REVIEW

How not to be seen: immune-evasion strategies in gene therapy

A Zaldumbide and RC Hoeben

Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

The development of efficient and safe vectors for gene delivery paved the way for evolution of gene therapy as a new modality for treatment of various inherited disorders and for cancer. The current vectors, viral and non-viral, have their limitations. Innate and adaptive immune responses to vector particles and components may restrict the efficiency of gene transfer and the persistence of expression of the transgene. Results from preclinical studies in animals and more recently data from clinical studies have demonstrated the potential impact of the cellular and the humoral immune response on the therapeutic efficacy. Not only the vector components, but

also the transgene products may induce an immune response that negatively affects the therapeutic efficacy. The induction of a cytotoxic T-cell response to transgene-encoded peptides, as well as the production of antibodies directed against secreted proteins have been reported in preclinical and clinical studies, and these may thwart those applications that require long-term expression. Here we will review some of the options to blunt the acquired immune responses to transgene-encoded polypeptides.

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The immune system as foe of the gene therapist

During maturation, thymocytes undergo a quality control process to eliminate clones that recognize self-antigens. Gene therapy may lead to the expression of neo-antigens, resulting in the induction of an immune response and cytotoxic destruction of the modified cells. Many preclinical and clinical studies have demonstrated the magnitude of this problem and identified a number of variables that can affect the immune response, as outlined below.

Responses against the transgene product

In Duchenne's muscular dystrophy, dystrophin gene delivery appears to be a promising treatment to restore the cytoskeletal integrity in the muscle fibers. However, the induction of an immune response against dystrophin was observed after cardiac transplantation of myoblasts in dystrophic patients.^{1,2} A dystrophin-specific cytotoxic T-cell response was noted after injection of the human dystrophin gene as naked DNA into *mdx* mice, although human and mouse dystrophin proteins are more than 90% identical.³ It may therefore not be surprising that intramuscular injection of an adeno-associated virus (AAV) vector encoding a human microdystrophin

into *mdx* mice triggered an immune response against dystrophin.⁴

An immune response may even be induced in patients expressing alleles with missense mutations, or with small in-frame deletions. In a recent study, Figueredo *et al.*⁵ used a major histocompatibility complex (MHC)-binding prediction program to estimate the probability of a cellular immune response against the $\Delta F508$ product of the cystic fibrosis transmembrane conductance regulator gene (CFTR), which is the most prevalent mutant protein found in cystic fibrosis disease patients. Several high-scoring MHC class I binders were identified within the specific sequences. These data suggest that there is a significant probability that the host mounts a cellular immune response against CFTR, even if the difference between therapeutic and host's CFTR is only a single amino acid.

Obviously, if gene replacement therapy with a gene that is highly similar to the one of the host can trigger a transgene-specific immune response, the synthesis of a foreign gene product is likely to generate a similar response. This has been evident from a clinical study involving donor lymphocyte infusion. Donor T cells were administered to leukemia patients to induce a graft-versus-leukemia (GvL) immune response. This effect should eradicate residual leukemic cells that survived the cytoablative therapy. At the same time, however, the recipients are at risk to develop a graft-versus-host disease (GvHD). In order to selectively eradicate the infused lymphocytes in case GvHD develops, the donor lymphocytes are modified to express the herpes simplex virus (HSV) thymidine-kinase (tk) gene. The product of the HSV-tk gene converts the prodrug ganciclovir (GCV) into a phosphorylated derivative.^{6,7} GCV phosphates are

Correspondence: Professor RC Hoeben, Leiden University Medical Center, Postal Zone S1-P, PO Box 9600, Leiden 2300 RC, The Netherlands.

E-mail: R.C.Hoeben@lumc.nl

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active compounds that induce cell death by inhibiting DNA synthesis. Therefore, HSV-tk-modified T cells can be selectively killed by administering GCV to the patients.^{8,9} However, in a clinical study 7 out of the 23 patients treated developed a strong cytolytic T-lymphocytes (CTL) immune response against the HSV-tk protein,¹⁰ even though, in this particular example the immune response did not affect the efficacy of the therapy.

