retroviruses are the key components of the infectious viral particles to transduce the targeted cells from ecotropic (infecting only rodent cells) to xenotropic (infecting all mammalian cells), and pantropic (infecting various species). Because of cellular modifications of viral envelope protein and the properties of these proteins themselves, they could elicit the human serum complement attacking to them.[4] Similarly, lentiviral vectors containing vesicular stomatitis virus glycoprotein G (VSV-G), which is the most common heterologous Env protein,[5] could also activate the component system. Regarding the immunogenicity of recombinant adeno-associated vector (rAAV), previous studies demonstrated that rAAV vectors are immunoincompetent, allowing sustains transgene expression *in vivo*.[6–8] Nevertheless, primary target of the immune response is the capsid of the vector particle even these vectors do not encode any viral proteins for lacking viral DNA sequences in their genomes,[9] because rAAVs require forming viral particles with packaging cells to infect targeted cells. For example, following administration to immunocompetent mice and rhesus monkeys, rAAV-2 vector could induce activation of Th2 subsets and B cells to viral capsid proteins.[10] Furthermore, AAV-2 specific IgM and IgG responses were observed in this experiment. Some data proved that the immune response to rAAV depends on the route of administration. Brockstedt et al.[11] have used rAAV encoding ovalbumin (OVA) to investigate C57BL/6 mice following different administration routes, such as intraperitoneal, intravenous, subcutaneous, or intramuscular delivery, they found that all routes led to not only the cell immune response but also the humoral response to vectors with the exception of the intramuscular delivery.

Since the recombinant adenoviral vectors are able to transduce both quiescent and proliferating cells very efficiently, they have been shown to be promising vectors for gene therapy. A major disadvantage of adenoviral vectors (Adv), however, lies in the activation of both the innate and adaptive parts of the recipients' immune system when applied *in vivo*. The inflammatory responses induced by adenovirus particles are very strong. In fact, some studies have proved that even the empty capsid alone derived from adenoviruses could induce an adaptive immune responses.[12,13] The adenovirus capsid activates a number of signaling pathways following cell entry including p38 mitogen-activated protein kinase and extracellular signal regulated kinase that ultimately lead to expression of pro-inflammatory genes. Various cytokines, chemokines, and leukocyte adhesion molecules are induced by the adenovirus particle in a wide range of cell types providing a molecular basis for the inflammatory properties of these vectors.[14] The first generation Adv in which the *E1*-gene is deleted can induce a cytotoxic T-lymphocyte (CTL) mediated immune response by the host against the vectors, since some host cellular proteins can complement E1-deleted vectors.[15] Moreover, *E4*-gene product substantially affects cell toxicity and the host immune response.[16,17] Additionally, another important barrier in the application of rAdv for gene therapy is that the majority of the human population has been exposed to various adenoviral serotypes during their life. For

example, up to 50% of human subjects have a detectable immune response to adenovirus type 5.[18] Therefore, they may have circulating antibodies against Adv proteins from different serotypes. Using rAd should lead to a strong B cell mediated immune response which will contribute to the rapid diminishing of adenoviral particles from periphery.

B. Transgene Products

Besides the immunogenic proteins of viral particles and newly synthesized viral proteins, the transgene products may also contribute to the immunogen of gene therapy. Because the transgene products are never made in the hosts resulting from either complete gene mutation or absent expression, so an entirely new protein could be considered as ''foreign'' by the host immune system. Protein replacement gene therapy for some genetic diseases, which are caused by the absence of a functional protein, has revealed that neutralizing antibodies to the therapeutic products can be formed. For example, rAAVs expressing secreted transgenes, such as OVA and factor IX (FIX), have been shown to elicit immune responses against the transgene products. Intramuscular administration of rAAV encoding human FIX (hFIX) into C57BL/6 mice also induced a humoral response directed against the transgene products.[19] It has been reported that there are approximately 10–15% of hemophilia A and 2% of hemophilia B chronic transfusion patients producing antibodies to FVIII and FIX, respectively.[20] Administration of rAAV expressing OVA into C57BL/6 mice by intramuscular or intravenous routes induced anti-OVA antibodies.[11] Immunization of C57BL/6 mice with a lacZ-expressing adenovirus also elicited CTL responses to the transgene product, beta-galactosidase.[21]

