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THE IMMUNOLOGY OF GENE TRANSFER: AN OVERVIEW

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1.1 INTRODUCTION

As the science of gene transfer has progressed over the past two decades it has become apparent that certain obstacles to the success of this therapeutic approach are substantial in nature. Attaining clinical benefits from gene transfer requires the effective delivery of a therapeutic transgene to cells of the host and the establishment of levels of transgene expression that are sufficient in both magnitude and duration to result in a therapeutic benefit. Although these objectives are straightforward in principle, their attainment has required the interaction of life scientists from a variety of backgrounds to optimize each of the many steps involved in a gene transfer protocol [Anson and Fletcher 2007; Cardone 2007; Pierce et al. 2007].

While the development of efficient transgene delivery systems and transgene expression cassettes has occupied a considerable amount of time during the evolution of gene transfer protocols, it has long been clear that the host immune response to gene transfer will present a formidable obstacle to the attainment of therapeutic goals. The aim of this introductory chapter is to summarize the current status of knowledge pertaining to the immunological challenges facing gene transfer and to illustrate specific aspects of this challenge with examples from the gene transfer literature. Detailed discussions of various subject areas follow in the succeeding chapters.

1.2 COMPONENTS OF THE IMMUNE RESPONSE TO GENE TRANSFER

There is very good evidence that recipients of gene transfer develop responses that involve the full spectrum of innate and adaptive immunity. Initial responses to transgene delivery can be seen within hours of administration, depending on the means and route of delivery. These early responses involve mechanisms that include the detection of pathogen-associated molecular patterns (PAMPs) present on the transgene particle (i.e., viral structural proteins or nucleic acids) by pattern recognition receptors (PRRs) on cells of the innate immune system (i.e., macrophages and dendritic cells) and the subsequent elaboration of pro-inflammatory cytokines that can up-regulate later adaptive immune responses. These adaptive responses can include the generation of antibodies to the transgene delivery vehicle and to the transgene product, as well as cell-mediated responses to these entities. Overall, depending on the context of the gene transfer protocol and a variety of factors pertaining to the details of the protocol, the host immune system can elicit adverse reactions that can span an interval from within hours of vector administration to months after gene transfer.

1.3 THE CONCEPT OF IMMUNOLOGICAL DANGER AND GENE TRANSFER

Two nonexclusive theories of immunologic responsiveness have emerged in the past few decades. The first of these is the *self–nonself theory*, which predicts that adaptive immune responses will be generated against any antigen to which the organism has not been exposed during early development [Janeway 1992]. In this instance, self-antigens presented in the thymus to T cells that are reactive to these self-antigens are selected for elimination by apoptosis. This form of centrally mediated immunological tolerance accounts for the limitation of autoreactive immune responses in normal physiology. However, the self–nonself theory of immune reactivity does not explain how self-neoantigens, which can be expressed within an organism after the development of initial central tolerance, are preserved without evoking a nonself adaptive response. This situation is best exemplified by the appearance of self-neoantigens, at the time of puberty and in pregnancy, that do not routinely generate adaptive immune reactions. To explain how these new nonself antigens are tolerated, the *theory of immunological danger* has been proposed and is now widely accepted as a complementary principle for the development of adaptive immunity [Matzinger 1994]. According to the danger theory of immune reactivity, antigens are tolerated unless they are presented to immune cells in the context of “dangerous,” inflammatory signals. The coexistence of these signals at the time of antigen presentation alters the maturation state of the antigen-presenting cell and predisposes the generation of effector cell responses rather than the acquisition of immunologic tolerance [Gallucci et al. 1999].

It has become clear that many aspects of gene transfer protocols result in the presentation of antigens in the context of inflammatory danger signals, and thus the likelihood that immunogenic responses are generated to the delivery vehicle and/or transgene product is enhanced significantly [Brown and Lillicrap 2002]. Various factors relating to the mode of transgene delivery, the nature of the delivery vehicle, and the way in which the delivery process affects host cells have all been implicated in the generation of inflammatory microenvironments, scenarios in which effector cell responses are readily generated.