Pharmacological gene regulation is a key issue for safe gene therapy but here again its *in vivo* use can be hampered by immune rejection. The archetypical systems for regulated gene expression are the so-called Tet-On and Tet-Off systems. In these systems, the transcription of the gene of interest relies on the binding of the tetracycline/doxycycline-activated transactivator (rtTA) to the Tet-operator-controlled promoter. Simple intramuscular injection of plasmid or adenoviral vectors encoding rtTA in primates generates a cellular and humoral immune response against the transactivator protein and results in the destruction of the gene-modified cells.¹¹ Although extensive evolution yielded improved transactivator proteins, which require lower amounts of the activator, the transactivator proteins remain very immunogenic.

Other examples come from the fluorescent and bioluminescent reporter proteins that are popular for tracing genetically modified cells *in vivo*. The green fluorescent protein (GFP) that originates from jellyfish and similar proteins from other organisms facilitate monitoring the fate of the grafted genetically modified cells. Again, their utilization is limited in immune competent hosts by the destruction of the grafted cells by antigen-specific immune responses.^{12–15} In non-human primates, hematopoietic stem cells genetically modified by lentiviral vectors for the expression of GFP or yellow fluorescent protein (YFP) reporter genes were lost as a result of a CTL response to the modified cells in four out of five recipients.

Transgene expression level and tissue specificity

Gene therapy can correct the bleeding diathesis in hemophilia B.¹⁶ However, the development of specific antibodies (inhibitors) against the clotting factor may be a serious complication. Several animal studies demonstrated that gene therapy can induce factor IX (FIX) inhibitors. Direct intramuscular delivery of a human FIX cDNA expression vector triggered a strong FIX-specific immune response in immunocompetent mice¹⁷ and resulted in the generation of anticanine FIX antibodies in hemophilic dogs.¹⁸ Follenzi *et al.* observed a specific cellular and humoral response upon lentiviral transfer of the FIX cDNA when expression was driven by a ubiquitously active promoter (that is, the human cytomegalovirus (hCMV) immediate early enhancer and promoter). In contrast, no immune response was detected if the FIX gene was expressed under the control of a liver-specific promoter.¹⁹

Immune response against *neo*-antigens can also be illustrated by several approaches tested for anemia treatment. While erythropoietin (Epo) gene delivery with an AAV vector and under the control of a CMV promoter could result in a long-term and stable expression of the transgene,^{20,21} a transgene-specific immune response was observed following the implantation of encapsulated

Epo-secreting myoblasts.²² This apparent discrepancy may be explained by the nature of the recipient. In the first studies, the authors explored the efficacy of Epo delivery in healthy animals, whereas the latter one was carried out in a homozygous mouse model for severe anemia (that is, Epo-TAgh) in which the wild-type Epo gene was absent (disrupted by SV40 large T-antigen insertion). A recent study showed that the transgene expression level could also be a determining factor. Indeed, in a macaque model, intramuscular administration of AAV containing an Epo gene driven by the hCMV promoter causes polycythemia shortly after injection, breaks immune tolerance and leads to the development of autoimmune anemia.²³

In addition to the transgene features, the gene-transfer vector used may affect the host's immune response (for review see^{24,25}). For viral vectors the tropism may be critical. In mice that received the adenoviral vector, a transgene-specific CTL response was observed, whereas no CTL were detected after AAV2-mediated delivery.²⁶ In an elegant study, Jooss *et al.*²⁷ attributed the differences to the fact that AAV2 vectors, in contrast to adenovirus type 5 vectors, do not transduce the dendritic cells that function as antigen-presenting cells. The CTL response resulting from the activation of the CD4⁺ T-helper cells eradicated the transduced cells and prevented long-term expression of the transgene.