C. CpG Dinucleotide or CpG Motifs

CpG dinucleotide or CpG motifs (CpGs) are not uniformly distributed in the human genome. There are approximately 1/80 CpGs present in 98% of the human genome, but CpG islands distributed in 1–2% of the genome are approximately 200 bp to several kb in length.[22] CpG island in the human genome is always comprised of gene promoters and (or) exons. CpGs within CpG islands are normally unmethylated whereas most CpGs outside CpG islands are methylated. The genomic DNAs of bacteria and vertebrates differ from the frequency and methylation of CpGs, which are relatively common in bacterial DNA. The frequency of unmethylated CpGs in bacterial DNA has 3–4 times greater than methylated CpGs in verterbrate DNA.[23–25] So the vectors for gene therapy based on plasmid derived from bacterial DNA also have higher frequency of unmethylated CpGs. Although nearly all DNA viruses and retroviruses appear to have evolved to reduce their genomic content of CpGs by 50–90% from that expected based on random base usage,[25] there is higher frequency of CpGs in these viral genomes. To determine the frequency of CpGs in 11 lentiviruses derived from 8 host species and to compare them with several other retroviruses and a set of human genes, a group found that the percentages of CpGs in all 11 lentiviral *gag* genes taken together is 1.05, 1.08% in *env* and 0.43% in *pol*, and CpGs are 3.26% in *env*, 3.68% in *pol*, and 4.62% in *gag* genes in the three retroviruses; and that noncoding region in the genome of retrovirus, such as LTR or between 5′LTR and *gag*, the frequencies of CpGs are from 0.9–5.0%.[26] It has been proved that the cause of retrovirus vector silencing has been partly attributed to *de-novo* cytosine methylation of CpG-rich region located in the LTR promoter.[27] Similarly, the frequency of CpGs in the adenovirus type-2 and type-12 are more than 1.2 and 0.3%, respectively.[25] Though viral structure genes are partly or totally deleted for the preparation of gene therapy vectors, all viral vectors are generally engineered to contain the gene of interest, as well as various regulatory sequences, such as promoters, enhancers, and genes used to select transfected cells. So there are somewhat CpGs in these viral vector genomes.

It is well known that unmethylated CpGs in bacterial and plasmid DNA are recognized by the immune systems as a dangerous signal or immunostimulant, and the recognition depends on the toll-like receptors on the surface of the immune cells, such as B-lymphocytes, natural killer cells (NK cells), monocytes, macrophages, and dendritic cells (DCs).[28] CpG oligodeoxy-nucleotides (ODNs)

or bacterial DNA can stimulate DCs and macrophages to secrete cytokines (e.g., IL-2, IL-6, IL-8, IL-12, and TNF-α) and to increase expression of co-stimulatory molecules (e.g., MHC-II, CD80/B7-1, and CD86/B7-2).[29,30] Furthermore, CpGs can not only activate NK cells to secrete IFN-γ but also induce murine B-cells to proliferate and secrete Ig *in vitro* and *in vivo*.[23,31] Current results also demonstrated that ODNs can induce human monocytes to mature into functional DCs which present antigen to T-cells.[32] The immune responses induced by CpG DNA mainly depend on the toll-like receptors pathway and DNA-PKs pathway which can activate transcriptional factors NF-κB and (or) AP-1.[33–35] Activated NF-κB and AP-1 up-regulate expression of cytokine genes or co-stimulatory molecule genes.