1.4 TARGETS OF THE IMMUNOLOGIC RESPONSE TO GENE TRANSFER

Delivery of transgenes through a variety of means can result in immune responses to several components of the gene transfer system. These responses can take the following forms:

- Innate immunity
- Fever

- Thrombocytopenia
- Systemic cytokine elevations
- Multiorgan inflammation leading to death
- Activation of the adaptive immune response
- Adaptive immunity
 - Antibodies to the vector, compromising vector readministration
 - Antibodies to the transgene product, nullifying transgene expression
 - Cytotoxicity to vector and/or transgene product, leading to loss of transduced cells

Although the efficiency of nonviral delivery protocols continues to be inferior to viral vector-mediated approaches to transgene delivery, there are still instances where nonviral delivery may prove efficacious. In these cases, immune responses may be generated to the transgene nucleic acid depending on how and where this is delivered and the nature of the nucleotide sequence. In particular, unmethylated CpG sequences, which are found more frequently in prokaryotic genomes (i.e., plasmid backbones), can incite innate immune responses through interaction with Toll-like receptor (TLR) 9 [Verthelyi 2006].

In contrast to nonviral delivery protocols, viral-mediated gene transfer offers the potential for a significantly wider array of immunological responses. While the various forms of disabled viruses used for this purpose are unable to replicate, the delivery to host cells utilizes very large numbers of viral particles to optimize the delivery efficiency [Lowenstein 2003]. The result of these high-multiplicity-of-infection protocols on the immune system are twofold: The large viral load is a potent stimulus for PRR signaling, and there is good evidence that receptors such as TLR7 are activated by exposure to engineered viral genomes. Furthermore, once inside the transduced host cells, the viral proteins are disassembled and presented on the cell surface in the context of MHC class I molecules and can incite potent CD8⁺ cytotoxic T-lymphocyte (CTL) responses that will, in turn, eliminate the transduced cells. Peptides derived from the viral vector will also be presented after uptake by antigen-presenting cells in the context of MHC class II molecules, and effector CD4⁺ T-cell responses will ultimately lead to the generation of antibodies to the vector that will limit vector readministration.

Aside from immune responses to the delivery vehicle and the transgene particle, in gene transfer protocols for the replacement of proteins in monogenic inherited deficiencies (e.g., lysosomal storage diseases, hemophilia), the transgene product may be recognized as a neoantigen, and adaptive immune responses may be generated to the protein [Lillicrap 2000]. These can either target destruction of the cell of synthesis through a CTL-mediated response or, more often, result in the formation of antibodies through a CD4⁺ T-cell-facilitated mechanism. In either instance it is clear that the beneficial effects of the gene transfer protocol will be compromised significantly by the host's adaptive immune response.

1.5 FACTORS AFFECTING THE IMMUNE RESPONSE TO GENE TRANSFER

1.5.1 Host Influences

Not surprisingly, factors inherent to the host can have a profound influence on the nature and extent of the immune response to gene transfer. Both genetic and acquired factors, such as the following, can significantly alter the innate and adaptive responses to various forms of gene therapy:

- Host factors
 - Recipient immunogenotype
 - Prior exposure to virus
 - Prior exposure to transgene product
 - Maturity of immune system at time of transfer
 - Coexistent inflammation
 - Coexistent immunosuppression
- Gene transfer protocol factors
 - Route of transgene delivery
 - Type of delivery system
- Transgene delivery vehicle factors
 - Type of viral vector
 - Vector dose
 - Pseudotype of vector particle
 - Type of transgene promoter
 - Presence of vector preparation contaminants

As one would anticipate, these influences can best be dissected in inbred strains of laboratory animals, where genetic homogeneity enhances the contributions of genetic loci involved either directly or indirectly in generation of the immune response [Rawle et al. 2004]. Thus, testing the immunogenic potential of a particular gene transfer protocol should take into account the inherent immunogenic phenotype of the test animals so that immunogenicity is neither significantly under- or overestimated. In outbred species such as humans, the analysis of genetic factors influencing immunity (the immunogenotype) is considerably more complex and may rely on indirect evidence of immune dysregulation obtained through personal or family histories of disorders with immune contributions. The situation is somewhat less complicated in the case of the adaptive immune response to newly delivered proteins in inherited protein-deficient states (e.g., hemophilia). In these circumstances, there is an incidence of antibody development to the protein neoantigen, even with standard protein replacement therapy. This complication is especially likely in patients in whom the underlying genetic defect results in a null phenotype [Schwaab et al. 1995], although this risk can be reduced by prior tolerance induction to the protein in question.