It should be noted that although in most of the aforementioned examples viral vectors had been used for gene transfer, similar immune responses against the transgene products might be anticipated if efficient non-viral vectors are employed. Indeed, some DNA liposome complexes may be highly immunostimulatory.^{28,29}

The immune system, our 'bodyguard', may frustrate many gene therapy applications by selectively eliminating the genetically modified cells. Thus, it is pivotal for the further advancement of gene therapy that the immune system is tamed and that the genetically modified cells are protected from the actions of the adaptive immune system.

So what avenues are open to evade the effects of the immune system?

Reducing innate immune responses

In addition to the acquired immune response, the innate immune system acts on viral pathogens.³⁰ Toll-like receptors (TLR) found for example in dendritic cells and macrophages recognize pathogen-associated molecular patterns (PAMPs) in DNA, mRNA or double-strand RNA. Immediately after infection, the PAMPs-TLR interactions induce secretion of proinflammatory cytokines (mainly interferon α and β) and chemokines, which activate an adaptive immune response.

Recently, Yang and colleagues demonstrated the importance, in therapy, of inhibiting interferon signaling pathways.³¹ Mice pretreated with anti-IFN α or anti-IFN β 6 h before an adenoviral vector infusion show an increase of the viral DNA copy number, a decrease in the virus-specific CTL response, and a long-term expression of the transgene. In their evolution, viruses developed various strategies to reduce the innate

immune responses by inhibiting the interferon-signaling pathway. Examples of viral proteins that tamper with these pathways include A46R from vaccinia virus,^{32,33} the NS3/4A protease from hepatitis C virus,³⁴ and the RTA ubiquitin ligase from the Kaposi sarcoma herpes virus.³⁵ Adopting such viral-derived strategies for use in gene therapy vectors may allow blocking innate immune sensors and affect communication with the adaptive immune system and may constitute an attractive approach to reduce undesirable immune responses.

Affecting T-cell function/anergy induction

The most robust ways to prevent or suppress the immune responses are those currently used to prevent graft rejection in clinical organ transplantation. These rely on the utilization of general immunosuppressive treatment regimen, which can affect several T-cell subpopulations: inhibition of the regulatory T cells (that is, CD4⁺/CD25⁺) by cyclophosphamide treatment or blockage of T-cells activation by inhibiting interleukin production (cyclosporin, methotrexate).^{36–38} However, such treatments may have serious side effects, such as severe anemia, neutropenia, kidney or/and liver dysfunction, and lead to increased sensitivity to viral infections.

An insight in the relevant processes could provide new options to interfere with T-cell activation. Complete T-cell activation requires at least two signals: the first one originates from the direct interaction between the T-cell receptor (TCR) and the MHC class I peptide complex, and the second from the interaction between accessory molecules at the cell surface (CD40L/CD40 and CD28/CTLA4-B7).

Interestingly, synthesis of soluble human CTLA4Ig, generated by fusing the extracellular domain of CTLA4 with the Fc domain of the IgG, leads to a prolonged state of T-cell unresponsiveness by blocking the CD28 signaling pathway *in vitro*.^{39,40} The feasibility of this approach was demonstrated by Kay *et al.*⁴¹ who showed that the co-administration of recombinant adenovirus with soluble murine CTLA4Ig yielded high expression of the transgene for up to 5 months after injection. In a transplantation protocol for diabetes treatment, transduction of pancreatic islets of Langerhans with adenovirus or lentivirus vectors expressing CTLA4Ig attenuate transplant rejection and prolongs glycemia maintenance in streptozotocin-treated mice.^{42,43} The importance of these co-stimulatory proteins in T-cell activation has been illustrated in a comparative study in which several immunosuppressive treatments were evaluated after human factor VIII (hFVIII) naked DNA transfer into hemophilia A mice. Ochs and co-workers demonstrated that whereas blockage of either CD28/B7 or CD40L/CD40 interaction is sufficient to delay FVIII-inhibitor formation, only the combined treatment CTLA4Ig/anti-CD40 yielded a lasting immune tolerance to the *neo*-antigen and supraphysiologic levels of hFVIII in the mice.⁴⁴