D. RNA Interference and the Immune Responses

In recent years, an RNA-based silencing mechanism has emerged that is ancient, conserved among species from different kingdoms (fungi, animals, and plants). These intermediate products result from the cleavage of double-stranded RNA (dsRNA) and consist of 21–23 nt-long RNA duplex effecter molecules capable of recognizing and guiding the degradation of complementary mRNA sequences. RNA interference (RNAi) is the sequence-specific gene-silencing induced by dsRNA, and gives information about gene function quickly, easily and inexpensively. The use of RNAi for genetic-based therapies is widely studied, especially in viral infections, cancers and inherited genetic disorders. Small interfering RNA (siRNA) expression has been studied with siRNA from viral vectors and plasmid vectors, that efficiently deliver siRNA into both dividing and non-dividing cells, stem cells, zygotes, and their differentiated progeny.[36] Although RNAi strategies may be a powerful tool for gene therapy, little attention has been given to the potential non-specific effects that might be induced by siRNA. Recently, it was reported that a number of vectors, including lentiviral vectors and plasmid vectors, which express dsRNA from RNA polymerase III (*pol* III) promoters can trigger an interferon responses in mammalian cells.[37] Most important, Sledz et al. found that transfection of siRNA result in interferon (IFN)-mediated activation of the Jak-Stat pathway and global up-regulation of IFN stimulated genes. This effect is mediated by the dsRNA-dependent protein kinase (PKR), which is activated by 21-base pair siRNAs and required for up-regulation of IFN-β in response to siRNAs.[38] siRNAs are associated with silencing triggered by transgenes, microinjected RNAs, viruses, and transposons and hence can be considered intermediaries in host defense pathways against foreign nucleic acids.[39] Indeed, protection against viruses and transposons may be the mature function of the core of the RNAi pathway. RNA-based silencing mechanism acts very likely as the ''immune system'' of the genome.[40] Therefore, the induction of the IFN and possibly other, cellular signaling pathways indicates that siRNAs have broad effects beyond the selective silencing of homologous targets genes when introduced into cells.

4. CHALLENGES CAUSED BY THE IMMUNE RESPONSES IN GENE THERAPY

These potential immunostimulants could not only induce the cellular immune response by generating cytotoxic T cells, but also induce the humoral immune response by generating specific antibodies. In pursuing gene therapy, it is important to induce transgene expression for a long time in a tissue-specific manner without strong immune responses against vectors, transgene products, and transduced cells. Despite considerable progress over the past decade in the generation of gene transfer systems with reduced immunogenic properties, the remaining immunogen of many gene therapy vectors is still the major hurdle preventing their application in clinical trials, because the host immune responses induced by immunogen of the vectors for gene therapy lead to unexpected problems, including low level and short term of transgene expression, inefficient readministration of the same vectors, and severe side-effects in clinical trials.

The potential success of gene therapy will depend on long-term transgene expression to cure or slow down the progression of diseases. The duration of transgene expression is a complex function involving the transduced cell types, transfection methods, vector constructs, and the forms of transgene in nucleolus in the transduced cells (e.g., integration into the host genome or episomes). It is obvious that the host immune responses often reduce the level and duration of transgene expression: (1) it has been demonstrated that transduced cells are recognized and eliminated by CD8 + T cells from the recipient.[41–43] Although the specific immune responses against vectors or gene products can lead to elimination of transfected cells, antibodies responses did not play a major role in the loss of transgene. Infiltration of the transduced skin with CD4 + and CD8 + cells and induction of transgene-specific CTL implied a role for T-cell-mediated responses. Transduction of mice deficient in either MHC-I or MHC-II molecules resulted in transient transgene expression. Only in MHC (−/−) mice lacking expression of both MHC-I and MHC-II molecules was persistent transgene expression seen. These data indicate a primary role for T-cell-mediated responses in the immune-mediated loss of transgene expression.[44] Immunization of C57BL/6 mice with a lacZ-expressing adenovirus vector elicited CTL responses to both viral antigens and the transgene product, beta-galactosidase (beta-gal). Adoptive transfer experiments, as well as studies involving lacZ-transgenic mice revealed that CTLs to viral antigens are sufficient to destroy virus-infected hepatocytes, and that CTLs to beta-gal can not solely account for the observed hepatocyte destruction which has characterized the use of first generation viruses;[45] (2) non-specific immune responses have an inhibitory effect on gene expression. For example, cytokines, such as IFN-γ, TNF-α, could inhibit gene transcription or decrease the stability of mRNA.[46,47] Furthermore, cytokines, which are derived from both specific and non-specific immune responses, may down-regulate the promoters used for control of the therapeutic genes. In particular, the cytokines IFN-γ, TNF-α could inhibit the viral promoters usually used for the control of transgene expression in gene therapy based on viral vectors;[48,49] (3) the transduced cells could be induced to apoptosis because of both the humoral and cellular immune responses against the targeted cells. For example, cationic lipid-protamine-DNA (LPD) complexes, but not each component alone, can induce a high level of cytokine production (such as IFN-γ and TNF-α) after being injected into the mice by intravenous injection. These cytokines could partly trigger apoptosis in the lung. Treatment of mice with antibodies against the two cytokines prolongs the duration of gene expression and also improves lung transfection on a second injection of LPD.[50] Most importantly, transduced cells apoptosis could increase cytokines secreting. Therefore, both the activity of the promoters down-regulated and the transduced cells eliminated may impact on expression of the transgene.