In contrast to the complexity of the immunogenic phenotype and its variable influence on gene transfer outcomes, there is strong evidence to indicate that acquired coexistent pathologies (e.g., infection), which can act as inflammatory adjuvants at the time of gene transfer, may significantly enhance the host immune response, with occasionally catastrophic consequences [Raper et al. 2003]. Thus, the administration of any form of gene transfer to recipients in whom inflammatory pathologies coexist, or in whom the immune response is being manipulated, should be approached with caution.

1.5.2 Influence of Mode and Route of Transgene Delivery

The mode and route of transgene administration can influence transgene immunity through a number of mechanisms. As one distinct example, certain anatomical sites in the body, such as the anterior chamber of the eye, experience only attenuated immunologic responses to newly introduced antigens [Kaplan and Niederkorn 2007]. Thus, the delivery of transgenes to the eye through a variety of approaches may be significantly less complicated by immune compromise than delivery to many other sites. Similarly, transgene delivery to sites of mucosal uptake, such as the nasal and gut epithelium, may also predispose to the generation of a tolerogenic as opposed to an immunogenic outcome [Faria and Weiner 2006].

In contrast, systemic delivery of transgenes through the circulation will inevitably result in the involvement of cells of both the innate and adaptive immune systems at various sites. This problem can be overcome to some extent by directing delivery through means such as organ-specific vascular injections (e.g., hepatic artery injection) or through delivery directly into specific tissues (e.g., intramuscular injection). An additional level of tissue specificity for transgene expression can be achieved through the use of a targeted transcriptional regulatory element. In selected anatomical sites, different subsets of immune cells may be involved that may result in significantly different immunological outcomes. As one important example, limiting transgene expression to the liver by a combination of local vascular injection and the use of a liver-specific transgene promoter has recently been shown to promote the generation of tolerance to a secreted transgene product through the expansion of antigen-specific regulatory T cells [Cao et al. 2007].

In addition to regulating immunogenicity through altering the route of administration, the mode of transgene delivery also influences the host response. Thus, while viral vector-mediated delivery to skeletal muscle may be relatively non-immunogenic, potent host responses can be elicited if high vector doses are delivered to single sites [Arruda et al. 2004]. Similarly, while nonviral vector transgene delivery is generally better tolerated by the immune system, protocols such as hydrodynamic injection, which result in perivascular tissue trauma, are often associated with adaptive immune responses to the transgene product, due, at least in part, to the inflammatory environment in which the transgene is expressed [Miao et al. 2006].

1.5.3 Vector Influences

Viruses have evolved over millions of years to deliver nucleic acids efficiently to host cells, and thus represent ideal transgene delivery vehicles [Kay et al. 2001]. To date, despite intensive investigation, gene transfer scientists employing strategies that utilize components of the viral delivery system have not come close to emulating the efficiency of viral transduction. Nevertheless, despite their efficiency as delivery vehicles, all disabled viruses suffer from the inevitable consequence of being viewed as “foreign” and “dangerous” by the host immune system. Fortunately, the degree to which this immunologic limitation exists appears to vary significantly between types of viral vector.

A number of vector-related variables merit general comment prior to a more detailed consideration of each viral vector type. First is the issue of vector production and the potential for delivering immunogenic contaminants of the production system along with the vector itself. Viral vectors are generated through protocols that provide access to essential viral structural components that are deleted from the viral genome through their expression *in trans* from helper constructs in the producer cell lines. These helper constructs may be delivered through transient transfection or, in instances where larger vector yields are desired, through the generation of stably transfected cell lines in which helper proteins are usually under the control of inducible promoter elements. Whatever the details of helper protein expression, a potential consequence of the presence of these proteins is that they may be co-purified with the vector and can elicit independent innate and adaptive responses following gene transfer. During vector production, depending on the transgene promoter used, expression of the transgene product can occur from the producer cell line. Thus, in addition to the co-purification of viral structural helper proteins, vector isolation may also be complicated by the presence of contaminating transgene protein that will be co-administered at the time of transgene delivery and may elicit an adaptive immune response.