Similarly, administration of anti-inflammatory cytokines, such as IL-10 or TGF- β , can reduce the immune responses to allografts by inhibiting cytokine production and blocking T-cell activation.^{45–47} Intracoronary perfusion of the donor heart with an adenovirus expressing the Epstein-Barr virus IL-10 homologue (vIL-10), combined with a subtherapeutic dose of cyclosporine A, significantly prolonged graft survival from 10 to 36

days in a rat heart transplantation model.⁴⁸ Remarkably, IL-10 is also able to prevent autoimmune diabetes in NOD mice since a single administration of an AAV vector carrying the vIL-10 gene postponed the onset of diabetes in young (3 weeks old) and prediabetic (6 weeks old) NOD mice.⁴⁹ Moreover, the same approach protects transplanted islets from autoimmune destruction in a murine model.⁵⁰

Conditioning the recipient

A small population (1–2%) of CD4⁺ T cells controls immune homeostasis. The CD4⁺/CD25⁺/FoxP3 regulatory T cells (Tregs), stimulated by anti-inflammatory cytokines like IL-10 or TGF- β , induce self-tolerance by actively suppressing immune responses. Adapting Treg numbers could be an important approach for therapeutic treatment. Two possibilities should be considered: reducing Treg numbers for cancer therapy (in ovarian cancer treatment, clinical observations correlated the poor survival rate with an infiltration of these ‘inhibitory’ T cells),⁵¹ or activating Tregs to induce transplantation tolerance (for review see Wood and Sakaguchi⁵²) or self-tolerance to autoantigens.^{53,54} Experiments performed in NOD mice revealed that injection of islet-specific Tregs reversed hyperglycemia in 50% of the treated mice.⁵⁵ However, Tregs activation could be also an important issue to circumvent immune rejection of transgene-modified cells. Experiments performed in murine and canine models demonstrated that AAV-mediated hepatic FIX gene transfer can induce, by an as yet unidentified mechanism, tolerance to the transgene product.^{56–58} Interestingly when splenocytes from tolerized mice were transferred into naive mice, prolonged FIX gene expression was observed even after injection of a potent CTL activator (that is, Ad-FIX). In contrast, mice that received non-tolerized or CD4-depleted splenocytes lost FIX gene expression in the liver.⁵⁹ Recently, the same group points out the contribution of the Treg population by depleting the CD4⁺/CD25⁺ subset: four out of the five mice that received the mouse anti-CD25 antibody treatment developed FIX inhibitors within 6 weeks after AAV-hFIX injection into the liver.⁶⁰

Humanizing gene products

To prevent the immune responses against transgene products several groups investigated the feasibility of using autologous (self) gene products to reduce the cytotoxic immune response in human recipients. Among others, inducible ‘humanized’ caspase 8,⁶¹ FasL⁶² and CD20⁶³ were developed to circumvent the immune responses that may restrict the use of viral or bacterial suicide genes, such as the HSV-tk gene. However, it remains to be evaluated whether building the suicide genes from human components suffices for preventing the induction of an immune response.

Restricting expression of the transgene

The use of cell type-specific promoters to target transgene expression has been advocated as an efficient means to reduce transgene expression in professional antigen-presenting cells such as dendritic cells. Although several studies provided evidence for the effectiveness of this approach, others still reported immune reactions against the transgene product.^{19,64} More recently, the endogenous expression of specific microRNAs was used

to prevent expression of the transgene in the hematopoietic lineage. Indeed, endogenous non-coding RNA (20–23 nucleotides long) can assemble into a RNA-induced silencing complex (RISC) to post-transcriptionally repress gene expression (for review see Rana⁶⁵). A miRNA-regulated lentiviral vector was developed which contains the target sequence of the hematopoietic-lineage-specific miRNA mir-142-3p. This de-targets transgene expression in hematopoietic cells and results in an effective suppression of expression in intravascular and extravascular hematopoietic lineages, without affecting the expression level in non-hematopoietic cell types.⁶⁶ Recently, the same group further validated the potential of this approach by treating hemophilia B mice with a lentivirus carrying both FIX and the mir-142-3p target. Interestingly, the treated mice maintained FIX expression for over 9 months without detectable anti-FIX humoral immune responses.⁶⁷ Hence, the exploitation of miRNA may constitute a promising approach to prevent adapted immune responses against transgene products.