If the transgene can't replicate itself during cell division or lose its expression over time because of the immune response in gene therapy, it should be necessary to inject repeatedly with the same recombinant vectors to targeted tissues. However, re-injection of the same vectors into the targeted tissues may not be effective because of induction of host immune responses to transgene products or vectors. B cells and Th-2 cells activated by potential immunostimulants in the delivery systems can lead to secreting neutralizing antibodies (NAB) which block re-administration of vectors, so the host produces tolerance to transgene products and vectors. For example, rAAV expressing hFIX (rAAV-hFIX) was injected into the livers of mice which had been pre-exposed to AAV, the results demonstrated that none of the mice with pre-existing immunity can express hFIX, whereas all naive control mice expressed hFIX following administration of rAAV-hFIX.[51] There was further study to evaluate a blocking antibody to human CD4 in a biologically compatible mouse in which the endogenous *CD4* gene was functionally replaced with the human counterpart. CD4 antibody prevented an anti-vector response long after the effects of the CD4 antibody diminished; read-ministration of vector without inhibition of gene expression.[52] This study implied that the immune response may affect efficiency of the vectors re-injection.

Additionally, the immune responses induced by vectors or transgene products in gene therapy for clinical trials may lead to severe side-effect for the treated patients. Recent clinical trial proved that a

clinical inflammatory syndrome is attributed to aerosolized lipid-DNA administration in cystic fibrosis. The results proved that approximately half patients developed a pronounced clinical syndrome of fever, myalgias, and arthralgia beginning within 6 hr of aerosolized lipid-DNA administration, and serum IL-6 became elevated within 1–3 hr of gene administration because of systemic inflammatory response disseminated intravascular coagulation, and multiple organ failure.[53,54] Unfortunately, intrahepatic injection of an E1/E4-deleted adenovirus vector encoding the ornithine transcarbamylase gene (*OTC*) in an 18-year-old patient with OTC deficiency led to the death of the patient. According to a NIH report, this event occurred probably as a result of hyper-inflammatory immune responses induced by rAd. Postmortem finding suggested that the high dose of Ad vector, delivered by infusion directly to the liver, quickly saturated available receptors for the vectors within that organ and then spilled into the circulatory and other organ systems, including the bone marrow, thus inducing the systematic immune response.[57]

Although RNAi is a potent and specific tool for gene therapy, it can also induce the interferon response after introduced to mammalian cells. IFN-α/β is a rapidly inducible component of the innate responses to infection and provides a signal for initiation of the adaptive immune responses. IFN-α/β contributes to the immunostimulatory activity of microbial adjuvant and can itself act as an adjuvant. There are some evidences showing that IFN-α/β can promote the differentiation of human peripheral blood monocytes into DCs. Activation DCs express higher levels of CD83, MHC class I and II, CD40, CD80, and CD86, and have an increased ability to stimulate T cell proliferation.[56] So IFN-α/β can partly enhance immune responses *in vivo* through the stimulation of DCs. The participation of IFN-α/β in immune responses is allied to its production by a population of DC precursors and its stimulatory effects on DCs.