Upon delivery to the gene transfer recipient, the immunological consequence of viral vector exposure will depend on a variety of factors, including the history of prior contact with the virus, the route and mode of vector delivery, the vector dose, and the presence of any coexistent conditions within the recipient that might influence the ability to generate an immune response. Viruses possess structural elements, PAMPs, that will be recognized by receptors, PRRs, on cells of the recipient’s innate immune system. To date, most of our knowledge related to these interactions involves the family of Toll-like receptors (TLRs), which are expressed on the surface and within endocytic compartments of cells of the innate immune system [Seth et al. 2006]. Thus, the activation of TLR signaling pathways by a variety of ligands involved in gene transfer protocols, such as viral double (TLR3) and single (TLR7 and TLR8)-stranded RNA, envelope glycoproteins (TLR2 and TLR4), and unmethylated CpG nucleotides (TLR9), all have the potential to mediate downstream inflammatory cytokine and chemokine synthesis and release. Even though viral vectors have been engineered to remove many of their intrinsic structural elements, the vector particles will still possess peptide sequences that facilitate cell entry but at the same time signal to the

cell that it has been invaded by a potential pathogen. Removal or manipulation of these sequences may reduce innate immunogenicity but will probably also reduce transduction efficiency. Overall, the early interaction of viral vector elements with components of the innate immune system remains a major obstacle to the attainment of efficient delivery and long-term persistence of the transgene.

The ability of viral vectors to transduce cells varies significantly for different viruses and cell types. This variable has profound effects on the immunogenic potential of a particular vector type. More specifically, the transduction of and expression within professional antigen-presenting cells (APCs: e.g., dendritic cells) will generate situations in which vector particles and the transgene product are processed and presented on MHC class I molecules to cognate CTLs. In circumstances in which there is a coincident expression of co-stimulatory molecules by the APCs, an adverse CTL response will result in the potential of cytotoxicity for any host cell presenting the appropriate vector or transgene-derived peptide. Thus, the use of vectors that are inherently less inclined to transduce APCs or can be targeted away from APCs represent strategies that will very likely reduce cellular adaptive immunity.

Once inside the cell, the vector particle must gain entry to the nucleus and disassemble the protein elements that have facilitated entry into the cell. In many instances, the details of where, when, and over what duration of time this event occurs are still vague [Thomas et al. 2004], but the subsequent presentation of these vector-derived peptides on the surface of the transduced cell in the context of MHC class I receptors represents a significant limitation to vector-mediated gene transfer [Arruda and Xiao 2007]. Once presented at the transduced cell surface, the subsequent host response will depend on prior exposure to the virus (i.e., the potential presence of immunologic memory) and whether the presentation is occurring from an APC, in which co-stimulatory molecule expression can result in cognate CTL activation. In cases where the virus has been seen previously by the host immune system and an adaptive memory response has been generated, expansion of vector-specific CTLs and subsequent destruction of transduced host cells may ensue.

1.6 IMMUNOLOGIC CONSIDERATIONS FOR SPECIFIC VIRAL VECTORS

1.6.1 Adenoviral Gene Transfer

Although adenoviral vectors provide a very efficient transgene delivery system for most cell types, their excellent ability to transduce cells is matched by limitations imposed by the host immune response. Cellular entry by these vectors is mediated by an interaction of the viral capsid with several cell surface receptors, including the coxsackie and adenovirus receptor (CAR) and the family of α_v integrins. However, studies in which these interactions have been ablated suggest that the viral capsid can still promote associations with cells of the innate immune system through other, as yet unidentified mechanisms.