Protecting the modified cells

A radically different approach to protect the gene-modified cells may employ virus-derived immune-evasion mechanisms. Many viruses, and especially members of the herpes virus family, have developed very effective immune-evasion systems in particular by reducing generation of antigenic peptides and their presentation in the context of MHC class I molecules.

The presentation of antigenic peptides by MHC class I molecules and the recognition of this complex by CTLs is the final step in a cascade of processes starting in the cytoplasm. Here the proteins are degraded by the proteasome, generating small antigenic peptides. These peptides enter the endoplasmic reticulum via the transporter associated with antigen processing (TAP) proteins, where they are captured by the MHC class I complex. The peptide-loaded complex is routed via the Golgi system to be expressed at the cell surface. Viruses interfere with these process at all levels to evade the immune system (for review see Loenen *et al.*⁶⁸ and Vossen *et al.*⁶⁹). The US2 and US11 proteins from hCMV cause a rapid degradation of newly synthesized MHC class I heavy chains,^{70,71} the US3 protein retains the MHC-I in the endoplasmic reticulum,⁷² MIR1 and MIR2 from human herpes virus 8 (HHV8) and Nef from the human immunodeficiency virus downregulate MHC class I expression on the cell surface,^{73–76} ICP47 from HSV and UL49.5 from bovine herpes virus 1 act as competitive inhibitors of the peptide/TAP complex,^{77,78} and the US6 protein from hCMV inhibits peptide translocation.⁷⁹ These mechanisms to prevent *neo*-antigens presentation and to protect genetically modified cells are currently being evaluated. However, whereas some studies with ICP47 support its protective effect,^{80,81} the reduced amounts of MHC class I molecules may make the modified cells more sensitive to NK cell activity.^{82,83} Experiments in non-human primates, using adenovirus vector that co-express ICP47 and the CFTR gene, indeed suggest an increased NK cell sensitivity of the gene-modified cells.⁸⁴

The feasibility of this approach has also been elegantly demonstrated by Horwitz and colleagues in a diabetes model.⁸⁵ Type I autoimmune diabetes destroys islets by mechanisms that involve MHC class I molecules and

tumor necrosis factor α (TNF- α). In the well-studied lymphocytic choriomeningitis viral (LCMV) murine model of virus-induced autoimmune diabetes, an LCMV polypeptide expressed on the islets is a target for autoimmune destruction of β cells after LCMV infection. The authors studied whether the human adenovirus type 2 E3 genes, which code for proteins implicated in the MHC class I downregulation and in inhibition of TNF- α /Fas-induced cytotoxicity, could protect islets from immune destruction. To this end, transgenic mouse expressing Ad E3 genes under the control of a rat insulin promoter were generated and crossed with LCMV mice. Eight months after, it appeared that none of the ten double transgenic mice (RIP-LCMV $\times$ RIP-AdE3) developed diabetes. A similar strategy could also be exploited in a gene therapy setting, by co-expression of the therapeutic gene with one or more viral immune-evasion genes.

Generation of 'immuno-stealth' proteins

Rather than preventing the cells capacity to present peptides via MHC class I molecules altogether, the transgene product can be protected from proteasomal degradation. This effectively makes the gene product invisible to the immune system. Pioneering experiments of Masucci and co-workers demonstrated that a specific sequence from the Epstein-Barr virus nuclear antigen 1 (EBNA-1) has the capacity to inhibit proteasomal degradation of linked antigens.^{86,87} We and others have exploited this mechanism to generate 'immuno-stealth' forms of some gene products frequently used in experimental gene therapy research, such as *Escherichia coli* β -galactosidase, HSV-tk and GFP.^{88–90} This was achieved by fusing codons for the 238-amino-acid-long glycine-alanine repeat (GAR) at the N-terminus of the reporter or therapeutic genes. Although antigen-specific CTLs were induced in mice after adenovirus-mediated transfer of minigenes encoding GAR-modified model antigens, the CTL neither *in vitro* nor *in vivo* react to cells producing these antigens. This is consistent with the model that the GAR inhibits generation of antigenic peptide from the fusion proteins.⁸⁹ Importantly, the fusion proteins retained their biological activity.