5. STRATEGIES FOR CIRCUMVENTING THE IMMUNE RESPONSES UNDESIRED IN GENE THERAPY

It is now clear that the host factors, particularly the host immune responses to the transduced cells, transgene products and vectors, are important obstacles to gene therapy. Facing these challenges, a number of studies have been focused on taking measures to solve them. These strategies include immunosuppression, selection of different administration routes and dose of the vectors, using the tissue-specific promoters and modifying the vectors.

To date, induction of tolerance to a therapeutic systemic protein has broad implications for design of clinical trials and gene-based treatment strategies for genetic diseases and is of particular importance in treatment of patients. Immunomodulation of the host immune systems has been used to achieve somewhat satisfactory results. The use of immunosuppressive agents to prevent NAB against transgene products and vectors is a usual method for immunomodulation. These agents include immunosuppressive drugs and specific immune modulators that block the various pathways resulting from antigen presentation to T cells and B cells activation. Using the immunosuppressive drugs, such as cyclosporine, tacrolimus, and cyclaphosphamide,[57] could inhibit the synthesis and release of cytokines and prevent the differentiation of CD4 + cells, thereby blocking an immune response.[58] The other kind of immunosuppressive agents are the immune signal blocking monoclonal antibodies, such as anti-CD4, anti-CD80, anti-CD86, anti-CD40L, and CTLA4-Ig. Antigen presentation depends on interaction between MHC-I on the APCs and CD4 on the T cells. The studies of immunomodulation models in mice demonstrated that the formation of NAB to AAV-2 capsid proteins could be inhibited with antibody to CD4. The interaction between CD40 on the B cells and CD40L on the activated T cells is important for B cells differentiation in T cell-dependent humoral immune response. Readministration of Adv in rhesus monkeys lungs by blockade of CD40-CD40L interaction using anti-CD40L showed suppression of Adv induced lymph leukocyte proliferation, cytokine responses and humoral immune response.[59] Recent study found that combination of

anti-CD80 and anti-CD86 or anti-CD40L and anti-CD86 monoclonal antibodies resulted in strong inhibition of the immune responses against Ad.[60] Interaction between CTLA4 on surface of the T cells, CD80 and CD86 on the APCs can induce Ig class switching and DCs or macrophages activation. CTLA4-Ig can also block important co-stimulatory pathways and turn off T cells production of IL-2 involved in the initiation of cellular immunity.[58] Some works suggested that recombination of CTLA4-Ig and the other immunomodulators (such as anti-CD40L,[61] hUGT1A[62]) could permit long-term, repeatable transgene expression. For non-viral vectors, co-injection plasmid DNA with the immunosuppressive agent, such as dexamethasone could also avoid the immune responses induced by CpGs.[63] However, using specific inhibitors of CpGs signaling pathway may more efficiently block CpG-induced cytokine secreting by leukocytes. Endocytic uptake of DNA into an acidified intracellular compartment is the first required step in the pathway. Inhibitors of endosomal acidification, such as chloroquine and quinacrine hydrochloride, greatly reduced the secretion of IL-2 from mouse spleen cells *in vitro* and inhibited cytokine production in the lung by approximately 50% without affecting gene expression.[64] An animal experiment proved that expression of foreign gene products in bone marrow derived cells is capable of inducing T cells tolerance to proteins expressed exclusively in the cytoplasm. T cells from Balb/c mice reconstituted with mock-transduced bone marrow were able to kill target cells expressing enhanced green fluorescent protein (EGFP), in contrast, T cells from mice reconstituted with EGFP reduced bone marrow were unable to kill targets expressing EGFP.[65]