Although preexistent adaptive immunity to adenovirus will compromise vector administration and potentially result in targeted cytotoxicity of transduced host cells, it is the effects of adenoviral vectors on innate immunity that have limited the recent development of systemic adenoviral transgene delivery [Muruve 2004]. Systemic adenovector delivery is associated with avid uptake by Kupffer cells in the liver and the interaction and uptake by other cells of the innate immune system. This effect has two consequences: First, there is a threshold effect for adenoviral vector dosing such that when the innate immune cell “sink” has been saturated, rapid increments in parenchymal cell transduction can be achieved with minimal vector dose escalation. Second, and of major importance for the safety of *in vivo* systemic gene transfer employing adenovectors, is the fact that the interaction of these vectors with innate immune cells signals the expression and release of an array of pro-inflammatory cytokines and chemokines, including IL-1, IL-6, TNF- α , macrophage inhibitory protein-2, and RANTES. This effect results in a complex constellation of inflammatory manifestations within 3 to 4 hours of vector administration. These include fever, thrombocytopenia, and other signs of systemic inflammation. Although the extent of this pro-inflammatory cascade can be predicted to some extent based on variables such as vector dose, past clinical experience with adenovectors indicates that the inflammatory response may sometimes far exceed expectations and lead to catastrophic clinical outcomes [Raper et al. 2003].

There is strong evidence to indicate that the innate response against adenovectors is mediated to components of the viral capsid and their interaction with innate immune cells. Thus, while adaptive immunity against these vectors has been reduced substantially through the development of helper-dependent adenovectors that contain no adenoviral genes, the problems with innate responsiveness have remained unchanged [Muruve et al. 2004]. Although the innate immune response to systemically delivered adenovectors has significantly limited further development of gene substitution trials, this phenomenon has been beneficial for vaccine development and for cancer immunotherapy with this vector system. Indeed, the first commercially available gene transfer product, Gendicine, employs an adenoviral vector expressing p53 for the treatment of various forms of neoplasia [Peng 2005].

1.6.2 Adeno-Associated Viral Gene Transfer

Adeno-associated viruses (AAVs) are widely regarded as excellent vehicles for gene transfer. Their preferred selection as gene transfer vectors is due at least in part to the fact that they appear to be minimally provocative to the host immune system. While adenovectors efficiently transduce APCs and elicit rapid and significant pro-inflammatory responses from the innate immune system, the AAV serotypes that have been studied to date do not seem to transduce APCs effectively and do not generate innate immune reactivity [Sarukhan et al. 2001; Zaiss and Muruve 2005]. Thus, acute immunologic consequences of AAV gene transfer have been negligible.

However, while the interaction of AAVs with the innate immune system seems inconsequential, there are significant problems relating to the adaptive immune response to this vector. Natural, asymptomatic AAV infection in humans is common, and estimates of up to 80% of human populations possess neutralizing antibodies to some AAV serotypes [Moskalenko et al. 2000]. Furthermore, strong conservation of immunodominant capsid epitopes suggests that changing serotypes may meet with only partial success in attempting to evade existing anti-AAV immunity. The presence of anti-AAV antibodies will compromise vector delivery and adversely influence transduction efficiency.

Whereas the incidence of humoral immune responses to AAVs is relatively well documented, far less is known about the frequency with which cell-mediated reactivity to the vector is generated. Unfortunately, there does not appear to be a useful correlation between the finding of humoral responses to AAVs and the presence of a cell-mediated response. However, there is now strong evidence from the evaluation of AAV gene transfer in human clinical trials that CTL responses to AAV capsid antigens can result in significant cytotoxicity and the loss of cells that have been transduced by the vector [High et al. 2003; Manno et al. 2006; Mingozzi et al. 2007]. Whether these adverse CTL responses can be predicted from an analysis of circulating cells prior to gene transfer seems unlikely. Nevertheless, the result of these cytotoxic responses remains a critical obstacle to persistent AAV-mediated gene transfer, and the outcome of trials of immunosuppression administered around the time of transgene delivery is eagerly awaited [Herzog et al. 2001; Jiang et al. 2006].