However, degradation of normal proteins by the proteasome is only responsible for a small fraction of the antigenic peptides expressed on the cell surface. Most antigenic peptides are derived from defective products of proteins synthesis (DRiPs), including prematurely terminated products, misfolded proteins and frame-shifted products.^{91,92} We recently provided evidence that EBNA-1 harbors a mechanism that may reduce unconventional epitope generation. Translation of the EBNA-1 mRNA from another open reading frame would yield a product that contains repeat sequence consisting solely of glycine, glutamine and glutamic acid residues (GZr). This repeat sequence, too, is able to inhibit proteasomal degradation of linked antigens.⁹³ Intriguingly, neither the GAR nor the GZr elements prevent antigenic peptide generation *in trans*, that is, presentation of antigens derived from other proteins.

An important aspect to note is that the use of these inhibitory sequences may not be limited to the transgene product itself. In a phase 1–2 clinical study, Kay and collaborators demonstrated that infusion into the hepatic artery of a recombinant AAV vector carrying the human FIX cDNA into patients with hemophilia B, resulted in a

decline of FIX level 8 weeks after treatment in some patients.⁹⁴ A similar strategy gave very promising results in hemophilic dogs.^{95,96} IFN γ assays established that the transaminase elevation, a consequence of destruction of transduced hepatocytes, was not due to an immune response against the transgene product itself, although a response against unconventional epitopes can not be fully excluded. Rather the response may be directed against the AAV capsid proteins. The authors attributed the difference observed between experimental models and human to the fact that during childhood many humans are infected by AAV2 and this infection often accompanied by adenovirus infection results in the formation of CD8⁺ T cells directed against antigens from the helper adenovirus and against AAV-derived peptides.⁹⁷ Therefore, two attractive approaches could be envisioned to circumvent this immune response to capsid proteins: induction of tolerance, like it has been reported in non-human primates⁹⁸ and modification of AAV capsids proteins with sequences, such as the GAR or GZr elements, in order to generate an 'immuno-stealthed' therapeutic vector.

Although latency-associated proteins in other herpes viruses do not contain GAR or GAR-like motifs, the LANA-1 protein from HHV8, which has a function similar to EBNA-1, contains a protein domain with a high similarity with the GZr-domain. Also, this repeat can prevent presentation of antigenic peptides in the context of MHC class I molecules.^{99,100} Likewise, Bennett *et al.*¹⁰¹ identified that the ORF73 protein of the murine γ herpes virus 68 (which is functionally equivalent to EBNA-1 and LANA-1), can block the antigen generation from linked polypeptides.

So far, the application of the 'immuno-stealthed' technology was hampered by the lengths of the repeat sequences, and the problems associated with stably maintaining such repeat sequences in *E. coli*. Shorter versions of these inhibitory sequences still have to be developed as inhibitors of antigen presentation. Studies have shown that shorter repeats can be equally or even more potent than the full-length repeats in preventing presentation of model antigens. Using the very unstable protein Ikb, Sharipo *et al.*¹⁰² demonstrated a GAR as short as 24 amino acids could increase Ikb half-life, but it remains to be established whether such short elements are sufficient for immune evasion. Nevertheless, these *in cis* acting inhibitors of antigen presentation will be widely applicable for preventing the adaptive cellular responses against neo-antigens derived from transgene products and may enhance the long-term efficacy of many experimental gene therapy procedures.

The examples, mentioned above, provide several avenues for escaping the immune system. The immune-evasion strategy of choice may depend largely on the precise application for which it is intended, and may need further adaptation to provide optimal protection to the transduced cells. Nevertheless, the immune system should not be an insurmountable hurdle for long-term gene therapy anymore.

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