Secondly, because the route of administration of vectors for gene therapy may significantly contribute to the immune response to the transgene products,[66,67] the different injection routes of the gene delivery vectors may play somewhat roles in circumventing the immune responses in gene therapy. For example, a single injection of rAAV-OVA to C57BL/6 mice elicit both OVA-specific antibodies and CTL, as well as neutralizing antibodies to AAV, moreover, the strength of CTL response is correlated with the route of administration. Mice injected either intraperitoneal, intravenous, or subcutaneously with rAAV-OVA developed a strong OVA-specific CTL response, however, mice injected intramuscularly with the same virus developed minimal OVA-specific CTL response.[11] Many studies proved that the route of rAAV encoding FIX administration influence the extent and type of host immune responses against rAAV capsid proteins and transgene products, these evidences are reviewed by Su et al.[68] To prevent the immune responses against rAdv, feeding antigen or intrathymic administration of adenoviral vectors has previously been shown to be effective methods of tolerance induction in various animal models.[69,70] Therefore, it was interesting to see whether these mechanisms might also contribute to the prolongation of adenovirus-mediated transgene expression and facilitate readministration of rAd. The other experiment showed that the greatly reduced risk of anti-FIX formation following hepatic gene transfer with an AAV vectors is the result of FIX-specific induction of immune tolerance by this route of administration.[71] In fact, the different routes of administration determine which immune organs and cell populations will be involved in the response. For example, virus injected intravenously are taken up primarily by the spleen and liver; whereas, intramuscular injection results in the virus being directed to local lymph node. Difference in the lymphoid cell population of these organs may generate different quality of immune response. For the dose of administration, however, the vectors for gene therapy need to be produced in numbers (typically, $10^6 \sim 10^{13}$ viral particles, vp) for administration to patients,[72] the effect of gene therapy partly depends on the dose of administration of vectors to targeted tissues. For example, hFIX can't be detected under the low level of vector ($<2\times10^{10}$ vp) in C57BL/6 mice injected with rAAV-hFIX.[66] So lower dose of administration could not obtain gene therapy expected.

Thirdly, using specific promoters to drive transgene expression could circumvent the immune responses in gene therapy. The expression level of γ-sarcoglycan in mice after injected by rAAV under the muscle-specific promoter (e.g., muscle creatine kinase, MCK) is higher than that under the cytomegalovirus promoter (CMV). Most importantly, strong cellular and humoral immune

responses to transgene were measured in mice using CMV promoter.[73] Similar results can be obtained using plasmid DNA vaccines: HBsAg-specific humoral and cell-mediated responses are not induced in mice when the MCK promoter is used in place of the CMV promoter to drive expression of HBsAg.[74] Additionally, there are up-regulated promoters under inflammatory conditions leading to an effective approach for circumventing the immune responses in gene therapy. Some investigators found that the murine acute-phase protein (APP), including complement factor3 (C3) and serum amyloid A3 (SAA3), can normally or highly express under inflammatory conditions, using C3 or SAA3 promoter to control luciferase gene can avoid transient expression of the targeted gene induced by the inflammatory response.[75] These promoters could also show new light on circumventing the immune responses in gene therapy.

Finally, modifying the vectors for gene therapy by removing all the wild-type genes from the virus and by methylating CpGs in plasmid DNA or removing CpGs from the plasmid DNA may circumvent the immune responses. Viral vectors for gene therapy are based on viruses by deleting the wild-type gene from viruses. Lentiviral vectors are generated by deleting six of nine viral genes in HIV and leaving *gag*, *pol*, and *env*; ''gutless'' vectors, in which all the viral genes are removed and provided *in trans*, have also been generated. Such extensive deletions from original genome have led to a reduction in immune responsiveness and even long-term transgene expression. For example, new generation high capacity adenovirus vectors, which were deleted all adenoviral genes, have been shown to display reduced toxicity and prolonged transgene expression compared with first generation vectors after administration to peripheral organs of immunological naive animals.[76] Similar result was obtained from another study, in which the ''gutless'' adenoviral vector encoding mouse erythropoietin (mEPO) was injected into fully immunocompetent mice by using intramuscular administration, approximately 100% of treated animals showed expression of mEPO after 4 months;[18] another interesting development is the generation of viral vectors with modified cell tropism to lead recombinant vectors transducing into the targeted cells.[57] Modifications of cell tropism of recombinant vectors may also help to reduce the virus titer required for efficient transduction of certain cell types and consequently minimize undesired side effects and inflammatory responses to vectors. The arginine-glycine-aspartame (RGD) and polylysine (pK7) motifs have been shown not only to enhance Ad5 infection through an Ad5 receptor-independent pathway, but also to reduce Ad5-driven specific Th1 and antibody responses. Double-modified Ad5 with RGD and pK7 exhibited higher transfection efficiency and less toxicity, inflammation, and immune responses related to Ad5 infection than unmodified and singly-modified Ad5 vectors.[77] Recently, a study in which rAAV vectors expressing different genetic sequences (coding or non-coding) were repeatedly injected into rat brain implied that the AAV capsid structure is altered by the vector genetic sequence and that secondary structure of the single-strand genome has an impact on the antigen of the virus.[78]