1.6.3 Retroviral Gene Transfer

Recent efforts to utilize recombinant retroviruses as a means of transgene delivery have been complicated principally by concerns relating to their propensity to cause insertional mutagenesis. Detailed studies have now documented what may be important differences between the sites of insertion for onco-retroviral and lentiviral genomes [Bushman 2007]. However, aside from these complications, recent evidence has been presented to suggest that these vectors may also have immunologic consequences that were previously overlooked. Persistent high-level lentiviral-mediated transgene expression following *in vivo* delivery has been difficult to achieve and appears to be due, at least in part, to the fact that lentiviral particles pseudotyped with commonly used envelopes such as VSV-G transduce and activate APCs more efficiently than they transduce parenchymal cells. This propensity results in subsequent activation of the adaptive immune system and the frequent generation of humoral and cell-mediated responses to the transgene product [Follenzi et al. 2004]. As a further result of this predilection, lentivectors show the same type of threshold dosing effect seen with adenovirus. Saturation of reticuloendothelial cell "sinks" such as the Kupffer cell population will then allow significant increments in parenchymal cell transduction with relatively small increases in vector dose [van Til et al. 2005]. However, in addition to acting as a sink for lentivector distribution, the transduction of these innate immune

cells also generates a type I interferon response (i.e., IFN- α and IFN- β) [Brown et al. 2007]. These events have several adverse effects on the persistence of lentivector gene transfer. First, lentivector transduction of APCs activates these cells and thus establishes an environment in which expression and presentation of the transgene product (in the context of either MHC class I or II) will preferentially result in immunogenic as opposed to tolerogenic T-cell responses. As a result, lentivector delivery is often associated with the generation of a humoral response to the transgene product and the production of CTLs that act to remove the transduced parenchymal cells. In addition to the effects secondary to APC maturation, the elicitation of IFN- $\alpha\beta$ further inhibits transduction of parenchymal cells and stimulates the cytotoxic clearance of cells that have already been transduced. In response to these interactions with the innate immune system, future studies of in vivo lentivector delivery will need to find ways in which these early adverse events are abrogated or minimized.

1.7 PRECLINICAL MODELING OF GENE TRANSFER IMMUNOLOGY

While the challenges of the host immune response to gene transfer are now very well appreciated, developing preclinical strategies to determine the mechanisms underlying these responses and to evaluate potential means to circumvent them are not straightforward. Most in vivo gene transfer studies begin in laboratory mice. These animals offer significant advantages in that mouse immunology has been very well studied and reagents to assess immune responses in the mouse are abundant. However, the commonly used inbred mouse strains also have the disadvantage of generating immune reactions that are relatively homogeneous and to some extent predetermined by the particular immunophenotype of the inbred strain. As just one example, Balb/c mice have a much greater tendency to generate Th2 polarized responses than do C57BL/6 mice, in which Th1 responses are predominant [Rawle et al. 2004]. As a result of these differences, the prediction of immunological outcomes of gene transfer strategies in outbred species such as humans should be approached with caution.

The second complicating issue with the use of animals for preclinical testing of the immune responses to gene transfer is the separation of responses that are prompted by exposure to heterologous transgene products from those responses that are inherent to the gene transfer strategy itself. Even in animals with null mutations for specific proteins, the choice of a species-matched transgene replacement may prove essential to prevent a humoral response to the transgenic protein and limit the potential to assess long-term persistence of transgene expression [Connelly et al. 1996].

Finally, the natural infectivity of animal models with viruses that are now being used as candidate vectors for human studies is sometimes limiting. As a result, memory responses of humoral and cell-mediated adaptive immunity to the vector are difficult to assess [Herzog 2007]. This important limitation can be addressed by various approaches involving passive immunization in

which either immunoglobulin preparations possessing antivector activity are administered [Scallan et al. 2006] or adoptive transfer of cytotoxic memory T cells can be delivered prior to trials of gene transfer. However, all of these strategies are complex, and none of them recapitulate the details of the memory response in the naturally infected host.

1.8 APPROACHES TO ABROGATING THE IMMUNE RESPONSE TO GENE TRANSFER

As alluded to at the start of this overview, there is now a realization that the host immune response to various components of the gene transfer protocol represents the most challenging obstacle to the clinical implementation of this therapeutic paradigm. Therefore, the development of strategies such as the following, used to abrogate or at least minimize these responses, is essential for the future translation of gene transfer benefits:

- Reducing innate immunity
 - Utilizing an *ex vivo* transgene delivery approach
 - Minimizing the vector dose
 - Avoiding coexisting inflammation in the host
 - Using AAV vectors for *in vivo* delivery
- Reducing adaptive immunity
 - Delivery to prenatal and neonatal immune systems
 - Avoiding antigen-presenting cell transduction
 - Blocking co-stimulatory molecule interactions
 - Peri-gene transfer immunosuppression
 - Delivery to immunoprivileged sites (e.g., eye)
 - Delivery to the liver
 - Delivery to mucosal epithelium

Of course, as mentioned previously, immune reactivity can, in some instances, be a desirable effect of gene transfer, and thus studies aimed at using this approach for vaccination or immunotherapy of cancer have used this effect to their advantage [Li et al. 2005; Xue and Stauss 2007].