For non-viral vectors modification, the main strategies for avoiding the immune activation are focused on modification of CpGs in vectors, including methylation and elimination of CpGs. It was reported that certain sequence motifs specifically inhibit the recognition and signal transduction of CpG ODNs, for example, replacing a GCGTT or ACGTT motif with GCGGG or ACGGG, respectively, converted a stimulatory CpG ODN into an inhibitory ODN. Inhibitory ODNs blocked cell-cycle entry and protection from apoptosis induced by stimulatory ODNs.[79] The CpG-reduced plasmid vector was found to be significantly less immunostimulant, as the levels of IL-12, IFN-γ, and IL-6 in the serum 24 hr intravenous delivery were reduced by 40–75% compared to the unmodified vector. However, methylation or mutation of CpGs may affect activity of the promoter or make the plasmid replication far less efficient, because CpGs in the plasmid is usually closed to the promoter or the plasmid replication origin. Currently, a group found that calf thymus DNA specifically inhibits the immune activation by *E. coli* DNA,[80] therefore, co-administration of vertebrate DNA or CpG methylated DNA with gene therapy vectors may inhibit the immune response induced by the plasmid without affecting the gene expression.

6. FUTURE DIRECTIONS

Gene therapy, which depends on viral or non-viral gene transfer systems, is an interesting approach for the correction of defective genes. The ideal gene therapy vector requires that the vector components should not elicit immune responses after injection, since humoral antibody responses will make re-injection of the vector ineffective, whereas a cellular response will eliminate the transduced cells. The host immune responses induced by potential immunostimulants in gene delivery systems should provide challenges to gene therapy. The biggest challenge is transient expression of transgene in contrast to expected gene therapy of the correction of defective genes, severe side-effects caused by the immune responses, of course, should be concern of both basic research and clinical trials.

To circumvent the immune response in gene therapy, novel gene delivery system should be considered in the near future. The development of recombinant viruses of non-human origin as vectors for gene therapy may provide new hope. Using such vectors could avoid vector neutralization by pre-existing antibodies directed against the virus on which the vector is based.[81] Furthermore, side-effects caused by interaction between current viral vectors with a primed immune system or with blood components could also be reduced. However, future studies will be required to decide the most appropriate applications for non-human viral delivery systems, whether used as tools for functional gene expression *in vitro* or applied to the more ambitious goal of human gene therapy. In addition, immunosuppression might be used to circumvent the immune responses that would interfere with vector re-injection or development of anti-trangene antibodies, although, because of potential toxicity, this is certainly best avoided if possible. Co-expression of immunosuppressant (tolerance inducing) with vectors encoded transgene could avoid the undesired host immune responses.

Meanwhile, RNAi may act as gene therapeutic agent for suppression of specific gene expression, but it is timely to consider the interferon responses induced by siRNA not only in a basic research but also in therapeutic applications in clinical trials. The ability to efficiently and stably produce and deliver sufficient amount of siRNA to the proper target tissue require refinement before this new technology can be tried clinically.

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