From the discussion above it is clear that both innate and adaptive immune responsiveness need to be taken into consideration in terms of gene transfer protocols. The activation of either arm of the immune system can result in a failure to express the transgene for therapeutically relevant periods of time and can, in extreme circumstances, result in systemic inflammatory states that can have catastrophic outcomes.

1.8.1 Strategies to Limit Innate Immune Responses

One obvious option to limit innate responses to the transfer process is to deliver the transgene via an *ex vivo* approach. Here, there can be exclusive targeting of the vector to cells isolated *ex vivo*, and the exposure of the host innate immune system to the vector is negligible. However, adaptive humoral and cell-mediated responses to the transgene product and cell-mediated cytotoxicity to the disassembled vector can still ensue upon reintroduction of the transduced cells.

As indicated above, innate immune responses to adenovectors and, to a lesser extent, lentivectors are both significant. In both instances, keeping the vector dose to the minimum required to achieve a therapeutic response will limit innate responses, as will the transient depletion of reticuloendothelial cells. Efforts to target these vectors away from interacting with cells of the innate immune system have been attempted with strategies such as conjugation with polyethylene glycol and have shown some success in reducing the acute cytokine increments and thrombocytopenia [De et al. 2005]. Overall, and at the current time, the well-studied serotypes of AAV vectors seem to offer the best option in terms of minimizing innate immunity in the context of gene transfer.

1.8.2 Strategies to Limit Immune Responses to the Transgene Vector

Adaptive immune responses to the transgene vector compromise vector delivery through interaction with antivector antibodies in the circulation and limit the persistence of transgene expression through cytotoxic clearance of transduced cells by vector-specific CTLs. Both mechanisms are significant obstacles to achieving long-term gene transfer. Circumventing the memory humoral response to the vector is difficult, and while increasing the vector dose or attempting to reduce antivector antibodies transiently through strategies such as plasma exchange might facilitate vector delivery, this obstacle may always reduce transduction efficiency. Similarly, efforts to abolish or at least minimize the memory CTL response to vector epitopes presented at the transduced cell surface following capsid disassembly have not yet been explored adequately. There is some evidence, with certain vectors, to suggest that these capsid proteins may persist for several weeks following vector administration. In this context, the delivery of a T-cell immunosuppressive regimen for a period of time following vector exposure (i.e., for two to four months) may significantly reduce the loss of transduced cells. This type of approach requires systematic evaluation in clinical trials where memory T-cell responses are anticipated.

1.8.3 Strategies to Limit Immune Responses to the Transgene Product

The final area of concern relating to adaptive immune reactivity involves the potential for humoral and cell-mediated responses to the transgene product. In some instances, the gene transfer recipient may already have been exposed to the transgene product and have preexisting tolerance to this antigen. In these recipients, the likelihood of an adaptive response developing following gene transfer

is reduced significantly unless the transfer protocol involves a strategy that could break tolerance (e.g., an approach that is accompanied by inflammation). Thus, in most gene transfer recipients, in contrast to the adaptive responses to vector epitopes, memory cells specific for the transgene product will not be present and the immune response will be primary in nature. With this in mind, there is an opportunity to utilize gene transfer to establish tolerance to the transgene product. With existing knowledge concerning tolerance mechanisms, the goal would appear to be the attainment of peripheral tolerance through the induction of T regulatory cells. To facilitate this outcome, the transgene product must be presented to antigen-specific T cells in the context of a tolerogenic environment, in which the expression of co-stimulatory molecules on APCs is minimized, with the consequence that the likelihood of activating effector T cells is reduced markedly.

One strategy that appears to reduce adaptive responses to the transgene product is to target expression of the transgene away from APCs. Interestingly, while this approach would be expected to minimize CD8⁺ effector T-cell activation and subsequent cytotoxic responses to the transgene product, the avoidance of APC transgene expression also reduces humoral responses to the transgene protein, even though the protein will be presented on MHC class II molecules after uptake of extracellular material [Follenzi et al., 2004]. Thus, the effect of targeting transgene expression away from APCs on minimizing humoral responses suggests that there may be mechanisms through which proteins synthesized within APCs can somehow be presented on MCH class II molecules and interact with CD4⁺ effector T cells. Targeting transgene expression away from APCs can be achieved by a variety of approaches, including the following: through the use of a type of vector that transduces APCs inefficiently (e.g., an AAV); through pseudotyping the vector particle to avoid APC transduction; through the use of a tissue-specific transgene promoter that minimizes expression within APCs (this will still occur through “promoter trapping” with integrating vectors); and finally, through the use of a miRNA-based approach in which the 3' end of the transgene transcript contains binding sites for miRNAs expressed exclusively in hematopoietic cells [Brown et al. 2006]. The latter, elegant strategy targets transgene mRNA for degradation or blocks translation of the transcript through the binding of the hematopoietic miRNA to their cognate sequences in the mRNA.

Administration of immunosuppressive therapy at the time of transgene delivery may also eliminate the adaptive response to the newly expressed protein. However, the type of immunomodulatory agent, the degree of immunosuppression required, and the duration of treatment are details that require much further study.

In general, additional strategies for attaining tolerance to the transgene product involve expression of the protein in a tolerogenic context with the subsequent expansion of regulatory T-cell populations. There appear to be a number of approaches to achieving this objective. One strategy that has been tested successfully in animal models is to deliver the transgene during fetal or neonatal development, when the effector arm of the immune system is still relatively

underdeveloped and at a time when central tolerance mechanisms may still be invoked [Waddington et al. 2004; Xu et al. 2007]. Central tolerance to the transgene product can also be achieved by direct intrathymic delivery of vector [Marodon et al. 2006]. Nevertheless, given the significant differences between the time of maturation of the mouse and human immune systems, it is not easy to see how these approaches could be translated into clinical practice.

Aside from changing the stage of development at which the transgene is delivered, other approaches can be utilized to bias the tolerogenic expression of the transgene. Synthesis and presentation of the transgene product in the presence of co-stimulatory blockade will be successful in some instances [Haegel-Kronenberger et al. 2004; Jiang et al. 2004], although how long the blockade needs to be maintained and how complete the blockade should be remain unanswered questions. Finally, expression of the transgene within specific organs or at specific anatomical locations seems predictive of tolerogenic presentation of the protein to the adaptive immune system. Thus, AAV-mediated gene transfer to the liver has been associated with the generation of CD4⁺CD25⁺FoxP3⁺ transgene-specific regulatory T cells [Mingozzi et al. 2003; Cao et al. 2007]. Similarly, delivery of transgenes to cells of the nasal and gut mucosa also predisposes to a tolerogenic response to the transgene product through mechanisms that may involve generation of several regulatory T-cell populations, including TGF- β - and IL-10-producing cells [Cao et al. 2006].

1.9 CONCLUSIONS

In concluding this overview, it should be apparent that the immunological challenges to successful gene transfer are many and varied. In particular, the use of viral vectors for transgene delivery provides a significant provocation to the innate and adaptive immune systems that can result in major safety concerns and eliminate the potential for long-term benefit from the gene transfer process. Nevertheless, substantial progress has been made in the past decade in elucidating details of the immune response to gene transfer, and some advances have been achieved in developing approaches to circumvent or minimize these responses. Perhaps most important, substantial coincident advances have been made in our understanding of certain basic aspects of immunology, such as the evolving details of T-cell tolerance. Just as scientists and physicians in transplantation medicine have learned to manipulate the immune system to achieve cell and solid organ transplants, the gene therapy field is defining strategies to prevent immune-mediated rejection of vectors and therapeutic genes.

There is realistic optimism that gene transfer will provide clinical benefits within the foreseeable future. If these successes are to be achieved, strategies to minimize adverse immune responses to gene transfer protocols must be established. In the remainder of the book, details of our current knowledge are provided, together with future perspectives of this biologically fascinating and clinically important topic.

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